Stability of Tryptophan in Parenteral Amino Acid Solutions: Identification of Degradation Products and Development of HPLC Analysis Methods

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„Ein festes Ziel weckt die Begeisterung.
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1 Introduction

1.1 Impurity analyses in parenteral amino acid formulations

The guarantee of quality, safety and efficacy of pharmaceutical ingredients and finished pharmaceutical products is an utmost important issue in the pharmaceutical industries and regulatory authorities worldwide. Pharmacopoeias, administrative forces and guidelines provide standards and quality criteria for active pharmaceutical ingredients (API´s) and pharmaceutical formulations. Divergences from the accepted standard can set consumer´s health at risk severely. Monographs in pharmacopoeias define the substances and provide validated methods for identification, purity and content assessment. Especially the identification and the assessment of impurities in drugs and new drug products are a constantly discussed issue, due to its special importance, complexity and the advances in analytical performance.

The growing global demand for medication and the increased application of drug therapy goes along with an increased drug production and simultaneous development of quality control methods. Thereby, impurities and degradants in API´s and in pharmaceutical products were reported, which led to public, political and economic affairs in history and recently. The thalidomide-related embryopathy in the 1960ies [1, 2], the onset of epidemic-like Eosinophilia-Myalgia syndrome (EMS) after tryptophan (Trp) supplementation in the late 1980ies [3-7] , the heparin incident in 2007/08 [8, 9] and the recent discovery of potential cancerogenic impurities in valsartan and ranitidine drugs in 2018 [10-12] are exemplary cases for the implication of drug impurities. These incidents affected the safety of drug therapy detrimentally. Regulatory authorities like the Food and Drug administration (FDA) in the U.S., the European Medicine´s Agency (EMA) and national pharmacopoeia committees have been forced to introduce strict guidelines for the adoption, the compliance and the assessment of quality management. The “International Council for Harmonization of the Technical Requirements for Registration of Pharmaceuticals for Human Use“ established the so-called ICH guidelines, setting sophisticated standards for stability and impurity testing, and the validation of assessment methods, as well as thresholds for impurities in drug substances and products. The criteria are applied in pharmaceutical industry and research worldwide.

According to the ICH guidelines, impurities are defined as any components in the drug product or drug substance that is not defined as an excipient of the drug product or the drug substance itself [13]. In general, the ICH guidelines categorize impurities in organic,

inorganic and process-based impurities according to different origins [14]. Potential sources can be the synthesis route or the purification procedure, technological processing into the finished formulation, degradation during storage or chemical interactions of ingredients. Consequently, it is not possible to remove or impede the occurrence of impurities in the drug or drug product completely. The ICH guidelines provide general directions - depending on the maximal daily drug dose - for reporting, identification and qualification thresholds for each impurity [13, 15, 16]. The impurity assessment methods in the compendial drug monographs provide validated methods for the quantification impurities limiting the total amount of impurities in many cases additionally. The recommendations for the targeted development and validation of analytical impurity assessment methods are described in the ICH guideline Q2 (R1) [17].

Analytical performance and requirements advance steadily together with the introduction of more sophisticated methods. Thus, impurities can be assessed nowadays, which were unknown or have not been detected before [18-20]. Hence, the regulatory requirements in quality management of finished pharmaceutical products, especially long-time established products may need to be revised on impurity and safety aspects based on a current analytical approach.

In this context, pharmaceutical formulations consisting of a combination of APIs are rather complex. The formation of a variety of degradants during manufacturing processes or interactions between substances are thinkable. Parenteral amino acid (AA) formulations are exemplary pharmaceutical products consisting of several APIs, which are subject to strict requirements with regard to quality, safety and sterility due to intravenous administration. In parenteral AA formulations, every AA is an individual API and must comply to purity and stability regulations on its own. Finally, complex formulation must meet the quality demands during manufacturing, processing, storing and administration. *Formulation 1* and *2* (kindly provided by Fresenius Kabi AG, Bad Homburg, Germany) are parenteral AAs solutions, combining the challenges of a multi-component system with the named quality demands of parenteral formulations like sterility, compatibility and stability during manufacturing, administration and storage.

The standard parenteral AA formulations consists of the 9 essential AAs (EAA: His, Iso, Leu, Lys, Met, Phe, Thr, Trp, Val), several non-essential AA (NEAA: Ala, Arg, Gly, Pro, Tyr, Ser) and some AA considered as conditionally essential such as Glu, Cys and Taurine

with a standard dosage of 1.0-1.5 g per kg bodyweight per day. The composition of the formulation depends on the individual need of the patient, thus there are different ready-to-use formulations available. An alternative to premixed formulations is the preparation of individual formulations in the dispensary. Typical clinical conditions requiring parenteral nutrition are e.g. malnutrition, severe surgery, burns or traumas, gastrointestinal dysfunction and the supplementation in neonatology/paediatrics. The parenteral AA administration is a well-established form of nutrition, which can be even applied in ambulant supplementation of uncomplicated cases of malnutrition or digestive malfunctions. The evidence-based benefits of parenteral nutrition are the fast nutrient supply, the controllable administration, allowing individual adjustment and the complementation with nutrients such as carbohydrates, lipids, vitamins and/or further therapeutics. However, some proteinogenic AAs have been discarded from parenteral AA formulations due to stability issues; e.g. Cys (prone to oxidation to cystine and incompatibility with drugs, product yellowing) and Glu (Glu degradation). Anyway, occasional yellow discoloration was still reported in specialized information - without an explanation so far. The discolouration issue is a current issue in quality management leading to ongoing revaluation of stability testing and AA and impurity assessment methods, respectively.

AA analysis is very important in chemical and medical analyses, but it is demanding and thus constantly developing since the discovery of AAs. The high polarity, low molecular weight and the lack of a chromophore of most AAs impede the majority of conventional HPLC analysis methods. The demand for fast, easy and robust AA analyses continues to grow consistently with the increased application of AAs and peptides in therapy and the discovery of their ability to indicate metabolic malfunctions in body fluids. Advances in AA analysis in the last decades led to revision and harmonization of general methods in leading pharmacopoeias and literature.

Research done on the general stability of AAs and possible analyses methods is summarized in the following review and supplemented by a brief evaluation of AA compatibility with additives and typically used primary packaging.

1.2 Stability and the assessment of amino acids in parenteral nutrition solutions

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Abstract

Sterile amino acid solutions are applied in medical care as part of Total Parenteral Nutrition systems. Typical formulations consist of variable admixtures of essential and non-essential AA together with carbohydrates, electrolytes, vitamins, trace element solutions and lipid emulsions. The complexity of these formulations gives rise to stability and compatibility reflections. This review focuses on amino acid stability in pure amino acid solution and name methods of assessment. Incompatibilities of AAs with the other ingredients are matter of concern in clinical practice and evaluated for relevance.

Keywords

Amino acid, parenteral nutrition, stability, compatibility, quality assessment

Abbreviations: AA, amino acid; AiO, all-in-one; MCB multi-chamber bags; MLB, multilayered bags

1 Introduction

Parenteral nutrition is commonly used for sufficient nutrient supplementation in patient groups such as cancer patients, critically ill and elderly persons or preterm infants [1-4]. They are composed of amino acids (AAs), carbohydrates, lipids, vitamins, electrolytes and trace elements in variable proportions to meet the required clinical demands [5-7].

AAs are one of the most important building blocks of body tissues, enzymes and hormones, thus indispensable for vital body functions. When it comes to metabolic dysfunction, insufficient resorption, increased nutritional demands after severe surgical trauma, and medical care of preterm and neonates, there is beneficial prove for targeted supplementation or even complete substitution of AAs via the parenteral route of administration. Hence, there is an instant need for AA formulations that are quickly available, ready-to-use, and safe [8]. AAs can be administered separately or together with the afore-mentioned supplements as total parenteral nutrition (TPN) admixtures. It is possible to compound a mixture suitable for any clinical complication [9-11].

Quality has to be assured from the pharmaceutical point of view. Purity of each ingredient, stability, compatibility, and degradation considerations have priority, because safety of either AA as parenteral solutions and TPN admixtures must be guaranteed [12-14]. The quality of each ingredient can be assessed by corresponding monographs of the European Pharmacopoeia (Ph. Eur.) [15] or United States Pharmacopeia (USP) [16]. Recently, the AA monographs in Ph. Eur. were revised. In order to guarantee purity, the AA analysis was introduced in addition to special impurity assessment in the monographs of individual AAs.

However, this review shall give an overview over the stability profiles of the AAs used in parenteral nutrition solutions, as well as the possible interactions between additives, electrolytes, trace elements, excipients and packaging materials. Possible restrictions due to physical or chemical reactions are summarized and evaluated due to clinical relevance. Eventually this review will summarize the current recommendation on compounding procedures, since the process of compounding TPN admixtures does affect the quality and safety of the final product substantially.

Taken together this review will focus on relevant considerations about stability and degradation of AAs and the analytical state-of-the-art methods. The final conclusion will summarize the practical use of AAs in parenteral nutrition and corresponding solutions.

2 AAs in parenteral formulations

In the beginning of the 20th century first medical investigations were made about metabolism of AAs after intravenous administration. One of the first report in this field was published in 1913 by Henriques and Andersen, who infused a beef hydrolysate into a goat [17]. Rose determined the essential AAs and their overall importance for human health [18] and in 1937, Robert Elman published first successful studies about an intravenous infusion of AAs as a fibrinogen hydrolysate [19, 20]. In 1944, Wretlind invented the first enzymatically hydrolyzed and dialyzed intravenous formulation of AAs called Aminosol® [21]. These formulations already seemed to be promising although they were not complete from present day´s perspective, since intake did not match the sufficient physiological need. Each type of hydrolyzed protein has an unchangeable AA profile resulting in an abundance or lack of certain AAs measured against human requirements. It took some years until the first crystalline pure L-AA formulation was introduced in 1964 in Germany by Bansi [22].

The aim of AA supplementation is to achieve a positive nitrogen balance and to provide the body with all essential AAs for metabolic functions and tissue building. It took some time and a lot of research to find the optimal composition of AAs. Some AAs do provide sufficient nitrogen and some seem to be ineffective. Subsequently the task was to figure out whether there are stability problems or metabolic ones.

2.1 AA composition from clinical point of view

Today there are several guidelines on parenteral AA supplementation providing a clinical background about the necessity of AAs [23]. Organizations such as ESPEN (European Society for Parenteral and Enteral Nutrition) and the American counterpart A.S.P.E.N. (American Society for Parenteral and Enteral Nutrition), as well as the well-recognized JSPEN (Japanese Society of Parenteral and Enteral Nutrition) and BAPEN (British association for Parenteral and Enteral Nutrition), update recommendations of the

supplementation continuously and provide clinical and pharmaceutical evidence. Statements and guidelines are given for the treatment of e.g. metabolic disorders, the critically ill, preterms and neonates and for postoperative patients in severe trauma, which all have specific requirements with regard to dosage and composition of AAs.

All commercially available AA formulations provide nine essential AAs i.e. histidine (His), isoleucine (Ile), leucine (Leu), lysine (Lys), methionine (Met), phenylalanine (Phe), threonine (Thr), tryptophan (Trp), and valine (Val) in varying amounts between 38-57% of the total AA content and nonessential AAs are included in an amount of 43-62%, respectively. Explicit formulation of single AA content may vary in order to meet the individual demands of the patient. E.g. in case of hepatic encephalopathy, one may require a mixture with more branched chained AAs and reduced aromatic AAs [24]. The German Association for Nutritional Medicine recommends an AA dosage of 0.8 g/kg bodyweight/ day for adults with normal metabolism [25].

2.2 Commercially available formulations

Tab. 1 gives an overview of representative AA formulations. Bearing in mind, that requirements of the individual may vary, these formulations have different concentrations of single AAs, but they do not differ in the total AA content of 10%. To some extend these variations are based on specific patient groups´ demands, or they are set ad libitum by manufacturer. However, some are related to concrete stability issues. Especially the formulation of nonessential AA composition allows greater variability, without impact on quality but also with the possibility of adjustment to special requirements. In case of hepatic encephalopathy, treatment with increased branched chain AAs (Ile, Leu, Val) supplementation proved to be beneficial [26], patients under severe stress conditions receive additional high dose Gln formulations [27], and Arg and Cys are essential in pediatric care [28-30], additionally non-proteinogenic AAs like ornithine (Orn) or taurine may be part of pediatric formulations since they are considered to be conditionally essential in preterm infants [24].

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Amino Acid	Aminoven®	Primene®	FreAmine®	Aminoplasmal®	Aminosyn®
essential			**(g/L)**		
Ile	5.00	6.70	6.90	5.00	7.20
Leu	7.4	10.00	9.10	8.90	9.40
Lys	6.60	11.00	7.30	4.07	7.20
Met	4.30	2.40	5.30	4.40	4.00
Phe	5.10	4.20	5.60	4.70	4.40
Thr	4.40	3.70	4.00	4.20	5.20
Trp	2.00	2.00	1.50	1.60	1.60
Val	6.20	7.60	6.60	6.20	8.00
non-essential					
Arg	12.00	8.40	9.50	11.50	9.80
His	3.00	3.80	2.80	3.00	3.00
Ala	14.00	8.00	7.10	10.50	12.80
Gly	11.00	4.00	14.0	12.00	12.80
Pro	11.20	3.00	11.20	-	8.60
Ser	6.50	4.00	5.90	2.30	4.20
Tyr	0.40	0.45	-	0.40	0.44
Cys	-	1.89	<0.16		
Taurine	1.00	0.60	-	-	-
Glu	-	10.00	-	7.20	-
Asp	-	6.00	-	5.60	-
Orn	-	2.49	-	-	-

Table 1: Overview of commercially available AA formulations. All formulations contain 10% (g/L) AAs.

2.3 Stability of AAs in aqueous solution

Prescribing information of each formulation state *p*H value in the range of 5.5-6.5 adjusted with either acetic or citric or maleic acid, theoretical osmolality in the range of 850-1000 mOsm/l, and the optical appearance of the solution has to be clear, of no colour or slightly yellow.

The solutions are usually supplied in plastic bags or glass bottles, either for further compounding with other solutions or to be administered intravenously with an infusion set. The factors influencing AA stability are other AAs and ingredients in the formulation, light irradiation and temperature fluctuations while storage (and administration), residual oxygen in the container and the shelf life or respectively storage time until usage [31-35]. If aseptic production cannot be guaranteed, heat sterilization may be necessary, this high pressure, high thermal impact should also be considered.

One of the first studies designed to obtain data on the stability of such AA solution was performed in 1974 by a hospital pharmacist [36]. A formulation of 15 AAs in sterile water was compared to the same formulation dissolved in 25% dextrose solution after storage for a period of twelve weeks. The start AA concentration was 4.25% (g/L). The solutions were stored at 4 °C, 25 °C, and 37 °C, respectively. Changes in *p*H and colour formations were observed like strong darkening in the dextrose sample and slight yellowing of the control solution. Individual AA degradation was analyzed by means of the Amino Acid Analyzer. After 12 weeks of storage at 4 °C none of the AA concentrations fell below 90% of initial concentration in sterile water (control) solution, but at room and higher temperatures a slight degradation tendency is anticipated. AAs in dextrose solution showed even higher degradation rates, which will be discussed in the following chapter. Generally, the *p*H value of the solutions decreased slightly over storage period, again enhanced with higher temperatures. AA degradation in sterile water was moderate during 12 weeks of storage. Unfortunately, the most interesting AAs with regard to instabilities, Trp, Arg, and Cys, were excluded because of an unsuitable detection method. In 1974, Lien and Nawar [37] studied the thermal decomposition of the branched chain AAs, Val, Leu and Ile, under very harsh conditions. Solid samples of single AAs were heated at 180-270 °C for 1 h, volatile decomposition products were isolated and analyzed by GC-MS. Val, Leu, and Ile showed similar modes of decomposition: principal products were ammonia, carbon dioxide, carbon monoxide, and the inherent olefins, propane, isobutene and butane, respectively. Remaining compounds were identified as hydrocarbons, ketones, aldehydes, imines, and primary and secondary amines characteristic to parent substance. With increasing temperature, the colour changed from slightly yellow to orange and even black, also original

crystalline appearance altered (not described in detail). Besides decarboxylation and deamination, the authors suggested mechanisms including α-cleavages of aldehyde components, β-cleavages of amine components and partly McLafferty rearrangements; partly free radicals were involved.

The proposed *initial* decomposition mechanisms may be applied to all AAs theoretically, either solid or in solution, since the type of AA side chain was not claimed to be responsible for any particular reaction (Fig. 1). Sohn and Ho investigated ammonia generation of AA solutions during thermal degradation [38]. With every AA used, ammonia is the primary thermal degradation product pointing to deamination and/or deamidation. 19 AAs were tested in single solutions and generated ammonia was quantified by a gas sensing ammonia electrode. Nonpolar AAs such as Ala, Val, Leu, Ile, and Met released less than 5% ammonia; polar ones such as Thr and Ser released 5-6%. Most significant amounts were detected for Arg, Asn, Asp, Gln, and Cys. AAs containing more than one nitrogen atom, i.e. Arg, His, Trp and Lys, showed higher amounts of ammonia consistently. Cys releases ammonia from its α-amino group; furthermore, because the reactive thiol group is released even at low temperatures, molecular reactivity rises and promotes the nucleophilic attack on the α-carbon, thus initiating further release of ammonia.

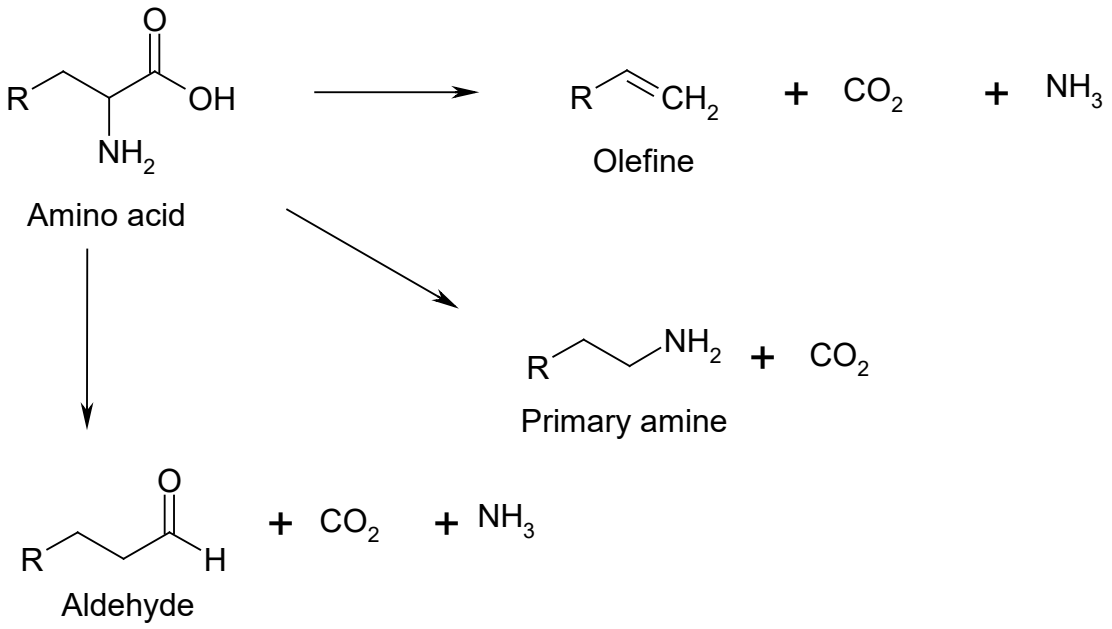
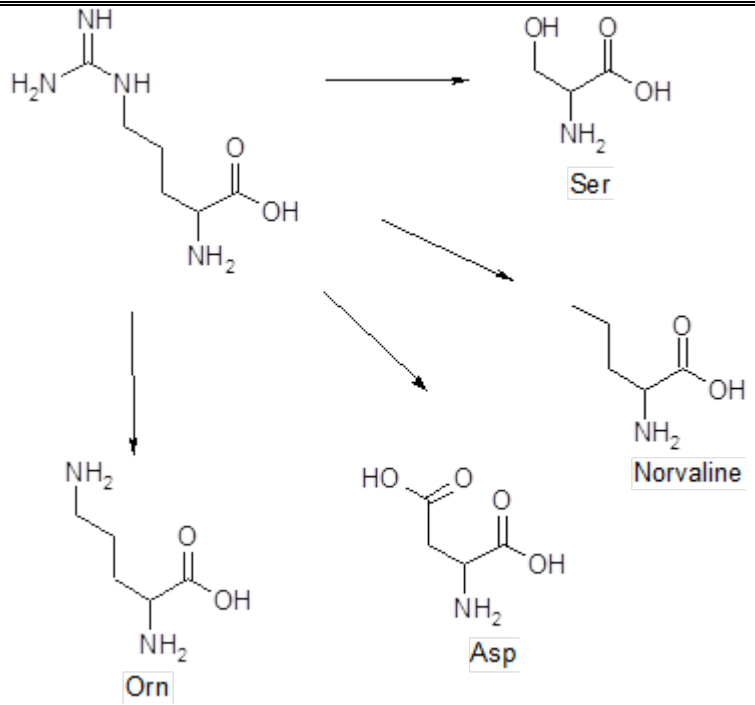


Figure 1: AA decomposition

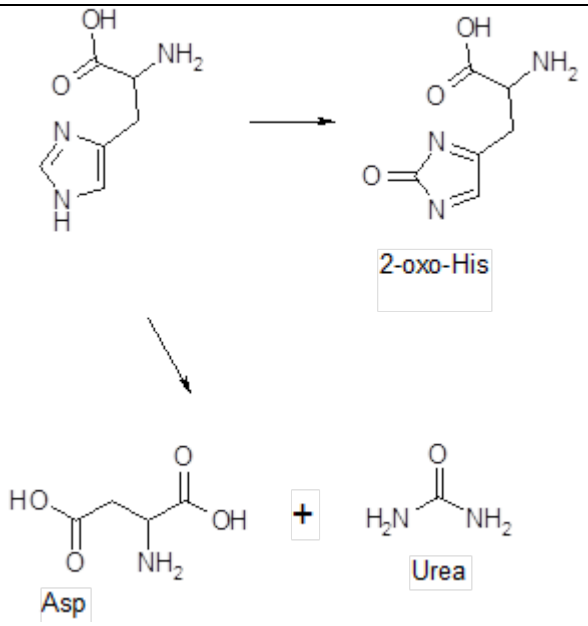
Light irradiation is another factor influencing the stability, non-ionization radiation like UV or visible light has only poor degradative potential [39]. However, interaction of non-ionizing radiation in the presence of substances with chromophores may generate oxidizing species, such as hydrogen peroxide, being a precursor of hydroxyl peroxide – one of the most potential oxidizing species. In this context the impact of hydrogen peroxide and hydroxyl radicals on AAs has to be discussed [40, 41]. Arg shows significant photo-degradation in presence of hydrogen peroxide, as studied by Ansari et al. [42]. Ammonia and urea are the major products formed as a result of synergistic action of UV light and hydrogen peroxide, depending on radiation duration. Further decomposition of Arg gave Asp, Ser, nor-valine and Orn (Fig. 2). An explanation may be the reaction of hydroxyl radical with Arg causing a radical dissociation of carbon-carbon single bonds, followed by recombination and group rearrangements. Photoexcitation of the α-amino group followed by deamination, and as well as cleavage of the guanidinium group lead to ammonia liberation. In association with reported decreased AA concentration after periods of light irradiation (though the focus of these studies was not to elucidate degradation products) [43, 44], an investigation of a model pediatric parenteral AA solution proved hydrogen peroxide generation after 90 min light irradiation in the 425-475 nm waveband, as used in pediatric phototherapy [45]. According to study results of Boreen et al. in 2008, most susceptible to photo-oxidation are Trp, Met, Tyr, His, Cys, and Phe in aqueous solution [46]. Formation of singlet oxygen ($^1O_2$) is related to the reaction mechanisms of photo-induced oxidizing species. Trp is oxidized to one and twofold hydroxylated Trp, as well as oxygenated species and kynurenine and/or formylkynurenine (Fig. 2). In the context of protein-bound Trp degradation the named decomposition products and metabolic glycosides are known to be of a slight yellow colour. They were identified as responsible substances for the yellowing of organic tissues, such as wool and lentils [47, 48]. (Nonetheless, instruction leaflets of available formulations tolerate slightly yellow appearance of the solutions). Tyr is oxidized to its dimer dityrosine or 3-,4-dihydroxy (DOPA) derivatives, His and $^1O_2$ form Asp and urea or it is oxidized to 2-oxo-His. Met is oxidized in the presence of $^1O_2$ to Met sulfoxide (Fig. 2). Phe is oxidized to *ortho*- or *meta*-Tyr (Fig. 2) [49]. Because of the very low solubility of Tyr and the fact, that the human body convert Tyr from Phe, parenteral nutrition solutions include Tyr only in very small concetrations, if at all. Usually AA formulations do not contain Gln because of concerns about its instability and toxicity of degradation compounds. Tested in typical formulations, Gln degradation rates amount to 0.8 %/d at room temperature or 0.1-0.15 % d at -4 °C. In the degradation process, the free pair of electrons of nitrogen in the α-amino group reacts with the δ C-atom to pyroglutamic acid and ammonia (Fig. 2). Rising ammonia

concentration was consistent with decreasing residual Gln concentration. Toxic effects of these compounds are unlikely, since small concentrations of pyroglutamic acid are also intermediates in mammalian γ-glutamyl cycle and the human body handles up to 14 g of ammonia per day [50]. The same mechanism is possible for Glu, but releasing equimolar water instead of ammonia, however to the best of our knowledge, no studies are available on this as stability concern in parenteral solutions. Coherently with these reactions, no interactions with other AAs were described.

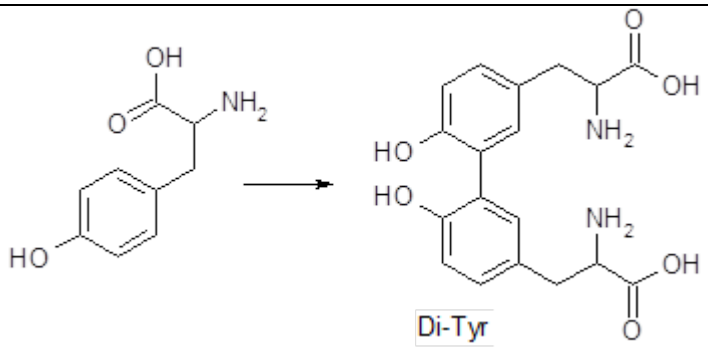
Cys is usually excluded from these formulations, because it is not essential and will be converted enzymatically from Met in the human body. The reactive thiol group is susceptible to oxidation to the disulfide Cystin, sulfinic, sulfenic and/or sulfonic acids, which cannot be reduced by metabolic cellular systems (Fig. 2) [49].

	Degradation products
Arg	

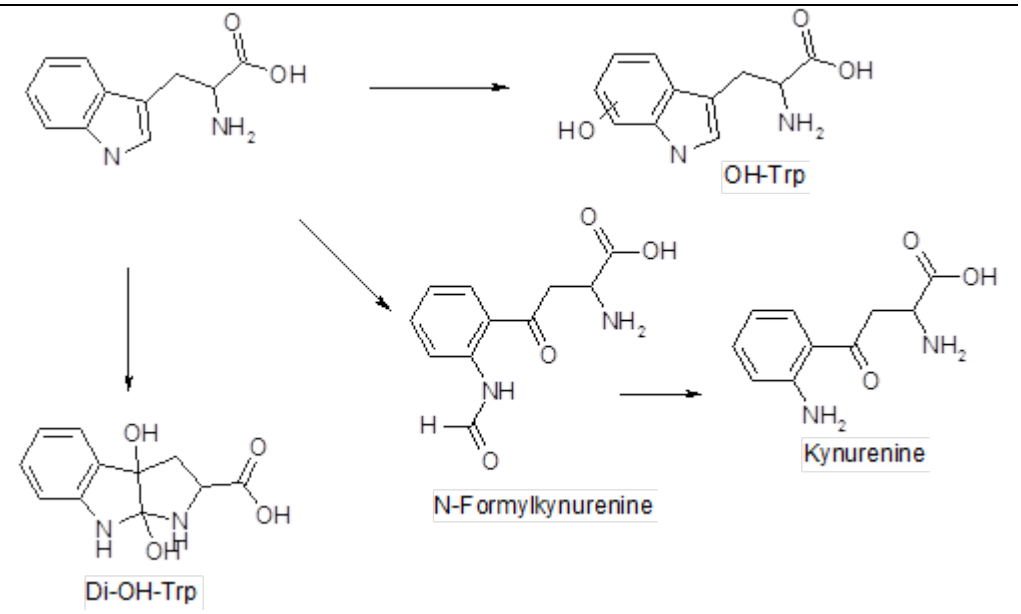
His


OH
NH2
O
N
N
H
2-oxo-His
HO
OH
NH2
Asp
+
H2N
Urea

Tyr


OH
NH2
HO
Di-Tyr

Trp


OH
NH2
N
OH-Trp
HO
NH
H
Kynurenine
N-Formylkynurenine
Di-OH-Trp

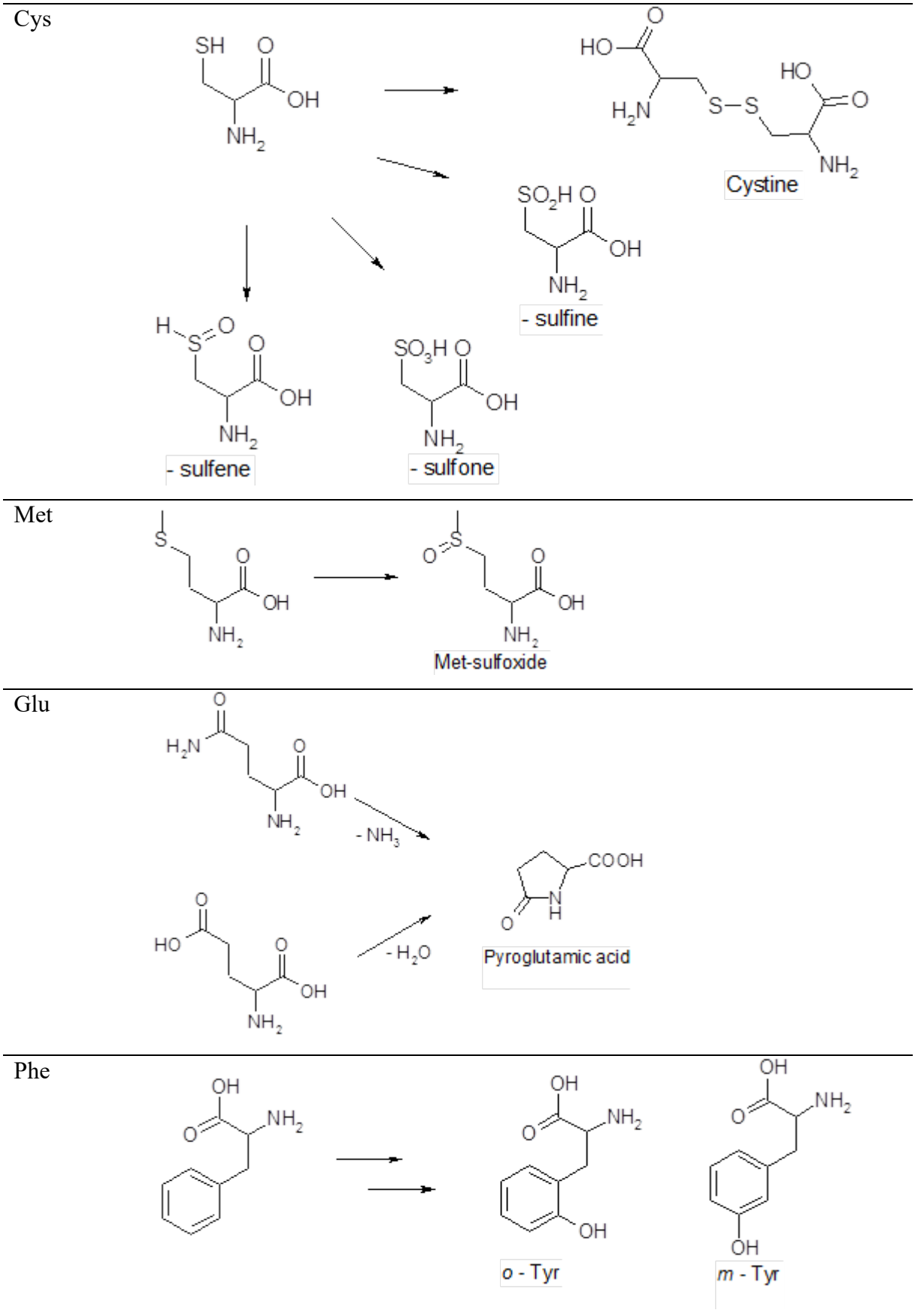


Figure 2: AA degradation after heat treatment and light irradiation in aqueous solution

Based on the facts, that concentration of AAs in parenteral solution cannot be considered as critically, free AAs are not highly reactive by nature, and they exhibit chemically buffering character, it can be said, that such formulations can be regarded as stable. Studies performed on stability usually apply very harsh and extreme conditions, which do not reflect the practical use, but it can help to elucidate possible instability problems. Taking together; safety and quality of parenteral AA solution in a closed system, made under strict controlled conditions and stored according to the instructions, are guaranteed over shelf life period. However, sterilization processes can challenge the stability of an AA solution (see below). The following table (Tab. 2) summarizes the stability facts of all AAs in use.

Amino acid	Stability	Notes	Ref.
essential			
His	Decline in presence of HCl in <24 h, depending on acid concentration; Long term stability >90% over 6 months at 4 °C; Decomposition to Asp	-	[2] [31] [46]
Ile	Stable under normalized conditions	Thermal degradation above 180 °C	[37]
Leu	Stable under normalized conditions	Thermal degradation above 180 °C	[37]
Lys	Long term stability >90%; over 6 months at 4 °C	Stable ingredient; no information about degradation products	[33]
Met	Decomposition at 25 °C in 30 d; Long term stability: loss >20% over 6 months at 4 °C; Photolysis to methionine sulfoxide	Sulfoxides are not problematic; Enzymatic reconversion to Met possible (human methionine sulfoxide reductase)	[34] [33] [46] [49]
Phe	Actually stable; possible hydroxylation to tyrosine	Deamidation possible; yielding benzene, toluene, *p*-ethylbenzene	[51]
Thr	Long term stability test: loss >25% over 6 months at 4 °C	No information available	[33]
Trp	Light and oxygen induced degradation; Incompatibility with sodium bisulphite (formerly used as antioxidant in PN); Long term stability: loss up to 13% over 6 months at 4 °C; Degradation to kynurenine, formylkynurenine,	Temperature and light-induced decomposition as synergistic factors; Most unstable amino acid; Degradation maybe prevented by use of ultraviolet light protective bags, nitrogen purging, oxygen scavengers	[33] [34] [35] [49] [52]

	hydroxylated, di-hydroxylated forms		
Val	Stable under normalized conditions	Thermal degradation above 180 °C	[37]
non-essential			
Ala	Long term stability: loss >20% over 6 months at 4 °C	No information available	[33]
Arg	Decomposition at 25 °C in 30 d; Long term stability: loss >20% over 6 months at 4 °C; Degrades to norvaline, Ser, Orn, ammonia, and urea	Synergistic effects of H_2O_2 and UV irradiation; Decomposition inhibited by hydroxyl radical scavengers – e.g. vitamin A/E given as additives	[34] [33] [42]
Asn	Dominant deamination/deamidation	Thermal decomposition	[38]
Asp	Deamination <5%	Thermal decomposition	[38]
Cys	Fast oxidation to cystine in presence of oxygen; Reacts to glucocysteine in presence of glucose; Precipitates with copper at high concentrations; Thermal deamidation, and release of thiol group.	Not used; if, only in low concentrations, because not essential in adults If added as Cys-HCl, it decreases pH, and is not biologically available	[38, 49, 53-57]
Gln	Dominant deamination/deamidation; Especially unstable in amino acid mixtures; Pure solution with glucose is apparently more stable; Upon short heating immediate degradation to pyroglutamic acid (5-oxoproline) and ammonia	Normally not included, but metabolically very important in special patient groups; may be given as biologically available and more stable dipeptide or as N-acetyl-glutamine	[38, 50]
Glu	Thermally stable; Deamidation <1.3%	Theoretically formation of pyroglutamic acid possible, not described in parenteral formulations	[38]
Gly	Long term stability: loss >20% over 6 months at 4°C	No information available	[33]
Pro	Decline in presence of HCl in <24 h depending on HCl concentration; Long term stability: loss >20% over 6 months at 4 °C	No information available	[31] [33]

Tyr	Precipitates at pH <3; Low solubility product Long term stability (>90%) over 6 months at 4 °C; Oxidation to dityrosine	Not used, if only in very low concentration due to low solubility product; Caution with acidic additives such as HCl; Photo-degradation to dityrosine	[31] [2] [33] [58] [46]
Ser	Stable	No information available	

Table 2: AA stability overview

2.4 Analytical methods assessing AA stability

All of these AA formulations may undergo various handling such as sterilization procedures like autoclaving processes, temperature fluctuations like freezing, thawing or refrigeration, exposition to sunlight or other forms of radiation (e.g. light therapy irradiation), transport and storage periods during their application in clinical practice. As discussed in the previous chapter, particular AAs are more eligible to (long time) storage and are robust to environmental changes, such as temperature fluctuations or radiation exposure, than other AAs degradation means possible loss of functional groups, molecular rearrangements, heat induced destruction and photo-oxidation, resulting in either reduced AA concentration, or even in critically toxic by-products. There is not much information about harmfulness or toxicity of degradation products, though animal studies suggest that oxidation products of e.g. Trp may be associated with hepatic dysfunction and complication during parenteral alimentation [59].

Many studies have been carried out to conduct stability prediction of the components throughout either rather conceivable or extreme conditions. One possible approach of stability assessment can be the measurement of the single AA concentration after defined storage periods or after exposure to named conditions, and comparison to the initial concentration. Another way might be the screening for toxic or problematic degradation products, thus creating a direct stability indicator. A less distinguishing option is to measure the whole ammonia concentration, since this is a decomposition product shared by all AAs. This may indicate the remaining AA concentration but gives no information about the final composition or individual degradation ratios. A great advantage of AA analysis in parenteral formulations is the simplicity of sample preparation. Absence of complex matrix, insoluble excipients or other intervening substances facilitates direct sample handling. Methods used in analysis of more complex formulations such as body fluids or protein hydrolysates usually need preparational steps such as extraction ahead

of analysis, which are dispensable in aqueous AA solutions and thus most of these methods become directly applicable in general.

The Ph. Eur. distinguishes between methods of protein hydrolysis and "methodologies of amino acid analysis", 8 methods are described [15]. The AA analyzer is a widespread way of analysis and meanwhile obtainable fully automated. Technically speaking, AAs are separated on a cation exchange resin using lithium or sodium based buffers as mobile phase, followed by post-column ninhydrin derivatization and UV detection of the purple coloured derivatization products [60-62]. Though not actually intended for this purpose, this method is also used for assessment of related (ninhydrin-positive) substances [63]. Ninhydrin positive substances are ammonia, AAs, imino acids, primary amines, yielding purple coloured products and secondary amines, yielding yellow coloured products, detectable at 570 nm and 440 nm, respectively. In the context of stability assessment, the advantages are good automation capacity and applicability for AA quantification. However, it is limited, since it does not acquire any substances, which do not react with ninhydrin, and some AAs react in multiple ways. If temperature, time, and *p*H are not maintained constant the accuracy of detection is low [64, 65]. Additionally, it is expensive and time consuming. Further methods prescribed in the Ph. Eur., as listed below (Tab. 3), include pre-column or post-column derivatization of AAs, yielding detectable derivatives by either UV/vis or fluorescence. Of noted, the USP applies analogous methodologies for the analysis of AAs.

Separation of polar compounds like (non-derivatized) AAs in classical RP-HPLC is inherently difficult, due to weak retention on the reversed phase [66]. More polar resins allow interaction and retention of such compounds; therefore, ion exchange chromatography was successfully introduced. Elution patterns of AAs on different resin types have been studied since the 1950ies [67]. Other chromatographic methods to separate polar compounds are the application of porous graphitic columns (PGC) and the HILIC technology. In the case of PGC retention it is assumed to be caused by complex electronic and π-π interactions, but it is not yet fully clarified [68, 69]. PGC are robust towards very low or high *p*H values and aggressive solvents. However, in order to enhance interaction often volatile ion-pair reagents are used, primarily perfluorinated carboxylic acids with n-alkyl chains. This increases the retention (of ion-pair reagents and analytes) so much, that complete desorption from the stationary phase can become time-consuming and eventually problematic [69, 70]. For HILIC the stationary phase is modified with amides, hydroxyl, cyano or amino groups to be polar outwards and thus exhibiting a basis for interaction with polar compounds [71]. Elution is usually carried out by acetonitrile/water

gradients; with increasing aqueous content, analytes desorb from the stationary phase. The use of volatile solvents and buffers allow effective coupling to mass detectors, for both PGC and HILIC columns, which is of great benefit for direct amino acid analysis [66, 72].

If using RP columns intentionally, the methods of choice are either a form of derivatization or use of ion-pair reagents. Pre-column derivatization, with OPA or ACC reagents, does not only facilitates UV/vis detection (for the most AAs), but also enhances interaction of the derivative with the nonpolar stationary phase [73, 74]. Optimized eluents and the use of gradients improve separation and reduce retention time of AAs and modified analogues as well. Depending on the stationary phase and derivatization technique, adjustment of *p*H, buffer concentration, and ratio of aqueous and organic phase influences the outward charge of molecules and thus the capability of interaction with the stationary phase. More or less of organic solvents leads to desorption or further adsorption to the resin, respectively [75].

The lack of chromophore, except Phe, Trp, and Tyr, makes derivatization for UV/vis detection indispensable. Though detection of impurities and underivatized AAs at wavelengths below 220 nm is reported [76, 77], the right choice of solvents is very important, e.g. due to UV cut-offs. Consequently, either a derivatization step or change of detector is required, alternatively electrochemical methods of detection, charged aerosol detectors (CAD), refractive index detectors and mass spectrometers are appropriate [78-82]. Here the advantages of mass spectrometric devices become apparent. Poor separation is compensable by targeted screening of explicit masses and, in the case of fragmentation studies, structural information can be retrieved on top. Restrictive conditions to mass spectrometric analysis are ionization ability of the analytes and strict use of volatile solvents and salts.

Tab. 3 and 4 sum up HPLC methods as applied in the Ph. Eur. and in applied science, respectively, the latter outlines rather recent methods as published in the field of research without derivatization but using either alternative separation or detection devices.

In the context of alternative separation methods besides chromatography, capillary electrophoresis has to be discussed. Both Ph. Eur. and USP itemize capillary electrophoresis in detail, however explicit application methods for AA analysis are not given. Anyway, in research capillary electrophoresis is applied successfully for AA assessment in biological matrices, in pharmaceutics e.g. parenteral solutions and in food chemistry [83-90].

Separation	Eluent	Detection	Analysis	Ref.
IEC	Lithium- or sodium based buffer, isocratic	ninhydrin post-column derivatization; UV/vis: 570 nm (amino acid derivatives) 440 nm (imino acid derivatives)	AAs, imino acids, related ninhydrin-positive-substances	[15, 60, 63]
IEC (SCX)	Borate buffer systems isocratic	OPA post-column derivatization; fluorometric: excitiation at 348 nm emission at 450 nm	Primary amines and derivatives, excluding Pro and other secondary amines	[15, 91, 92]
RP18- HPLC	Sodium phosphate buffer ACN, isocratic	PITC pre-column derivatization; UV/vis: 254 nm	AAs	[15, 93]
RP18- HPLC	Borate buffer ACN isocratic	ACC pre-column derivatization fluorometric: excitation at 250 nm emission at 395 nm	AAs 2 fol*D.* higher detection limit for Cys	[15, 94, 95]
RP18- HPLC	Sodium phosphate buffer ACN Gradient	OPA pre-column derivatization; fluorometric: excitation at 348 nm emission at 450 nm	Primary amines and derivatives, excluding Pro and other secondary amines	[15, 96]
RP18- HPLC	Sodium phosphate buffer ACN gradient	DABS-Cl pre-column derivatization; UV/vis: 436 nm	AAs	[15, 97, 98]

RP18- HPLC	Sodium acetate buffer ACN MeOH acetic acid gradient	FMOC-Cl pre-column derivatization; fluorometric: excitation at 260 nm emission at 313 nm	AAs, His derivatives prone to fast decomposition	[15, 96, 99, 100]
RP18- HPLC	Borate buffer ACN gradient	NB*D*.F pre column derivatization; fluorometric: excitation at 480 nm emission at 530 nm	AAs	[15, 101, 102]

Table 3: Analytical methods assessing AAs

Method	Separation technique	Mobile Phase	Detection	Detected compounds	Ref.
IP-RP-HPLC-UV	RP 18	0.1 % (v/v)TFA, in water and in ACN, sodium heptane, sodium sulfate, buffer *p*H 2.3	UV/vis: 210 nm	AAs, Not Cys but cystine, and cysteine-complexes with trace elements: Zn, Se	[76]
HILIC–ESI-MS	TSKgel Amide 80 column	0.5 mM/ 2.5 mM NH_4OAc buffer in 90 %/60 % aqueous ACN gradient *p*H 5.5	Triple quadrupole MS, ESI-source Q1 scan Neutral loss scan	AAs, hydroxylated Pro derivatives, Cys excluded for oxidation/ dimerization	[66]
IP-HPLC-MS/MS	octadecyl silica column	perfluorinated carboxylic acid solution* ACN gradient	triple quadrupole MS, APPI source MRM	AAs, biogene amines	[103] [104, 105]

IP-LC-ELSD	Porous graphitic column, Hypercarb®	perfluorinated carboxylic acids solution* ACN gradient	ELSD	AAs	[106]
IP-RP-HPLC-ELSD	RP-18	Pentadecafluoro-octanoic acid solution ACN gradient	ELSD	most polar AAs: Asn, Asp, Ser, Gly, Gln, Cys, Glu, Thr, Ala, Pro	[78, 105]

Table 4: AA analysis without derivatization.

*(*trifluoroacetic, heptafluorobutyric, nonafluoropentanoic, tridecafluoroheptanoic, pentadecafluorooctanoic acid test, range of 0.5 mM – 20 mM tested), Abbreviations: ODS, octadecylsilane; IP, Ion-pair; IEC, Ion exchange chromatography; SCX, strong acidic ion exchange chromatography; ACC, 6-amino-chinolyl-N-hydroxysuccininimidylcarbamate; DABS-Cl, 4-N,N-dimethylaminoazobenzene-4'-sulfonyl chloride; FMOC-Cl, 9-fluorenylmethyl chloroformate; NBD.F, 4-fluoro-7-nitro-2,1,3-benzoxadiazole; TFA, trifluoroacetic acid; HILIC Hydrophilic- interaction chromatography; MS, mass spectroscopy; ESI-MS, electrospray ionization – mass spectroscopy; MS/MS, tandem mass spectroscopy; APPI, atmospheric pressure photoionization; MRM, multiple reaction monitoring, ELSD, evaporative light scattering detector)*

3 Total Parenteral Nutrition

In clinical practice single AA formulations are more often used as a compound of so called Total Parenteral Nutrition (TPN) admixtures. In 1972, Solassol was the first one to apply the new form of parenteral nutrition [107]. The idea was to compound a formulation that provides the patient with AAs, carbohydrates, lipids, and micronutrients such as vitamins, trace elements and electrolytes, which is safe, effective and low in complication risks [108]. In historical context parenteral AA solution administration was a form of supplementation for all patients, where the enteral route for alimentation failed. Later it was found, that the combination of intravenous available carbohydrates and AAs had a sparing effect on the nitrogen balance resulting in better survival of the patients. The problems here were overfeeding and high blood sugar levels leading to weight gain and insulin resistance. With invention of a parenteral fat emulsion, it became possible to supply the required caloric intake in a form of a mixture of glucose and fat. So the idea of a balanced TPN was born. Many clinical trials confirmed the advantages of this administration route and continuous improvement in this field is still going on [109]. Compounding and administration of “All-in-One” (AiO) admixtures also reduce the

risk of error and contamination. There are different nutrition systems available nowadays which are ready to use directly or after flexible and convenient adjustment to clinical demands [13] .

Basically, there are two concepts of AiO mixtures. For one, an individual formulation of AAs, glucose, fat, additives, and medication is manufactured on order from the hospital pharmacy in a plastic bag container, then refrigerated at 4 °C until use within a couple of hours. These systems are compounded at volumes only up to daily required doses, in order to minimize the time of possible destabilizing interaction processes in the mixture or exposure to stressing conditions, e.g. exposure at room temperature is less favorable than storage in refrigeration [110]. Possible reactions are creaming and coalescence of a fat emulsion, sugars and AAs may react to yellow or brown substances, occurrence of precipitates of electrolytes or a non-preferred change in *p*H value. Though the reaction rate begins slowly, it takes up momentum with each alteration.

Secondly, a more economic and practical way is the multiple chamber bag (MCB) concept (Figure 3). MCBs are plastic bags divided into three compartments that are separated by a breakable seal, preventing reactions between the components. Each chamber contains either lipid, carbohydrate or an AA formula. Without disrupting sterility, just by break open the inner peelable seals, it is possible to mix the components to obtain a ready-to-use AiO nutrition mixture. It is also possible to add individual amounts of additives like vitamins, electrolytes or trace elements solution via a special port. If not required, the lipid chamber may remain unopened, so it is possible to compound only carbohydrate and AA formulation. However, this type of nutrition is less personalized, but the product provides much longer shelf life (compare 1 d to 6-18 months, given by manufacturer). Still once mixed, the formulation should be used within 24 h, due to the same physicochemical instability issues as reported for the first type of mixture [111].

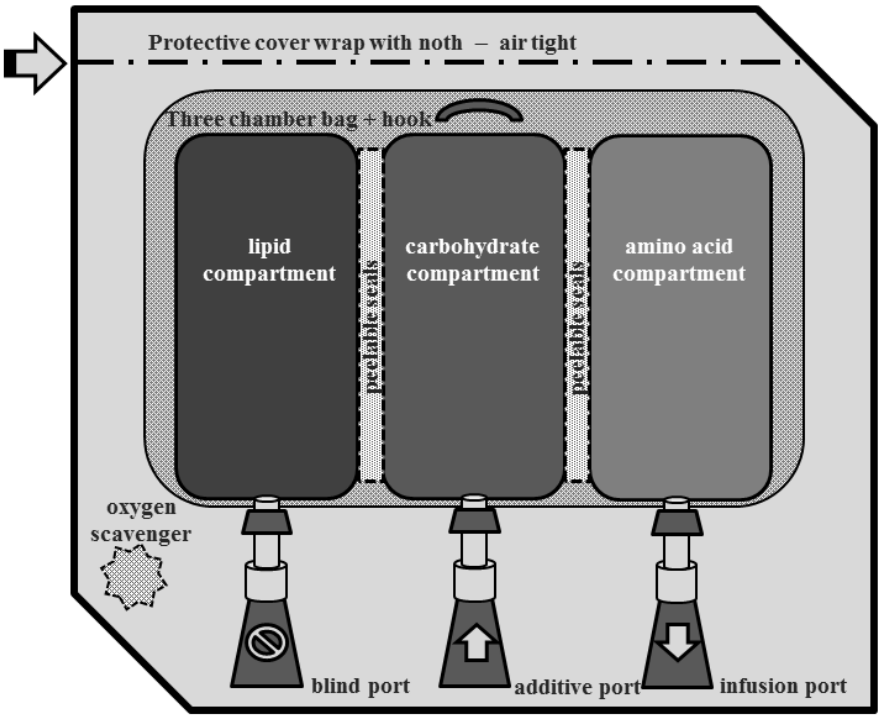


Figure 3: Scheme of a 3-in-1 multi chamber bag system (MCB)

The compounding order is crucial for the quality of the final product, thus preparation information and the guidelines of the associations mentioned before provide a step-by-step prescription of compounding steps in order to ensure a safe product according to Good Clinical Practice (GCP) [112]. First, AAs and glucose solutions have to be mixed; second, the addition of the lipid component is allowed; gently shaken into a homogenous emulsion avoiding creation of air bubbles. Additives shall be given in the AA compartment prior to any other steps [113]. The creamy opaque consistency of the complete admixture aggravates any visual observation, apart from colour change or vesication.

Tab. 5 displays the content of commonly used products; the greatest variability lies in glucose content, whereas lipids and AAs have rather little medical scopes depending on each patient group, metabolic benefits, and pre-existing illnesses.

Content	Aminomix®	Kabiven®	Clinimix® 5% G-E	Numeta® G16% E	NuTriFlex® Lipid Peri	NuTriFlex® Lipid plus
AAs [g/L]	50,00	33,10	50,00	26,00	32,00	38,00
Glucose [g/L]	200,00	98,00	350	155,00	64,00	120,00
Lipids [g/L]	-	39	-	31	40,00	40,00
Electrolytes [mOsm/L]	1779	1060	1625	1230	840	1350
Vitamins	-	-	-	-	-	-
pH*	5,5-6,0	5,6	6	5,5	n. s.	5-6
Additives	glacial acid	glacial acid, NaOH	glacial acid, NaOH	malic acid, NaOH, egg lecithin, glycerine, Na-Oleate	citric acid, NaOH, egg lecithin, glycerine, Na-Oleate	citric acid, egg lecithin, glycerine, Na-Oleate

Table 5: Overview of some commercially available MCB formulations, exemplary 2-in-1 and 3-in-1 products. (final pH in admixture)*

3.1 Compatibility and stability of TPN admixtures

Finally, at the point of administration both types of AiO admixtures share the same problems affecting stability, which restricts the application period. Though ideally looking like a homogenous system, the components are of very different nature, destabilizing the system back into an inhomogeneous. The instability can be classified, into thermodynamical and chemical processes (Tab. 6) [111].

Chemical instability	**Physical instability**
• hydrolysis	• thermal decompostion
• complexation	• photo-induced modification
• degradation	• adsorption
• oxidation/reduction	• interaction with primary packaging
• photolysis	• creaming/caking
• radical chain reactions	
• racemization	
• polymerisation	
• condensation	
affected by	**affected by**
• pH	• light exposure
• temperature	• bag material
• component concentration	• production process

Table 6: Classification of instabilities.

The following subchapters summarize compatibility issues between the substance groups with respect to AAs. The reported considerations refer to studies made on stability. So the applied study design may not always render real practical and clinical conditions. The actual probability must be evaluated individually for every system and conditions applied.

3.1.1 AAs and carbohydrates

The most common used source of carbohydrates is glucose, which has reducing properties; thus it can easily react with free AAs in solution. The reaction is referred to as Maillards reaction or non-enzymatic browning. First incompatibility considerations in parenteral solution based on reduced bioavailability of AAs and toxic reaction products were described by Fry and Stegnik in 1982 [114]. Especially during long time medication with parenteral admixtures insufficient nutrient levels are a matter of concern.

The initial step in Maillards reaction includes formation of 1-amino-1-deoxyketose (Fig. 4, **1**), with intermediate formation of the unstable Schiff´s base, leading to Amadori compounds, theoretically all AAs can react. This step is reversible, though following rearrangements are not, formation of not bioavailable decomposition products of AAs and potentially toxic by-products is the consequence (post-amadori compounds). Two degradation pathways (Fig. 4, *A/B*) are possible, leading to different products, characteristic for rather early or late stage of reaction pathway (Fig. 4, **2**/**3**). The challenging task is to analyse parent sugars, Amadori compounds and parent AAs, simultaneously. Davidek et al. established a method of simultaneous quantitative analysis of Maillard reaction precursors and products by means of high performance anion exchange chromatography. Anion exchange columns can retain AAs, Amadori compounds and sugars, consecutive elution was carried out by a gradient of water, sodium hydroxide and sodium acetate. Compounds were detected by coupling a diode array detector (DAD) with an electrochemical detector in amperometric mode [115].

Another study on AA loss in presence of reducing sugars tested 9 essential AAs and taurine in glucose solution to determine relative stability with regard to *p*H value, temperature and sugar concentration [116]. Enzymatic browning products were monitored by absorbance measurements using a photometer (λ =420 nm), in linear range up to 1.0 absorption units. Residual AA content was measured by means of RP-18 HPLC separation, after pre-column derivatization of AAs with OPA, coupled to fluorescence detector. To obtain AA losses, the content of treated samples was compared to the content of untreated samples. The most notably

losses of AAs were observed with Lys, Trp, and His. However, the authors concede that these losses may involve different reactions besides Maillard browning. Increasing *p*H value, temperature and sugar content leads to increased losses over time. Hence solution mixtures should be stored under controlled conditions. Similar results were obtained by Labuza and Massaro in 1990 by investigation of AA losses in model TPN. Separation of AAs was carried out by RP-HPLC with pre-column PITC derivatization and fluorescence detection. Cys was included and analyzed as oxidized cystic acid prior to derivatization. In this study a kinetic model for predicting the concentration of AA loss was suggested, since the observed systems exhibited a linear zero order behavior, equations can determine the kinetics theoretically, indicating a rapid loss initially followed by a steady state phase. AA losses were quantified up to a range of 20-45 %, recommending substitutional addition of 20-40 % extra to premixed formulation [117].

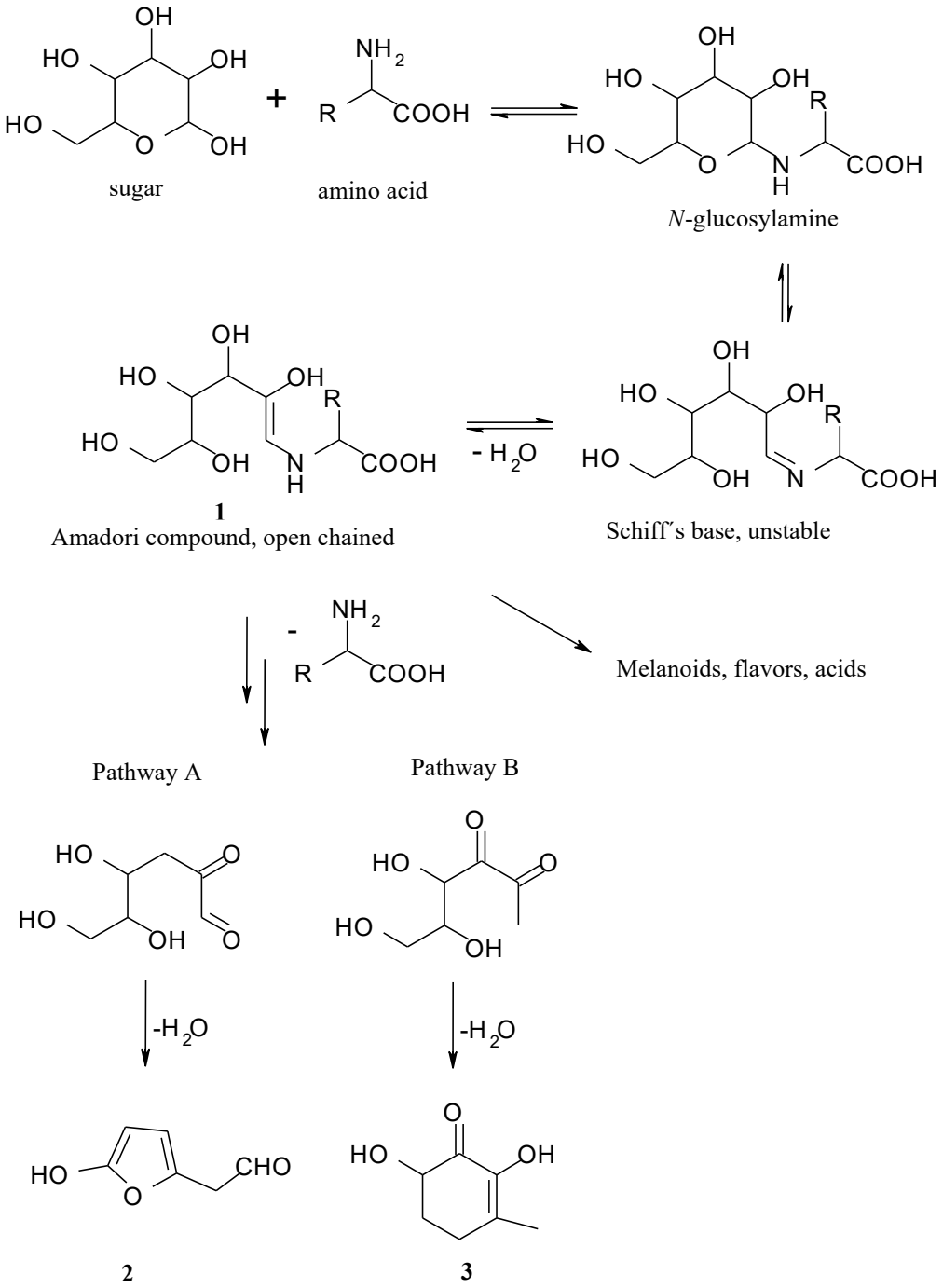


Figure 4: Maillard´s Reaction

3.1.2 AAs and lipids

It proved to be beneficial to meet the patient's requirements of essential fatty acids in form of lipid formulations consisting of a blend of vegetable oils emulsified in water [118-121]. Formulations contain variable amounts of long-chain and middle-chain triglycerides (LCT/MCT), mono-unsaturated and poly-unsaturated fatty acids (MUFA/PUFA) and an emulsifying agent, usually lecithin (an ionic egg phospholipid), as shown in Table 5. In the long process of emulsification each oil droplet is coated with a monolayer of phospholipids, orientating the negatively charged hydrophilic heads towards the water phase. Repulsive forces of the negative surface charge (zeta potential) of the droplets preserve stable oil-in-water (O/W) emulsion [113]. This "new" chemical ingredient in the system creates a whole range of further stability concerns [122].

The lipid emulsion is most stable when manufactured at *p*H of 8 and with surface potential of -35 mV [121]. Acidity of AAs and glucose formulations (Tab. 5) decreases *p*H and can potentially destabilize the emulsion. The consequences are a higher tendency to creaming, coalescence and cracking of emulsion. Special AA formulations, rich in branched chain AAs in neonatal and pediatric use, are more acidic, placing the admixture even more at risk. It has been shown, that especially for preterm infants, higher concentrations of branched chain AAs are beneficial for the development of the respiratory system [123-126].

On the other hand, AAs may also have a protective effect by facilitating buffering capacity, especially basic AAs enhance the electrostatic barrier, hence reducing the coalescence bias [113, 127]. Nevertheless, acidic AAs such as Glu and Asp may destabilize the formulation. Anyway, the effect of single AAs may be neglectable in the complete mixture because of repulsive effects of lipid globules and low concentration in the final formulation [10]. The optimum stability is claimed to be at a ratio of at least 1.5 of basic to acidic AA concentration [128].

Another critical matter is the lipid peroxidation of polyunsaturated fatty acids by radical autoxidation [129]. The formed lipid peroxides readily react with AAs giving degradation and oxidation products, which needs be monitored. In principle lipid peroxide values can be measured spectrophotometrically at 560 nm by ferrous oxidation-xylenol orange (FOX) assay [130] or by an iodometric assay as done by Steger and Muhlenbach in an intravenous lipid formulation [131]. Light dependent formation of lipid peroxides and hydrogen peroxide was described by Silvers et al. in lipid parenteral nutrition solutions admixed with a multivitamin

formulation; values were measured by FOX assay and oxygen release on catalase addition with an oxygen analyzer [132]. These investigations focused merely on the extent of peroxide formation, because concerns about peroxides are reasonable from the pharmaceutical and medical point of view; yet interactions between other ingredients apart from antioxidants such as vitamins were not subject of this study. One of the first HPLC methods to assess lipid peroxides was published by Yamamoto et al. in 1987 [133]. In presence of hydrogen peroxide, radical chain reactions and free radicals are likely to occur, whose by-products may further react with AAs causing dimerization, polymerization, and oxidation. Possible reactions are mentioned in reviews on lipid oxidation referring to old *in vitro* studies including His, Arg, Ser, Glu, Met, Tyr, Phe, and Thr [134-136]. However, to the best of our knowledge, no recent studies were reported about rather modern analytical methods to assess these types of reaction products in TPN. Since lipid emulsions and AAs are separated until administration, and the period of delivery is limited, these reactions are not that relevant in practical stability consideration anymore.

3.1.3 AAs and vitamins, electrolytes and trace elements

Patients depending on parenteral nutrition do not only need sufficient amount of all macronutrients, but all essential micronutrients, too, especially when total parenteral alimentation is prescribed for long time of administration [137]. As described before, the modern MCB systems have an extra port for additives. Thus, solutions of vital electrolytes (sodium, potassium, magnesium, chloride, phosphate) trace elements (ions of chromium, copper, iodine, iron, manganese, selenium, zinc, molybdenum), water soluble (vitamin B_1/thiamine; B_2/riboflavin; B_6/pyridoxine; B_{12}/cobalamin; pantothenic acid; niacin; biotin; folic acid) and lipid soluble vitamins (vitamins A, E, D,K) can be compounded into the formulation easy, fast, and safe [138]. Only few stability issues are described related to AA interaction, the most relevant ones are summarized below.

Among vitamins ascorbic acid is the least stable substance in TPN. It is readily oxidized reversibly to dehydroascorbic acid (DHAA) (Fig. 5), but still prevailing physiological antiscorbutic activity. DHAA degradation is irreversible being the actual loss of ascorbic acid. Cys, which has reducing capacity, can promote such a reaction, because of this character, Cys is usually excluded from AA formulations or at least applied only in very low concentrations. Rather the whole environment of formulation, e.g. presence of reducing sugars, oxygen content,

and bag materials, lead to degradation of ascorbic acid. The degradation can be monitored by an isocratic ion-pair reversed phase liquid chromatography coupled with a UV detector [139].

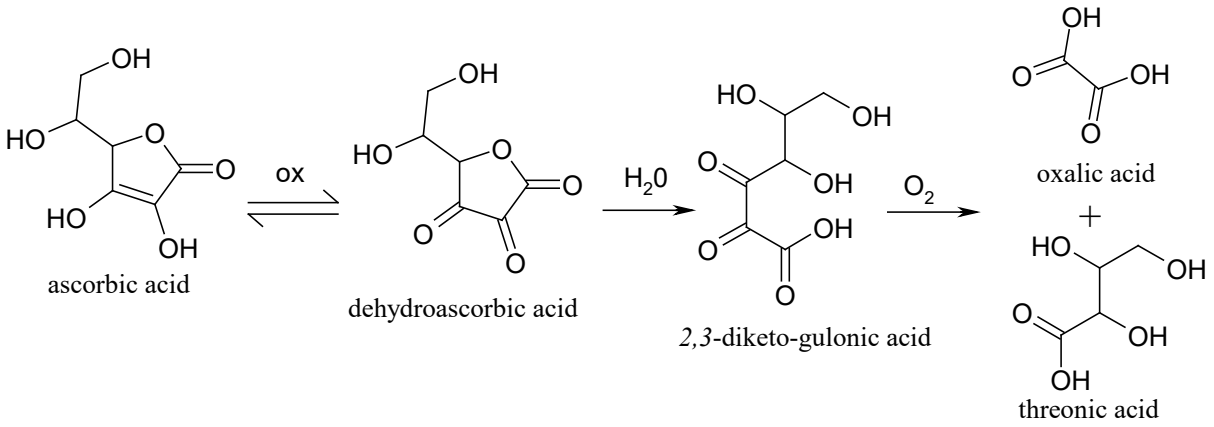


Figure 5: Degradation of ascorbic acid

Bhatia et al. studied the effect of multivitamins and light irradiation on parenteral AA solution [43, 44]. Garcia and Silva studied the photo-oxidation of AAs in the presence of the photosensitizer riboflavin in parenteral solutions [140]. Both obtained similar results; in fully-fledged AA formulations, His, Met, and Trp were significantly photo-oxidized by ambient light in the presence of riboflavin, a component of common multivitamin formulation. The aim was to deduce photo-oxidation reaction mechanisms, it was found that His and Met reacted differently than Trp. This fact was suggested by oxygen consumption measurements under various experiment conditions. The structural decomposition of His and Met remained unexplained. With reference to earlier studies [141, 142], Trp degrades to kynurenine, formylkynurenine and supposedly to hydroxylated or oxygenated products with higher molecular weight than Trp (Fig. 2) [141-143]. However, to complicate the issue, ascorbic acid was shown to have a protective effect; hence degradation of Trp (and other components) begins after depletion of ascorbic acid only [140]. This happens because of its anti-oxidative nature, nevertheless structural changes remained unexplained. Simultaneously other vitamins with anti-oxidative character, such as vitamin E were suggested to have a similar effect but were not tested.

Stability assessment of vitamins in parenteral formulations is another subject; it has to be assured, whether degradation products, oxidized or reduced forms of vitamins affect the stability of the residual components. HPLC methods are proposed to assess water soluble and lipid soluble vitamins and related substances. Lipid phase is separated by solid phase extraction

prior to analysis [144-146]. Sforzini tested vitamin A and E stability up to 24h during administration period, and declared decreasing vitamin content 24 h after compounding, confirming the recommended restriction of administration period [91]. Once again, these studies focused on degradation and assessment of the vitamin and did not investigate interaction with AAs.

Copper, iron and zinc are trace elements of relatively high physiological concentrations in organism; chromium, manganese and selenium are available at lower levels. Composition and admixture of trace elements are precisely defined, but interactions with AAs cannot be ruled out. Generally, AAs exert a positive effect on bioavailability by building chelates with the metals [147], zinc complexes particularly with His, Cys, Gln, and taurine. Iron and copper also form chelates with diverse AAs, however resulting in enhanced bioavailability and not in decreasing stability [148]. Results of a more recent study indicate the formation of copper and Cys complexes [149], found in blackened inline-filter membranes of the administration tube sets. Mainly sulfur containing AAs are affected by trace element interaction. In this study, measurements of individual elements were carried out by scanning electron microscopy and energy dispersive spectrometer analysis, providing qualitative and semi-quantitative (elemental) analysis. AA profiles were obtained by means of RP-HPLC coupled to tandem mass spectroscopy. Significant lower Cys levels were found after 24 h storage period of a model admixture [149]. Actually, Cys is not prevalent in TPN for adults, since it can be synthesized physiologically from Met, but for preterm infants Cys is essential, because neonates lack the required enzymes for metabolic transformation [150].

Electrolytes are used in AA solutions to adjust osmolality; the concentration is kept to a minimum. One significant problem might be the precipitation of calcium phosphate, depending on buffering capacity (titratable acidity) of AA formulation, which is largely determined by Arg, His, and Lys concentration and the final volume of mixture [151]. Complexation between calcium and Lys, but also Glu and Asp, Arg and His is reported *p*H dependent but not monitored [152].

3.2 Impact of primary packaging

In the broadest sense of system compatibility, one has to mention primary packaging as further component. All ingredients are in direct contact to the primary packaging material during the

whole shelf life, storage and administration. The influence of the materials shall be roughly considered for integrity.

First plastic bags replaced glass bottles for parenteral formulations in 1960 for economical and practical reasons. Polyethylene and polyvinyl chloride (PVC) were used as common material. In contrast to glass, these materials had increased gas and humidity permeation with polyethylene and adsorption effects with PVC. Nevertheless, higher particle load in inline-filters, adsorption capacities for lipophilic components and leachables in form of plasticizers (di-(2-ethylhexyl)-phthalate, (DEHP)) were a great drawback [111]. Especially the loss of fat-soluble vitamin (retinol) was associated with diffusion into the semipermeable membrane of the container [112]. DEHP is considered to be potential cancerogenic and disruptive to emulsion stability due to decomposition into phthalic acid, a strong acid. Thus, methods for rapid HPLC assessments were established [153, 154].

Replacement of PVC led to standard plastic container material ethyl vinyl acetate (EVA). EVA bags do not contain plasticizers, but gas permeation of oxygen into the admixture is not hindered. This allows steady gas diffusion encouraging oxidative reactions and decreased stability of sensitive chemicals. For that reason, MCB are protected by an air tight cover wrap provided with an additional oxygen scavenger. In EVA (and in PVC) bags oxidation of AAs is more likely to occur. Most affected AAs are, Trp, Cys, His and Met, despite the fact, that losses are rather nutritionally unimportant, degradation products are mostly unknown and toxic effects on long term feeding cannot be precluded [155].

Further development of primary packaging was the implementation of multilayered plastic bags (MLB). Polyamides or ethylvinyl alcohol - ethylvinyl acetate combinations are materials for coating film on inner and outer side of the container to prevent oxygen permeation. Polypropylene is a low adsorption plastic preventing water adsorption during heat sterilization [111].

In 2004, Balet et al. confirmed demonstratively the protective effects of MLB in contrast to EVA bags on oxidation of parenteral nutrition [156]. Lipid peroxides, hydroperoxides and vitamin content were evaluated by methods similar to the ones described in prior chapters of this review.

Finally, in the context of primary packaging the impact of light irradiation has to be evaluated. The primary packaging has to provide adequate protection to its content. Light sheath of parenteral solution is necessary to protect photo-sensitive AAs, vitamins and poly-unsaturated

fatty acids [157]. Artificial light is rather unproblematic, but daylight can cause degradation, because of absorption of UV light; generally speaking, the most sensitive ingredients are retinol and to a far less extend riboflavin, reactions may be of photolytic or photo-oxidative nature [158]. However, absorption of UV-light does not lead necessarily to decomposition; it can also be shielded off to some extent as it is the case with ascorbic acid [159]. Accurate care in terms of light protection is a rational and practical recommendation.

4 Conclusion

The practical application of AA solutions in parenteral nutrition over decades gives evidence to be beneficial and comfortable. Both pure solutions and TPN meet the requirements of international pharmacopoeias of efficacy, safety and quality. Many studies elucidated and confirmed stability in the prescribed period of administration. Studies beyond the scope of application showed relative stability for even longer periods. Theoretically many diverse incompatibility incidents are thinkable, but whereas variety increases, clinical significance does not. Establishment of explicit guidelines and instructions to medical staff contributes to high qualitative and safe use. Whensoever stability is affected in explicit consideration the named methods can provide an analytical basis to deduce specific analysis.

In the context of recent developments in modified HPLC, HILIC technique seems to be particularly suitable for analysis of AAs. It offers the possibility to separate small polar molecules, e.g. AA-related substances and degradation products (and also sugars) without derivatization or use of ion pairing reagents. Separation and analysis of charged substances allows coupling to mass spectrometric detector types. Application of mass spectrometers makes derivatization dispensable with increased detection sensitivity. Unknown compounds can be discovered, and fragmentation studies can support structural elucidation. Most HILIC methods are not time-consuming, offer easy sample handling with typical solvents e.g. acetonitrile and water, volatile additives, low buffer and salts concentration. It is in no way inferior to RP-HPLC, which is still the common and classical chromatographical approach. HILIC-MS is used in food chemistry and clinical analysis due to its large application area and named practical advantages [160].

Since the possible degradation products are so various, detection of unknown or unexpected peaks during analysis is challenging. Surmounting these difficulties allows accomplishment of

targeted screening procedures for thinkable problematic decomposition products and declaration of instability indicating substances.

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1.3 Tryptophan in pharmacy and medicine

1.3.1 Chemistry and biochemistry of tryptophan

2-Amino-3-(1H-indol-3-yl)propanoic acid is the chemical name of the α-AA Trp (Fig. 6). It is an α-Alanine with an indol-3-yl substituent. The stereochemistry of the α-position determines the AA as either *D*- or *L*-Trp. Only L-AA are able to pass into the human organism by active transport mechanisms, and thus becoming substrates of metabolism and protein biosynthesis. L-Trp is one of the 20 obligatory genetically encoded (proteinogenic) AAs. It is one of nine essential AAs and must be provided by diet obligatorily [21-23]. Bacteria and fungi use the shikimate-pathway for the synthesis of aromatic AAs such as Trp, Phe and Tyr [24]. To some extent, plants are also able to synthetize Trp. It plays an important role as metabolic precursor for plant development and regulation [25].

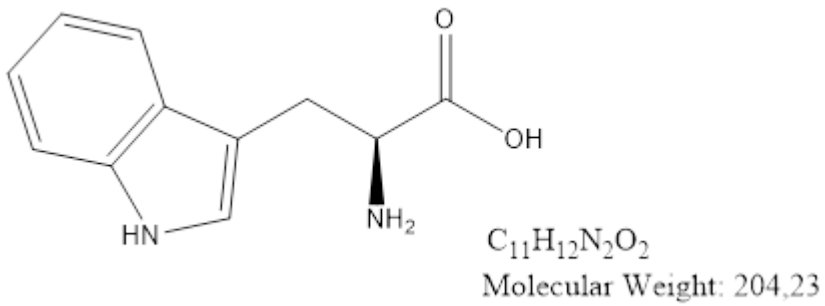


Figure 6: Chemical structure, formula and molecular weight of L-Trp

AAs are small, charged and very polar molecules (Fig. 7). The side chain of each AA determines its individual chemical and physicochemical property and classifies each AA as either acidic, basic, neutral, polar/non-polar, aliphatic or aromatic. The indole moiety attributes Trp as aromatic, large, neutral AA (LNAA) with rather hydrophobic behaviour. The aromatic system has a delocalized π-system enabling interactions with electrons and positively charged groups. It is prone to chemical reactions, such as oxidation or ring cleavage, as well as non-covalent interactions with polar and non-polar chemicals. The chromophore indole is UV-active and fluorescent - adding to the unique properties of Trp.

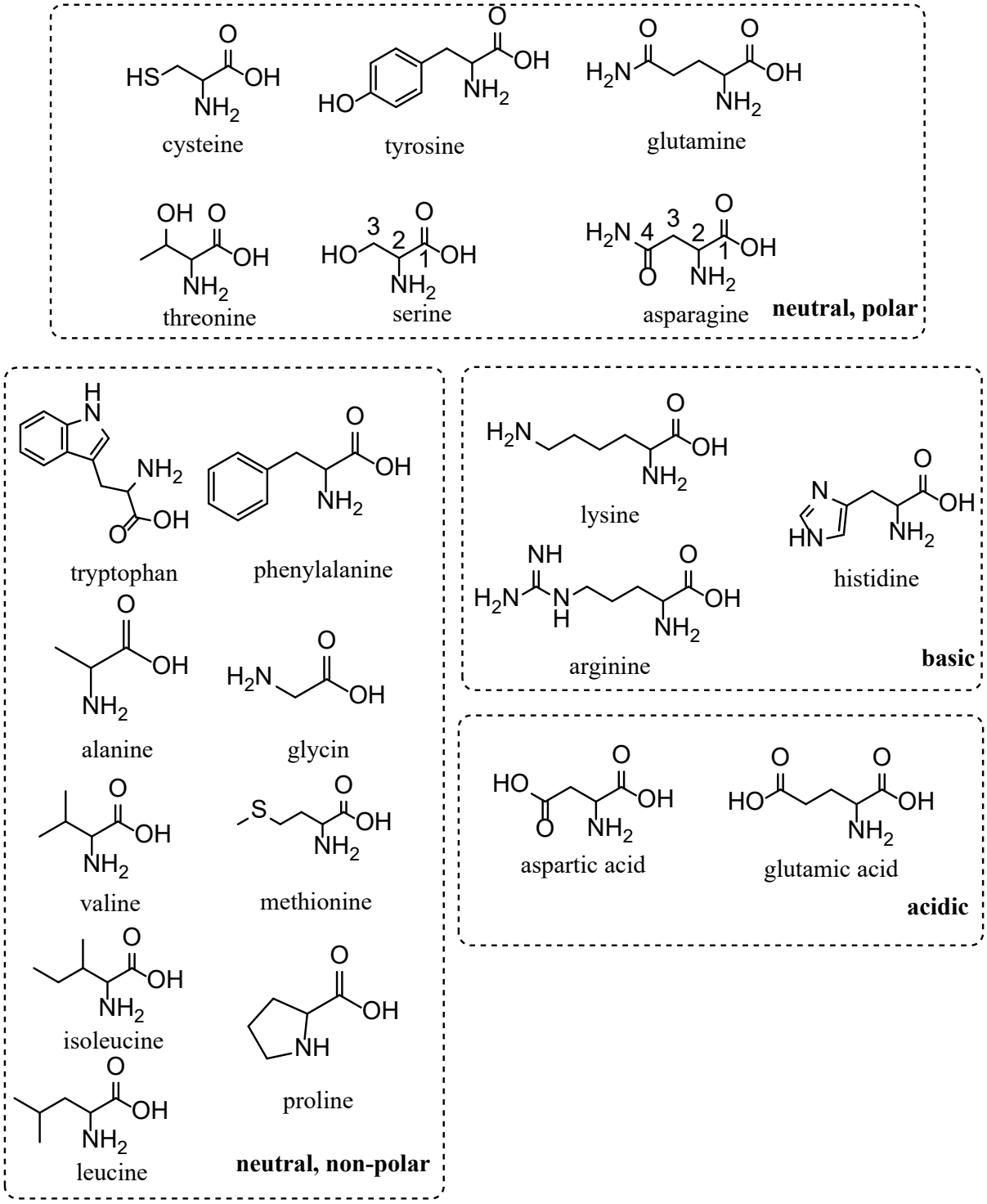


Figure 7: Overview of the 20 proteinogenic AAs. Classified according to side chain properties: neutral, polar; neutral, non-polar; basic; acidic

Trp is an indispensable AA for the human organism [26-28]. Only the L-form is bioavailable and can pass the blood brain-barrier [29, 30]. Among the 20 proteinogenic AAs, Trp has the lowest plasma concentration and lowest tissue storage [31]. Trp is needed for protein synthesis and it is the rate limiting key factor for the synthesis of neurotransmitters such as serotonin [26,

28], tryptamine [32, 33], and melatonin [21, 34]. Other important metabolic pathways of Trp include the kynurenine/quinolinic acid synthesis [23, 34-38] and the formation of biogenic (indole-)amines [33]. Serotonin is a neurotransmitter strongly related to mood and behaviour processes. Melatonin is the hormone of circadian rhythm and influences the immune system and gastrointestinal function [21, 39, 40]. Tryptamine is the decarboxylated Trp, which is a neuromodulator with excitatory and inhibitory functions [32]. The enzymatic formation of kynurenines and quinolinic acid is another function of Trp, which is initiated by the enzymatic cleavage of the indole ring [21]. This pathway is strongly stimulated by inflammatory molecules and it is up-regulated when the immune system is activated [38]. The biological significance relates to the modulatory role of kynurenines in immune response and the neuroactivity of kynurenic acid, 3-hydroxykynurenine and quinolinic acid. Quinolinic acid is the precursor for the synthesis of the electron transfer coenzymes nicotinamide adenine dinucleotide (NAD) and NAD phosphate (NADP) [41, 42]. Fig. 8 gives an overview over the two most important metabolic conversion routes of Trp: the kynurenine and serotonin/melatonin pathway.

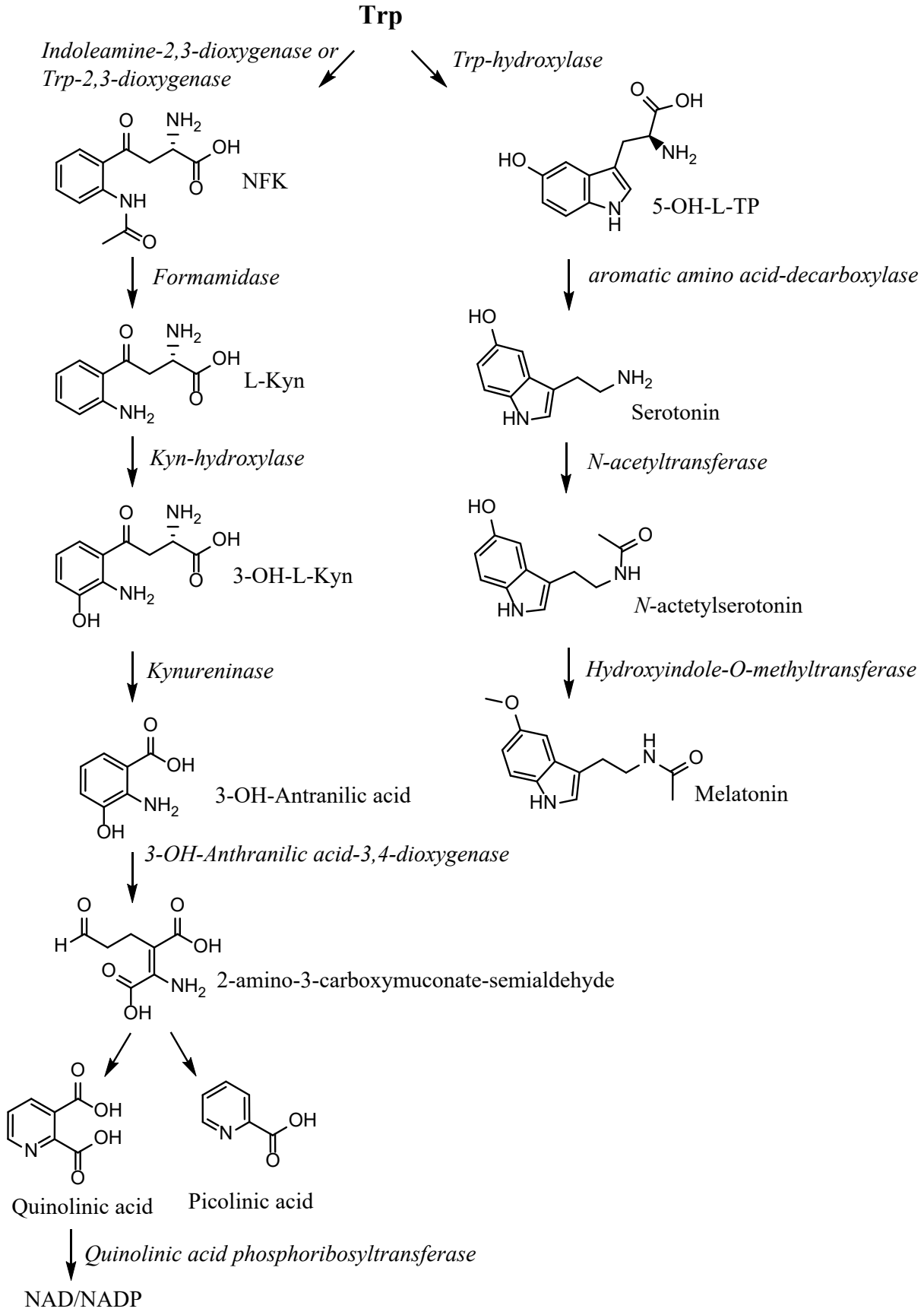


Figure 8: The enzymatic formation of kynurenines, serotonin and melatonin. Abbreviations: NFK: N-Formyl(L-)kynurenine, L-Kyn: L-Kynurenine, 3-OH-L-Kyn: 3-hydroxy(L-)kynurenine [34, 43, 44].

1.3.2 Therapeutic use of tryptophan

The metabolic relevance of Trp initiated many studies and clinical trials carried out in the last 35 years. Trp depletion or high dose supplementation was tested and found to be imperative for the modification of serotonin and melatonin plasma levels [23]. The supplementation with Trp showed adjuvant function especially in the treatment of light depressive episodes [45-47]. Trp proved to be an alternative medication for the treatment of (chronic) insomnia, short-term sleep disorders or jet-lag symptoms without the adverse effects of typical hypnotic medication such as hang-over effects or impaired cognitive performance [23, 48, 49]. Thus, Trp supplementation became a popular over-the-counter (OTC) drug for self-medication of insomnia and depressive episodes in the beginning of the 1980s. Daily intake varied between 250 – 450 mg and accumulated easily up to 1000 mg Trp per day together with the Trp uptake from food [23, 37].

Beside the specific indications, Trp is a fixed component in enteral and parenteral AA formulations [50-54]. The proportion of AAs vary according to the daily demands, the medical indication or the patient group, i.e. new-borns, infants, adults, elderly people, critically ill patients or trauma patients. However, the World Health Organisation (WHO) recommends a daily intake of 4-5 and 8.5 mg * kg body weight per day for adults and infants, respectively [55].

1.3.3 The EMS incident

In November 1989 the FDA issued a warning of Trp intake when contemporary studies suggested a significant association between OTC Trp supplements and the onset of the previously unknown Eosinophilia-Myalgia syndrome (EMS). More than 1500 EMS cases and at least 27 deaths were reported until mid-1990´ies [56]. The prevalence of EMS stopped with complete recall of Trp food supplements with a daily dose of more than 100 mg. EMS is characterized by severe muscle pain and profound elevated eosinophilic count without any evidence for substantial inflammation processes [57]. Intensive research on possible causes proposed two hypotheses: either individual cases of alternated Trp metabolism or EMS-triggering contaminants in Trp bulks [58, 59]. Years of Trp medication without reports of EMS disqualified the first hypothesis. Eventually, all EMS cases could be traced down to contaminated lots of Trp produced between October 1988 and June 1989 from one single manufacturer Showa Denko K.K., (Tokyo, Japan) [57]. Concerned lots were produced with modified microbial biosynthesis leading to unknown and unassessed impurities. Several

impurities have been identified in the decade after the incident. The most prominent contaminants were 3-phenylaminoalanine (3-PAA) and 1,1´ Ethylidene(bis)-Trp (EBT), which were associated with the outbreak of EMS. However, until now there is no medical evidence proving the triggering effect of the suspected substances, because the pathophysiological implications of EMS are still unclear. On the other hand, other studies suggest a toxic condensation product of Trp and bacterial fatty acids as the most significant EMS-triggering impurity [7, 60-63].

1.3.4 Industrial tryptophan production

Industrial bulk substance is produced by microbial fermentation processes. The most relevant industrial bacterial strains are *Escherichia coli* or *Corynebacterium glutamicum.* The biosynthesis of L-Trp is rather long and complicated [64], thus genetic engineering efforts are carried out constantly to lower production cost, increase Trp yield and decrease bacterial mechanisms of down-regulation and the accumulation of by-products, respectively [65-68]. Presumably, alterations of the bacterial Trp fermentation process used by Showa Denko led to elevated levels of by-products and contaminated the batches, which were related to the outbreak of the disease later on. Frequent intake of contaminated Trp supplements led to an accumulation of impurities, which caused onset of EMS [69]. Consequently, the revision of the compendial analysis methods for Trp degradation products and related substances was initiated. However, the contaminant EBT (a Trp-dimer) was defined as a marker for EMS-related lots. Therefore, the impurity assessment method in the Trp monograph (Ph. Eur. 9th edition) prescribes the assessment of EBT. Other related impurities are limited to a total amount of 100 ppm and are not assessed individually.

2 Aims and objectives

The pharmaceutical products *Formulation 1*and *Formulation 2* (8.1. Supporting information, Tab. A 1) are parenteral nutrition solutions and thus must guarantee stability and quality criteria. Parenteral formulations are demanding pharmaceutical products in with regard to purity, mutual compatibility of ingredients, stability across manufacturing and sterilization processes, and long-term stability. In general, parenteral nutrition (PN) solutions are ready-to-use and can be administered directly, for example in medical conditions [51, 70, 71]. Therefore, the solutions must be in an impeccable condition unexceptionally. But, in some cases yellow discolouration, precipitates and turbidity were observed during manufacturing, processing, or shelf-life [72-77].

The yellow discolouration of biological tissues, such as the age-related yellowing of wool or the eye lenses, are associated with degeneration of AAs, peptides and proteins and have been studied in research before [77-81]. However, the reviewed literature suggests the AAs Trp, Tyr and Cys as the most prevalent precursors to coloured degradation products. The ingredients of *Formulation 1* and *Formulation 2* (Tab. A 1) contain Trp in concentrations of 2 g/L and 1.4 g/L, respectively. Tyr is poorly soluble in water and therefore it is included only in very small amounts and Cys is only included in *Formulation 2*, respectively. Consequently, Trp becomes the main target for a stability study in steam-sterilized aqueous solutions with emphasis on the identification and the assessment of degradation products responsible for the yellowing.

The aim was to identify and quantify Trp degradation products causing the yellow discoloration. The degradation products were identified and quantified in pure Trp solutions, as well as in the exemplary finished products. Therefore, a stability-indicating method was developed, optimized and validated in accordance to the ICH guidelines ICH Q2 (R1).

The Trp degradation products were identified and characterized. A selective stability-indicating method was developed, optimized and validated, and used for the analyses of finished pharmaceutical products. The assessment of Trp degradation products in the exemplary products is complemented by the comparison the influence of two different types of primary packaging, both commonly used for parenteral solutions.

The demonstration of an instability and discolouration issue after steam sterilization of pure Trp solutions was documented by sample footage and assessed by UV/Vis spectroscopy. The identification and characterization of degradation substances in highly stressed samples was done by preparative LC, HPLC-UV and HPLC-UV/MS/MS, respectively. The identification of degradation substances was supported by the compilation of a substance library with theoretical and known degradation products. The proposed degradation products were confirmed by their

synthesis and used as reference substance for method optimization and validation. Method development and optimization included the testing and comparison of different separation techniques such as ion pair (IP)-RP-HPLC and RP-HPLC with a modified column material. The validated RP-HPLC-UV method indicates Trp degradation substances selectively next to Trp and other AAs in stressed parenteral nutrition solutions. Furthermore, common primary packaging material, i.e. glass bottles and plastic bags for injectables were tested and evaluated for their impact on Trp degradation.

3 Results and discussion

3.1 Preliminary tryptophan stability tests

The stability of Trp is an important issue in research. For example, in medicinal chemistry, the deterioration of Trp and Trp side chains at the active site would affect the functionality of enzymes, hormones, antibodies, peptides and APIs. Consequently, metabolic pathways and therapeutic actions may be ineffective. Hence, possible reactions and degradation must be known and prevented, if possible. However, most of the influencing factors tested include the presence of enzymes or potent reaction partners. Additionally, influencing factors such as (strong) peroxides, metals, strong acids and bases were tested. Thus, the transfer to parenteral AA solution is only limited, because parenteral formulations are enzyme-free, comply to narrow ranges of pH values and receive "gentle" and as least as possible processing during manufacturing and storage. For this reason, it was essential to clarify, if the stability of aqueous Trp solutions (pure Trp and in presence of other AA) may be affected negatively by processing, such as steam-sterilization as used in the manufacturing procedure. Additionally, technical deficits during the manufacturing procedures, such as fluctuations of oxygen content, can affect Trp stability.

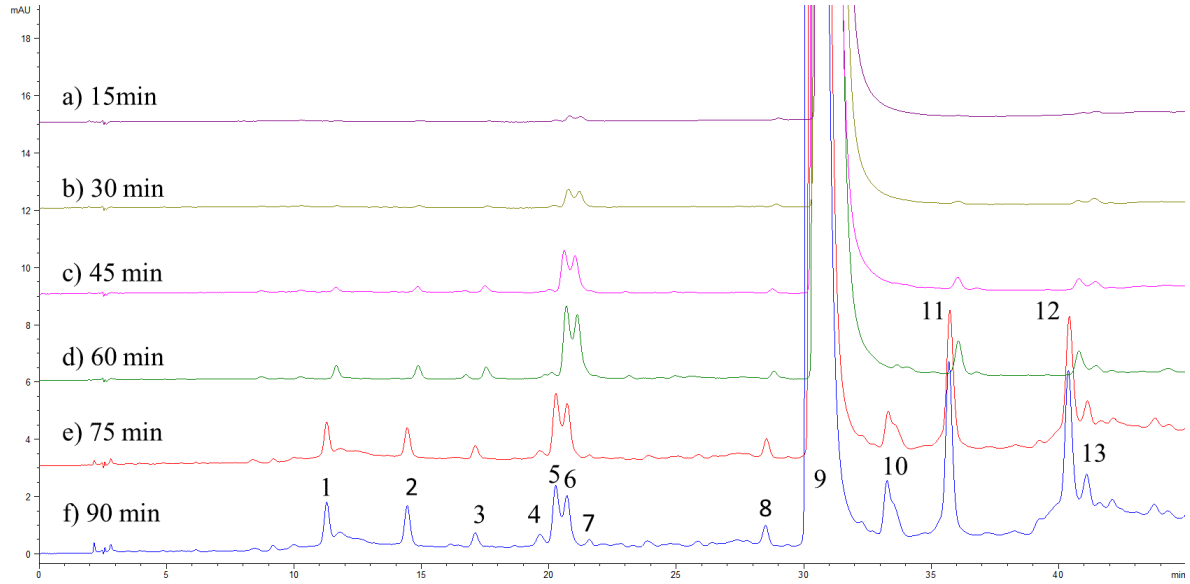
Therefore, preliminary Trp stability tests were conducted focusing on the stability of pure Trp solutions after heat stress by autoclave. The aim of the preliminary test was to demonstrate if steam-sterilization is sufficient to induce Trp degradation. Furthermore, few related substances are commercially available and were tested for their presence in the stressed Trp solutions. Finally, preparative LC was applied for the characterization of emerging peaks in the stressed Trp solutions.

3.1.1 Induced Trp degradation

The initial Trp stability screenings were conducted by repeated stressing of aqueous Trp solutions (50 mL, 2 g/L Trp) by steam-sterilization in an autoclave (7.4 Sample stressing). The solutions were kept in brown glass vials and were subjected to 6 consecutive autoclaving cycles of 15 min at 121 °C, 2.1 bar. Aliquots were taken in between the cycles and subjected to HPLC-UV/MS analysis (7.5 HPLC methods, *Method A),* which is a modification of the compendial Trp impurity assessment method (9th edition Ph. Eur.) [82]. Modifications were necessary in order to facilitate linearly coupled MS detection. Fig. 9 (a-f) is an overlay of the HPLC-UV chromatograms with the corresponding ion peaks *(m/z)* after each autoclave cycle.

The numerous peaks in the chromatograms demonstrate the increase of degradation products with each autoclave cycle applied. Nearly every UV peak could be assigned according to a certain *m/z* ion and showed the presence of several isomers with *m/z* 221 and 237, respectively.

Despite a visible UV-peak, it is not always possible to trace a definite mass signal due to high background noise, or improper detection settings, like it was in the case of peak 10. Especially the increasing organic amount of the mobile phase, which is necessary for the elution of Trp, promoted elevated and disruptive background signals.



Peak	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**
m/z	237	237	209	-	221	237	-	-	205	-	177	147	175
Rt [min]	11.2	14.5	17.3	19.8	20.2	20.8	22.5	28.6	30.9	33.3	35.8	40.5	41.1

Figure 9: Overlay of UV chromatograms (254 nm) of stressed 2 g/L Trp solution after consecutive autoclaving cycle a)-f) (15 min, 121 °C, 2.1 bar). Peak No. 9 is Trp.

This stressing set up was repeated with a triple AA solution, containing Trp, Tyr and Phe (7.3 Sample preparation, *Trp-Tyr-Phe-solution*), which are common ingredients in parenteral AA solutions. The aim was to investigate, if there are any interactions taking place between Trp, Trp degradation products and Tyr or Phe, which may be induced by the autoclaving process. Amongst all the other AAs, Phe and Tyr where chosen first due to their side chains; the benzol and phenol group, respectively. In comparison to the other AAs, they are more reactive, and they provide chromophores for UV-detectability and adequate retention on a reversed phase. The HPLC-UV/MS analyses showed an identical pattern of Trp degradation products beside

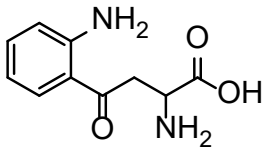
the peaks of Trp, Tyr and Phe. No UV and no MS peaks could be observed, which would indicate the degradation of Tyr and Phe or any interactions between them and Trp degradation products. In order to monitor the Trp degradation in a "complete" parenteral AA solution, a solution was prepared identical to *Formulation 1* (7.3 Sample preparation, *AA test solution,*). The *AA test solution* was subjected to 5 consecutive autoclave cycles of 15 min (121 °C, 2.1 bar). The HPLC-UV/MS analyses demonstrated the same pattern of Trp degradation products as observed in pure Trp solutions and in the triple AA solution. This showed that the presence of other AAs does not influence the formation nor the elution of Trp degradation products. Therefore, further experiments focused on the identification and characterisation of Trp degradation products only in pure Trp solutions.

3.1.2 Commercially available references and related substances

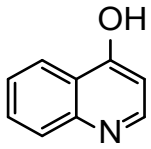
The compendial Trp impurity assessment method specifies related substances A-L. The method provides the quantification of the potentially toxic by-product 1,1´-EBT (impurity A) and the limitation of other impurities B-L limited in sum to a total of 100 ppm, referenced with *N*-Ac-Trp (internal standard IS) by peak area. However, for the impurities B-L there is no indication of individual R_t or any other information about their differentiation. Among the impurities B-L, four substances were commercially available and are demonstrated in Fig. 10. The substances are Kynurenine (Kyn), *N*-Acetyl-Trp (*N*-Ac-Trp, IS), 5-Hydroxy-Trp (5-OH-Trp) and 4-Hydroxy-Quinoline (4-OH-Qn) [82-85].

Method A was applied for HPLC-UV/MS analyses of *reference mix-1* and the UV chromatograms (λ 220, 254, 280, 340 nm) are shown in Fig. 11. Comparison of the retention time and the *m/z* ions in the *reference mix-1* with stressed Trp solutions confirmed the presence of the substance Kyn only (compare: Fig. 11, Kyn, Rt 17.3 min and Fig. 9, Peak **3**). *N*-Ac-Trp, 5-OH-Trp and 4-OH-Qn were not found in the highly stressed Trp solutions and were therefore excluded from further consideration. According to chemical considerations and research (8.3 Substance library), hydroxylation in position 5 of the indole ring is rather unlikely under applied conditions, because this position is not activated and would require e.g. harsh oxidative conditions or enzymatic reactions. The same holds true for the acetylation of the amino group, which requires an e.g. enzymatic transformation [85-87]. Chemically seen, 4-OH-Qn is rather a downstream decomposition product of earlier occurring Trp degradation products (Fig. 8 and 8.1 Supporting information, Fig. A 1). Assumingly, the formation may involve the cleavage of

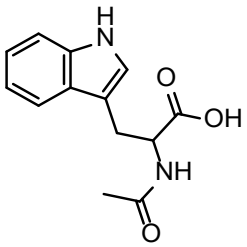
the aliphatic chain and the pyrrole moiety followed by a recyclization into the quinoline and hydroxylation in position 4.



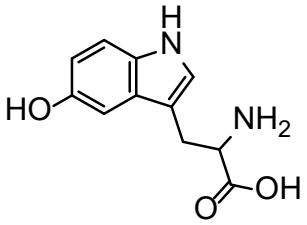
Kyn
$C_{10}H_{12}N_2O_3$
208.2 g/mol



4-OH-Qn
C_9H_7NO
145.2 g/mol

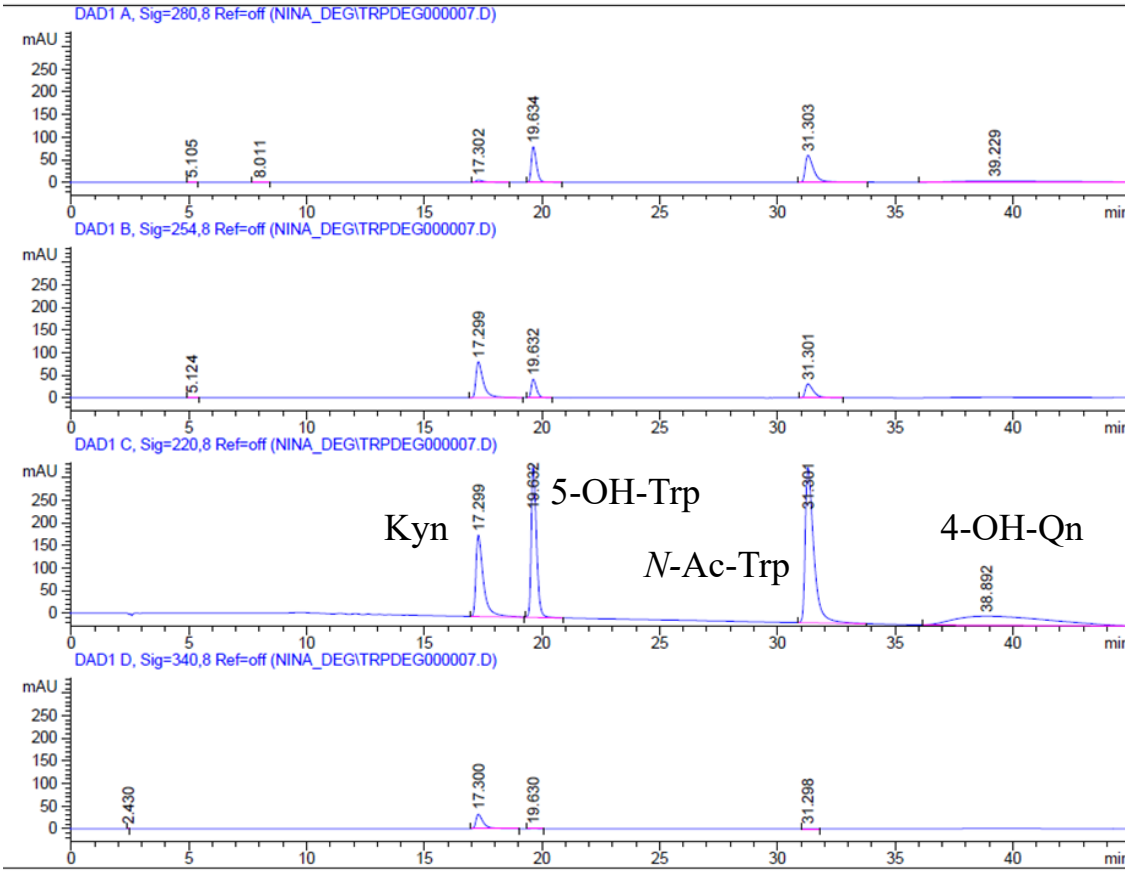


N-Ac-Trp
$C_{13}H_{14}N_2O_3$
246.3 g/mol



5-OH-Trp
$C_{11}H_{12}N_2O_3$
220.2 g/mol

Figure 10: Commercially available reference substances used in the compendial Trp impurity assessment method.



Substance	Rt [min]	*m/z*
Kyn	17.3	209
5-OH-Trp	19.6	221
N-Ac-Trp	31.3	247
4-OH-Qn	38.9	146

Figure 11: HPLC analysis of reference mix-1 *(*Method A*), λ = 220, 254, 280, 340 nm.*

3.1.3 Preparative LC

In order to isolate and identify the degradation products in a highly stressed Trp solution, preparative LC was used for the separation and extraction. Subsequently, the extracted fractions were analysed by LC-MS/MS.

Therefore 100 mL of the *Trp prep solution* (7.3 Sample preparation) were concentrated by water evaporation and submitted to preparative LC. For the separation of Trp degradation products an analytical test method (7.5 HPLC methods, *Method Prep 1)* was developed and subsequently upscaled for the preparative LC purpose (7.5 HPLC methods, *Method Prep 2*).

Fig. 12 shows an UV chromatogram (λ 254 nm) of the *Trp prep solution*, analyses with *Method Prep 2.* The peaks A-F were assigned according to the fractions collected.

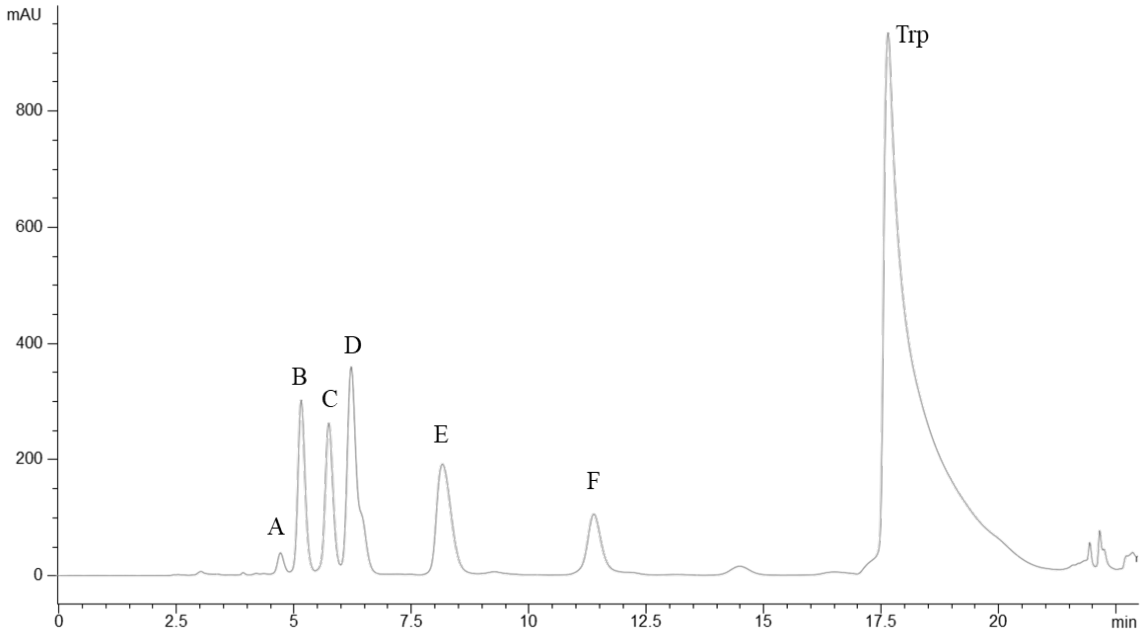


Figure 12: UV chromatogram (λ 254 nm) of Trp prep. solution, analysed by Method Prep. 2. *Peaks A-F were collected (fractions A-F)*

Baseline separation with at least 1-2 min time distance is the minimal requirement for efficient fractionation. The desirable time distance between the eluting peak could not be achieved, mainly because the substances are too similar in size, polarity and thus retention behaviour. Furthermore, because the presence of diastereomers is suspected, separation thereof is rather challenging by means of preparative LC. The upscaling of the analytical test HPLC method into a preparative method usually results in a loss of selectivity and therefore is not reliable for the

separation of such substances. However, six peaks (A-F) were collected as fractions accordingly. The fractions were condensed and submitted to LC-MS/MS analyses (*method A 1*). In order to check, if there any substances without a chromophore, additional fractions, eluting 2 min prior to and after Trp, were also collected. The additional fractions were concentrated by water evaporation and subjected to LC-UV/MS analyses by *Method A 1*. However, no MS signals were detected. The following chapter discusses the results of the LC-MS/MS analyses of each fraction.

The separation performance and fractionation of the preparative LC was successful only in parts due to close elution of the peaks, which goes along with a high probability of contamination. Furthermore, the initial experiments indicated the presence of several isomers and presumably diastereomers making the separation by preparative LC more challenging. Finally, the working up of the fractions; i.e. water evaporation/condensation, is additional heat stress, which may lead to further decomposition or conversion of the extracted substances.

3.1.4 LC-MS/MS analysis of fractions A - F

The fractions collected A-F and the *Trp prep solution* were analysed by LC-UV/MS/MS *Method A 1.*

Figures 13 a) and b) show the TIC (total ion chromatogram) and UV chromatogram (λ 254 nm) of the *Trp prep solution*, respectively. Figures 13 c-h) show the TICs of single fractions A-F. The signals in the TICs mirror the purity of each fraction.

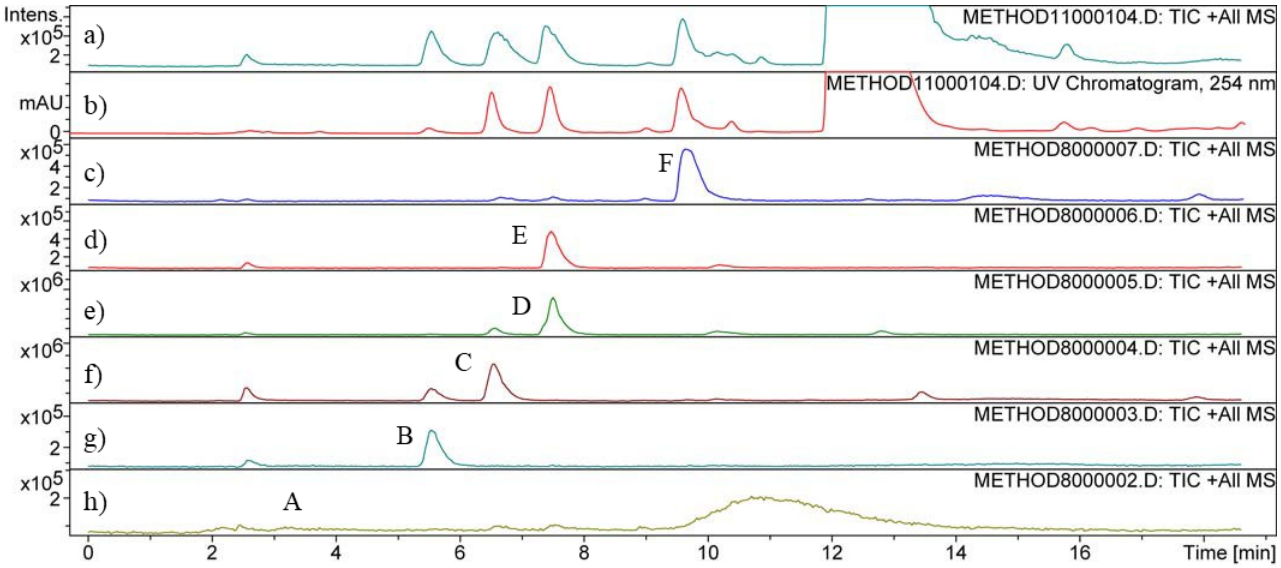


Figure 13: Analysis of Trp prep solution and fractions A – F (Method A1), a) TIC Trp prep solution, b) UV chromatogram (λ 254 nm) Trp prep solution, c) TIC fraction F, d) TIC fraction E, e) TIC fraction D, f) TIC fraction C, g) TIC fraction B, h) TIC fraction A.

In Fig. 13 c-g) the TICs of the Fractions B – F show one predominant MS peak. The MS and MS/MS spectra of the main peak of each fraction B-F are shown in Fig. 14-18, respectively. In each fraction, the most abundant MS signal, the parent ion, was fragmented (MS^2) into the daughter ions accordingly. The MS^2 spectra show the MS signals of the daughter ions and a blue rhombus, which represents the actual parent ion.

Fraction A (Fig. 13 h)) was lacking any significant UV or MS peak. The elevated baseline in 10-12 min could not be assigned to any MS signal and is most probably due to the elevated organic content of the gradient elution. Although, as shown in Fig. 12, fraction A was the first eluting peak in the analysed sample, LC-MS analysis indicated no substances. Presumably, the amount of the compound in fraction A was too low or it is instable and thus was lost during fraction work-up, storage or sample preparation.

Fraction B contains the parent ion with *m/z 221.2* and two daughter ions with *m/z 203.9* and *175.0,* respectively (Fig. 14). Oxidation or hydroxylation of Trp (*m/z 205*) results in a mass increment of +16 u, being a possible explanation for the parent ion *m/z 221.2*. 5-OH-Trp would be a possible structure, but it was excluded as described in 3.1.2. Hydroxylation of the indole moiety in another position, giving e.g. 2-OH-Trp or 3-OH-Trp are rather likely. Oxidation of Trp may be followed by an intramolecular rearrangement into a three-ring system is thinkable and has been described in literature [88]. The presumed structure, a pyrrolo-indole-2-carboxylic acid (PIC) is shown in Fig. 14. The fragmentation indicates a facile loss of the carboxy group

(-46 u) and water (-18 u), respectively. The daughter ions speak for the three-ring-structure and do not fit to the fragments reported for OH-Trp isomers [88]. The formation of PIC was reported yet only in the presence of enzymes, strong peroxides or photosensitisers [89-92]. According to the information retrieved from the substance library, the formation of PIC could be explained by presence of a photosensitizing substance like e.g. Kyn [93-96]. The preliminary tests (3.1.2.) already proved the presence of the substance Kyn in autoclaved Trp solutions.

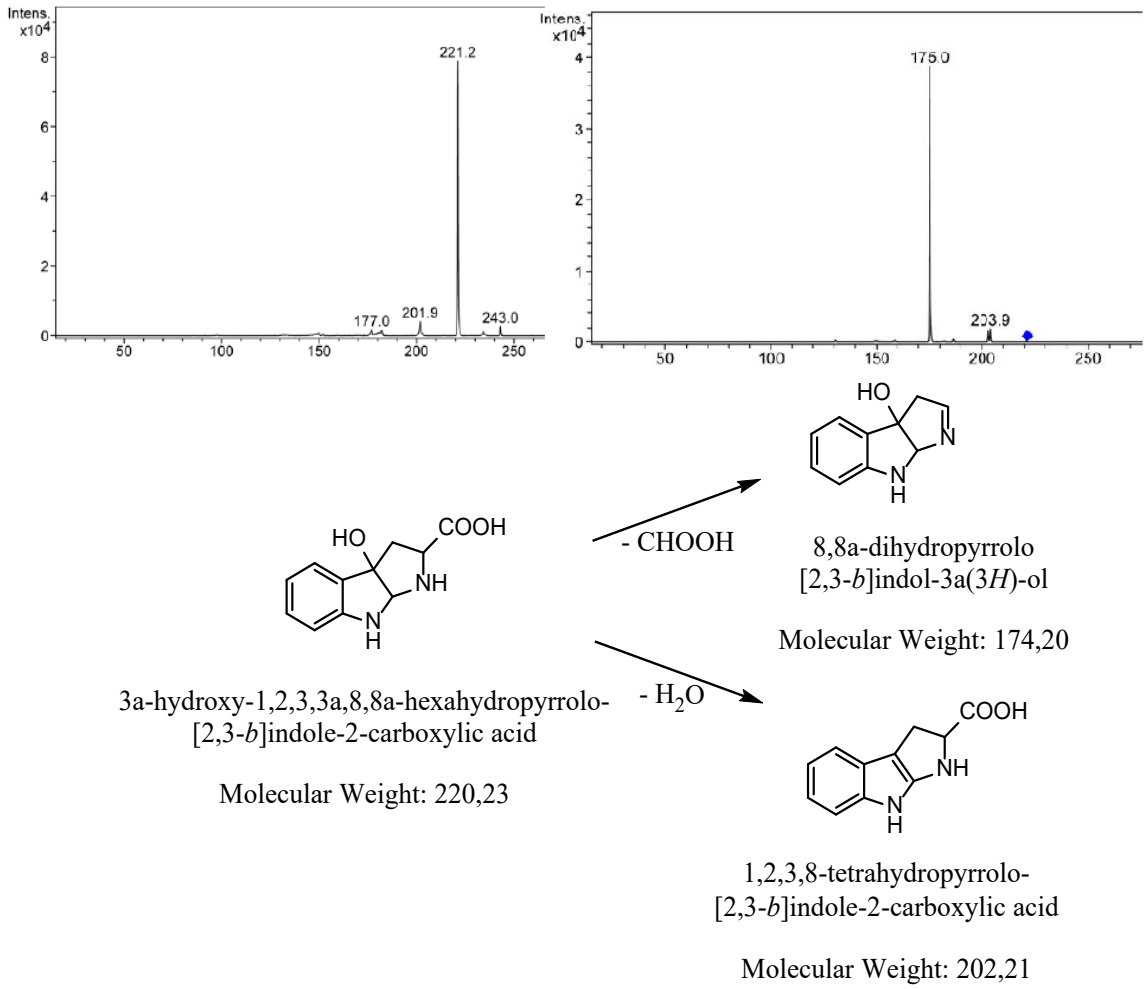


Figure 14: Fraction B, MS (left) and MS^2 spectra (right) and proposed structures of parent and daughter ions, respectively.

The main ion with *m/z 237.0* was found in fraction C and was fragmented as shown in Fig. 15. According to the molecular weight, the substance is equivalent to a Trp oxidation product, which took up two oxygen molecules (+32 u). The diastereomers DiOia, (R,R/R,S 2-amino-3-(3-hydroxy-2-oxoindolin-3-yl)propanoic acid and NFK (*N*-formylkynurenine) are highly relevant substances as Trp degradation markers. Both are listed as related substances in the Trp monograph and have been described in literature (8.3 Substance library, Tab. A2). Fig.15

proposes possible molecular structures for the observed daughter ions. The most abundant daughter ion with *m/z 146.4*, may be 1-*H*-indole-3-carbaldehyde, with the intact indole moiety. Side signals of fragment ions with *m/z 218.9 (~219)* and *m/z 191.0* derive most probable from the loss of water (-18 u) and the loss of carboxylic acid (-46 u), respectively. These findings are consistent with fragmentation studies of Trp oxidation products [97, 98].

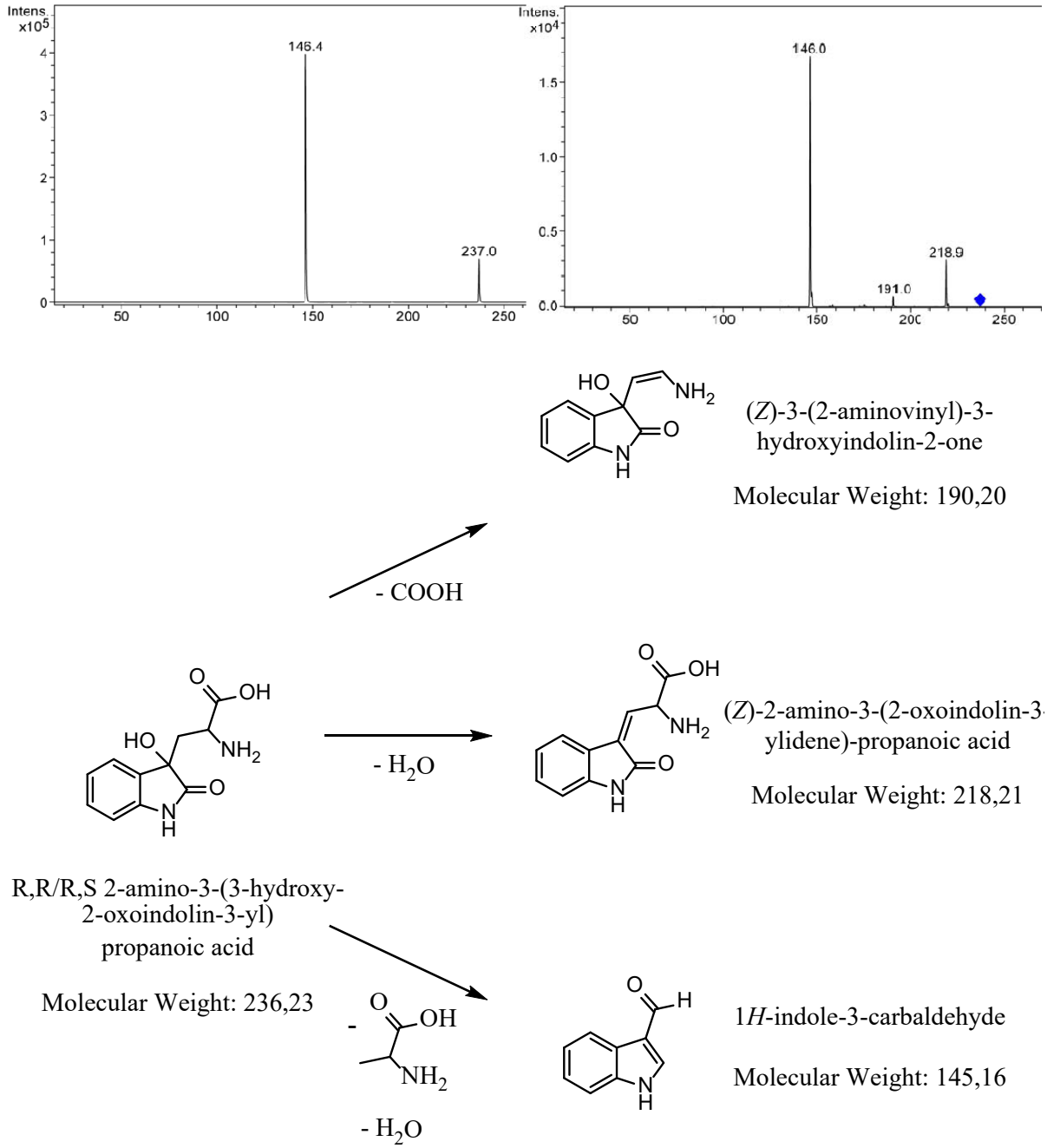


Figure 15: Fraction C, MS (left) and MS^2 spectra (right) and proposed structures of parent and daughter ions, respectively.

MS spectra of fraction D (Fig. 16) is similar to fraction C, both having the parent ion *m/z 237.*0. However, different retention times of the main peaks, shown in Fig. 13 e) and 13 f), indicate that they are two different substances. It is possible, that fraction C contains the DiOia diastereomers and fraction D NFK, respectively. Fragmentation resulted in one common daughter ion with *m/z 146.0.* It is possible, that the applied fragmentation settings are rather harsh for an instable substance such as NFK. The settings applied may lead to molecule cleavage and intramolecular rearrangement into a more stable common downstream degradation substance, the 1-*H*-indole-3-carbaldehyde. In general, NFK is a well-known Trp oxidation product of Fenton´s reaction, γ-irradiation or enzymatic treatment [97, 99, 100]. Again, NFK is an eligible precursor to Kyn, whose presence has already been shown in 3.1.2. According to cited literature, the stability of NFK is limited in acidic aqueous solutions.

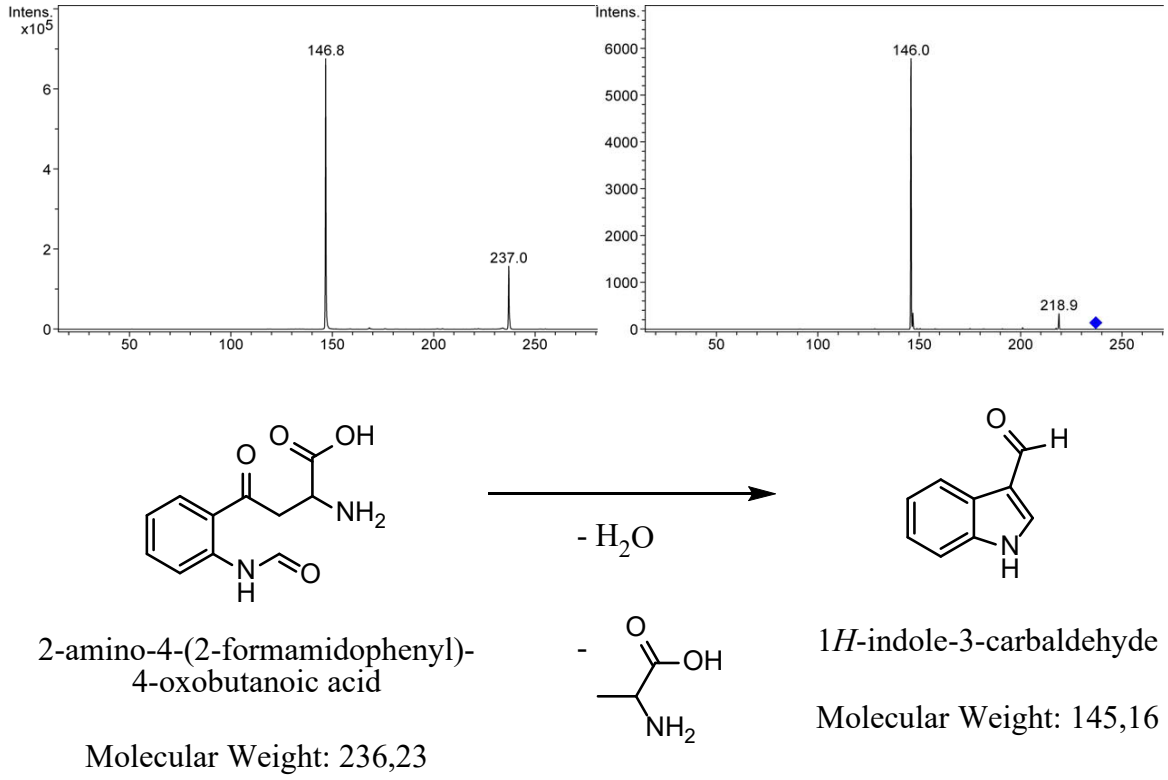
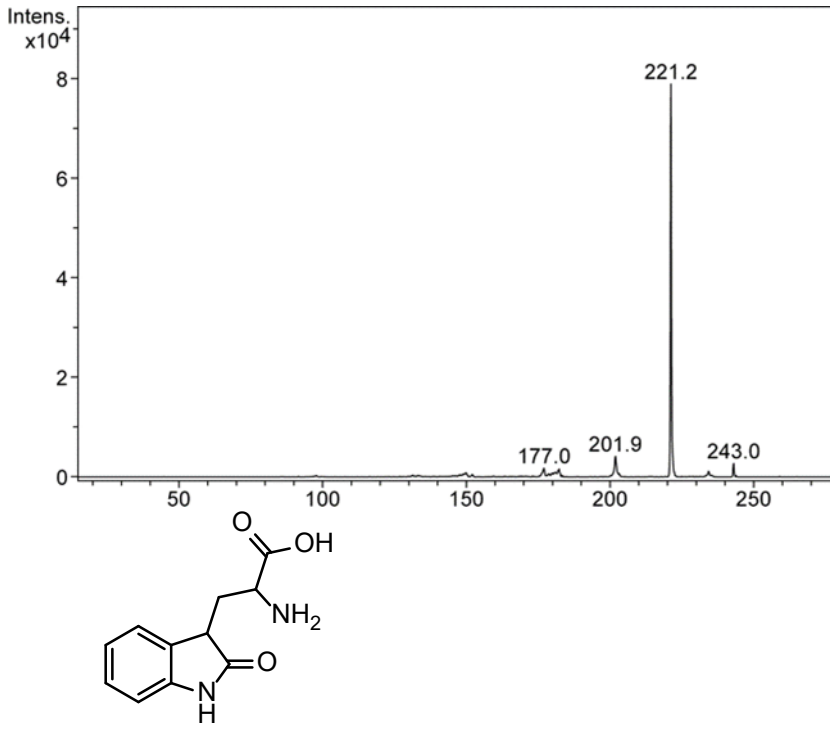


Figure 16: Fraction D, MS (left) and MS² spectra (right) and proposed structures of parent and daughter ion, respectively.

As shown in Fig. 31 d) and 13 e) the TICs of fraction D and E are nearly identical. It is thinkable, that preparative LC was not successful in efficient separation and thus the fractions are contaminated by each other. In both fractions, the ion with *m/z 237.0* was the most abundant one the fragmentation results showed an identical MS^2 spectrum (Fig. 16). However, MS

analyses demonstrated the presence of one further ion with *m/z 221.2,* eluting directly after the *m/z 237.0* ion. Obviously, the difference of retention time differentiates this compound with *m/z 221.2* from the isomeric ion found in fraction B. In fraction E, the abundance of this ion was too low for fragmentation, hence there is only the MS spectrum, shown in Fig. 17. The molecular weight corresponds to a further single oxygen oxidation product and according to the substance library (8.3. Substance library, Tab. A2) this may be the diastereomer Oia (R,R/R,S 2-amino-3-(2-oxoindolin-3-yl)propanoic acid) [101-103].

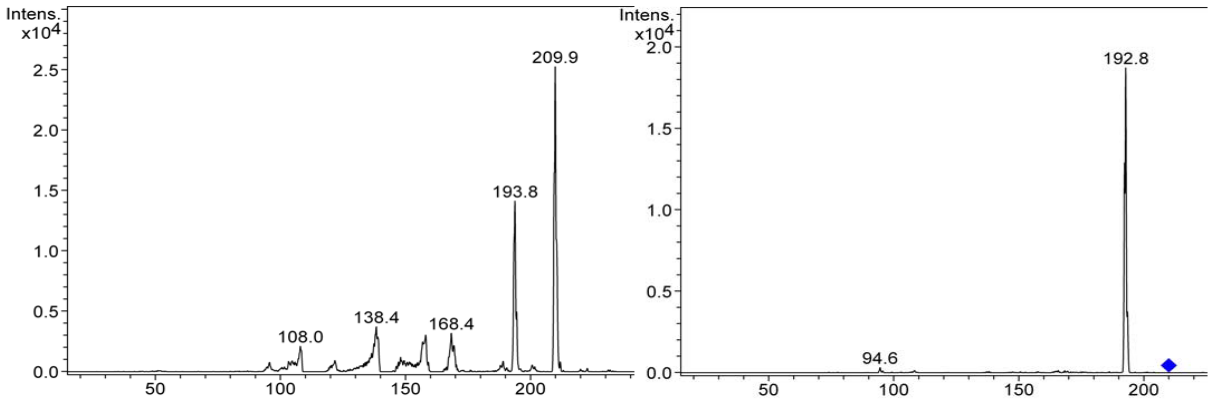


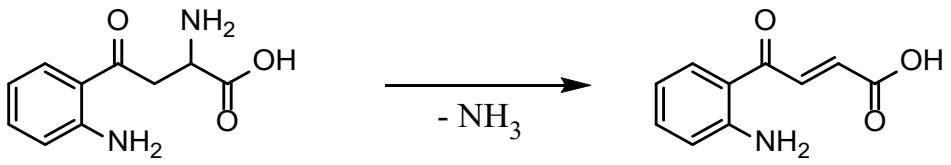
2-amino-3-(2-oxoindolin-3-yl)
propanoic acid

Molecular Weight: 220,23

Figure 17: Fraction E, mass spectra and proposed structure.

The parent ion with *m/z 209.0* detected in fraction F can be assigned to the substance Kyn. MS and MS/MS spectra of the ion and the fragmentation structure are shown in Fig 18. The small side signals in the MS spectra indicate impurities of the fraction, which may be due to partial decomposition of Kyn during fraction handling. The side signals, i.e. ions with *m/z 168.4* and *138.4* are in accordance to literature about Kyn fragmentation [104-106]. The MS^2 spectra shows the predominant mass transfer due to the loss of ammonia (-17 u) resulting in the fragment ion with *m/z* 192.





2-amino-4-(2-aminophenyl)-4-oxobutanoic acid

Molecular Weight: 208,22

(*E*)-4-(2-aminophenyl)-4-oxobut-2-enoic acid

Molecular Weight: 191,19

Figure 18: Fraction F, MS (left) and MS^2 spectra (right) and proposed structures of parent and daughter ion, respectively.

Summary

Preparative LC provided the fractions which were subsequently submitted to LC-MS/MS analyses. However, the efforts of extraction by preparative LC were only successful in parts due to a lack of purity and fraction contamination. Additionally, the fraction treatment and sample preparation may have induced the decomposition of the separated compounds, which led to ambiguous results. Nonetheless, some information about possible Trp degradation products could be retrieved from these experiments and the most probable products are summarized in Fig. 19. Referring to the information provided by literature research and the substance library about Trp degradation products, the following statements about Trp degradation can be pointed out. Trp degradation products are closely related and mutual conversion is likely to happen. Consequently, the degradation substances extracted may have transformed from “early” to “downstream” degradation products during sample treatment. Especially heating and day light irradiation during water evaporation may have induced oxidation and rearrangement of the substances. Thus, fraction contamination or the alteration of the compounds made substance identification more difficult. Nonetheless, the close relationship between the (proposed) degradation substances was reported in literature so far.

The substances NFK and the DiOia diastereoisomers derive probably from a common precursor due to Trp oxidation [98]. Consequently, the substance NFK is likely to decompose to Kyn by a simple deformylation in an acidic environment [107]. Both, Kyn and NFK comprise photo-sensitizing properties. Hence, if these substances are present even at trace levels and exposed to UV-light, they may induce further oxidation of Trp and Trp degradation substances [83, 84, 108, 109]. Two isomers with the molecular mass of 220 g/mol (*m/z ~ 221)* were found and differentiated by retention times and fragmentation pattern. The earlier eluting substance, assigned as PIC, has a fragmentation pattern consistent with literature [62] and is different from a hydroxylated Trp-derivative. Thus, the later eluting isomer, which was found in fraction E, is probably *R,R/R,S* Oia. This is supported by the presence of another closely related diastereoisomer, *R,R/R,S* DiOia. However, it remains unclear, which of the substance occurs first and if there is any conversion between DiOia and Oia. The proposed substances were compared to the list of related substances in the compendial Trp impurity assessment method. Although the proposed substances are listed partly among the specified related substances, there is no information provided for the characterization or differentiation of these. Furthermore, there is no information available about Trp degradation products in aqueous solutions after steam sterilization treatment so far. Thus, the fractionation and MS^2 experiments provide a rough idea about the characteristics (and chemical relationships) of actual Trp degradation products as well as upcoming identification and assessment challenges, respectively.

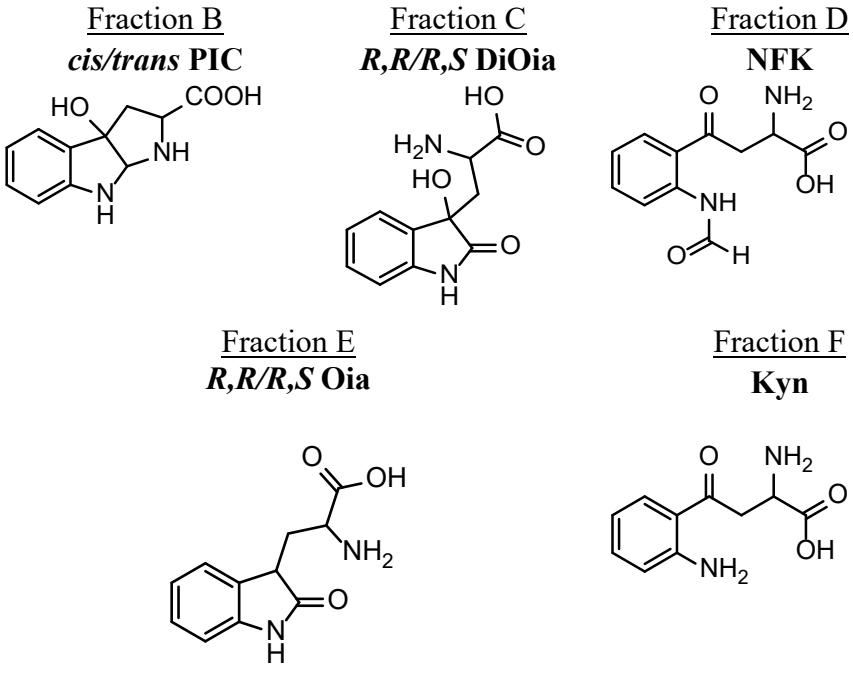


Figure 19: Overview of proposed Trp degradation substances found in the fractions B-F.

3.2 Investigation of tryptophan-related yellowing in parenteral amino acid solution: development of a stability-indicating method and assessment of degradation products in pharmaceutical formulations

Nina Unger, Antonio Ferraro, Ulrike Holzgrabe

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Abstract

Parenteral amino acid solutions containing tryptophan tend to develop a yellow colouration upon storage. Hence, the aim of the present study was to find out whether tryptophan degradation products are the reason for the yellowing. The degree of discolouration and tryptophan degradation was examined by visual examination and UV/Vis measurements with respect to oxygen presence, pH value, and duration of steam sterilization. LC-UV analyses of autoclaved tryptophan solutions indicated eight degradation products, namely *R,R/R,S* 2-amino-3-(oxoindolin-3-yl)propanoic acid, *R,R/R,S 2*-amino-3-hydroxy-2-oxoindolin-3-yl)propanoic acids*, cis/trans* 3a-hydroxy-1,2,3,3a,8,8a-hexahydropyrrolo[2,3-b]indole-2-carboxylic acid, *N′*-formylkynurenine, and kynurenine. The proposed degradation products were confirmed by spiking of synthesized degradation products and LC-UV/MS analyses. The LC-UV analysis method was optimized and validated according to the ICH guideline Q2 (R1). Tryptophan stability in commercially available parenteral amino acid formulations was evaluated over a storing period of 12 months in two common types of primary packaging after autoclave procedure.

*Abbreviations***:** AA, amino acids; DiOia, (*R*,*R/R*,*S*) 2-amino-3-(3-hydroxy-2-oxoindolin-3-yl)propanoic acid (diastereomers); Kyn, kynurenine; NFK, N′-formylkynurenine; NP, nitrogen purged; Oia (*R,R/R,S*) 2-amino-3-(2-oxoindolin-3-yl)propanoic acid (diastereomers); OC, oxygen-containing; Ph. Eur., European Pharmacopoeia, PIC, (*cis/trans)* 3a-hydroxy-1,2,3,3a,8,8a-hexahydropyrrolo[2,3-b]indole-2-carboxylic acid (diastereomers)

1 Introduction

Amino acids (AA) formulations for parenteral nutrition (PN) are a convenient, reliable, and important way of nutrient administration in paediatrics or for patients suffering from severe clinical conditions. Typical solutions consist of up to 15 to 20 AA, electrolytes, and excipients, depending on the affected patient group and their respective clinical symptoms. In parenteral AA formulations, each AA is considered as an active pharmaceutical ingredient (API) which must comply with strict quality requirements. In general, parenteral formulations are challenging pharmaceutical preparations underlying a gapless and permanent quality control. The demands for purity, stability, and sterility have to be controlled throughout all manufacturing processes, storage, and application. We summarized general instability implications of AA in different systems of parenteral formulations and coherent AA assessment methods in a recently published review [1].

The inactivation of AAs after prolonged autoclaving in the presence of sugars was studies already in 1949 by Evans and Butt and found significant decrease for some AAs, most probable due Maillard´s reaction [1, 2] However, occasional yellowing of parenteral pure AA formulations occurring during manufacturing, storage, and the application period, has been reported in prescribing information, clinical nutrition guidelines, and several research papers [1, 3]. According to the literature, peptide-bound Tryptophan (Trp) and Trp metabolites are involved in various discolouration processes of biological tissues, like e.g. the yellowing of the eye lenses and wool [4]. Because of the relatively reactive side chain moieties of cysteine (Cys; thiol), tyrosine (Tyr; phenol), and Trp (indole), these AAs are most likely prone to oxidation resulting in a detrimental peptide and protein alteration [5, 6]. In the case of the essential AA Trp it is known that the indole moiety is prone to oxidation, ring opening, and electrophilic substitution [7-9]. The aliphatic chain may react in an intra- or intermolecular manner with the amine in the pyrrole ring, reduction, cleavage, loss of ammonia or condensation reactions resulting in Trp dimers [10, 11]. To our knowledge, degradation of Trp and Trp-containing peptides has only been reported under harsh, non-physiological or not (pharmaceutical) product-related stressing conditions such as the presence of strong peroxides, enzymes, photosensitizers (e.g. dyes), with Fenton´s reagents, near UV- or γ-irradiation, or strong thermal treatment [7, 12-16]. According to cited literature, the most probable degradation products of Trp represent several oxidation products being mono- and di-hydroxylated Trp derivatives with modifications of the indole backbone [7, 17, 18]. It is well-known that mammalian Trp metabolism involves the formation of so-called kynurenines by enzymatic opening of the pyrrole ring, oxidation of the aliphatic chain, and a partial formation of the aromatic amine

group [19-21]. Thus, kynurenines have an *o*-aminoacetophenone backbone and considerably differ from hydroxylated Trp derivatives with an intact indole backbone. Three predominant types of Trp degradation are shown in Fig. 20. Condensation and dimerization of Trp molecules into e.g. 1,1´ Ethylidene(bis)-Trp, as well as the Pictet-Spengler reaction, a carbonyl condensation of the aliphatic chain and the pyrrole site, are described as possible sources for Trp impurities [22]. Compiling a substance library of the most probable Trp degradation products according to reviewed literature was beneficial for LC-UV/MS assisted substance identification (8.3. Substance library).

This study demonstrates that steam sterilization by means of an autoclave, as a commonly applied method for liquid sterilization, is already sufficient to induce Trp degradation with the presence of oxygen at trace level. Stressed samples were used for identifying putative Trp degradation products starting with an adjusted compendial HPLC-UV method from the European Pharmacopoeia (Ph. Eur.) [23]. Adjustments allowed the separation of degradants, a run time reduction and MS detection. The HPLC-UV method was optimized with regard to the identification of the degradation products and subsequently validated according to the ICH guideline Q2 (R1). Eight degradation substances could be identified and characterized. To our knowledge, the formation of all of these substances under applied conditions has not been described before but can be considered relevant in terms of quality control of solutions containing Trp. The extent of degradation and apparent discolouration was studied considering pH value, oxygen presence, and autoclaving duration. The aim of the current study is the application of the stability indicating HPLC-UV method for the assessment of Trp degradation in two commercially available parenteral AA formulations. The results suggest that the discolouration of Trp-containing solutions due to Trp decomposition can be impeded to a great extent by strict oxygen deprivation as well as light protection by the utilization of (brown) glass bottles for manufacturing and storage.

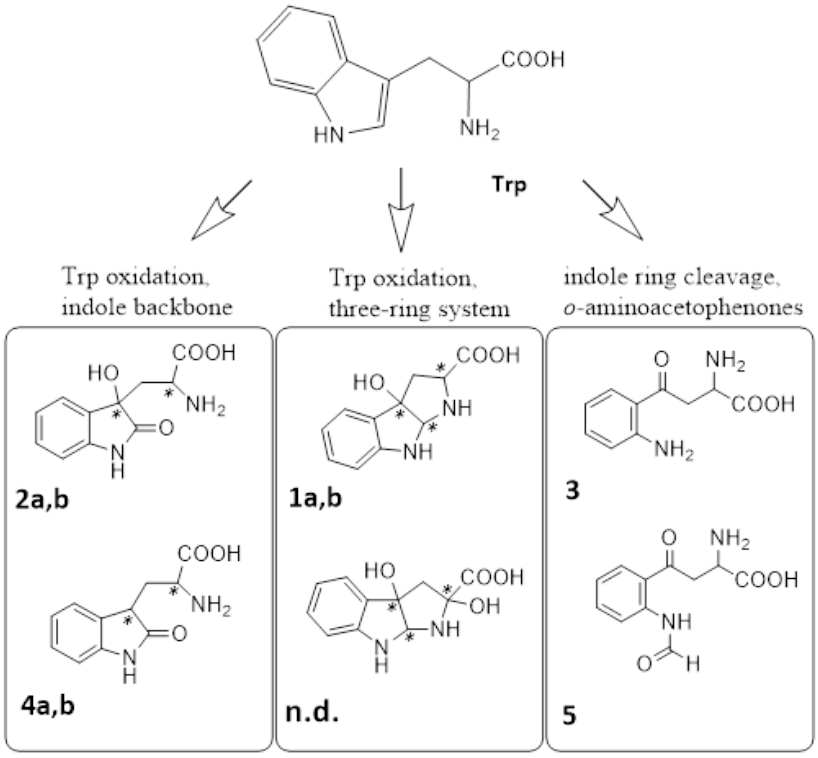


Figure 20: Three types of Trp degradation reactions with most probable degradation substances. Oxidation of indole: 2a,b:R,R/R,S 2-amino-3-(3-hydroxy-2-oxoindolin-3-yl)propanoic acid (DiOia) diastereomers, 4a,b: R,R/R,S 2-amino-3-(2-oxoindolin-3-yl)propanoic acid (Oia) diastereomers, 1a,b: cis/trans 3a-hydroxy-1,2,3,3a,8,8a-hexahydropyrrolo[2,3-b]indole-2-carboxylic acid (PIC), n.d.: not detected; hydroxylated PIC. O-aminoacetophenones: 3: kynurenine (Kyn), 5: N′-formylkynurenine (NFK)

2 Experimental

2.1 Chemicals and material

All aqueous solutions were prepared with water from a Merck Millipore® water purification system (Merck, Darmstadt, Germany). Acetic acid, sodium hydroxide (40 wt.-%), dimethyl sulfoxide, hydrochloric acid (36 wt.-%), triethylamine, acetic anhydride, diethyl ether, deuterium hydroxide (99.9 % D-atom, contains 0.01 wt-% 3-(trimethylsilyl)propionic acid (TSP)), ethanol, trifluoro-acetic acid, ammonium formate, and formic acid were purchased from Sigma-Aldrich Chemie GmbH (Taufkirchen, Germany). HPLC-MS grade acetonitrile (ACN) and methanol (MeOH), L-Trp and DL-Kynurenine (DL-Kyn), were purchased from VWR international (Darmstadt, Germany). L-Kyn (**4**), L-Tyr, and L-Phe were purchased from TCI chemicals (Eschborn, Germany). Nitrogen gas was provided by Linde AG (Linde, Gases Division Germany, Pullach, Germany). All chemicals used for HPLC analysis were of analytical grade, all chemicals used for mass analysis were of LC-MS grade. Prior to use, all

solutions were filtered through 0.25µm polypropylene or 0.45µm cellulose acetate particle filters purchased from VWR international (Darmstadt, Germany). *Formulation 1* and *formulation 2*, and PVC-free plastic containers for injectables were kindly provided by Fresenius Kabi AG (Bad Homburg, Germany).

2.2 Apparatus

Autoclaving was performed using a programable autoclave from Systec (Systec GmbH, Linden, Germany), autoclave duration was chosen as needed, temperature was set at 121 °C, 2.1 bar. The pH was measured with a calibrated Metrohm pH electode (Metrohm International, Herisau, Switzerland). UV/Vis absorption spectroscopy experiments were performed on a Shimadzu UVmini-1240 UV/Vis instrument (Shimadzu Deutschland GmbH, Duisburg, Germany). LC was performed on an Agilent 1100/1200 chromatography system equipped with online vacuum degasser, binary pump, thermostatted auto sampler, column thermostat compartment, and a varialbe wavelength UV/Vis detector (Agilent Technologies, Waldbronn, Germany). MS analyses were performed on an LC-MSD Trap G2445D ESI ion trap (Agilent Technologies, Waldbronn, Germany). NMR experiments were carried out on a Bruker Avance®; ^{1}H 400.132 MHz ^{13}C 100.613 MHz (Bruker, Karlsruhe, Germany). The spectra were processed by using the Bruker TopSpin v3.0 software program. The samples were measured in deuterium hydroxide (99.9 % D-atom) referenced with TSP internal standard.

2.3 Chromatographic conditions

Preliminary HPLC-UV/MS

A preliminary separation and identification method was performed on a LiChrospher® LiChroCART® RP_e-18 column 250 x 4.6 mm i.d. with 5µm particles (Merck KGaA, Darmstadt, Germany) with a methanol gradient using the following solvent and gradient settings: mobile phase A 0.1 % TFA/MeOH (95:5), mobile phase B 100 % MeOH, gradient 0-5 min: 0 % B, 5-40 min: 0-35 % B, 40-45 min: 35 %B, flow rate 1 mL/min, and UV detection at λ = 254, 280, 340 and 430 nm,. The MS detector was linearly coupled with settings applied as described below.

HPLC-UV

Separation was performed on a LiChrospher® LiChroCART® RP_e-18 column, 125 x 4 mm i.d. with 5 µm particles (Merck KGaA, Darmstadt, Germany) using gradient conditions. Mobile phase A consisted of 0.25% formic acid in a mixture of MeOH and 30 mM ammonium formate buffer (5:95, v/v) adjusted to pH = 3 with formic acid. Mobile phase B consisted of 0.25% formic acid in a MeOH/60 mM ammonium formate buffer (50:50, v/v). The flow rate was set at 1.0 mL/min, autosampler temperature at 8 ± 2 °, column temperature at 15 °C, and UV detection was carried out at λ = 254 and 340 nm, respectively. Injection volume was 10 µL and the needle was washed with isopropanol/water (50:50, v/v). The gradient was as follows: 0-10 min: 0 % B, 10-15 min: 0-30 % B, 15-20 min: 30 % B, 20-22 min: 30-90 % B, 22-28 min: 90 % B, 28-30 min: 90-0 % B and re-equilibration time of 5 min.

HPLC-UV/MS analyses

The linearly coupled MS detector was used with MS settings as follows: electrospray ionisation (ESI), monitoring in positive mode, nebulizer 50 psi, ultrapure nitrogen dry gas 9 L/min, probe temperature 350 °C, mass range *m/z* 15-450, capillary -3500 V.

Visual inspection and UV/Vis spectroscopy

The stressed samples were subjected to visual inspection and spectrophotometry every week for a total period of eight weeks and finally once again after 16 weeks. The sampling was non-destructive to the closed lid system. Visual inspection included checking for particles and observing any colour change and comparison of yellowing levels with control samples. UV/Vis analysis was performed at λ 280, 340, and 430 nm with samples were diluted to 1:20 and measured in triplicate.

2.4 Synthesis of reference substances

*Synthesis of 2-amino-3-(3-hydroxy-2-oxoindolin-3-yl)propanoic acid diastereomers **2a,b***

The diastereomers **2a,b** were synthesized according to reference [22]. 1.55 g of **4a,b** were dissolved in 5 mL water and the pH was adjusted to 10-12 using triethylamine. The solution was supplied with oxygen and stirred at RT for 5 h. The reaction progress was monitored by LC-UV/MS. Purification was carried out by Flash Chromatography (PuriFlash, Interchim, Los Angeles, US), using an endcapped RP column, RS 40 C_{18}, 43 g (Macherey Nagel, Düren,

Germany). Elution conditions: linear water/MeOH gradient, from 0 % B to 3 % B in 5 min, hold for 5 min. **2a,b** were collected, dried, and recrystallized from ethanol, yielding 350 mg (0.2%). 2 mg was dissolved in 1.5 mL purified water and subjected to LC-UV/MS analysis indicating 2 peaks (51:49), λ = 254 min (R_t: 3.3/4.2 min), *m/z* 237, purity > 99%. ^{1}H (400 MHz, D_2O); NMR data are in accordance with reference [22].

*Synthesis of 2-amino-3-(2-oxoindolin-3-yl)propanoic acid diastereomers **4a,b***

The diastereomers **4a,b** (Fig. 20) were synthesized according to directions in references [17, 22, 24]. 5.0 g of Trp were suspended in 25 mL of acetic acid. A mixture of 4.5 mL of dimethyl sulfoxide and 12.5 mL of 36 wt% hydrochloric acid was added. The mixture was stirred for 2 h at RT. After pH adjustment to 5-6 with 0.5 M sodium hydroxide, a bright precipitate was formed. The precipitate was collected, washed with diethyl ether and dried, yielding 1.75 g of **4a,b** (0.35%). 2 mg was dissolved in 1.5 mL purified water and subjected to LC-UV/MS analysis. 2 peaks (57:43) were detected λ = 254 min (R_t: 8.9/10.6 min), *m/z* 221, purity >98%.^{1}H NMR data (400 MHz, D_2O) are in accordance with [22].

*Synthesis of N′-formylkynurenine **5***

Synthesis of **5** was conducted as described in references [22, 25]. 100 mg of L-Kyn (**4**) was dissolved in 220 µl formic acid, and a mixture of 96 µL formic acid and 48 µL acetic anhydride was added. The solution was stirred at 25 °C for 2 h. 500 µL of diethyl ether was added, and the cream-coloured solid precipitate was subsequently isolated after centrifugation and washing with diethyl ether. 2 mg was dissolved in 1.5 mL purified water and subjected to LC-UV/MS analyses indicating compound **5** (λ 254 and 340 nm, R_t: 9.9 min, *m/z 237*) and starting material (λ 254 and 340 nm, R_t: 6.13 min, *m/z 209*); the purity of **5** was determined as 79%. ^{1}H NMR data (400 MHz, D_2O) are in accordance to reference [22].

2.5 Sample preparation

Stressed Trp solutions

The Trp stock solution was prepared by dissolving 10.0 g of L-Trp in 5.0 L water (2 mg/mL), followed by 10 min degassing in the ultrasonic bath. The solution was divided into four aliquots for pH adjustment which were individually adjusted to pH 5.0, 5.5, 6.0, and 6.5 with 0.01 M acetic acid or 0.01 M sodium hydroxide, respectively. Each aliquot was distributed into 12 x

50 mL autoclavable glass vials for injectables. Analogously prepared samples were purged with N_2 for 10 min prior to vial sealing additionally. All samples were made in triplicate and subjected to autoclave for 30, 60, and 90 min (121 °C, 2.1 bar) or no heat treatment (control group). This procedure was repeated for a second set of pure 2 mg/mL Trp solutions with reduced autoclaving times of 10, 20, and 30 min without nitrogen purging (degassing only). The solutions were stored collectively at a dry and dark place at RT and retrieved for sampling as needed.

Preparation of parenteral AA formulations

50 mL aliquots of *formulation 1* (AAs: Ile, Leu, Lys, Met, Phe, Thr, Trp, Val, Arg, His, Ala, Gly, Pro, Ser, Tyr, Taurine) and *Formulation 2*(AAs: Ile, Leu, Lys, Met, Phe, Thr, Trp, Val, Arg, His, Ala, Gly, Pro, Ser, Tyr, Asp, Glu, Cys; 8.1. Tab. A2) were distributed either into glass vials or autoclavable non-PVC plastic bags for injectables in duplicate. *Formulation 1* and *formulation 2* are electrolyte free, pH is 5.5-6.5 and 5.4-5.8, respectively, adjusted with glacial acid (according to prescribing information). Autoclaving times were set to 10, 20, and 30 min. Additional control samples remained not autoclaved. All samples were exposed to daylight while cooling down to RT prior to analyses. After sampling, the solutions were stored at a dry and dark place at RT and taken for analyses as needed.

HPLC sample preparation

The *reference stock solutions* were prepared by dissolving 10.0 mg of each of **2a,b, 3, 4a,b** and **5** in 10.0 mL purified water (1 mg/mL). *The stressed reference solution* was an aqueous 2 g/L Trp solution, pH 5.5, autoclaved for 30 min (121 °C, 2.1 bar, glass bottle). For the *spiked stressed reference solution,* 5.0 mL of the *stressed reference solution* were spiked with 100 µL of each *reference stock solution* and diluted to 10.0 mL with mobile phase A (conc.: Trp 1 g/mL, spiking conc. **2a,b, 3, 4a,b, 5** each: + 10 µg/mL). The *standard model solution* (used for validation procedures) was prepared by weighing of 20 mg L-Trp, adding 250 µL of **2a,b-,** 65 µL of **3-,** 330 µL of **4a,b-** and 100 µL of **5-** *reference stock solutions* and diluting the resulting solutions to 10.0 mL with purified water. All solutions were stored at 4 °C in the refrigerator and were used within 7 days.

For LC, 2 mL aliquots of sampling material were extracted from the sealed containers using a syringe and filtrated through 0.25 µm cellulose syringe filters. 500 µl of the filtrate was diluted to 500 µL with mobile phase A. 1 mL of the *spiked stressed reference solution* was filtrated

used for LC directly (no further dilution). The samples were kept at approx. 4±2°C in brown glass vials until use. LC samples of stressed *formulation1* and *2* were prepared accordingly.

HPLC calibration sample preparation

Calibration curves were made by weighing separately 10.0 mg of the substances **2a,b, 3, 4a,b** and **5,** and dilution to 10.0 mL (*calibration stock solutions 1,* 1 mg/mL*).* 1.0 mL of each *calibration stock solution 1* was diluted to 10.0 mL to obtain each *calibration stock solution 2 (0.1 mg/mL)*. The *calibration stock solutions 2* were diluted with mobile phase A to obtain the following ranges of calibration curves: from 0.025 to 2.5% for **2a,b** (0.5, 1.0, 2.5, 7.5, 10.0, 25.0, 50.0 µg/mL), from 0.025 to 0.5% for **4a,b** (0.5, 1.0, 2.5, 7.5, 10.0 µg/mL), from 0.025 to 0.5% for **3** (0.5, 0.75, 1.0, 2.5, 5.0, 7.5, 10.0 µg/mL), from 0.0375 to 0.375% for **5** (0.75, 1.0, 2.5, 5.0, 7.5 µg/mL). Every level was injected in triplicate.

2.6 General procedure

In the first sample set the aqueous nitrogen-purged (NP) and oxygen-containing (OC) solutions of L-Trp were exposed to controlled heating cycles in a laboratory autoclave at a 121 °C, 2.1 bar for 30, 60, and 90 min, respectively. NP samples represent the best possible oxygen deprivation. In a second sample set, nitrogen purging was omitted, focusing on Trp degradation in the presence of oxygen with reduced autoclave durations of 10, 20, 30 min. Autoclaving was chosen as heat source, because it is routinely used for the sterilization of intravenous formulations in industry and in hospital pharmacies. Immediate (1-4 days after autoclave) and long-time effects (1 and 3 years) of heat stress were examined at solutions of pH values of 5.0, 5.5, 6.0, and 6.5. The samples were subjected to UV/Vis analysis and visual inspection, for the development of an LC-UV method and for identifying Trp degradation products.

3 Results and discussion

3.1 Colour inspection and UV/Vis analyses

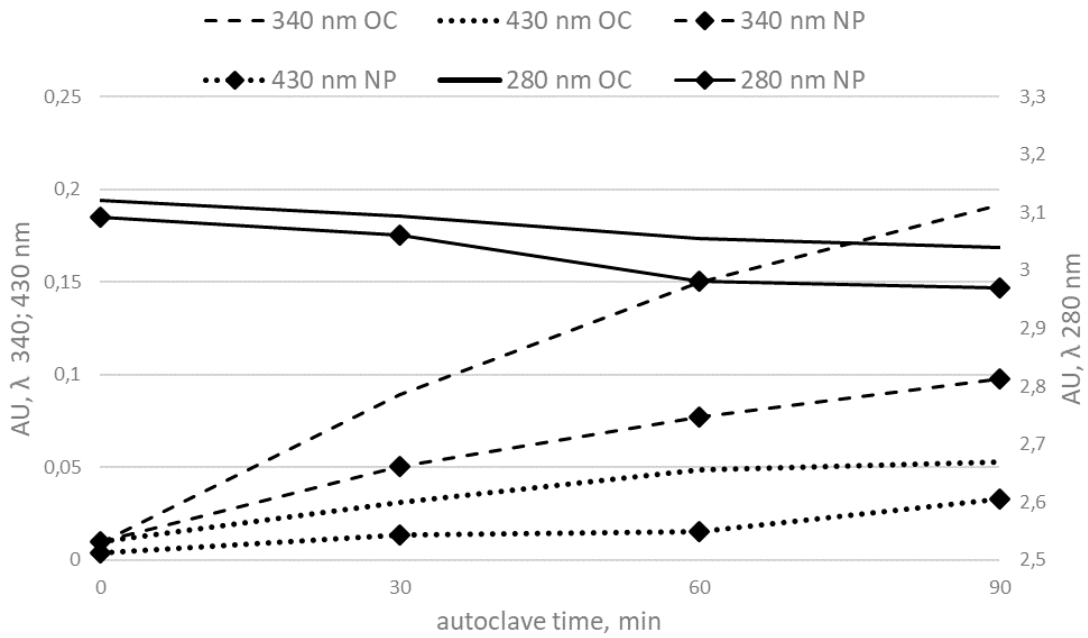
Stressed OC and NP samples were withdrawn for weekly colour inspection and UV/Vis analysis for a period of 8 weeks, and once again after eight additional weeks. The discolouration was evaluated by direct comparison with control samples and augmented with pictured documentation.

Directly after autoclaving, all OC samples showed a yellow discoloration in different degrees depending on the autoclave duration which intensified throughout the observation period. In contrast, all NP samples remained as uncoloured as the unstressed NP/OC control samples. Stressed NP also the unstressed OC solutions slowly developed a yellowish colour over time, whereas the unstressed NP control solutions remained uncoloured until the end of the inspection period. Effects of different pH values upon the degree of colouration could not be observed. All samples remained without precipitates throughout the whole observation period. Turbidity was observed in all 60 and 90 min stressed OC samples towards the end of the inspection period and brown particles were found only in OC samples within one year after the inspection period. All stressed NP samples were significantly brighter than the OC samples. Hence, oxygen may be a main factor for the yellowing of Trp-containing solutions along with elevated temperatures having a rather accelerating impact.

In order to monitor the coloration process, the following wavelengths were chosen: λ = 280 (A_{max} of Trp), 340, and 430 nm (no UV activity of Trp). The wavelengths were chosen according to internal discussions and preliminary test results with the objective to monitor non-indole derivatives and/or coloured substances. The influence of storage time period and the immediate impact of the autoclaving duration on the UV absorption of NP and OC samples are displayed in Fig. 21 a and b, respectively. Within the inspection period, no influence can be seen in both groups at λ = 280 nm which is due to the fact that structural changes upon degradation do not take place at the corresponding chromophore, hence resulting in derivatives with an intact indole (indole A_{max}: 216, 273, 288 nm in H_2O/EtOH (9:1) [26]). In contrast, at λ = 340 nm the absorbances of OC samples are increasing, indicating that new compounds might have been formed with a chromophore being different from indole. The NP solutions were found to possess a constant absorbance after an initial small increase at λ = 340 nm. The changes of the UV-spectra of the OC solutions upon autoclave duration and storage are mirrored in the λ = 340 nm absorbance increments. The reason for the increasing absorbance at λ= 340 nm may be due to the cleavage of the indole ring at the pyrrole site and would be consistent with UV characteristics of derivatives with an *o*-aminoacetophenone moiety [27]. Likely structures are **3** or **5**, which are known products of mammalian Trp metabolism including pyrrole cleavage and oxidation [19, 20, 28]. Both substances exhibit relatively strong absorbances at λ > 320 nm [29, 30]. The changes are similar at λ = 430 nm with regards to autoclave duration and storage time. In contrast to NP solutions, the absorbance increments are consistently and unequivocally greater in the OC solutions.

Comparison of NP and OC samples indicate that heating is sufficient to induce measurable alterations during storage.

a)



b)

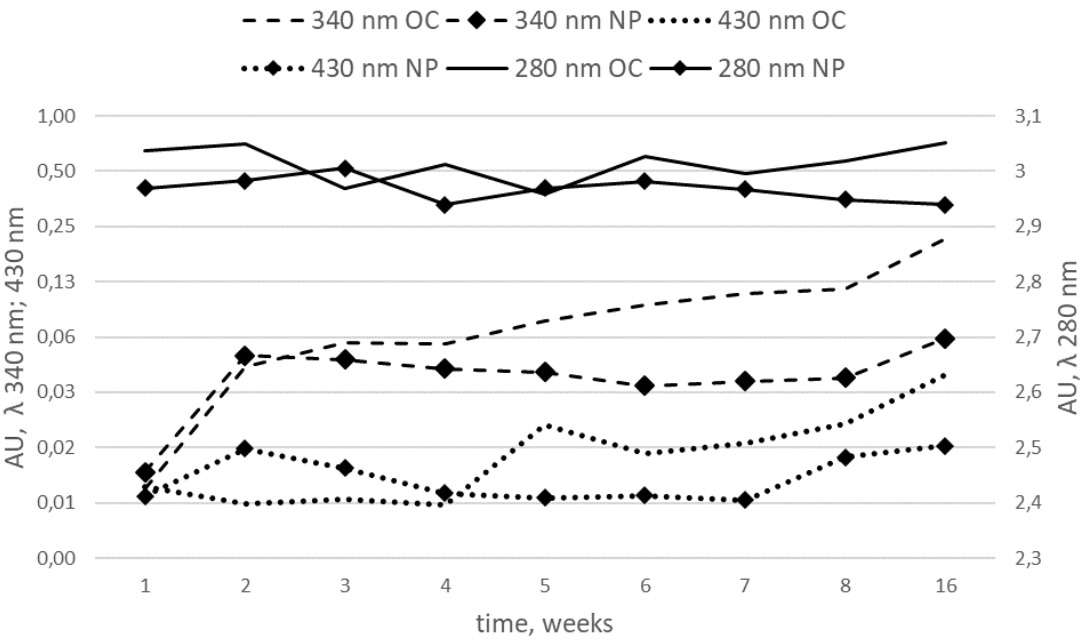


Figure 21: a) UV absorbances of stressed NP and OR Trp solutions (30 min, 121 °C, 2.1 bar, pH 5.5, glass bottles) after week 1-8 and 16. b) Mean UV absorbances of stressed NP and OR Trp solutions (30, 60, 90 min, 121 °C, 2.1 bar pH 5.5, glass bottles) with regard to autoclave duration, measured 4 weeks after autoclaving.

3.2 Method development and validation

3.2.1 Chromatographic development

Method development was based on the HPLC method reported in the Ph. Eur 9.0 for related substances [23], which makes use of a RP-18 column and gradient elution with an ACN/phosphate buffer, and a run time of 65 min. The original impurity assessment is focusing on the limitation of 1,1-Ethylidene(bis)Trp[1]. Here it was aimed to find a faster method, which is also applicable to mass detection. Thus, we exchanged the acetonitrile with methanol and replaced the phosphate buffer with TFA. Applying a gradient resulted in a baseline separation of at least 5 components eluting prior to Trp in the *stressed reference solution* (Trp: R_t 30.8 min) and a shorter runtime, with the exception of a double peak at R_t 20.3 and 20.8 min, respectively. Detector sensitivity was tested at λ = 220, 254, 280, and 340 nm and found to be best at λ = 254 nm.

Two other peaks, separated from each other and from Trp, are UV-active at λ = 340 nm additionally. In order to monitor if any impurities are eluting after Trp, run times with increased amounts of organic solvent (45-60 min, 35-55% B) were tested. Since no further peaks were observed, the maximal run time was set to 35 min.

3.2.2 Method optimization

Method optimization focused reduction of the analysis time while improving separation of components. This included the testing of different columns parameters (length, particle size, diameter), eluent composition, different methanol content, acidic modifiers (TFA, acetic acid, formic acid), introduction of volatile buffers (10, 15, 20, 30 mM ammonium formate), gradient modification, flow rates, and column temperature. Application of a shorter column (125 x 4.6 mm, 5µm) was successful for reducing run times significantly (Trp R_t < 20 min). Adverse effects on retention and separation performance were mitigated by utilization of a flat gradient and reduced MeOH portion in mobile phase B (MeOH/60 mM ammonium formate buffer, 50:50, v/v). Mobile phase A was changed to 30 mM ammonium formate buffer with 2.5% formic acid adjusted to pH 3 resulting in good separation, symmetrical peak shapes, repeatability, and good MS sensitivity. Retention was improved at a lower temperature of 15 °C. Finally, optimization efforts were successful in separating 8 substance peaks in the following elution order: **1a**, **2a**, **1b**, **2b**, **3**, **4a**, **5**, **4b** in less than 12 min followed by the elution

* other related impurities are limited to 100 ppm in total.

of Trp at R_t = 16 min (Fig. 22). Peaks **3** and **5** were the only substances which are UV active at λ = 254 and 340 nm. Baseline separation could not be achieved between the peak pair **1b** and **2b** (R_t 3.9, 4.2 min, peak-to-valley ratio: 0.7). The triplet of peaks **4a, 5,** and **4b** could be separated successfully, but still with a rather close elution of **5** and **4b**. In both cases, for quantification purposes the analysis of peak heights was preferred instead of using the peak area. Peak height was also found beneficial for the assessment of degradation products in the presence of other AA in *formulation 1* and *formulation 2*. The method is directly compatible to MS devices (settings 2.3.). However, MS detection is not required for the assessment of the identified Trp degradation products, thus the LC-UV method was validated.

3.2.3 Method validation

The LC-UV method was validated regarding specificity, linearity, range, LOD/LOQ, accuracy, precision, repeatability, and robustness in accordance to the ICH guideline Q2 (R1) [31]. Ruggedness was tested by running the method on another HPLC system.

Specificity of the method was proven by comparison of *spiked stressed reference solution* and the *standard model solution*. The resolution was at least 1.75 for baseline separated peaks and 0.5 and 1.1 for the peak-pairs **1b**-**2b** and **4b**-**5**, respectively.

Linearity and range were determined by evaluation of the slope, coefficient of determination, (R^2) and residual sum of squares (RSS) obtained from constructed calibration curves. The ranges of calibration curve were chosen considering the varying amounts of the degradation products **2-5**, depending on the age of samples. Correction factors (**F**) were obtained by the slopes of the calibration curves with regard to the calibration curve of the only commercially available reference **3** (DL-Kyn). Linearity of the detector response was evaluated by plotting concentration levels against concentration obtained with calibration curves. R^2 was higher than 0.999 and the slope was 1.000 ± 0.008. *LOD* and *LOQ* were calculated from the calibration curves according to the Ph. Eur. LOD and LOQ were determined as signal to noise (S/N) ratio of 3 and 10, respectively. Noise ranges were determined by injection of mobile phase A in triplicate. Substance concentration (see calibration levels) was plotted against S/N ratio, R^2 were higher than 0.995. Correction factors, linearity and LOD/LOQ data are summarized in Tab. 7.

	Linearity			LOD/LOQ		F
λ = 254 nm	**R^2**	**slope**	**RSS**	**µg/mL**		
2a	0.999	0.952	$6.79 * 10^{-6}$	0.27	0.86	0.4931
2b	0.998	0.956	$1.62 * 10^{-13}$	0.35	1.10	0.3588
4a	0.9995	0.998	$5.03 * 10^{-6}$	0.38	1.21	0.2191
4b	0.9995	0.998	$5.07 * 10^{-7}$	0.61	1.90	0.1414
3	0.9992	1.006	$5.54 * 10^{-8}$	0.11	0.48	-
5	0.9996	1.003	$1.6 * 10^{-9}$	0.19	0.42	0.6088
λ = 340 nm						**F**
3	0.9993	0.9443	$5.63 * 10^{-8}$	0.18	0.67	-
5	0.9995	0.9984	$1.7 * 10^{-7}$	0.51	1.89	0.321

Table 7: Linearity: LOD/LOQ and correction factors (F), for Trp degradation substances 2-5, determined at λ 254 nm and additionally at λ 340 nm for 3 and 5.

Accuracy was assessed by sextuple injection of the *standard model solution* (n = 6, intra-day) and was repeated on day 2 (n = 12, inter-day). Recovery rates were between ± 3-8 %, RSD = 0.11-0.5 (Tab. 8)

		Intra-day		Inter-day		
λ = 254 nm	**conc. (µg/mL)**	**measured conc.(µg/mL)**	**RSD n=6**	**measured conc.(µg/mL)**	**RSD n=12**	**recovery**
2a,b	25.0	25.9	0.11	25.4	0.85	100 % ± 4 %
3	6.5	6.99	0.22	6.5	8.6	100 % ± 8 %
4a,b	33.3	33.9	0.14	33.4	2.9	100 % ± 3 %
5	10.0	9.89	0.12	9.87	6.6	100 % ± 4 %
λ = 340 nm						
3	6.5	6.15	0.30	6.35	0.61	100 % ± 5 %
5	10.0	9.8	0.50	9.45	0.97	100 % ± 6 %

Table 8: Intra- and inter-day accuracy determination at λ 254 nm, with n=6, n=12 injections of standard model solution, respectively. Weighed-in and measured concentration, RSD and recovery of each substance. 3 and 5 are assessed additionally at λ 340 nm.

Precision and repeatability were determined by peak height evaluation for sextuple injections and six-fold sample preparations of the *standard model solution* on two consecutive days. The RSD intra-day was between 0.1 and 0.22 for injections (n = 6) and between 0.27 and 1.09 for sample preparation (n= 6), respectively. The RSD inter-day was between 1.02 and 8.64 for

injections (n = 12) and between 0.54 and 1.89 for sample preparation (n= 12), respectively. The data including SD, RSD, and confidence interval (CI, p=0.05) is summarized in Tab. 9.

	6-fold injection						**6-fold sample preparation**					
	intra-day			**Inter-day**			**Intra-day**			**Inter-day**		
	SD	**RSD**	**CI**	**SD**	**RSD**	**CI**	**Sd**	**RSD**	**CI**	**SD**	**RSD**	**CI**
2a	0.012	0.13	0.010	0.066	0.67	0.04	0.057	0.59	0.05	0.054	0.56	0.03
2b	0.008	0.11	0.006	0.075	1.02	0.04	0.035	0.49	0.03	0.041	0.56	0.02
3	0.012	0.22	0.010	0.433	8.64	0.25	0.013	0.27	0.01	0.089	1.89	0.05
4a	0.007	0.12	0.006	0.145	2.94	0.08	0.032	0.63	0.03	0.027	0.54	0.02
4b	0.004	0.13	0.003	0.099	3.06	0.06	0.024	0.73	0.02	0.02	0.59	0.01
5	0.004	0.12	0.004	0.269	6.58	0.15	0.046	1.09	0.04	0.062	1.47	0.03

Table 9: Precision and repeatability evaluation of peak heights for sextuple injection and 6-fold sample preparation of standard model solution. Intra-day; n = 6, inter-day; n = 12, SD, RSD and 95 % confidence interval (CI), p=0.05, λ 254 nm, determined on two consecutive days.

Robustness was examined by the following variation of operation parameters: temperature ± 2 °C, flow rate ± 0.1 mL/min, eluent A methanol content ± 20 % (rel. v/v), and buffer concentration ± 10 % (rel. v/v). The *spiked stressed reference solution* was injected under either condition in triplicate and evaluated on peak symmetry and resolution. Methanol content and column temperature were found to be critical for the separation of **1b** and **2b**. The percentage of 6 % (v/v) methanol in eluent A and 17 °C has a negative effect on the resolution (< 0,8). Since diastereomer **2a** is separated from **1a** and **1b**, and the ratio between **2a** and **2b** is known, this does not affect quantification. However, the separation of closely eluting **4a, 5** and **4b** and all remaining peaks is not affected. Therefore, the method is considered robust against the tested influences.

Ruggedness was tested by performing analyses of *spiked stressed reference solution* on two different Agilent HPLC systems on two consecutive days. Analyses performance of both HPLC systems were compared with regard to retention time, peak symmetry and resolution, and demonstrated reproducible results.

3.3 Substance identification

3.3.1 Degradation substances in stressed Trp solutions

The LC-UV/MS analysis of stressed Trp solutions (OC, 90 min, 121 °C, 2.1 bar, pH 5.5) revealed the masses of eight Trp degradation substances. The chromatograms of *stressed reference solution*, *standard model solution* and *spiked stressed reference solution* are shown in Fig. 22. MS analyses assigned the following ions to peak: **1a,b** *m/z 221*, **2a,b** *m/z 237* , **3** *m/z 209*, **4a,b** *m/z 221*, **5** *m/z 237,* Trp *m/z 205.*

The mass spectra of each pair **1a,b** and **4a,b** exhibit both *m/z 221* being the Trp mass (204 u) plus an increment of 16 u. This indicates an oxidation in different positions. Since **1a,b** and **4a,b** elute rather close to each other, they might be pairs of diastereomers. The early elution of **1a,b** (R_t:2.8, 3.9 min) in contrast to **4a,b** (R_t:8.9, 10.6 min) may indicate differences in polarity. Two highly relevant Trp oxidation products have been reported before: oxidation of the pyrrole ring in position 2 leads to the formation of the 2-OH-Trp diastereomers (*R,R* and *R,S* 2-amino-3-(2-oxoindolin-3-yl)propanoic acid (**4a,b,** Oia, Fig. 20) and has been reported e.g. as a preferred product of Trp treatment with H_2O_2 [32] or trichloromethyl-peroxide [33].

Additionally, the formation of Oia diastereomers **4a,b** in a ratio of approximately 1:1 has been pointed out in RP-HPLC analyses [22]. High abundances of the *m/z 221* ions for **4a,b** (Fig. 23a)) indicate the formation of rather stable ions. Sample spiking with synthesised **4a,b** confirmed the peaks to be Oia diastereomers.

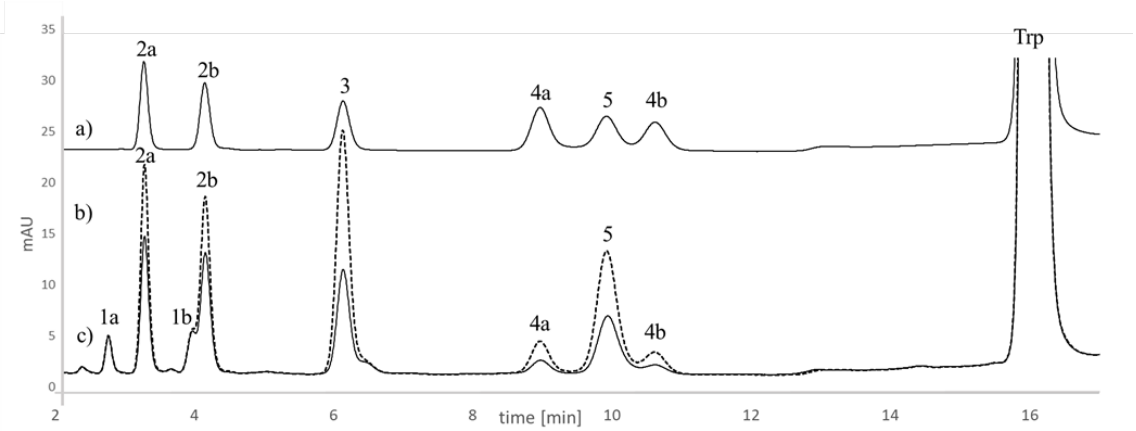


Figure 22: LC/UV chromatogram overlay of a) standard model solution, b) spiked stressed reference (dashed line), and c) stressed reference: peak, substance name (R_t): 1a,b; PIC diastereomers (2.8/ 3.9min), 2a,b; DiOia diastereomers (3.3/ 3.9 min), 3; Kyn (6.1 min), 4a,b; Oia diastereomers (8.9/ 10.6 min), 5; NKF (9.9 min), Trp (15.9 min).

The second Trp oxidation product reported with *m/z* 221 is the diastereomer *cis/trans* 3a-hydroxy-1,2,3,3a-8,8a-hexahydropyrrolo[2,3b]indole-2-carboxylic acid (**1a,b**, PIC, Fig. 20) [34], which is an three-ring system due to incorporation of the amine group into a second pyrrolidine ring. The increment of +16 u to Trp is due to the addition of a single oxygen as a hydroxy group. **1a,b** can be assigned to PIC because of the facile loss of -18 u and -46 u, which was has not been observed for **4a,b,** representing the loss of the water and the carboxylic acid group (*m/z 203* and *175,* Fig. 23 b)), respectively, being in accordance with literature [11]. The synthesis of PIC was made according to literature [35]. In-process LC-UV/MS analyses of the reaction mixture indicated low yields of the target substances **1a,b**, and purification was only partially successful. Using the mixture of 1a,b revealed the clear assignment of the degradation products at RT: **1a,b**: 2.8/3.9 min, respectively (Fig. 22). Nevertheless, the mixture could not be used for validation purposes.

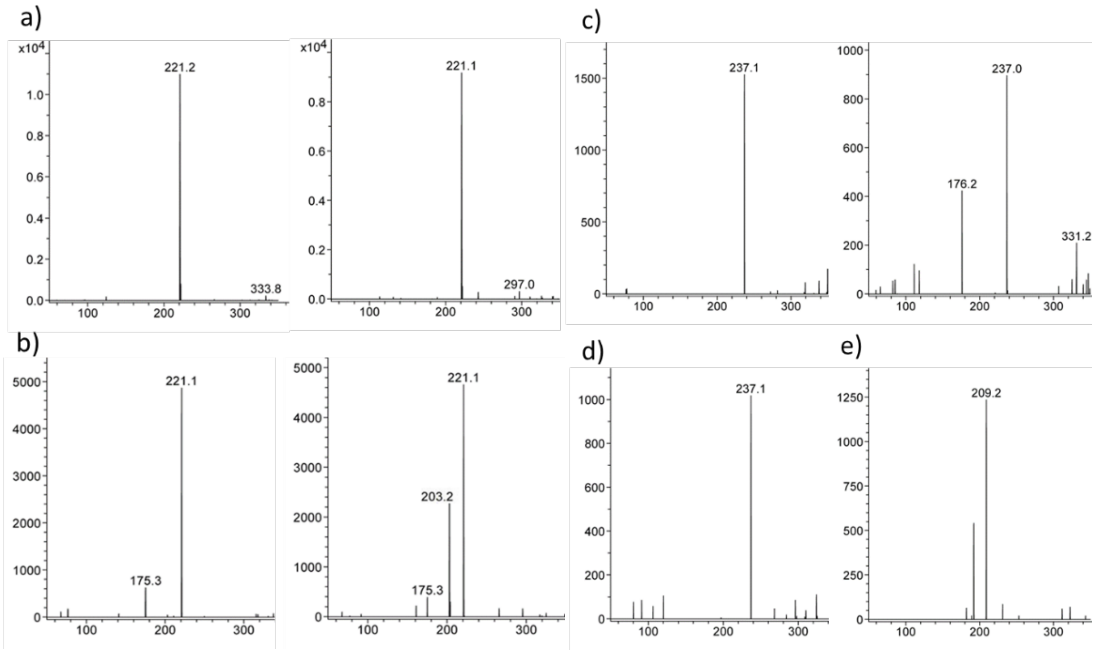


Figure 23: MS spectra of a) 4a,b ions with m/z 221.2 and 221.1, respectively. b) 1a,b ions with m/z 221.1, additional ions with m/z 175.3 and 203.2 indicate the loss of -COOH and -H_2O, respectively. c) 2a,b ions with m/z 237.1 and 237.0, respectively. d,e) 5 nd 3 ions ith m/z 237.1 and 209.2, respectively. The additional ion next to m/z 209.2 (m/z 192) indicate the loss of -NH_3, possibly caused by ionisation process.

Differentiation of the three ions with *m/z 237*, namely **2a,b** and **5** (Fig. 23 c, d) was supported by comparison of the UV-response. Only **5** was UV-active at λ = 254 and 340 nm, indicating a structural difference of **5** in comparison to derivatives with an intact indole backbone, as explained in section 3.2. The UV absorbance of **5** is in accordance with an *o*-

aminoacetophenone chromophore [27]. However, according to cited literature, three ions with *m/z 237* must be expected, depending on applied Trp stressing methods [7, 12, 15, 17, 36, 37]. One of them is *N*-formylkynurenine (2-amino-4-(2-formamidophenyl)-4-oxobutanoic acid; **5,** NFK, Fig. 20)) being an intermediate in Trp metabolism and a Trp oxidation product with Fenton´s reagent or photooxidation. [7, 37]. NFK has an *o*-aminoacetophenone backbone and thus corresponds to the chromophore characteristics of the peak of **5** [29, 30]. UV/Vis data of NFK are consistent with observed UV absorbances of **5** and can be assigned as NFK. Thus, the formation of **5** involves cleavage of the indole ring accompanied by the insertion of two oxygen atoms according to the mass increment of +32 u (Fig. 23 d)). The two other detected ions with *m/z 237* are a different kind of double Trp oxidation products, and were described as *R,R* and *R,S* 2-amino-3-(3-hydroxy-2-oxoindolin-3yl)propanoic acid diastereomers (DiOia; **2a,b**, Fig. 20). The formation of DiOia diastereomers was reported with e.g. UV-irradiation of oxygen saturated, alkaline aqueous Trp solutions in the presence of photosensitizers [15, 17]. Due to elution proximity of **2a** and **b**, the peaks relate to the DiOia diastereomers (Fig. 23 c)) [16] and were confirmed by spiking of stressed Trp solutions with the synthesised reference substances as shown in Fig. 22.

The proposed Trp degradation mechanism for the formation of NFK and DiOia comprises a shared precursor. The insertion of two oxygen atoms in positions 2 and 3 results in a 2-,3-Trp dioxetane intermediate (Fig. 24). The dioxetane is not stable and readily rearranges either into *R,R* and *R,S* DiOia or it undergoes pyrrole ring cleavage between C2 and C3, followed by the oxidation of C3 atom and formylation of the aromatic amine, resulting in **5**/NFK [15, 16, 38].

Peak **3** is also UV-active at $\lambda = 254$ and 340 nm, pointing to an *o*-aminoacetophenone structure similar to the degradation product **5**. The *m/z 209* ion of **3** (Fig. 23 e)) indicates a loss of the formyl group in **5**. Thus, **3** can be assigned to Kyn and was proven by reference substance. The *m/z 192* ion represents the loss of $-NH_3$ (-17 u), which is an expected reaction during ionization process [39]. *Kyn-* and *NFK reference solutions* were brightly yellow, and their colour intensified during storage. Hence, the discolouration of a Trp (-containing) solution can be assigned to Kyn and NFK formation. According to literature, NFK and Kyn are photosensitizers and may induce photodegradation of molecules including Trp and Trp oxidation products and themselves [40]. Thus, the photosensitizing properties may result in a reaction of unknown compounds of yellow colour and contributing to the progressing discolouration and degradation of aqueous Trp solutions.

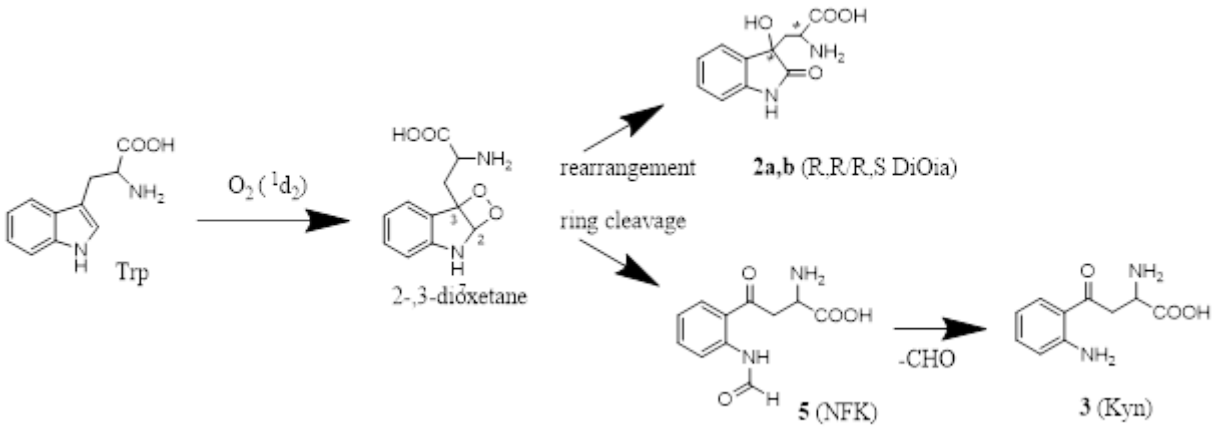


*Figure 24: Proposed degradation pathway of Trp. Insertion of molecular oxygen leads to the 2,3-dioxetane intermediate which readily rearranges into **2a,b** and/or **5,** which is a product of pyrrole ring cleavage. Deformylation of **5** results in the formation of **3** (Kyn).*

3.3.2 Assessment of Trp degradation products in *formulation 1* and *formulation 2*

The LC-UV method was used for the analysis of the parenteral AA *formulations 1* and *2*. Therefore, 50 mL aliquots of each product were transferred into glass bottles or freeflex® plastic containers for injectables which are multi-layered bags with functional films for stability and flexibility consisting of different blends of polyethylene and polypropylene. Glass bottles and plastic containers were subjected to steam sterilization by autoclave (10, 20, and 30 min,121 °C, 2.1 bar). Fig. 25 shows the chromatograms of a) unautoclaved *formulation 1* spiked with reference solutions **2a,b**, **3**, **4a,b**, **5** (100µg/mL), b) unautoclaved *formulation 1*, and 30 min autoclaved *formulations 1* and *2,* c) and d), respectively, sampled from glass bottles. Not autoclaved *formulation 1* shows exactly three peaks being the UV-active AA Tyr, Phe, and Trp. The chromatograms of stressed *formulations 1* and *2* samples only show seven out of eight identified degradation products (**1a,b**, **2a,b**, **4a,b**, **5)** because the Phe peak overlaps with compound **3** (Fig. 25 c, d). In order to assess **3**, the detector was set at λ = 340 nm, because Phe does not absorb above λ > 280 nm. At λ = 340 nm, the recovery of **3** was determined as 100 ± 0.4 % in spiked *formulation 1* solutions. Baseline separation could not be achieved for Tyr and **2a** (Fig. 25 a, c, d). However, the presented method provides symmetrical peak shapes at λ = 254 and 340 nm, and the evaluation of the peak calibration demonstrated, that peak heights are eligible and therefore chosen for the analyses of all degradation substances, respectively. The presented method facilitates a direct assessment of Trp degradation products in finished parenteral AA formulations. The results indicate that autoclaving of finished *formulation 1* and

formulation 2 in the presence of oxygen leads to Trp degradation as observed in pure Trp solutions. The evaluation of degradants with regard to product, stressing duration, and primary packaging after one year of storage is described in chapter 3.6.3.

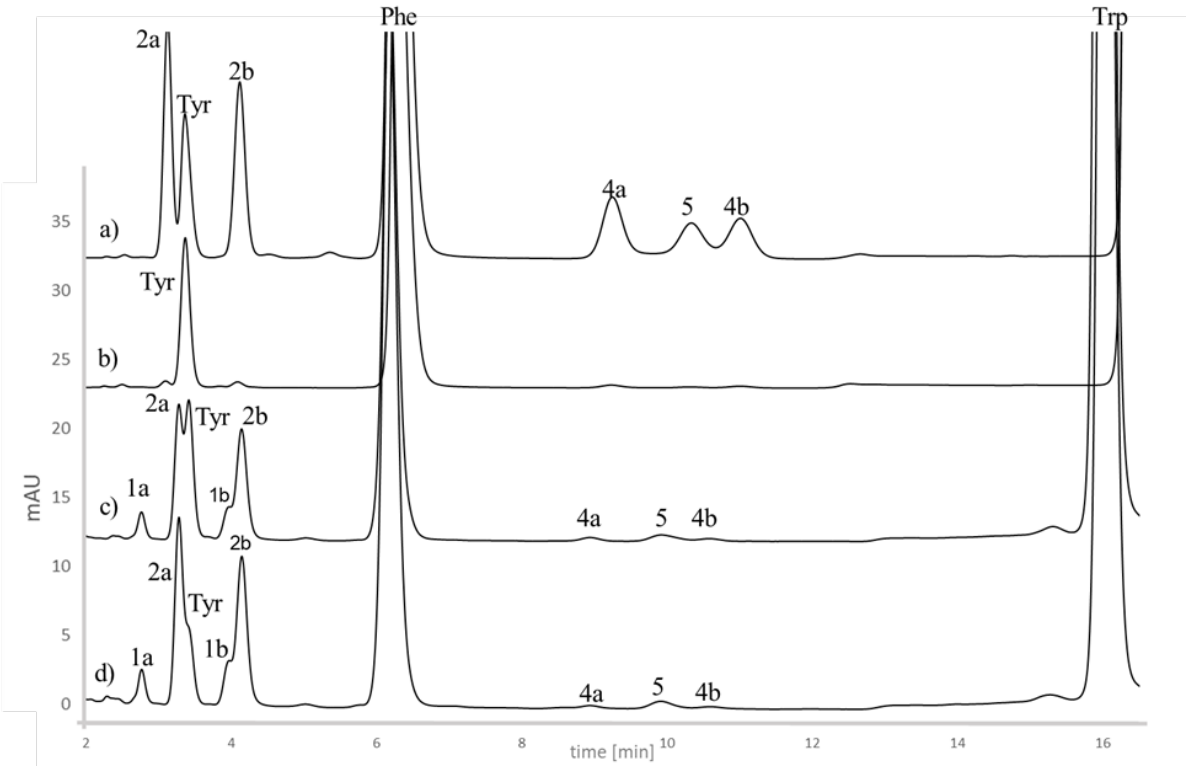


*Figure 25: Chromatograms of a) spiked, not-autoclaved formulation 1 (spiked with reference solutions **2a,b**, **3**, **4a,b**, **5** (100µg/mL), b) not-autoclaved formulation 1, c) stressed formulation 2, d) stressed formulation 1 (30 min, 121 °C, 2.1 bar, in parenteral plastic bags).*

3.4 Quantification of Trp degradation products

3.4.1 Degradation products in *stressed reference solutions*

The amount of each degradation product (**2a,b**, **3**, **4a,b**, and **5**) developing in the *stressed reference solution* were followed-up in freshly prepared samples, and four days, one and three years after autoclaving and are summarized in Tab. 10. The given percentages of impurities refer to a content of 2 mg/mL Trp solutions.

Therefore, the *reference solution* was autoclaved for 30 min at 121 °C, 2.1 bar in glass bottles and subsequently stored at RT and exposed to daylight. Analysis of the autoclaved samples was performed after cooling to RT and indicate the 8 degradation products **1a,b-5.** Thereof**,** only **4a,b** exceed the LOQ.

	conc. (µg/mL)							
Substance	**direct**	**SD**	**4 days**	**SD**	**1 year**	**SD**	**3 years**	**SD**
2a,b	<LOQ	-	21.7	0.01	37.9	0.001	37.2	0.003
3	<LOQ	-	8.0	0.06	12.5	0.002	11.1	0.001
4a,b	2.8	0.021	3.2	0.07	4.3	0.001	3.6	0.001
5	<LOQ	-	7.3	0.04	16.1	0.001	4.6	0.001
Σ	2.8		40.2		70.8		56.5	
% of Trp	0.14		2.0		3.5		2.8	

Table 10: Total amounts of degradation products in stressed reference solution, measured on the day of autoclaving and after 4 days, 1 and 3 years of storage, respectively. Samples were measured in triplicate (n=3).

Quantification of degradation products was done by comparison of peak heights using external calibration curves. The total amount of the diastereomers **4a,b** was determined as 2.77 µg/mL and equals 0.14 %. LC analyses demonstrated a time-dependent increase of all degradation products above the LOQ in 4 days, which continued during the first year of storage. The increase of total amounts of degradation products was determined at 2.0 % after four days and 3.5 % after one year, respectively. However, the major part of degradation products consists of substances **2a,b**, **3** and **5**, namely 92 % and 94 % after four days and one year, respectively. The significant increase of these three substances hints to the degradation mechanism as proposed in Fig. 24, being the preferred Trp degradation pathway with regard to long time storage. After three years of storage, the total amounts of degradants decreased to 2.8 %, mainly due to the loss of **5** (-71.4 %). According to the literature, the total loss of the degradation products can be chemically explained with facile deformylation of **5** into **3** in an acidic environment. Subsequently **3** is prone to photo-induced deamination and decarboxylation resulting the loss of **3** by the formation of 4-hydroxyquinoline, kynurenine yellow and kynurenic acid, explaining the loss of degradation substances in total [41].

3.4.2 Evaluation of the influence of pH, heating duration, and presence of oxygen on Trp degradation

The impact of pH value (5.0, 5.5, 6.0, 6.5) on Trp degradation was examined in OC and NP samples. Non-adjusted aqueous Trp solutions exhibit a pH of 5.3-5.5. Slightly elevated amounts of degradation products were found randomly either in solution of pH 5.5 and/or 6.5, but total

amounts of degradation products were roughly identical. Thus, the impact of the pH value is neglectable because of the narrow pH range of parenteral AA formulations.

In the OC solutions, the degradation products **2a,b-5** were formed after autoclaving. Applying HPLC-UV analysis, most of the degradation products were below the LOQ. By means of LC-UV/MS analysis (Fig. 26 a-c), the relationship between autoclave duration and degradation can clearly be seen from the extracted ion chromatogram and it is obvious that a longer autoclave time results in a higher amount of degradation products which is not surprising. The assessment of degradants after one year of storage demonstrates converging amounts of degradation substances, regardless of initially applied autoclave duration, as summarized in Tab. 11, the total sum of degradants eventually equals 3.5-3.6% and applies to autoclave durations of 10, 20, and 30 mins. Hence, heating duration may accelerate Trp degradation in the short term, but it is not the most influencing factor for Trp degradation in long-time storage periods in the presence of oxygen.

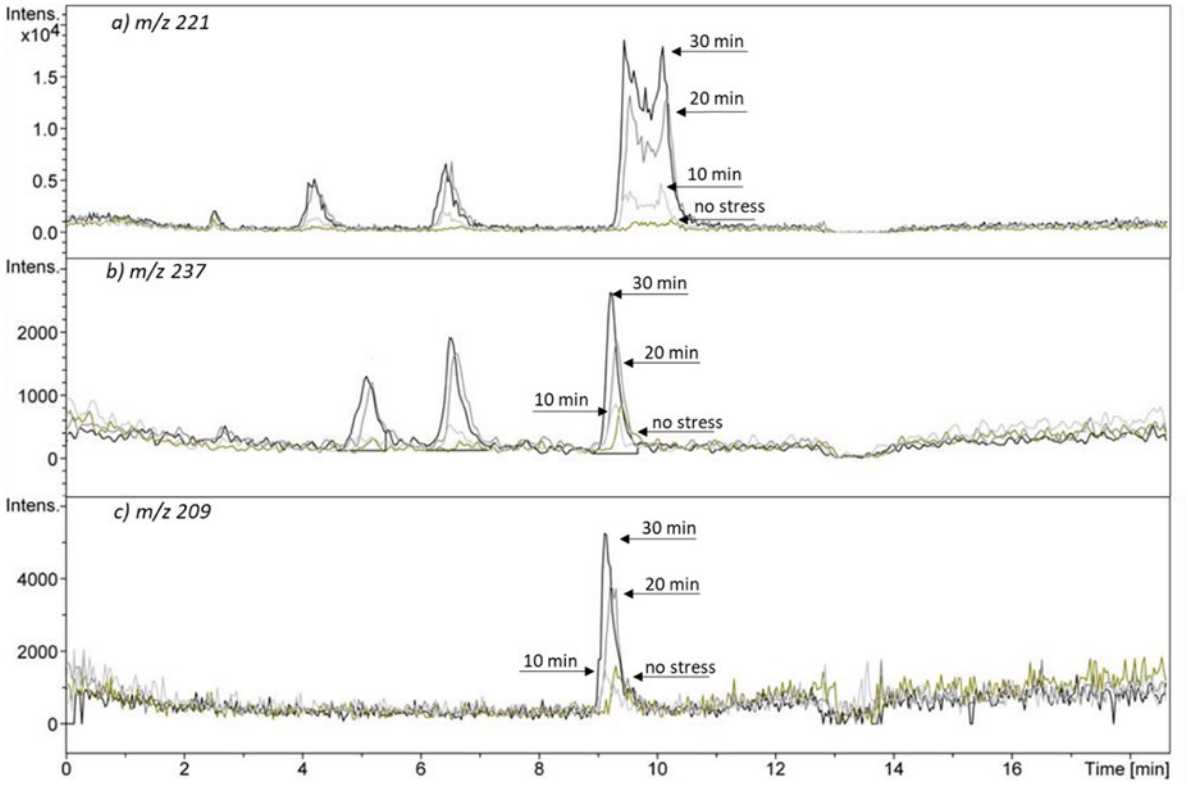


*Figure 26: Overlay of EICs of a) m/z 221 (**4a,b**), b) m/z 237 (**2a,b**) and c) m/z 209 (**3**). The increasing ion intensities correlate with stressing duration (no stress, 10, 20, 30 min) of the reference solutions.*

The degradation products in NP samples were evaluated and compared to OC samples. Therefore, samples were analysed which were autoclaved for 30, 60, and 90 min and which have already been stored for three years and compared to unautoclaved control samples. The

results are summarized in Tab. 12. After three years of storage, not autoclaved NP samples showed no degradation products. In general, Trp degradation is strongly reduced in NP samples being 0.75-1.7 % vs 2.3-4.4 % in OC samples, mainly because of significantly lower amounts of **2a,b**, **3,** and **5**. Despite this, the amounts of **4a,b** are still similar being 2.2-3.3 % in OC and 2.8-3.6 % in NP samples, respectively. Thus, nitrogen purging as a method of nearly complete oxygen expulsion impairs Trp degradation.

	conc. (µg/mL)						
Substance	**10 min**	**SD**	**20 min**	**SD**	**30 min**	**SD**	**Ø %**
2a,b	38.9	0.001	37.6	0.001	37.5	0.001	1.9
3	12.7	0.004	12.3	0.004	12.6	0.003	0.6
4a,b	3.4	0.002	4.9	0.001	4.5	0.002	0.2
5	15.6	0.003	14.7	0.002	17.9	0.002	0.8
Σ	70.6		69.5		72.5		3.5
Σ %	3.5	±0.31	3.5	± 0.27	3.6	± 0.34	

Table 11: Degradation products in stressed reference solutions autoclaved (121 °C, 2.1 bar, 10, 20, 30 min) analysed 1 year after autoclaving, stored at RT, exposed to day light. The sum (Σ) gives the total amount of degradation products with regard to Trp concentration (Σ %). The mean degradation rate is 3.5% in a 2 mg/mL Trp solution. During the storage of 1 year, the degradation amounts are similar regardless of applied autoclave duration.

OC			conc. (µg/mL)					
Substance	**0 min**	**SD**	**30 min**	**SD**	**60 min**	**SD**	**90 min**	**SD**
2a,b	28.2	0.0013	45.9	0.0041	46.6	0.0062	61.3	0.0033
3	15.6	0.0002	14.4	0.0008	13.9	0.0007	18.1	0.0013
4a,b	1.0	0.0002	2.2	0.0002	2.5	0.0002	3.3	0.0001
5	0.5	0.0012	3.6	0.0012	3.5	0.0013	5.1	0.0001
Σ	45.3		66.1		66.5		87.8	
Σ %	2.3	± 0.12	3.3	± 0.25	3.3	± 0.18	4.4	± 0.21
NP				conc. (µg/mL)				
2a,b		-	10.1	0.0029	10.7	0.0032	23.9	0.0019
3		-	1.7	0.0005	1.8	0.0004	4.3	0.0004
4a,b		-	2.8	0.0004	2.4	0.0005	3.6	0.0007
5		-	0.4	0.0001	0.4	0.0002	2.1	0.0004
Σ		-	15.0		15.3		33.9	
Σ %		-	0.75	± 0.43	0.77	± 0.41	1.7	± 0.22

Table 12: Total and percentual amount of Trp degradation products in not stressed and autoclaved OC and NP samples (2 g/L Trp, 121 °C, 2.1 bar, 30, 60, 90 min). Samples were measured in triplicate (n =3).

Though, it has to be mentioned that 100% oxygen exclusion could not be realised since minimal ventilation during sampling was unavoidable. However, the amounts of **2a,b** are prevailing among the degradation products in all samples, regardless of the their treatment. The data suggests that Trp degradation follows two concurrent degradation pathways. The first pathway is Trp oxidation to **4a,b**, which does not depend on autoclave duration, high or low levels of oxygen or storage time, but may be accelerated by heat impact anyway. The second pathway (Fig. 24) results in **2a,b**, **3,** and **5**, depends strongly on higher levels of oxygen and less on autoclave duration. **2a,b** is the most abundant of all degradation substances, but reference solutions using **2a,b** were not coloured. The high abundance may be explained by the photo-sensitizing properties of **3** and **5**, which favours the double oxidation of Trp and the formation of **2a,b** in aqueous solutions [40]. Hence, UV-light may further induce Trp degradation already in the presence of small quantities of **3** and **5** [42]. Taken together, UV irradiation in day light affects the oxidation of Trp and should be considered during manufacturing, storage, and administration of aqueous AA solutions containing Trp.

3.4.3 Evaluation of Trp degradation products in formulation 1 and 2 stored in glass bottles and plastic bags

Formulation 1 and 2 samples, stored for one year after autoclave (10, 20, 30 min, 121 °C, 2.1 bar) either in glass bottles or plastic bags for injectables, developed a yellow discolouration proportional to autoclave duration and similar to pure Trp solutions. No difference in colour was observed with regard to the type of container used, but the yellowing was more intense in *formulation 2* than in *formulation 1*. HPLC analysis showed increased Trp degradation to substances **2a,b-5** within one year storage after autoclave. No new peaks were detected neither at $\lambda = 254$ nor at $\lambda = 340$ nm. The degree of degradation in glass bottles was found to be only slightly higher in *formulation 2* than in *formulation 1* (2.8-6.4 % and 2.5-4.0 %, respectively) which is in line with the findings of 3.5% for pure Trp solutions.

Furthermore, storage in plastic bags led to elevated amounts of degradation, i.e. 5.2-5.3% and 7.1-7.7 % in *formulation 1* and *2* respectively, as summarized in Tab. 13. Due to the similar degradation rates of pure Trp solutions, *formulation 1* and *2*, it can be said that the total amount of Trp degradation is not per se affected by the presence of other AAs. Taken together, both glass bottles and plastic bags were stored under the same conditions at RT exposed to day light.

Trp degradation was found to be significantly increased when autoclaved and stored in plastic bags. The average percentual degradation was 1.6 times higher in plastic bags than in glass bottles which equally applies to *formulation 1 and 2.* The reason for increased degradation in plastic containers might be a time-depended alteration of oxygen permeability of the plastic material over time, especially when exposed to UV-light.

The more intense yellowing of *formulation 2* can be explained by the small but consistently higher Trp degradation rate in comparison to *formulation 1*. However, the reasons for this observation are not clear and there is no evidence that kynurenines (**3** and/or **5**) support a photosensitized cysteine oxidation (included in *formulation 2* only) which has been supposed in the literature [41, 43].

	10 min	**S.D.**	**20 min**	**S.D.**	**30 min**	**S.D.**
Formulation 1 bottle						
Σ 2a,b-5; (µg/mL)	50.1	0.11	67.3	0.08	80.2	0.01
Degradation rate	2.5 %	± 0.21	3.4 %	± 0.12	4.01 %	± 0.01
Formulation 1 bag						
Σ 2a,b-5; (µg/mL)	103.2	0.08	105.9	0.37	105.1	0.25
Degradation rate	5.2 %	± 0.08	5.3 %	± 0.35	5.3 %	± 0.23
Formulation 2 bottle						
Σ 2a,b-5); (µg/mL)	39.5	0.21	54.1	0.08	89.5	0.13
Degradation rate	2.8 %	± 0.52	3.9 %	± 0.14	6.4 %	± 0.16
Formulation 2 bag						
Σ 2a,b-5; (µg/mL)	99.7	0.16	100.6	0.33	107.9	0.16
Degradation rate	7.1 %	± 0.16	7.2 %	± 0.32	7.7 %	± 0.15

Table 13: Total and percentual amount of Trp degradants in formulation 1 and 2 stressed in glass bottles and in polyolefin bags, assessed after 1 year (121 °C, 2.1 bar, 10, 20, 30 min, n =2)

Typical plastic bags for parenteral formulations are multilayers of different polyolefin blends (i.e. polypropylene/polyethylene), each with different properties and functions [44]. Advantages of plastic bags over glass bottles are lower production costs, safety and flexibility. Additionally, the utilization of plastic containers requires impact resistance, heat/steam sterilizability, high transparency, compatibility with pharmaceutical ingredients and safety with regard to leachables or extractables and must be free from additives and plasticizers like PVC

[45]. The multilayer principle includes at least two inner layers consisting of a polymer blend with high flexibility and lower melting points (<121 °C), whereas the polymer blends used in the outer layers have stabilizing and supporting functions with higher melting points (>121 °C). Inner and outer layers must be arranged alternately. The layering combines required container demands and preserve high product quality. Material testing includes steam autoclavation at 121 °C for 35 min and demonstrated a general loss of tensile impact, toughness only of the inner layer alterations due to an increase of polymer crystallinity [44]. However, the outer layers are heat resistant and satisfy requirements towards the product, in general [44]. In this context, it may be possible that alterations of the multi-layer system due to heat sterilization increase over storage time and facilitate oxygen permeability to a certain degree. To our knowledge, the impact of UV light irradiation has not been evaluated in the long term. Eventually, clinical guidelines restrict the administration of injectables to a maximum of 24 h due to hygienic reasons [46, 47]. Thus, administration of steam sterilized solutions in plastic containers may be considered safe within short time periods if used with a light protection sheath. Despite the economic advantages of plastic containers, general processing and bulk preparation of parenteral AA formulations in plastic bags cannot be recommended at this point.

4 Conclusion

The essential AA Trp is an important compound in parenteral AA solutions, but it is prone to degradation, which is reflected by yellow discolouration. Thus, Trp degradation in aqueous solutions after autoclaving with regard to the presence of oxygen, heating duration, and storage time was studied. The results are applicable to parenteral AA formulations containing Trp which were steam sterilized and stored in two common types of primary packaging for parenteral drug formulations. It could be shown that the discolouration of stressed solutions is related to the decomposition of Trp and is rather caused by an increased oxygen level than by autoclaving duration in the given pH range, all over an observation period of three years. The method developed provides validated results about the extent of Trp degradation under specific conditions and is appropriate for quality testing of parenteral AA solutions. Characterization of degradation products suggest two competing Trp degradation pathways favoured by the presence of oxygen and/or light irradiation. Especially the formation of the two photosensitizing substances Kyn and NFK (**3** and **5**), is strongly associated with the yellowing process which might further induce Trp degradation under light exposure. Hence, unconditional UV-light protection during manufacturing, processing, and storage may be as important as strict oxygen deprivation for Trp stability in product quality control. Instructions for administration of

parenteral AA formulations shall be complemented likewise. Eventually, the findings do not indicate any influence of other AA on Trp degradation per se. However, different degrees of discolouration are noticeable with varying AA composition in *formulation 1* and *formulation 2* and thus may be subject of further investigation.

5 Acknowledgement

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3.3 Application and comparison of different RP-HPLC separation techniques and column materials

For the separation of Trp degradation products, three different RP-HPLC methods were developed and compared with regard to their suitability and performance.

First, a stability indicating HPLC method was developed and validated using a traditional endcapped C18-column (*Method A 1,* [110]). A second separation technique makes use of ion pairing (IP) reagents on a reversed phase with the same column parameters. Two IP-RP-HPLC methods were developed using the IP reagents perfluorinated butanoic and pentanoic acid, respectively. IP-RP-HPLC promises improved retention and separation of small, charged and polar compounds such as AAs and related substances and was therefore chosen for the development of the HPLC methods.

The third method was developed on a pentafluorophenyl (PFP) phase. The PFP phase is a modified reversed phase. The C-18 alkyl-chains are replaced by perfluorinated phenyl groups. The PFP phase offers an alternative mode for the retention and separation of polar and charged molecules in comparison to IP-RP-HPLC.

All three developed methods exclude derivatization steps, use UV/Vis detection and deliberately facilitate MS detection. The aim of the method development is the comparison and the evaluation of different (orthogonal) RP separation techniques upon practicality, suitability and performance pointing out the challenges, advantages and disadvantages. Furthermore, the application of orthogonal methods is important in order to demonstrate, if there are any other degradation products, which would stay undetected using only one analysis method. Obtaining similar results, in turn, confirms the conclusions done so far. The results will be described and evaluated in the following section.

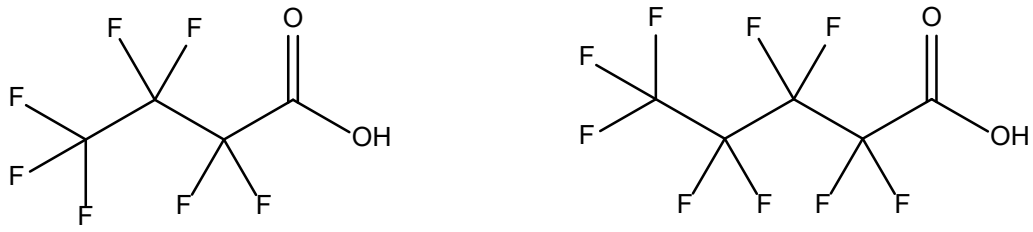
3.3.1 IP-RP-HPLC

IP-HPLC is advantageous technique for the analyses of small, organic, polar and charged molecules such as AAs. Successful applications of IP-RP-HPLC for the assessment of AAs have been described since the pioneer work developed by Eksborg and Schill in 1973 [111-113]. Other IP-HPLC methods for the assessment of AAs are covered in the introduction [77]. Typical IP reagents are salts or charged compounds (depending on pH of the mobile phase) with a variable *n*-alkyl-chain, thus they combine lipophilic and hydrophilic properties. The actual mechanisms working in IP-RP-HPLC are still unknown, but a dual interaction between

IP and the stationary phase (adsorption model) and between IP reagents and the analyte (partition model) is presumed. IP reagents exert ionic interactions with the polar and/or oppositely charged analytes and lipophilic interactions between the alkyl-chains and the stationary phase. Here, the AAs or Trp degradation products form ion pairs with the IP reagents. These IPs are kind of neutral on the surface and the lipophilic rest enhances interactions with the stationary phase resulting in better retention and selectivity [114]. Furthermore, the *n*-alkyl-chains of free IP reagents interact with the C-18 alkyl-chains of the stationary phase, so that the charged moiety is free for ionic interaction with free analytes, resulting in a kind of ion exchange mechanisms, adding to the retention performance. A variety of additives and buffers can be used in water-rich mobile phases, considering the specifications of used instruments and analytes [115].

The ion-pairing reagents must be volatile, in case of online-coupled mass detection or other evaporative detectors such as the evaporative light scattering detector (ELSD), or the corona charged aerosol detector (CAD). Perfluorinated carboxylic acids are strong acids, they are volatile and have been described in several studies as suitable IP reagents for the assessment of AAs on different column materials and named detectors [116-119].

Heptafluorobutyric acid (HFBA) and nonafluoropentanoic acid (NFPA) (Fig. 26) were chosen as IP reagents for the development of the HPLC-UV/MS *Method B* and *C* (7.5. HPLC methods), respectively.



HFBA *NFPA*

Figure 26: IP reagents used for the development of an IP-RP-HPLC-UV/MS method for the separation of Trp degradation products.

Fig. 27 and 28 show comparative overlays of a) the UV chromatogram (λ 280 nm), b) the TIC and c-f) the BPCs (base peak chromatograms) of *Method B* and *Method C,* respectively. The analysed sample was a stressed 2 mg/mL Trp solution, pH 6.5, 30 min, 121 °C, 2.1 bar, diluted 1:10 with mobile phase A.

The developed methods give similar chromatograms. However, differences regarding peak shapes, separation quality, as well as the practicality are discussed in the following section.

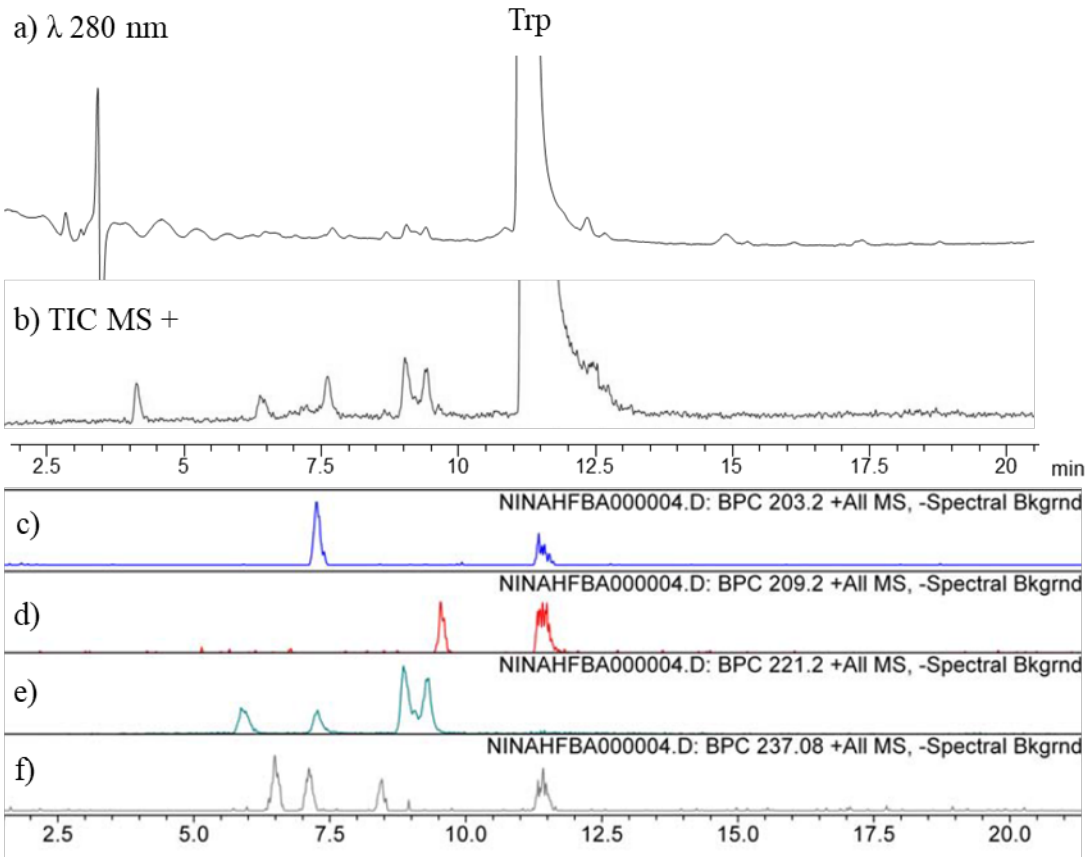


Figure 27: Method B: a) UV chromatogram, b) TIC and c-f) BPC (base peak chromatogram) of c) m/z 203.2; dehydrated cis PIC, d) m/z 209.2; Kyn, e) m/z 221.2; trans/cis PIC and Oia a,b, f) m/z 237.08; DiOia a,b and NFK. Sample: stressed Trp solution (2 mg/mL, pH 6.5, 30 min, 121 °C, 2.1 bar)

Trp elutes at 11.2 min and Trp degradation products elute between 5.8-9.1 min with following order: *trans* PIC, DiOia a, *cis* PIC, DiOia b, NFK, Oia a,b and Kyn (Fig. 27). The peak in Fig.27 b) at 4.1 min is an unknown peak and most probable due to contamination of the sample and was not found in other samples or sequences.

The ion with *m/z* 203.2 at R_t 7.4 min is no individual Trp degradation product, but it emerges due to facile water loss (-18 u) during ionization process of the *cis* diastereomer of PIC (*trans/cis* PIC R_t 5.8/7.4 min, *m/z* 221.2) and has been discussed in chapter 3.2. (3.3.1. Substance identification) and in ref. [62]. Therefore, an individual screening for the *m/z 203.2* ion was not included in *Method C* anymore (Fig. 28). The emergence of MS signals

simultaneously with Trp at R_t 11.2-12.5 min (Fig. 27 c, d, f)) may be due to decomposition reactions during the ionisation process and/or co-elution of remaining compounds due to the rising organic content of the gradient and were neglected hereinafter. The comparison of the elution times of the degradation products demonstrates the close structural relationship of the diastereomers *trans/cis* PIC and DiOia a,b, eluting between 5.8-7.4 min and of NFK, Oia a,b and Kyn between 8.0-10.0 min, respectively. In the latter group the UV signals are hardly to differentiate at λ 280 nm. Though the absorption maxima of Trp is at wavelength λ 280 nm, it is inferior for the assessment of Trp degradation products. The wavelength λ 254 nm would have been the better choice with regard to Trp degradation products but was not tested at the time of analyses.

However, in terms of detection, the shortcomings of UV detection and the separation performance can be mitigated in parts by MS detection. Here it is limited to MS signals of rather low intensity. <10.000, which may be related to both, the low abundance of the Trp degradation products and quenching effects because of ion pairing, being a known disadvantage of IP reagents for MS detector sensitivity [116, 120].

Method C was developed using NFPA in order to investigate the effects on the selectivity and separation of an analogue IP reagent with a longer *n*-alkyl chain. Due to stronger adhesion of NFPA itself and NFPA ion pairs, increased retention and run times, and even peak tailing would have to be expected. The composition of the mobile phases had to be adjusted with regard to increased organic content, adjustment of the pH and buffer molarity. Acetonitrile was added to methanol for its greater elution capacity, 30mM ammonium formiate buffer was introduced, and the gradient was adjusted by increasing the organic content. The adjustments were successful in terms of comparable analyses time and results.

As shown in Fig. 28, Trp elutes at 13 min with *Method C* and the Trp degradation products elute between 5.5-11.8 min, which are similar retention times compared to *Method B*.

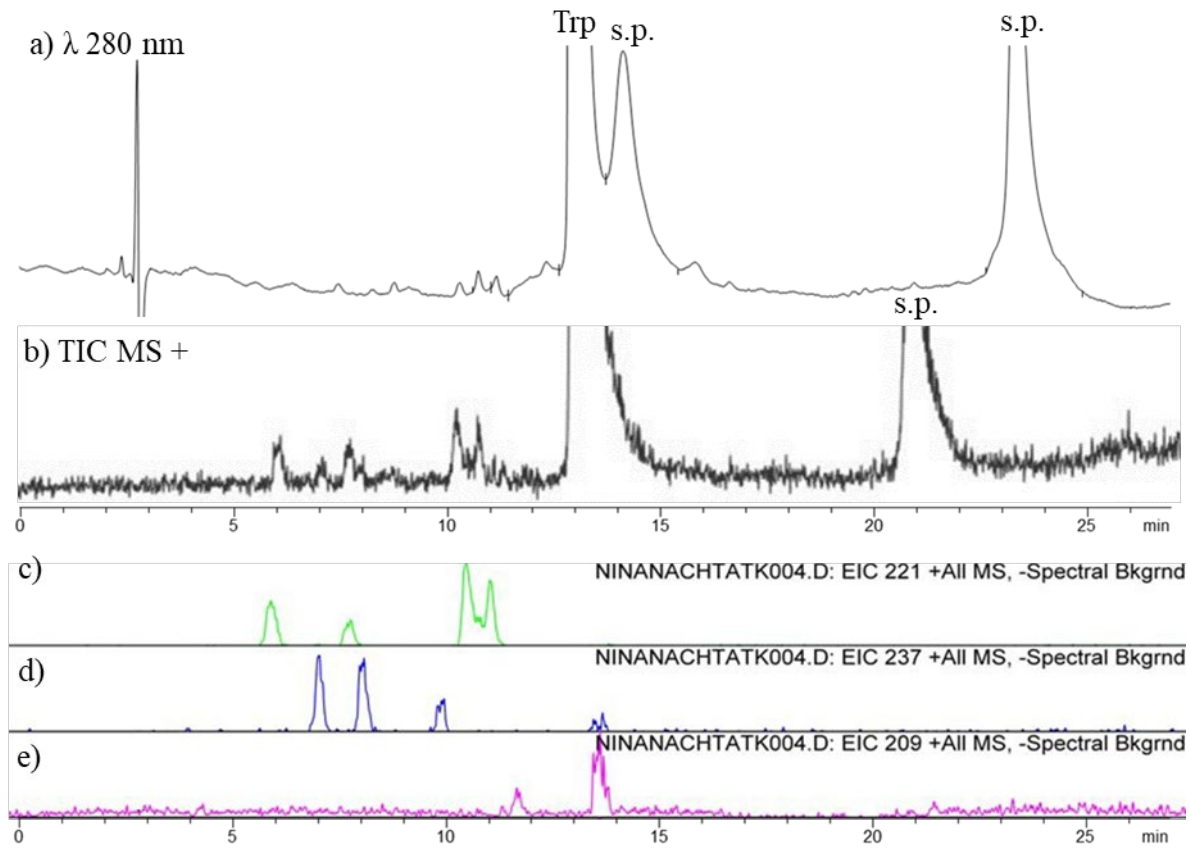


Figure 28: Method C: a) UV chromatogram, b) TIC and c-e) EIC (extracted ion chromatogram) of c) m/z 221.2; trans/cis PIC, d) m/z 237.08; DiOia a,b and NFK, e) m/z 209.2; Kyn, in a stressed Trp solution (2 mg/mL, pH 6.5, 30 min, 121 °C, 2.1 bar) analysed with Method C. Abbrev.: s.p.: system peak.

The comparison of elution shows that the degradation substance elutes more closely, and the elution order switched for *cis* PIC and DiOia b only. Other differences were observed with regard to overall feasibility and method performance. The baseline noise with UV/Vis and MS detection was significantly higher with NFPA as with HFBA. Furthermore, three system peaks emerged at R_t 14.6, 21.1 and 23.9 min, which could not be assigned to any other substance. The re-equilibration time needed to be increased for *Method C* and regular flush runs with high organic contents were necessary to clean the LC system. The ion suppression in MS detection increased during the cumulation of analyses runs and more frequent cleaning and signal calibration of the MS source was required despite of the volatile character of the used IP reagents.

3.3.2 RP-HPLC with PFP-stationary phase

Pentafluorophenyl (PFP) columns belong to the group of RP-stationary phases with modified properties due to covalently bonded perfluorinated phenylpropyl groups (Fig. 29). PFP-columns are a promising alternative to traditional C-18 or C-8 phases because the modification allows the combination of several polar and non-polar interaction mechanisms. The proposed mechanisms are claimed to provide an orthogonal selectivity with normal and reversed phase characteristics, which are especially suitable for the analyses of polar pharmaceuticals and biological samples [121, 122]. Hydrophobic bonding, electrostatic, hydrophobic, aromatic π-π charge-transfer, as well as steric/planar mechanisms are discussed [123]. PFP-phases provide larger capacity for the separation of aromatic, polycyclic and heterocyclic compounds [122], thus being eligible for the separation of the identified Trp degradation products DiOia, Oia and PIC diastereomers, Kyn and NFK, respectively. The low surface energy of fluorinated phases reduces interaction between hydrophobic compounds; and this contributes, together with the proposed retention mechanisms, to sharper peak shapes and enhanced selectivity.

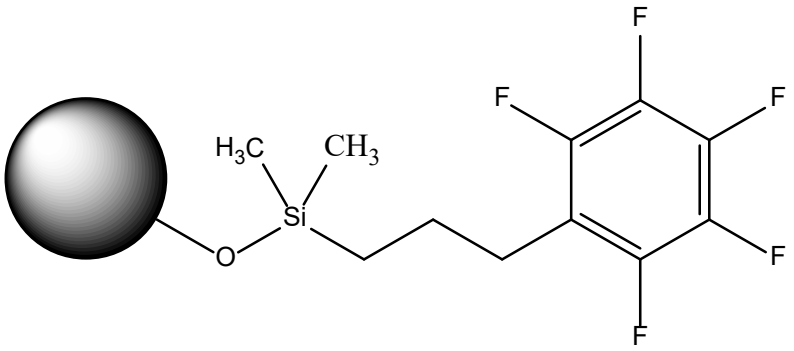


Figure 29: Scheme of a PFP stationary phase. The pentafluorophenyl ligand is bonded covalently via a propyl-spacer to the silica/polymer column.

The PFP column can interact as a reversed phase or as a normal phase, depending on the mobile phase composition used, i.e. high water or high organic content, respectively. This phenomenon is the so-called the "u-shape" retention of perfluorinated phases [124]. Using PFP-phases as a normal phase is comparable to HILIC techniques, which are applied for the assessment of small organic and polar compounds [77, 123]. However, several LC and/or LC-MS methods using perfluorinated stationary phases have been published recently for the assessment of amines, phenolic compounds, vitamins and small acids [125-128].

A Kinetex™ Core-Shell silica PFP-column with 100 Å, 2.6µm particle size and 150 x 4.6 mm i.d. (Phenomenex® Inc., Aschaffenburg, Germany) was used for the development of *Method D 1*. The elution was performed using gradient elution with water and methanol and 0.15 % formic acid as acidifying agent. The *spiked stressed reference solution* (7.3 Sample preparation) was analysed with *Method D 1* and the UV chromatograms at λ 254 and 340 nm, as well as the EICs of Trp degradation products are shown in Figure 30.

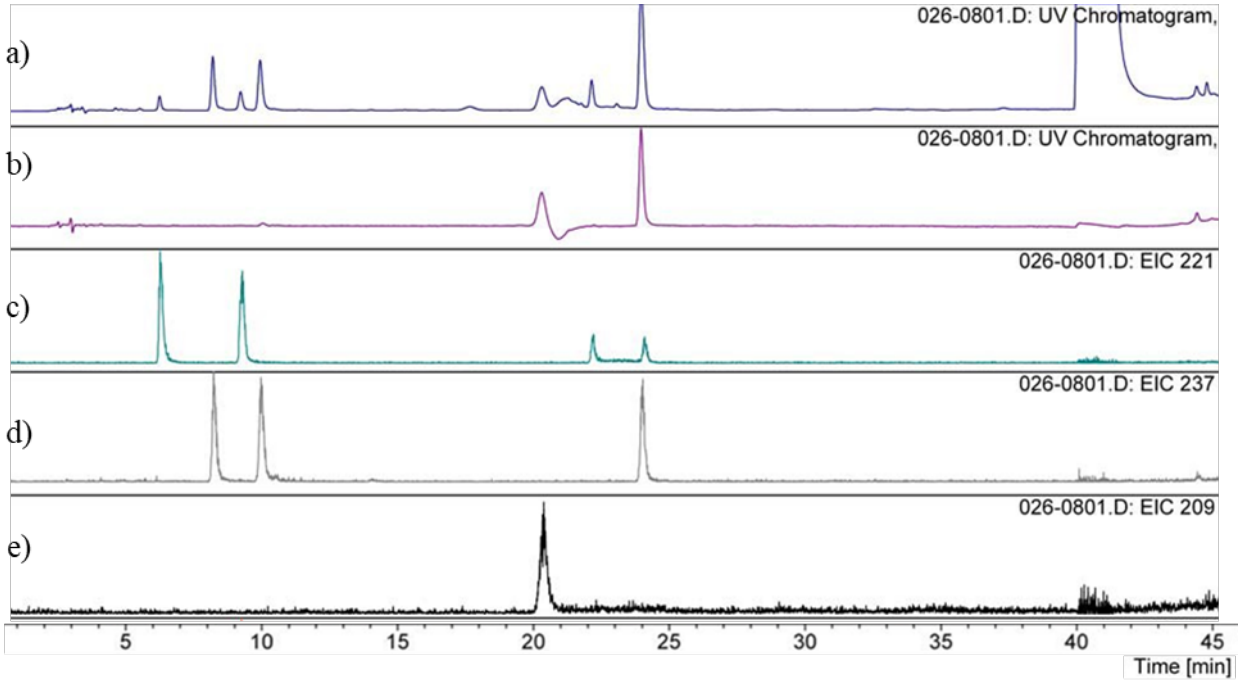


Figure 30: a) λ 254 nm UV chromatogram, b) λ 340 nm UV chromatogram, c-e) EICs (extracted ion chromatogram) of c) m/z 221; trans/cis PIC and Oia a,b, d) m/z 237; DiOia a,b and NFK, e) m/z 209; Kyn, in the spiked stressed Trp solution (2.3.1. sample preparation) analysed with Method D 1.

The wavelengths λ 254 and 340 nm were chosen, due to overall best sensitivity at λ 254 nm for all Trp degradation substances and additionally at λ 340 nm for Kyn and NFK. The degradation products *cis/trans* PIC, Oia a,b, DiOia a,b, Kyn and NFK are separated from each other and from Trp with one exception. Oia b and NFK coelute at R_t 24.3 min. In comparison to IP-RP-HPLC, the elution order of Kyn and NFK changed. With IP-RP-HPLC analyses NFK was eluting prior to Oia a and b, which were followed by Kyn. Probably, the polarity and electronegativity of the formylgroup of NFK is responsible for stronger retention due to hydrogen bonding and dipol-dipol interactions with the PFP groups. However, the predominant

retention mechanism for all degradation products is most likely the π-π interaction between the PFP groups and the heterocyclic groups, aromatic indole and/or *o*-aminoacetophenone groups, respectively. Furthermore, the UV chromatograms in Fig. 30 a) and b) show a striking baseline drift at 21-22 min, which may be explained as a delayed consequence of pressure fluctuations due to the increased methanol content (mobile phase B) in gradient elution. Baseline fluctuations were no longer observed after method optimization. Eventually, other possible reasons for the baseline drift can be contaminated eluents or contaminants on the stationary phase, which eluted gradually.

The method was optimized with regard to column temperature, introduction of buffers and pH adjustments aiming at the separation of coeluting NFK and Oia b. The optimized method *Method D 2* was tested for the analysis of Trp degradation products in the finished pharmaceutical products and is shown in Fig. 31. Fig. 31 a) shows the UV chromatogram at λ 254 nm of the *Formulation 1* as it was obtained from Fresenius Kabi AG (Bad Homburg, Germany), b) additionally stressed *Formulation 1* (glass bottle, 30 min, 121 °C, 2.1 bar) and c) *spiked stressed reference solution.* However, only little improvement could be achieved with regard to the separation of Oia b (4b) and NFK (5). Optimization efforts included a temperature change to 7 °C, the introduction of 30 mM ammonium formate buffer and pH adjustment with formic acid to an apparent pH of 3. Methanol content was kept the same as in *Method D 1* at 5 % in mobile phase A. Formic acid content was increased from 0.15 to 0.25 % in mobile phase A and B, respectively.

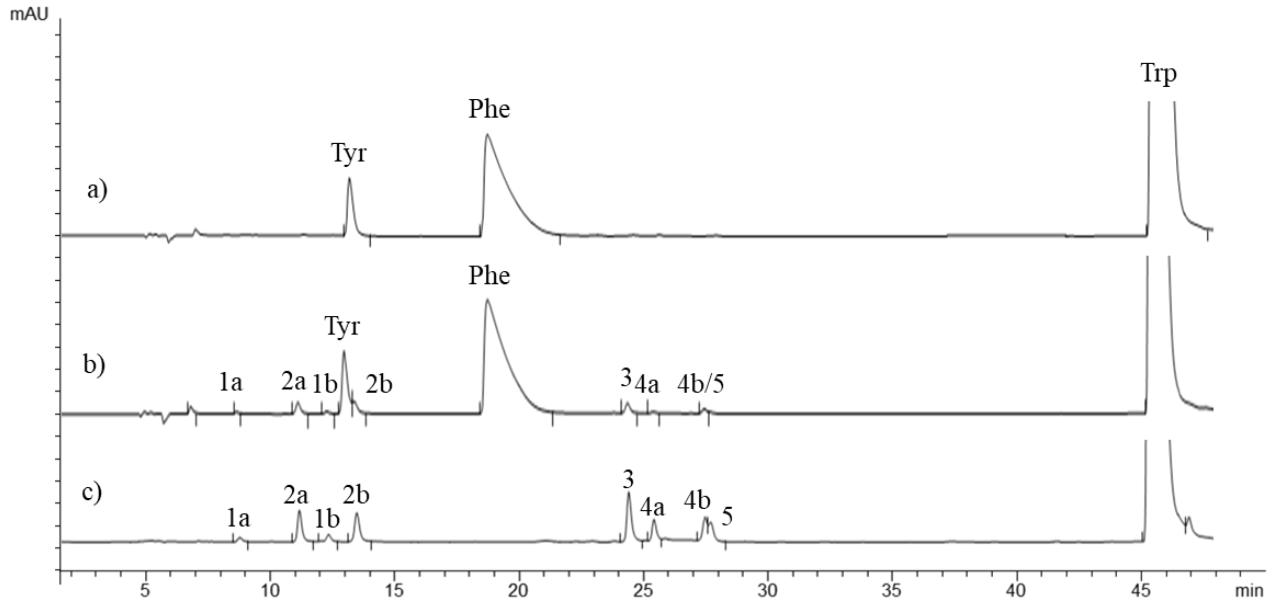


Figure 31: Overlay of the UV chromatograms at λ 254 nm analysed with Method D 2: a) Formulation 1, b) additionally stressed Formulation 1 (glass bottle, 30 min, 121 °C, 2.1 bar), c) spiked stressed reference solution (glass bottle, 30 min, 121 °C, 2.1 bar).

Fig. 31 b) shows that the AA Tyr elutes between *trans* PIC (1b) and DiOia b (2b) and impedes correct peak area assessment of 2b. However, this is neglectable, because the formation of the diastereomers DiOia a and b was repeatedly observed with a peak ratio of 52:48. Thus, the assessment of only one diastereomer is sufficient.

The Phe peak shows noticeable peak tailing in comparison to Tyr, although the structural difference is only an additional hydroxyl group at the phenol moiety in Tyr. This peak tailing may be explained by the high concentration of Phe - being 5.1 g/L in comparison to Tyr 0.4 g/L and Trp 2.0 g/L in F*ormulation 1*. Furthermore, the gradient is not steep enough and the organic content is too low for a sharp peak elution. Changing to a steeper gradient is not an option, because a small increase in organic content will lead to an accelerated and simultaneous elution of the substances Kyn, Oia diastereomers and NFK.

Lower column temperatures (RT, 20, 15, 10, 7 °C) pronounces rigidity of the stationary phase and showed to be beneficial for better peak shape and resolution. Better results were observed at 7 °C. However, the consequences of low temperatures were elevated backpressure and consequently long re-equilibration times and compromised robustness.

Method development and optimization showed that the PFP-column material is powerful and suitable for the separation of Trp degradation products in pure Trp solutions and in the presence

of other AAs (*Formulation 1).* However, the applied conditions implied long analysis and re-equilibration times, consequently relatively high mobile phase consumption, high backpressure and a lack of robustness.

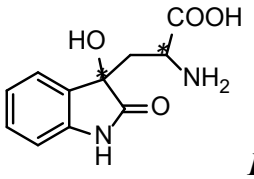
4 Final discussion

The studies performed aimed at the evaluation of Trp stability. This included the establishment of a degradation substance library and the development of a stability indicating method, which might be used in the manufacturing, processing and quality control of aqueous formulations containing Trp, such as parenteral nutrition solutions. The investigation of Trp degradation was studied under set conditions, limited to practical aspects and process-orientated, the identification of degradation substances and the development and comparison of suitable assessment methods. Special attention was paid to the isomeric Trp degradation products (i.e. diastereomers). Eventually, the methods were intended to be applied to pharmaceutical formulations.

4.1 Stability of tryptophan

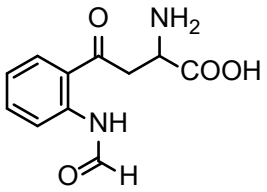
Literature research resulted in the compilation of a substance library (8.3. Substance library). Only little information was found with regard to Trp stability in aqueous formulations after heat treatment comparable to steam sterilization by autoclaving. Most reports on Trp stability were conducted with peptides and proteins containing Trp using harsh treatment and/or non-physiological conditions, which do not apply for parenteral AA formulations. On the other hand, several stability studies of parenteral formulations focused mainly on the interaction with other ingredients such as glucose, fatty acids, vitamins or other drugs and have been covered in the review. Hence, the information applicable to aqueous Trp formulations was limited. Thus, the preliminary tests performed demonstrated the stability problem of Trp after autoclaving, which has not been reported to our knowledge so far. A reproducible yellow discolouration was observed in Trp solutions, in the presence of (dissolved) oxygen after autoclaving process. The discolouration intensified with accumulation of heating cycles and during storage time. Consequently, the presence or the deprivation of oxygen were pointed out as the most contributing or impeding factor for the degradation of Trp, respectively. The pH range, in this case pH 5.5 -6.5, being the specified pH range of *Formulation 1* - was shown to be neglectable for Trp stability. The duration of applied heating periods has an accelerating function and was shown to be proportional to the formation of each degradation product, assessed directly after heat treatment (3.2, Fig. 26). The differences in quantity are compensated within several days of storage at room temperature, as demonstrated by the analyses within 1 week and 1 year after autoclaving. The quantification showed similar amounts of degradation products (3.2 Tab. 11, 1 year storage: 69.5-72.5 µg/mL, 3.5-3.6 %,), regardless of applied autoclaving duration.

Eventually, according to the quantification, the amounts of Trp degradation products reached a maximum after 1 year of storage and decreased to 2.8 % after 3 years of storage in total.



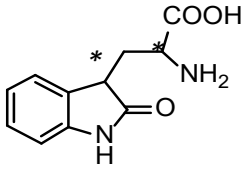
***R,S/R,R* DiOia**

236.23 g/mol



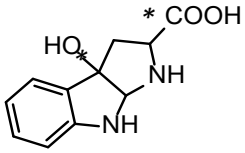
NFK

236.23 g/mol



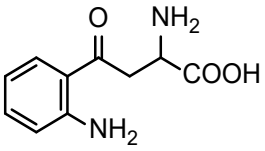
***R,S/R,R* Oia**

220.23 g/mol



***cis/trans* PIC**

220.23 g/mol



Kyn

208.22 g/mol

Figure 32: Overview of identified Trp degradation substances

The identified degradation products are summarized in Fig. 32. Two Trp degradation products, namely NFK and Kyn are photosensitizers, which contribute to further Trp decomposition under day-light or UV-irradiation, respectively. Thus, UV-light protection of parenteral formulations during processing and storage should be obligatory.

This study characterizes the development and concentration of Trp degradation products in the short term, namely directly after autoclaving and after one week, and in the long term, i.e. after one and three years of storage (after autoclaving). The percentage of degradation products changed during storage period. It was highest one year after autoclaving and decreased after three years of storage. Thus, the formation of Trp degradation products is not linear. An explanation might be that the substances Kyn and NFK and the Oia diastereomers are not stable and prone to decomposition, further oxidation or conversion, respectively. Additionally, the instability of prepared Kyn solutions was observed during sample preparation, e.g. the bright yellow colour of the solutions intensified within several days after preparation , and LC-UV/MS analyses demonstrated the decrease of the Kyn peak. Reported Kyn degradation products are

e.g. kynurenic acid, kynurenine yellow and 4-hydroxyquinoline, which were found by LC-MS analyses (8.1 Supporting information, Fig. A 1) [104, 129-132].

Presumably a major part of the "early stage" degradation products *R,S/R,R* Oia is oxidized to the DiOia diastereomers, because the DiOia diastereomers are predominant among all degradation substances after the storage periods (3.2, Tab. 13).

The conclusions made upon Trp decomposition and, in particular the percentual degradation rates in pure Trp solutions, were shown to be transferable to parenteral AA formulations.

An important additional factor, which influences amounts of degradation products in the long-term, is the type of primary packaging used. Glassware was shown to be superior in comparison to autoclavable multi-layered plastic bags (MLB) for injectables, examined within a one-year of storage after autoclaving. Trp degradation was significantly higher in plastic containers and can be attributed to increased oxygen levels inside the container. An increased oxygen diffusion through the plastic material can be explained by the time-dependent alteration of some of the single layers, induced by heating, steam saturation and pressure during the autoclave process. It has to be discussed, if steam sterilization of products made in advance and stored in MLBs is appropriate. The oxygen permeability in relation to storage time of MLBs is a highly relevant issue for the stability of Trp-containing formulations. However, the preparation of parenteral solutions for immediate use in MLBs, like it is used e.g. in hospital pharmacies, is convenient and can be considered safe, because any parenteral administration is restricted to a maximum of 24 h.

Taken together, Trp stability strongly depends on oxygen content and less on heating duration. Light irradiation and primary packaging used contribute to Trp stability as secondary factors. However, the heat induced by steam sterilization is an acceleration force, but the total amount of degradation products is controlled by oxygen concentration. Anyway, in aqueous solutions, Trp degradation and/or oxidation takes places even at trace levels of dissolved oxygen without heating but at much slower rates. Glassware is to be preferred over plastic containers for the preparation of bulk ware. The use of plastic material cannot be recommended, until the long-term alterations of the single layers in the plastic material have been evaluated with regard to oxygen permeability.

4.2 Impurity assessment methods

Diverse AA assessment methods were covered in the review, some amongst them might be suitable for the separation and assessment of Trp degradation products. However, the method developed should be direct, fast, robust and reliable for the detection and the quantification of impurities or Trp degradation products in bulk Trp and Trp-containing AA formulations.

The compendial Trp impurity assessment method focuses on the quantitative determination of the by-products of the Trp synthesis. 1,1´ Ethylidene(bis)-Trp is especially limited due to its toxicity, whereas the other related impurities are summarized and limited altogether to a total amount of 100 ppm by other purity assessment methods beside the HPLC analysis. Thus, the compendial method does not facilitate the determination, nor the separation of Trp degradation products and is not suitable for the assessment thereof [82]. Based on the compendial method, a preliminary RP-HPLC method (*Method A)* was developed allowing MS detection additionally. The optimization efforts resulted in *Method A 1* and succeeded in the separation and the assessment of Trp degradation substances within 17 min. *Method A 1* uses a 125 mm instead of a 250 mm C-18 RP column and optimization showed best results with ammonium formiate buffer and formic acid as acidifier instead of trifluoroacetic acid. The method was validated for UV-detection, although it is compatible with linearly coupled MS-detection. However, UV-detection was shown to be sufficient for the assessment of the substances, hence the extensive validation of a HPLC-MS method was omitted. The separation of the degradation substances was challenging due to their structural similarities and the presence of several (three pairs) of diastereomers. The adjustment of the organic content and buffer strength of the eluents was decisive for a successful separation. The separation of the degradation substances is highly sensitive to minimal alterations of methanol content of mobile phase A (5 % v/v) and the gradient steepness, respectively. Absolute deviations of 1-3 % methanol in mobile phase A would lead to a loss of resolution and co-elution of the PIC and DiOia diastereomers and of the Oia b and NFK, respectively. The gradient steepness needed to be established carefully, due to the same reason. This problem was managed successfully by finding the optimal buffer and strength (30/60 mM ammonium formate in mobile phase A/B, respectively) and pH adjustment (apparent pH 3, adjusted with formic acid), resulting in a robust method with reliable results.

Two alternative HPLC methods using ion pairing reagents (IP-RP-HPLC; *Method B* and *C*) and a modified column material (PFP-column; *Method D 1* and *2*) were developed, which are orthogonal techniques to traditional RP-HPLC. The alternative techniques are appropriate for the separation of small polar and charged compounds in general with a promising selectivity –

highly important in case of structural similarities. The IP-RP-HPLC was disadvantageous with regard to feasibility, because of long equilibration times, contamination of the HPLC system and high consumption of eluents. Moreover, system peaks appeared and diminished the quality of the chromatograms. The PFP-column showed good separation and excellent peak shapes of the Trp degradation products and was tested with stressed parenteral AA formulations. However, broad peaks were observed for the AA Phe, which overlapped the peaks of some degradation products. In addition, the method showed low robustness and was thus rejected from further optimization efforts.

5 Summary

The stability of Trp in pure solutions and in parenteral AA formulations was evaluated with regard to typically used manufacturing processes, storage conditions and primary packaging. Therefore, thorough stability studies on Trp solutions were conducted beforehand. The applied stressing method, i.e. steam sterilization by autoclave, are chemically seen relatively mild but showed to be efficient to induce Trp degradation in the presence of oxygen. Subsequent identification, separation and characterization were challenging due to similar substance properties, numerous stereoisomers and pairs of diastereomers found amongst them. However, the identified *o*-aminoacetophenone compounds, Kyn and NFK, are associated with photo reactivity and have photo-oxidizing properties. Thus, best possible protection from UV-light, together with strict oxygen expulsion, are the most important criteria to impede Trp degradation after autoclaving.

The identification of Trp degradation products was assisted by the compilation of a substance library, which included manifold reported and chemically plausible Trp degradation substances. The substances were classified for priority and their early or late-stage occurrence. The large number of possible substances and stereoisomers was narrowed down with the information retrieved from LC-UV/MS experiments. However, final identification was achieved by the synthesis of proposed substances as references. The following eight substances were characterized as Trp degradation substances: Kyn, NFK and three pairs of diastereomers *R,R/R,S* DiOia, *R,R/R,S* Oia and *cis/trans* PIC. Fig. 33 shows the proposed degradation pathway and demonstrates the close chemical relationship, which may be an explanation for the conversion of some substances into each other during the storage period. The proposed pathway brings together the results of different Trp stability and stressing studies, respectively [89, 94, 97, 98, 103, 133]. To our knowledge, the simultaneous formation of the identified degradation substances has not been reported before and especially not under the stressing conditions applied.

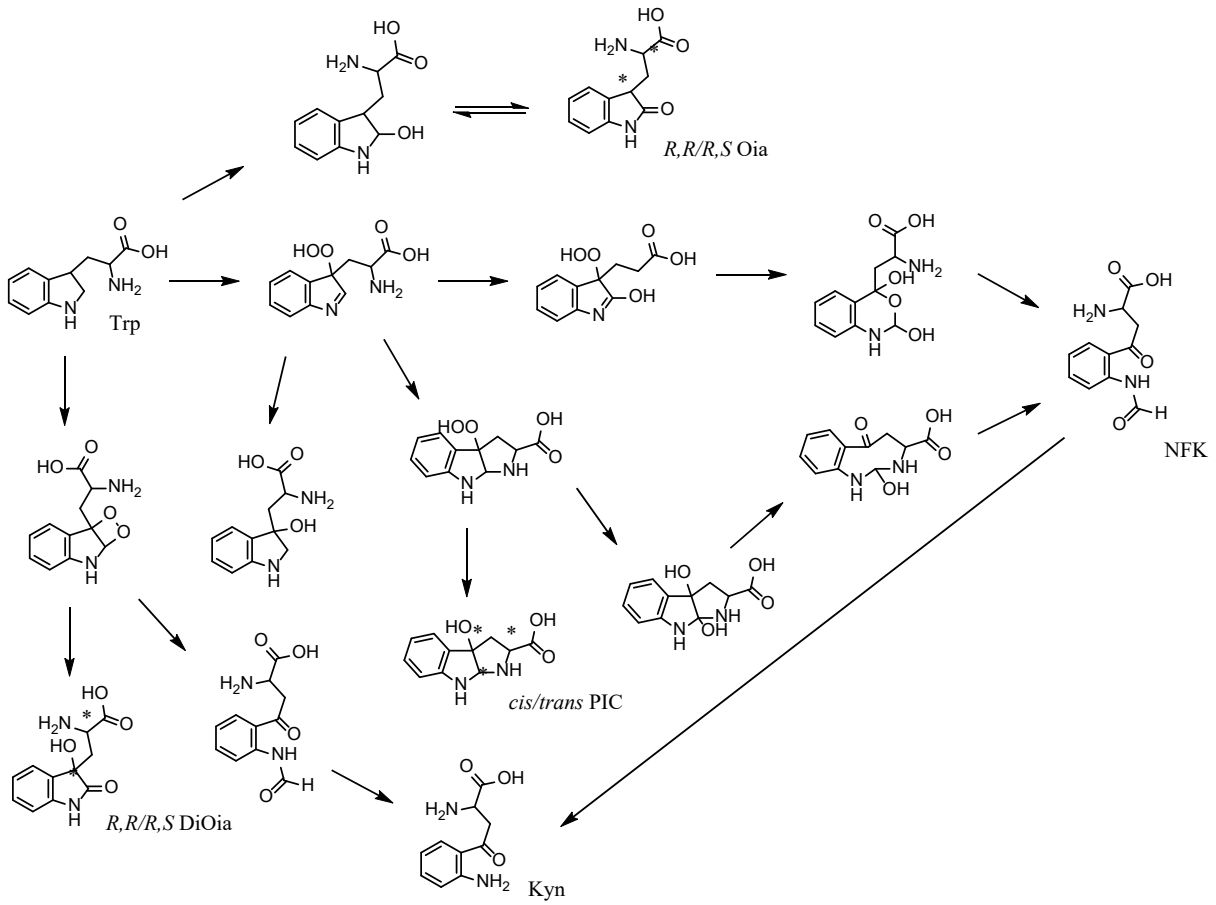


Figure 33: Proposed Trp degradation pathway.

The application of a traditional RP-HPLC method was compared to two developed IP-HPLC methods and a RP-HPLC methods using a modified perfluorinated column. Orthogonal analyses methods and especially the combination of UV and MS detection are necessary in order to indicate potentially undetected degradation substances. Main evaluation criteria were the separation performance, analyses time, reproducibility and feasibility. The best results upon assessment of all Trp degradation products, in both; pure Trp solutions and pharmaceutical formulations, were obtained by a traditional RP-HPLC. The optimized method was validated according to ICH guidelines Q2(R1) and meets the criteria of a stability-indicating HPLC-UV method. The validated method has a sufficient separation performance with an adequate selectivity indicating the Trp degradation substances next to each other and next to other AAs in finished pharmaceutical formulations.

The detailed knowledge of Trp degradation and the method presented may be transferred practically to the pharmaceutical industry processing Trp-containing products. In general, the findings might contribute to the quality management of such pharmaceutical products during

manufacturing and storage. Additionally, the study results provide basic information for the establishment of an impurity consideration following the ICH guidelines Q3B (R2) (impurities in new drug products) for products containing Trp. However, further development of the method applying more sophisticated detectors or more potent HPLC techniques like e.g. UHPLC and the implication of more sensitive (MS) detectors like ToF-MS would be advantageous with regard to economic and practical aspects.

6 Zusammenfassung

Diese Arbeit dient der Stabilitätsbeurteilung von Tryptophan (Trp) in parenteralen Aminosäurelösungen, insbesondere im Hinblick auf Einflussfaktoren wie der Herstellungsprozess, z.B. der Sterilisationsvorgang, Lagerungsbedingungen, sowie die Art der verwendeten Primärverpackung. Zunächst wurde die Stabilität von reinen Trp-Lösungen untersucht, die mehreren aufeinanderfolgenden Sterilisationszyklen im Autoklav ausgesetzt wurden. Generell stellt der Autoklavierprozess eine Vergleichsweise milde und kontrollierte Art der Hitzebelastung dar. Dabei wurde zwischen Lösungen unterschieden, die Sauerstoff enthielten und Lösungen, in denen der gelöste Sauerstoff mittels Stickstoffgas ausgetrieben wurde und die anschließend luftdicht verschlossen wurden. Es konnte festgestellt werden, dass der Autoklavierprozess, in Anwesenheit von Sauerstoff, zu einem Abbau von Trp führt, welcher sich außerdem auch durch eine Gelbfärbung der Lösungen zeigt. Die Identifizierung und Charakterisierung der Abbauprodukte erwies sich als schwierig aufgrund von sehr ähnlichen Substanzen, die eine Trennung mittels HPLC und die UV-Detektion alleine erschwerten. Die Massenspektroskopie zeigte erst, dass einige Abbauprodukte zeitgleich eluieren und einige isomere Formen vorliegen. Mithilfe von preparativer HPLC und Fragmentierung in der Ionenfalle konnten drei Diastereomeren-Paare gefunden werden, *R,R/R,S* Oia und DiOia, *cis/trans* PIC und zwei weitere Substanzen, Kyn und NFK. Die beiden letztgenannten Stoffe haben eine Sonderstellung, denn sie besitzen jeweils ein *o*-Aminoacetophenon-Grundgerüst anstelle des Indols, und absorbieren dadurch zusätzlich bei Wellenlängen von > 320 nm, und wirken photosensibilisierend, wodurch die Stabilität von Trp (unter Lichteinstrahlung) zusätzlich nachteilig beeinflusst wird. Daraus lässt sich ableiten, dass der Abbau von Trp in Lösungen maßgeblich durch strengen Sauerstoff- und Lichtausschluss verhindert werden kann. Die Abbildung Fig. 33 zeigt schematisch, wie die einzelnen Abbauprodukte möglicherweise entstehen und zusammenhängen könnten. Die Aufstellung der chemischen Zusammenhänge beruht auf den Ergebnissen verschiedener Trp-Stabilitätsstudien und bringt diese auf einen Nenner [89, 94, 97, 98, 103, 133]. Soweit durch die Literaturrecherche bekannt, wurde das zeitgleiche Auftreten aller hier identifizierten Abbauprodukte bislang noch nicht dokumentiert. Insbesondere wurden keine Studien über Stabilitätsprobleme, bedingt durch die Wasserdampf-Sterilisation gefunden. Des Weiteren zeigten die quantitativen Untersuchungen von Lösungen, die eine Woche, ein und drei Jahre (nach einmaligem Autoklavieren) eingelagert wurden, dass die Abbauprodukte nicht linear entstehen und zunehmen, sondern, dass sich deren prozentuale Anteile dynamisch verändern (Kapitel 3.2.).

Für die Identifizierung der Abbauprodukte von Trp war die Zusammenstellung einer Substanz-Bibliothek äußerst hilfreich. Sie beinhaltet chemisch plausible Trp-Abbauprodukte, sowie aus

der Literatur bekannte Abbauprodukte, die durch verschiedenste Stressmethoden hervorgerufen werden. Diese Substanzen wurden nach Plausibilität und Priorität kategorisiert, um ein gezieltes Screening in gestressten (autoklavierten) Trp-Lösungen durchzuführen. Zusammen mit den Ergebnissen der LC-UV/MS Analyse konnte die Auswahl auf einige wenige Abbauprodukte begrenzt werden. Da es sich dabei um Isomere handelte, gelang die Identifizierung letztendlich erst durch die Synthese der in Frage kommenden Stoffe.

Mithilfe der Synthese der Referenzsubstanzen konnte eine HPLC-UV Methode entwickelt, optimiert und nach den ICH Q2(R1) Richtlinien validiert werden, die eine Quantifizierung der Substanzen in reinen Trp-, und in handelsüblichen parenteralen Aminosäurelösungen ermöglicht. Für die validierte Methode wurde als stationäre Phase eine herkömmliche C18-Säule verwendet. Zu Vergleichszwecken wurde eine Methode auf einer Pentafluorophenyl (PFP)-Säule entwickelt und optimiert (*Method D 1* und *D 2)*, sowie zwei RP-Methoden mit zwei analogen Ionen-Paar-Reagenzien (*Method B* und *C).* Verglichen und beurteilt wurden dabei die Trennleistungen, Analysendauer, Reproduzierbarkeit und die praktische Anwendbarkeit der jeweiligen Methoden. Die besten Ergebnisse wurden aber mittels der traditionellen RP-HPLC erreicht.

Die Ergebnisse könnten für die Herstellung, Lagerung und Beurteilung von Trp-haltigen Lösungen durchaus relevant sein. Eine strenge Kontrolle der Sauerstoffwerte sowie ein kontinuierlicher Lichtschutz während und nach der Verarbeitung sind unverzichtbar. Die Ergebnisse erlauben außerdem ein gezieltes Screening nach Abbauprodukten, bzw. „Markern“. Die Erstellung von Beurteilungen, wie es z.B in den ICH Q3B(R2) Richtlinien gefordert ist, wird erleichtert, da die Identität bestimmt wurde und eine validierte Quantifizierungsmethode entwickelt wurde. Die Methode könnte für industrielle Zwecke noch weiter optimiert werden, indem z.B. eine UHPLC entwickelt wird oder sensiblere Detektoren, wie z.B. ein ToF-Massendetektor, verwendet werden. Letztendlich sollte allerdings von der Arzneibuchmethode abgegrenzt werden, die Verunreinigungen aus dem Trp-Herstellungsprozess erfasst (1,1´ Ethyliden(bis)Trp). Die hier entwickelte Methode erfasst die Abbauprodukte von Trp in reinen Trp und in Trp-haltigen Aminosäurelösungen, die typischerweise durch Fehler bei der Herstellung oder den Autoklavierprozess hervorgerufen werden können.

7 Experimental section

7.1 Chemicals and material

The chemicals and material used are described in chapter 3.2. (2.1. Chemicals and material).

Further chemicals and reagents used are 5-OH-(DL)-Trp, L-Ile, L-Leu, L-Lys, DL-Met, L-Phe and L-Thr, were purchased from VWR International (Darmstadt, Germany), N-acetyl-(DL)-Trp, L-Val, L-Arg, L-His, L-Ala, L-Gly and L-Tyr were from Sigma Aldrich, (Darmstadt, Germany) and 4-hydroxyquinoline, DL-Pro, L-Ser, and Taurine were received from TCI Chemicals (Eschborn, Germany), respectively.

7.2 Apparatus

HPLC-UV

Apparatus	Agilent HPLC 1100 series
Degasser	JP82039721-G1379B
Binary Pump	DE63059738-G1312A
Autosampler	DE64763639-G1329A
Column thermostat	DE63062591-G1316A
VWD	JP240018922-G1314A

HPLC-UV/MS

Apparatus	Agilent HPLC 1100/1200 series
Degasser	JP6375118-G1379B
Binary Pump	DE23912089-G1312A
Autosampler	DE13203341-G1329A
Column thermostat	DE90374172-G1316A
DAD	DE43626774-G1315B
MSD – ESI-Ion Trap	DE24205141-G2445D

Preparative HPLC

Apparatus	Agilent HPLC 1100/1200 series
Prep Pump A	DE43601133-G1361A
Prep Pump B	DE43601127-G1361A
Autosampler	DE64755192-G2260A
MWD	DE43603402-G1365B
Fraction collector	DE43601287-G1364B

7.3 Sample preparation

The sample preparation is described in the experimental section in chapter 3.2. (2.5. Sample preparation).

Other test solutions

Stressed Trp reference solution

The *stressed Trp reference solution* was prepared by dissolving 100 mg L-Trp in 50.0 mL water (2 mg/mL) followed by 10 min degassing in the ultrasonic bath. The pH was adjusted to 5.5 with 0.01 M acetic acid. The solution was distributed into 50 mL autoclavable glass vials and subjected to 30 min autoclave (121 °C, 2.1 bar). The solution cooled down at RT and was stored at a dry and dark place at RT and retrieved for sampling as needed.

Trp-Tyr-Phe solution

The *Trp-Tyr-Phe solution* was prepared by dissolving 200 mg of L-Trp (2 mg/mL), 40 mg of L-Tyr (0.4 mg/mL) and 510 mg of L-Phe (5.1 mg/mL) in 100.0 mL water followed by 10 min degassing in the ultrasonic bath. The solution was distributed into one 50 mL autoclavable glass vial and subjected to 30 min autoclave (121 °C, 2.1 bar).

Stressed AA test solution

The *stressed AA test solution* was prepared by dissolving of : 5.0 g Ile, 7.4 g Leu, 6.6 g Lys, 4.3 g Met, 5.1 g Phe, 4.4 g Thr, 2.0 g Trp, 6.2 g Val, 12.0 g Arg, 3.0 g His, 14.0 g Ala, 11.0 g Gly, 11.2 g Pro, 6.5 g Ser, 0.4 g Tyr, 1.0 g Taurine in 1000.0 mL water followed by 15 min degassing in the ultrasonic bath. Aliquots of 50 mL were distributed into autoclavable glass vial and subjected to autoclave (121 °C, 2.1 bar) as needed. The solution was cooled down to RT and kept in at 4 °C in the refrigerator.

Reference mix 1

The *Reference mix 1* was prepared by dissolving 1000 µg of 5-hydroxy-(DL)-Trp, N-acetyl-(DL), Trp 4-hydroxyquinoline and L-Kyn in 10.0 mL water (conc. each: 100 µg/mL) followed by 10 min degassing in the ultrasonic bath. The solution was kept at 4 °C in the refrigerator and used within 4 weeks.

HPLC sample preparation

For HPLC analyses, 1000 µL of each specific sample was filtrated by 0.22 µm cellulose or polypropylene syringe filter. Thereof 500 µL were diluted with 500 µL mobile phase A (according to the applied method) into a brown glass vial and analysed immediately or kept at 4 °C until use.

Preparative LC samples

Trp prep solution

The *Trp prep solution* was prepared by dissolving of 200 mg of Trp in 100 ml water (2 mg/mL), followed by 10 min in the ultrasonic bath. The pH was adjusted to 6.5 with a 0.1 M sodium hydroxide solution. The solution was distributed into two 50 mL autoclavable glass vials and autoclaved for 90 m (121 °C, 2.1 bar).

The autoclaved solutions were combined and condensed by water evaporation. The dry yellow residue was dissolved in 5 mL water followed by 15 min in the ultrasonic bath. The solution was distributed into four 1.5 mL brown glass HPLC vials and submitted to preparative LC.

Fraction sample preparation

Each collected fraction was condensed by water evaporation, the yellowish residues were dissolved in 1.5 mL of a mixture of water/methanol (95:5, v/v) and subjected to LC-MS/MS analyses (*Method A 1*).

Pharmaceutical formulations

Formulation 1 and *Formulation 2* were kindly provided from Fresenius Kabi (Bad Homburg, Germany). The products were delivered and kept in glass bottles in a dry and dark container at RT. The measured pH of the *Formulations 1* and *2* were within the given ranges of prescribing information, pH 5.5 - 6.3 and 5.4 - 5.8, respectively. *Formulation 1* and *Formulation 2* HPLC samples were prepared by dilution of 500 µl of the solutions with 500 µL of mobile phase A according to used analysis method.

Primary packaging

The primary packaging material used for sample autoclaving was glassware: 50 ml autoclavable glass vials (Zscheile u. Klinger GmbH, Hamburg, Germany) and 100 mL plastic containers for injectables: Freeflex® (Fresenius Kabi, Bad Homburg, Germany). The glass vials are closed with a rubber (chlorbutyl) plugs and cramped aluminium lids. Freeflex® bags are made multi-layered polyolefin consisting of different blends of polypropylene, polyethylene and thermoplastic elastomer. The bags are free from polyvinylchloride (PVC), latex and phthalates and can be used for steam sterilization at 121 °C [134]. The plastic bags have a port system for aseptic filling and closing.

7.4 Sample stressing

Sample stressing were conducted by autoclaving. The heating durations of 30, 60 or 90 min were accumulated by subsequent 2, 4 or 6 autoclaving cycles (15 min in net sterilization phase), respectively.

Autoclaving method

Sample stressing was conducted by steam sterilization in a laboratory autoclave (Systec, Linden, Germany). The autoclave procedure comprises a heating phase (H-phase), the actual sterilization phase (S-phase) and a cooling phase (C-phase). The typical temperature for the sterilization of liquids or solids is 121 °C. Therefore, water vapor is pressurized to 2.1 bar and heated to the required temperature during the H-phases. When stable pressure and temperature are reached, the sample is in the S-phase, the actual net time of autoclavation. In the following C-phase pressure and temperature decrease to 1 bar and unloading temperature, respectively. The net time in the S-phase is programable and was set to the duration as needed. Settings of H or C-phase are not adjustable.

7.5 HPLC methods

Method A

HPLC settings	
Column:	LiChroSpher® 100 LiChroCart® 250-4 (Merck, Darmstadt, Germany)
	4.6 x 250 mm RP-18e, particle size: 5µm
Mobile phase A	H_2O/MeOH (95:5 v/v), 0.1 % TFA
Mobile phase B	MeOH, 0.1%TFA
Flow rate	1 mL/min
Injection volume	15 µL
UV-detection	λ = 254, 280, 340 nm
Gradient	0 – 5 min: 0% B
	5 – 40 min: 0 → 35% B
	40 – 45 min: 35% B
	Re-equilibration time: 5 min

MS settings	
Ion source	ESI
Polarity mode	positive
Dry temperature	350 °C
Nebulizer	50.0 psi
Dry gas	nitrogen, 10.0 L/min
Scan range	120 - 400 *m/z*

Method A 1

HPLC settings

Column:	LiChroSpher® 100 LiChroCart® 250-4 (Merck, Darmstadt, Germany)
	4.6 x 250 mm RP-18e, particle size: 5µm
Mobile phase A	30 mM NH_4HCOOH/MeOH (95:5 v/v), 0.25 % FA
Mobile phase B	60 mM NH_4HCOOH/MeOH (50:50 v/v), 0.25 % FA
Flow rate	1 mL/min
Column temperature	15°C
Injection volume	10 µL
UV-detection	λ = 254, 340 nm
Gradient	0 – 10 min: 0 % B
	10 – 15 min: 0 → 30 % B
	15 – 20 min: 30 % B
	20 – 22 min: 30 → 90 % B
	22 – 28 min: 90 % B
	28 – 30 min: 90 → 0 % B
	Re-equilibration time: 5 min

MS settings (optional)

Ion source	ESI
Polarity mode	positive
Dry temperature	350 °C
Nebulizer	50.0 psi
Dry gas	nitrogen, 10.0 L/min
Scan range	50 - 400 *m/z*

Method B

HPLC settings

Column:	LiChroSpher® 100 LiChroCart® 125-4 (Merck, Darmstadt, Germany)
	4.6 x 125 mm RP-18e, particle size: 5µm
Mobile phase A	10 mM HFBA, H_2O/MeOH (97.5:2.5 v/v), 0.1 % FA
Mobile phase B	MeOH/ACN (2:1 v/v), 0.1 % FA
Injection volume	10 µL
Flow rate	0.5 mL/min
UV-detection	λ = 254, 280, 340 nm
Gradient	0 – 15 min: 5 → 15 % B
	15 – 20 min: 15 → 45 % B
	20 – 25 min: 45 % B
	25 – 30 min: 45 → 5 % B
	Re-equilibration time: 15 min

MS settings

Ion source	ESI
Polarity mode	positive
Dry temperature	325 °C
Nebulizer	50.0 psi
Dry gas	nitrogen, 8.0 L/min
Scan range	50 - 350 *m/z*

Method C

HPLC settings

Column:	LiChroSpher® 100 LiChroCart® 125-4 (Merck, Darmstadt, Germany)
	4.6 x 125 mm RP-18e, particle size: 5µm
Mobile phase A	10 mM NFPA, 30 mM NH_4HCOOH/ACN (90:10 v/v), 0.1 % FA
Mobile phase B	ACN, 0.1 % FA
Injection volume	10 µL
Flow rate	0.5 mL/min
UV-detection	λ = 254, 280, 340 nm
Gradient	0 – 15 min: 5 → 35 % B
	15 – 20 min: 35 → 45 % B
	20 – 25 min: 45 → 5 % B
	Re-equilibration time: 15 min

MS settings

Ion source	ESI
Polarity mode	positive
Dry temperature	325 °C
Nebulizer	50.0 psi
Dry gas	nitrogen, 8.0 L/min
Scan range	50 - 350 *m/z*

Method D 1

HPLC settings

Column:	Kinetex® (Phenomenex®, Aschaffenburg, Germany)
	Core-shell PFP
	100 Å, 150 x 4.6 mm i.d.
	particle size: 2.6 µm
Mobile phase A	H_2O/MeOH (95:5, v/v) 0.15 % FA
Mobile phase B	MeOH, 0.15 % FA
Injection volume	10 µL
Flow rate	0.4 mL/min
UV-detection	λ = 254 nm, 340 nm (VWD)
Gradient	0 – 15 min: 0 % B
	15 – 16 min: 0 → 15% B
	16 – 30 min: 15% B
	30 – 35 min: 15 → 20% B
	35 – 40 min: 20 → 40% B
	40 – 45 min: 40 % B
	45 – 50 min: 40 → 0% B
	Re-equilibration time: 15 min

MS settings (optional)

Ion source	ESI
Polarity mode	positive
Dry temperature	350 °C
Nebulizer	50.0 psi
Dry gas	nitrogen, 10.0 L/min
Scan range	50 - 400 *m/z*

Method D 2

HPLC settings

Column:	Kinetex®, Core-shell PFP, (Phenomenex®, Aschaffenburg, Germany) 100 Å, 150 x 4.6 mm i.d. particle size: 2.6 µm
Column temperature	7 °C
Mobile phase A:	30 mM NH_4HCOOH/MeOH (95:5, v/v) 0.25 % FA
Mobile phase B:	MeOH, 0.25 % FA
Injection volume	10 µL
Flow rate	0.4 mL/min
UV-detection	λ = 254 nm, 340 nm (VWD)
Gradient	0 – 15 min: 0 % B 15 – 16 min: 0 → 15% B 16 – 30 min: 15% B 30 – 35 min: 15 → 20% B 35 – 40 min: 20 → 40% B 40 – 45 min: 40 % B 45 – 50 min: 40 → 0% B Re-equilibration time: 10 min

Method Prep 1

HPLC settings	
Column:	Gemini® NX-C-18 (Phenomenex, Aschaffenburg, Germany) 110 Å, 250 x 10 mm i.d. particle size: 5µm
Mobile phase A	H_2O, 0.1 % FA
Mobile phase B	ACN, 0.1 % FA
Injection volume	15 µL
Flow rate	1 mL/min
UV-detection	λ =254 nm
Gradient	0 min: 5 % B 0 – 2 min: 5 % B 2 – 4 min: 5 → 10 % B 4 – 7 min: 10 % B 7 – 9 min: 10 → 15 % B 9 – 12 min: 15 % B 12 – 14 min: 15 → 100 % B 14 – 17 min: 100 % B 17 – 19 min: 100 → 5 % B 19 – 20 min: 5 % B Re-equilibration time 2 min

Method Prep 2

HPLC settings	
Column:	Gemini® NX-C-18, (Phenomenex, Aschaffenburg, Germany) 110 Å, 250 x 4.6 mm i.d. particle size: 5µm
Mobile phase A	H_2O, 0.1 % FA
Mobile phase B	ACN, 0.1 % FA
Injection volume	150 µL
Flow rate	4 mL/min
Column temperature	RT
UV-detection	λ =254 nm
Gradient	0 min: 5 % B 0 – 2 min: 5 % B 2 – 4 min: 5 → 10 % B 4 – 7 min: 10 % B 7 – 9 min: 10 → 15 % B 9 – 12 min: 15 % B 12 – 14 min: 15 → 100 % B 14 – 17 min: 100 % B 17 – 19 min: 100 → 5 % B 19 – 20 min: 5 % B Re-equilibration time: 2 min
Fractions	A: 4.6 – 4.9 min B: 5.0 – 5.5 min C: 5.6 – 5.9 min D: 6.1 – 6.4 min E: 7.8 – 8.3 min F: 11.0 – 11.6 min

8 Appendix

8.1 Supporting information

AA	Formulation 1	Formulation 2
	conc. (g/L)	
Ile	5.0	4.92
Leu	7.4	5.9
Lys	6.6	6.8
Met	4.3	4.2
Phe	5.1	5.9
Thr	4.4	4.2
Trp	2.0	1.4
Val	6.2	5.5
Arg	12.0	8.4
His	3.0	5.1
Ala	14.0	12.0
Gly	11.0	5.9
Pro	11.2	5.1
Ser	6.5	3.4
Tyr	0.4	0.17
Taurine	1.0	-
Asp	-	2.5
Glu	-	4.2
Cys	-	0.42
excipients: glacial acid, water for injection		

Tab. A 1: The ingredients of Formulation 1 and 2; concentration in g/L.

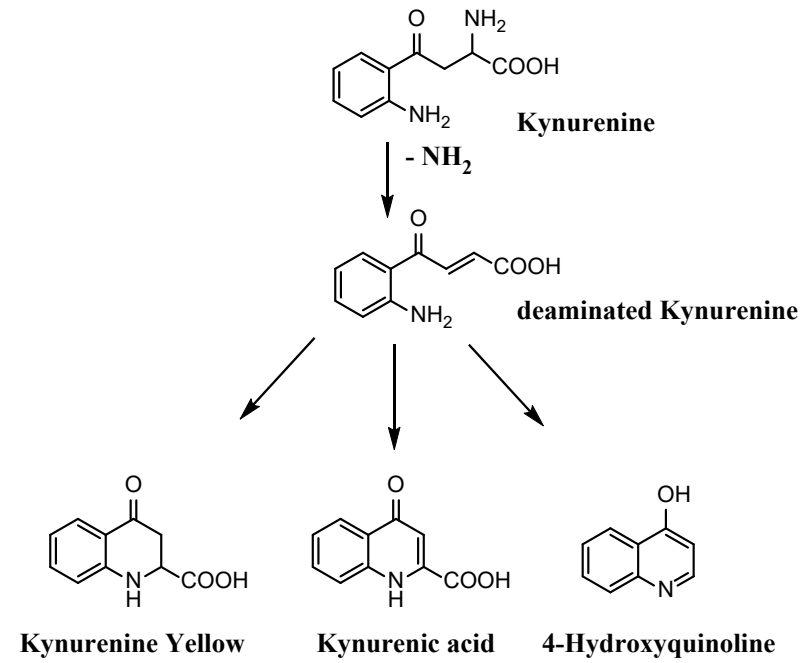


Fig. A 1: Degradation products of Kynurenine [104; 129-132].

8.2 Sample images

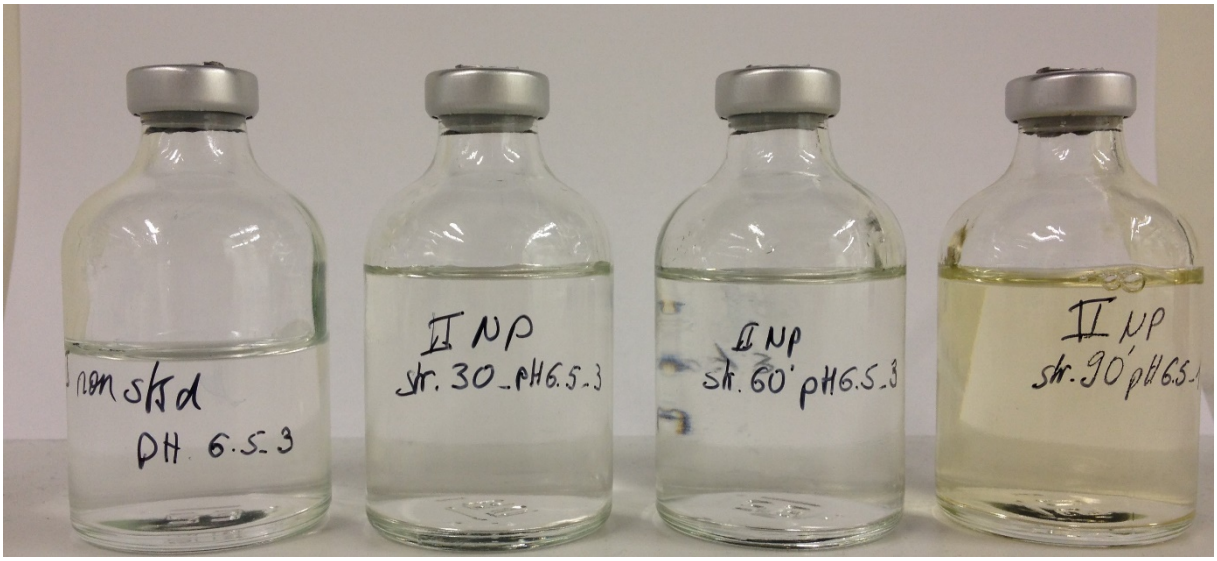


Figure A.2: Image of nitrogen purged Trp samples, pH 6.5, stored for 2 months after preparation and autoclaving, respectively: From left to right: not stressed (control), 30 min, 60 min, 90 min autoclaving at 121 °C, 2.1 bar

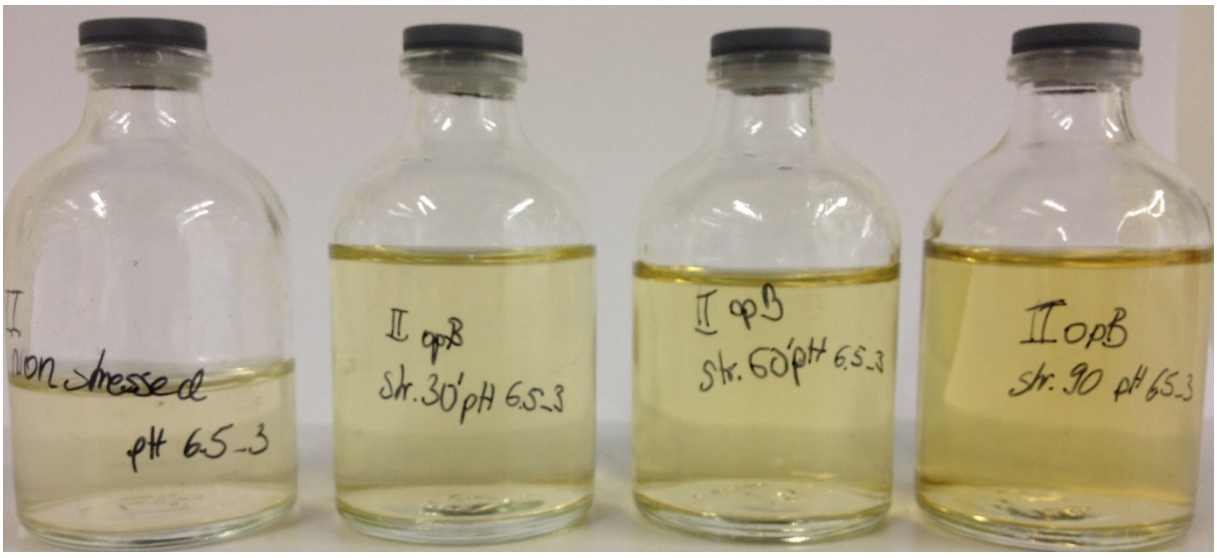
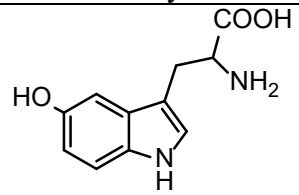
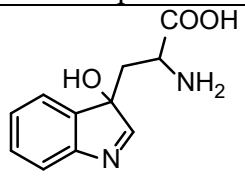
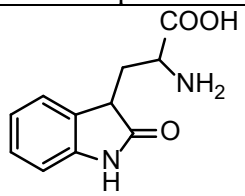
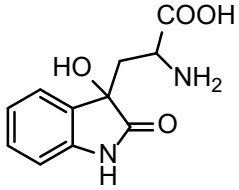
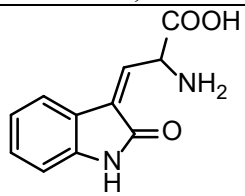


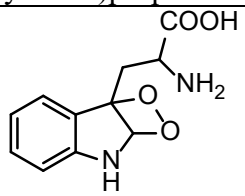
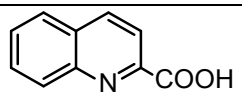
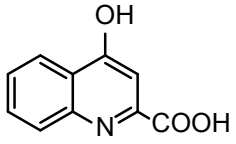
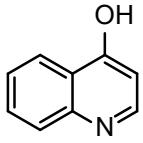
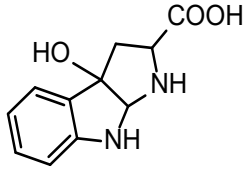
Figure A.3: Image of Trp samples with not controlled oxygen content (higher oxygen content), pH 6.5, stored for 2 months after preparation and autoclaving, respectively: From left to right: not stressed (control), 30 min, 60 min, 90 min autoclaving at 121 °C, 2.1 bar

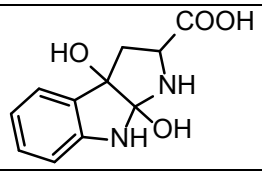
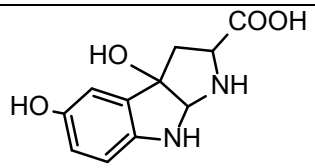
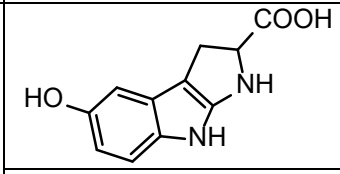
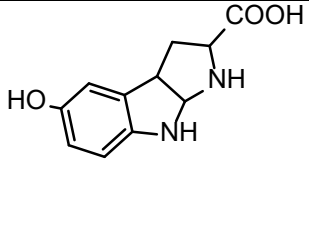
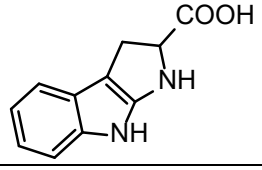
Figure A.4: Image of Trp duplicate samples with not controlled oxygen content (higher oxygen content), pH 5.0- 6.5 (front to back), 1 week after preparation and autoclaving, respectively: From left to right: autoclave time 10 min, 20 min, 30 min, at 121 °C, 2.1 bar

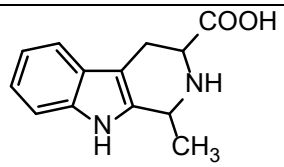
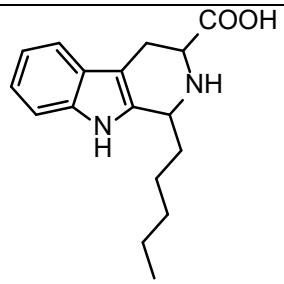
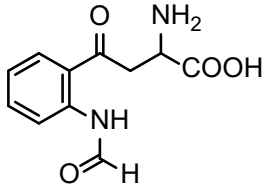
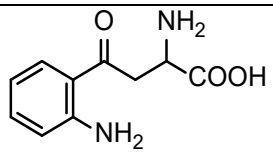
Figure A.5: Image of formulation 1 *and* 2 *(right/left) in glass vials and in plastic bags for injectables. The received pharmaceutical products were redistributed and subjected to the autoclaving procedure (actually a second autoclaving step after the sterilization during manufacturing). From left to right: autoclave time 10 min, 20 min, 30 min, at 121 °C, 2.1 bar. The left samples with formulation 2 showed significantly stronger yellowing than formulation 1, as explained in chapter 3.2.*

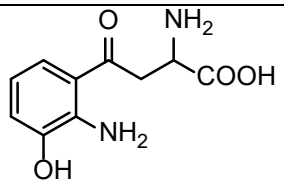
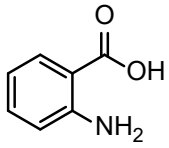
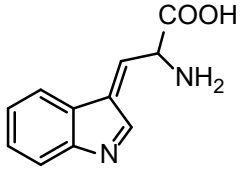
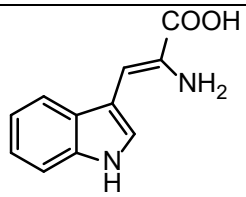
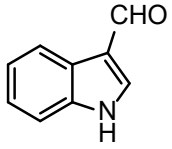
8.3 Substance library

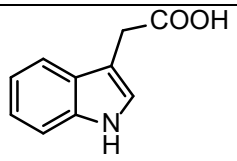
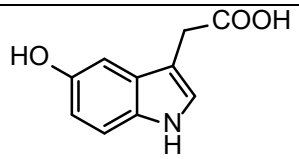
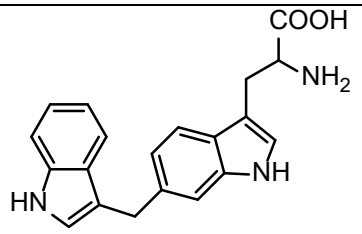
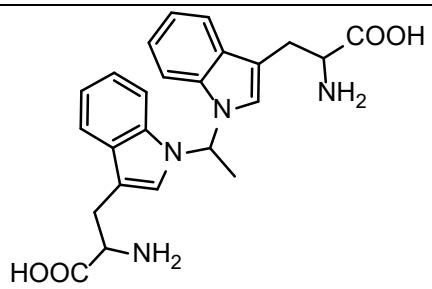
Indole/pyrrole oxidation and/or hydroxylation products			
	Structure, exact mass, IUPAC/ acronym	Comments	Reference
1		III Hydroxylation in position 5 is not preferred. Known Trp metabolite due to enzymatic conversion. Not main degradation product. Lower relevance.	[1-3]
	220.08 2-amino-3-(5-hydroxyindolin-3-yl)propanoic acid 5-OH-Trp		
2		I Position 3 is reactive and prone to oxidation or hydroxylation. Probably poor (MS) stability and followed by pyrrole ring opening to NFK. Highly relevant, if detectable.	[4-6]
	220.08 2-amino-3-(3-hydroxyindolin-3-yl)propanoic acid 3-OH-Trp		
3		I Position 2 is very reactive and prone to oxidation or hydroxylation. Diastereomers expected. Stabilized by keto-enol-tautomerism. Likely blue coloured. Highly relevant.	[3, 4, 7-9]
	220.08 2-amino-3-(2-oxoindolin-3-yl)propanoic acid 2-OH-Trp, Oia		
4		I Hydroxylation of Oia in position 3 gives equally stable DiOia diastereomers. Most abundant degradation product. Highly relevant,	[4, 8, 10-12]
	236.08 2-amino-3-(3-hydroxy-2-oxoindolin-3-yl)propanoic acid 3-OH-Oia, DiOia		
5		III Loss of water from DiOia can occur under natural conditions. Stable conjugated system. Maybe observed, but downstream product.	*

	218.07 (Z)-2-amino-3-(2-oxoindolin-3-ylidene)propanoic acid	Low relevance.	
6	 236.08 2-amino-3-(2a,3-dihydro-7bH-[1,2]dioxeto[3,4-b]indol-7b-yl)propanoic acid	III Dioxetane derivate, very like an intermediate. Extremely poor stability and thus not detectable by MS. Analytically not relevant.	[10, 11]
Quinolones			
7	 173.17 quinoline-2-carboxylic acid QCA, quinolinic acid	II Well known in Trp metabolic degradation. Potent chromophore and stable substance. Rather downstream product. Potentially a relevant marker in the long-term with high chemical stability	[13-15]
8	 189.04 4-hydroxyquinoline-2-carboxylic acid HQCA	III Oxidized QCA, a downstream degradation product. Would be stable and detectable, if formed. Low relevance.	*
9	 145.05 quinolin-4-ol 4-OH Quinoline	II Decarboxylation of HQCA is thinkable. Maybe a downstream degradation product of DiOia with high probability and a potent marker.	[2, 11]
Intramolecular rearrangement, three ring systems			
10	 220.08	I Oxidation in position 3 and subsequent intramolecular rearrangement to three-ring system but likely prone to eliminate water or rearrangement to more stable Oia. Cis/trans PIC isomers are expected, chemical stability is in question.	[7, 11, 12]

	3a-hydroxy-1,2,3,3a,8,8a-hexahydropyrrolo[2,3-b]indole-2-carboxylic acid PIC	Theoretically a first-line degradation product, highly relevant.	
11	 236.08 3a,8a-dihydroxy-1,2,3,3a,8,8a-hexahydropyrrolo[2,3-b]indole-2-carboxylic acid 2-OH-PIC	I Probably a rearrangement of DiOia or reduction of dioxetane intermediate. Expected as cis/trans diastereomers. Stable, detectable, and highly relevant.	[10, 11]
12	 236.08 3a,5-dihydroxy-1,2,3,3a,8,8a-hexahydropyrrolo[2,3-b]indole-2-carboxylic acid 5-OH-PIC	III Hydroxylation of PIC in position 5 is not very likely due to low abundance of PIC and low position reactivity. Low relevance.	*
13	 218.07 5-hydroxy-1,2,3,3a,8,8a-hexahydropyrrolo[2,3-b]indole-2-carboxylic acid	III Formed by water elimination of 5-OH-PIC. Would be stable and detectable, if formed. Rather not expected and low relevance.	*
14	 220.08 5-hydroxy-1,2,3,3a,8,8a-hexahydropyrrolo[2,3-b]indole-2-carboxylic acid	III Formed either by rearrangement of 5-OH-Trp or hydroxylation of PIC. Substance is not expected, low relevance.	*
14		II Stable substance and relevant, in presence of PIC. Targeted screening	[11]

	202.7 1,2,3,8-tetrahydropyrrolo[2,3-b]indole-2-carboxylic acid	recommended, maybe formed in MS ionisation process of PIC.	
17	 230.11 1-methyl-2,3,4,9-tetrahydro-1H-pyrido[3,4-b]indole-3-carboxylic acid	II Reported impurity in L-Trp products. Pictet-Spengler reaction of Trp in water. Detectable, therefore screening recommended	[7, 16-18]
18	 286,17 1-pentyl-2,3,4,9-tetrahydro-1H-pyrido[3,4-b]indole-3-carboxylic acid	III According to ref. possible impurity in L-Trp products. Lower relevance.	[16, 17]
Pyrrole cleavage			
19	 236.08 *(*S)-2-amino-4-(2-formamidophenyl)-4-oxobutanoic acid N-formylkynurenine, NFK	I Earlier degradation product. Most probable yellow coloured. De-formylation of anilines occurs rather slowly, depending on pH and/or temperature: intermediate stability due to self-condensation of keto-amines. Highly relevant.	[1, 3, 4, 10-12, 19-21]
20	 208.08 S)-2-amino-4-(2-aminophenyl)-4-oxobutanoic acid Kynurenine, Kyn (	I Kyn Early stage and well-known degradation product (e.g. metabolic, electrolysis). Highly relevant. Isolated product may not be stable (see NFK).	[1, 4, 12, 20-22]

21	 224.08 2-amino-4-(2-amino-3-hydroxyphenyl)-4-oxobutanoic acid 3-OH-Kyn	II May be formed due to Kyn oxidation, at higher Kyn concentration. Stable product and most probable contributing to yellowing. Down-stream Trp degradation marker. Intermediate relevance.	[1, 16, 20, 21]
22	 137.05 2-aminobenzoic acid Anthranilic acid	III Further downstream degradation product of Kyn, downstream product. Low relevance.	[4]
Aliphatic chain oxidation, reduction, and/or cleavage			
23	 202.07 (Z)-2-amino-3-(3H-indol-3-ylidene)propanoic acid	II May be formed by dehydration of 3-OH-Trp. Chromophore shifted towards visible spectrum. E/Z isomers possible. Interesting, but lower relevance.	[19, 21]
24	 202.07 (Z)-2-amino-3-(1H-indol-3-yl)acrylic acid	II Isomer of the substance above, probably thermodynamically most favourable form. E/Z isomers possible. Interesting, intermediate relevance.	[19, 21]
25	 145.05 1H-indole-3-carbaldehyde IC	II Aromatic aldehyde, a known degradation product, albeit downstream. Probably a strong chromophore. Quite reactive to many functional groups. Relevant as mechanistic marker but not as early Trp degradation marker. Intermediate relevance.	[11]

26	 175.06 2-(1H-indol-3-yl)acetic acid IAA	I De-aminated, reduced product. May participate in internal redox reaction. Relevant since it eliminates need for an external oxidant (e.g. free radical). Albeit, chemically not common reaction of amino acids. Highly relevant for mechanistic consideration.	*
27	 191.06 2-(5-hydroxy-1H-indol-3-yl)acetic acid 5-OH-IAA	III 5-OH product of substance above. Hydroxylation in position 5 is not most favourable. Low relevance.	*
Dimerization products			
28	 333.15 3-(6-((1H-indol-3-yl)methyl)-1H-indol-3-yl)-2-aminopropanoic acid	II Unexpected dimer characterized by MS studies. Attributed to oxidation in solution, not an analytical artefact. Medium relevance.	[2, 7, 19]
29	 434.20 3,3'-(ethane-1,1-diylbis(1H-indole-1,3-diyl))bis(2-aminopropanoic acid) 1,1-EBT	III Known impurity in Trp manufacturing. May be formed due to oxidative reaction, strongly depending on temperature and pH value. Low relevance.	[16, 18]

Tab. A 2: Theoretical Trp degradation substances. Degradation substances categorized for relevance due to probability and analytic aspects (I: high, II: intermediate, III: low). Comments give a brief assessment with regard to literature and internal communication/discussion with Fresenius Kabi (Bad Homburg, Germany).

**no supporting literature found; comments refer to internal discussions and information provided by Fresenius Kabi.*

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8.4 List of publications and documentation of authorship

I. Stability and the assessment of amino acids in parenteral nutrition solutions

Unger, N., Holzgrabe, U.

Journal for Pharmaceutical and Biomedical Analysis, Volume 147 (2018), pages 125-139

II. Investigation of tryptophan-related yellowing in parenteral amino acid solution: development of a stability-indicating method and assessment of degradation products in pharmaceutical formulations

Unger, N., Ferraro A., Holzgrabe U.

Journal for Pharmaceutical and Biomedical Analysis, Volume 177 (2020), 112839

This section contains a list of the individual contributions of each author to the publications reprinted in this thesis.

I. **Unger, N., Holzgrabe U.;** Stability and the assessment of amino acids in parenteral nutrition solutions

Journal for Pharmaceutical and Biomedical Analysis, Volume 147 (2018), 125-139

Author	**1**	**2**
Literature review	x	
Manuscript planning	x	x
Manuscript writing	x	
Correction of manuscript	x	x
Supervision of Nina Unger		x

III. **Unger, N., Ferraro A., Holzgrabe U.;** Investigation of tryptophan-related yellowing in parenteral amino acid solution: development of a stability-indicating method and assessment of degradation products in pharmaceutical formulations

Journal for Pharmaceutical and Biomedical Analysis, Volume 177 (2020), 112839

Author	**1**	**2**	**3**
Study design/concept development	x	x	x
Experimental planning	x		
HPLC method development/validation	x		
Data analysis/interpretation	x		
Mass spectroscopy	x		
Substance synthesis/work-up	x	x	
NMR data analysis and interpretation	x	x	
Manuscript planning	x		x
Manuscript writing	x		
Correction of manuscript	x	x	x
Supervision of Nina Unger			x

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Prof. Dr. Ulrike Holzgrabe:

Nina Unger:

8.5 Abbreviations

1,1-EBT	1,1′ Ethylidene(bis)-Trp
4-OH-Qn	4-Hydroxy-Quinoline
5-OH-(DL)-Trp	5-Hydroxy-(DL)-Tryptophan
AA	amino acid
ACN	acetonitrile
AiO	all-in-one
API	active pharmaceutical ingredient
BPC	base peak chromatogram
C18	octadecyl carbon chain
CI	confidence interval
DiOia	Di-Oxindolylalanine (*R,R/R,S 2*-amino-3-hydroxy-2-oxoindolin-3-yl)propanoic acid)
DL-Met	(D/L)-Methionine
DL-Pro	D-/L-Proline
EIC	extracted ion chromatogram
EMA	European Medicines Agency
EMS	Eosinophilia-Myalgia syndrome
ESI	electrospray ionization
FDA	Food and Drug Administration
HPLC	high performance liquid chromatography
HPBA	heptafluorobutyric acid
ICH	International Conference of Harmonisation
IP	ion pair
IS	internal standard
IV / i.v.	intravenous
Kyn	Kynurenine
L-Ala	L-Alanine
L-Arg	L-Arginine
L-Gly	L-Glycine
L-His	L-Histidine
L-Ile	L-Isoleucine
L-Leu	L-Leucine
L-Lys	L-Lysine

LOD	limit of detection
LOQ	limit of quantification
L-Phe	L-Phenylalanine
L-Ser	L-Serine
L-Thr	L-Threonine
L-Trp	L-Tryptophan
L-Tyr	L-Tyrosine
L-Val	L-Valine
MCB	multi-chamber bags
MeOH	methanol
MLB	multi-layered bags
MS	mass spectroscopy
N-Ac-Trp	N-Acetyl-(DL)-Trp
NFPA	nonafluoropentanoic acid
NFK	N´-formylkynurenine
NMR	Nuclear Magnetic Resonance
NP	nitrogen-purged
OC	oxygen-containing
Oia	Oxindolylalanine (*R,R/R,S* 2-amino-3-(oxoindolin-3-yl)propanoic acid)
PFP	Ppntafluorophenyl
Ph. Eur	European Pharmacopoeia
PIC	*cis/trans* 3a-hydroxy-1,2,3,3a,8,8a-hexahydro**p**yrrolo[2,3-b]**i**ndole-2-**c**arboxylic acid
Prep LC	preparative liquid chromatography
PVC	polyvinylchloride
R^2	coefficient of determination
RP	reversed phase
R_t	retention time
s.p.	system peak
TIC	total ion chromatogram
ToF-MS	time-of-flight mass spectrometry
Trp	tryptophan
UV/Vis	ultraviolet/visible (light)

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