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Master Thesis

Master of Science (M.Sc.)

Establishment of RP-HPLC and SDS-PAGE analytics of an antiseptic peptide to investigate its elimination on a model waste water plant

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Abstract

The use of antibiotics in the human health care leads to serious issues. Frequent use and often misuse of antibiotics results in multi - resistant bacteria (Bérdy, 2012). An incomplete degradation of the antibiotics in communal waste water plants supports the development of resistances (Kårelid et al., 2017). Thus, it is not only necessary to establish new ways of waste water purification to eliminate pharmaceutical drugs but as well to find new drugs to overcome the problematic of resistance. A promising approach is the development of peptide drugs.

The aim of this study was the characterization of the antiseptic peptide Aspidasept®. For this purpose analytical methods such as the RP-HPLC and the SDS-PAGE were established. Afterwards, the behavior of Aspidasept® on three stages of a lab scale sewage plant was examined to investigate in the purification of local waste water contaminated with pharmaceutical peptides. The adsorption on granulated activated carbon at different conditions, the filtration performance and the behavior on the biological stage were observed.

The characterization of the peptide showed the formation of dimers in aqueous solutions. Both established methods were suitable for the analysis and showed equal results. An antimicrobial activity was not observed. The adsorption on granulated activated carbon showed no significant temperature dependent behavior. With increasing activated carbon concentration the adsorption equilibrium decreased. The adsorption behavior can be well described using the *Freundlich*-Isotherm. For the adsoption in dem. water, the *Freundlich* parameters K = 0.187 $[(\mu g/g_{GAC})/(\mu g/L)]^n$ and n = 0.985 were obtained. For the filtration with a 1000 Da nanofiltration membrane a retention of 100 % were achieved. The filtration results in low recovery rates of the peptide (between 40 % and 85 %). The recovery is depending on the ionic strength and the pH of the solution. The trials in synthetic waste water showed a complete adsorption of the peptide onto the particles of the waste water. A chemical recovery of the peptide from the particles was feasible. The obtained results indicate that the purification of peptide contaminated waste water with the described methods is possible.

Contents

I Abbreviations

AMP	Antimicrobial peptide
APS	Ammonium per sulfate
CV	Column volume
DMSO	Dimethylsulfoxid
DTT	Dithiothreitol
GAC	Granulated activated carbon
LOD	Level of detection
LPS	Lipopolysaccharide
MIC	Minimal inhibitory concentration
MW	Molecular weight
PAC	Powdered activated carbon
RDA	Radial diffusion assay
RO	Reverse osmosis
RP-HPLC	Reversed-phase-high performance liquid chromatography
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SMX	Sulfamethoxazole
SOP	Standard operating procedure
SPE	Solid-phase extraction
TEMED	Tetramethylethylenediamine
TFA	Trifluoroacetic acid
TMP	Trimethoprim

II Symbols

Ab_{feed}	Absorbance of feed	[AU]
Ab_{per}	Absorbance of permeate	[AU]
Ab_{ret}	Absorbance of retentate	[AU]
c	Concentration of the adsorbing substance	[µg/L]
c_0	Start concentration of adsorbing substance	[µg/L]
c_{eq}	Equilibrium concentration of adsorbing substance	[µg/L]
K	Isotherm-parameter	$[(\mu g/g_{GAC})/(\mu g/L)]^n$
m	Adsorbent mass	[g]
m_{GAC}	Granulated activated carbon mass	[g]
n	Isotherm-parameter	[-]
q	Loading of the adsorbent	[µg/g]
q_{eq}	Loading of adsorbents at equilibrium	[µg/g]
R	Recovery	[%]
T	Temperature	[°C]
V_L	Experimental volume	[L]

III Appendix index

1 Introduction

Sepsis is one of the leading causes of death worldwide. In Germany, with 154,000 infections and 54,000 deaths annually, it is the third often deaths cause (Deutsche Sepsis-Hilfe, 2004). Thus, it results more often in death than colon cancer, breast cancer and AIDS (Deutsche Sepsis-Hilfe, 2004). With an estimated amount of approx. 800,000 infections per year in the United States it will increase to 1,600,000 infections by 2050 (Kumar, 2014). For the treatment of sepsis no specific drug is available (Brandenburg and Schürholz, 2015). In most cases the first therapeutic approach is the treatment of the bacterial infections which otherwise will result in sepsis. Commonly, antibiotics are used as therapy. However, this approach fails more often due to a developing resistance of bacteria against conventional antibiotic (Brandenburg and Schürholz, 2015). To overcome the crucial issue of sepsis treatment and the problematic of bacterial resistance, new approaches are required.

The first approach is to stop the progress of new bacterial resistance. These resistances are developed due to the fact that bacteria are not only coming in contact with antibiotics if they are used for a therapeutic issue but also in the whole environment. The way of antibiotics leads from the patient (or animal farm and other industries) to the waste water treatment plants. With the conventional 3-stage treatment methods a complete degradation of the antibiotics is not achievable. New methods for the waste water purification are required.

Investigations in methods for a 4^{th} stage such as activated carbon, ozonation, the removal with ferrate, advanced oxidation processes or nanofiltrations were made. Some of these methods made their way from the research to the economic application in local waste water plants (KOMS, 2018). The adsorption of micro-pollutants pharmaceuticals on activated carbon is already implemented in a lot of German waste water plants. But as well the realization of the ozonation is planned in some treatement plants.

Nevertheless, even though a complete degradation of antibiotics is possible, the treatment of sepsis and the increasing number of multi-resistant bacteria is still an issue to be discussed. New pharmaceuticals, not only new antibiotics, could be the

answer. Substances with a great opportunity are antimicrobial peptides. These show trends of a high therapeutic index and a low tendency of bacterial resistance (Brandenburg and Schürholz, 2015). The substance Aspidasept® belongs to the class of antimicrobial peptides. Aspidasept® shows as well an effect against septic shock. By binding free lipopolysaccharides which normally would cause an increased immune response a sepsis is prevented (Brandenburg and Schürholz, 2015). Even if Aspidasept® shows a great possibility as pharmaceutical, the elimination after the usage as drug has to be examined to exclude negative effects on the environment.

The aim of this study was the establishment of RP-HPLC and SDS-PAGE analytics of the antiseptic peptide Aspidasept®. The developed methods were used for the investigation of stability and behavior in various environments. For the concentration of samples the solid phase extraction should be examined. An antimicrobial activity was evaluated with the radial diffusion assay. After characterization, the antiseptic peptide was used for an investigation of elimination on a model waste water plant. The adsorption on granulated activated carbon (GAC) with variation in temperature and GAC amount was examined. With the obtained data the *Freundlich*-Isotherm was determined to decide about the economic efficiency. Furthermore, the behavior of Aspidasept® during filtration and on the biological stage of the waste water plant was investigated.

2 Theoretical background

2.1 Occurrence and toxicity of antibiotics

There are various ways antibiotics get into the environment (Wang et al., 2007). Beside the human health care where antibiotics get into waste water by human excretion or by the waste of hospitals and pharmaceutical industries, agriculture and livestock farming are the major ways (Wang et al. 2007; Hollis and Ahmed, 2013). Farm animals were treated with antibiotics to increase growth and health (Stephenson, 2001; Hollis and Ahmed, 2013). Only parts of the antibiotics were metabolized (Ebert et al, 2014). The excrements of the animals were distributed on field and heavy rain leads to a wash out. Thereby, the antibiotics get into surface water which leads to an entrance into ground and drinking water. A direct entry into the surface water is the treatment of fishes in aquacultures (Hollis and Ahmed, 2013). In the USA more than 80 % of the used antibiotics were consumed in the agriculture and aquaculture to increase the food production (Hollis and Ahmed, 2013).

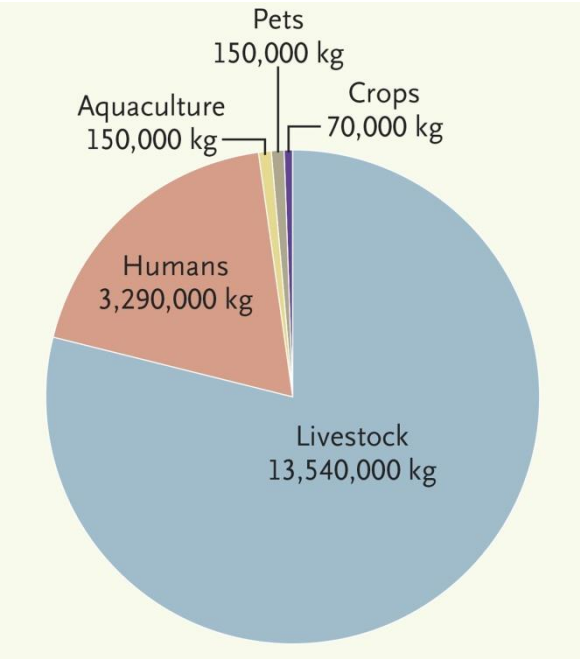


Figure 2.1: Annual amount [kg] of antibiotics used in the USA. Approx. 80 % of antibiotics are used in the agriculture (crops and animals) and aquaculture. Only 20 % of antibiotics are used in the human health care (Modified from Hollis and Ahmed, 2013).

The above specified circumstances indicate that annually several tons of antibiotics get into the environment, mostly in the aquatic surrounding. Two specific occurrences can be defined. The direct leads trough agriculture and aquaculture. In the indirect antibiotics are treated in local waste water plants (Ebert et al., 2014). The release of antibiotics into the environment results in antibiotic resistance in bacteria (Kumar et al., 2012). Resistant bacteria show a better chance of survival which increases the pressure on bacteria to develop these resistances.

Three main mechanisms can be defined to explain the impact of antibiotics on the human health (Chang et al., 2014). The first one is the direct infection with resistant bacteria coming from an animal without further spreading of the pathogen. A second mechanism can be described by an infection of a human and an ongoing transmission to other humans (Hollis and Ahmed, 2013; Chang et al., 2014). In this scenario only some of the human will get ill. As maybe most important and most difficult to study the third mechanism can be named. The mechanism described the transfer of resistance genes coming from animal farms into human pathogens (Chang et al., 2014). This horizontal gene transfer between the species is difficult to trace. A rising number of antibiotic resistant bacteria lead to an increase of problems in the human health care.

2.2 Peptides as pharmaceuticals

To overcome the dramatic developments in the human health care caused by increasing resistances against established antibiotics and a lack of new pharmaceuticals, an advanced research in novel drugs is in urgent need (Bérdy, 2012). Part of a solution of this concern in healthcare can be substances from plant, animal or microbial origin. Until today, more than 1 million natural compounds are discovered (Bérdy, 2005). Among them, only 5 % of the substances have been obtained from microbes. From all discovered natural compounds 20 – 25 % were identified as substances with biological activity. Only 10 % of the biological active substances are coming from a microbe origin (Demain and Sanchez, 2009).

Beneath compounds from natural origin, synthetic, chemically produced substances are under research or rather on the market.

Some of these natural or synthetic compounds with a great potential to stop the antibiotic crisis are antimicrobial peptides (AMPs). These peptides can be found in all living kingdoms as a product of the innate immune system (Mangoni et al., 2016). AMPs show various favorable effects suitable for the usage in the human healthcare system. The small peptides (generally 10 to 50 amino acids) were first recognized for their antimicrobial activity (Mangoni and Shai, 2011). Most AMPs share a majority of basic residues resulting in a cationic charge and an amphipathic structure in membrane-mimicking environments (Mangoni and Shai, 2011). With their simple secondary structure of α – helices, β – sheets and extended structures AMPs are considered as very stable in terms of heat treatments and other denaturation effects. The cationic structure helps to interact and unspecifically bind to the anionic phospholipids of cell membranes. This binding leads to the formation of pores and the destruction of the cell (Brogden, 2005). Due to this unspecific mode of action, microbes are rather unlikely to evolve resistances as it is when it comes to antibiotic targeting enzymatic receptors (Hale and Hancock, 2014; Fjell et al., 2011).

But, beside an antimicrobial activity of the AMPs various other effects were discovered showing a great opportunity for new pharmaceuticals for e.g. wound healing (Mangoni et al., 2016). Additionally to the wound healing some peptides have antiviral (e.g. anti-HIV) or anticancer properties (Wang et al., 2016). The synthesis of collagen, cytokine release, angiogenesis, chemotaxis, cell migration and proliferation and lipopolysaccharide (LPS) neutralization are known as favorable effects for wound healing (see figure 2.2).

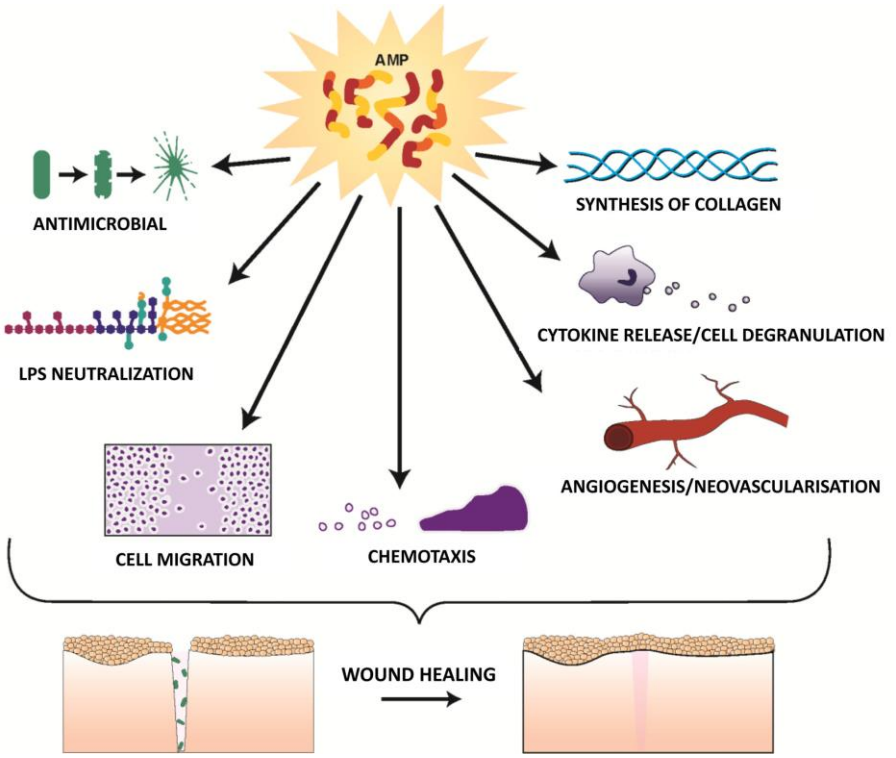


Figure 2.2: Schematic representation of the spectrum of activity of antimicrobial and antiseptic peptides relating to wound healing. The effect of AMPs includes an antimicrobial activity, LPS neutralization (sepsis prevention), cell migration and activation, chemotaxis, angiogenesis/ neovascularization, cytokine release/ cell degranulation and synthesis of collagen. (Mangoni et al., 2016)

2.3 Aspidasept® as antiseptic peptide

A promising approach for an alternative of antibiotics shows the peptide Aspidasept® (Pep19-2.5). Aspidasept® was developed based on the anti-LPS factor of the horseshoe crab (*Limulus*) (Gutsmann et al., 2010). The synthetic peptide consists of 20 amino acids with a total molecule weight of 2711.3 Da. The detailed amino acid sequence is showed in table 2.1.

Table 2.1: Amino acid sequence of the antiseptic peptide Aspidasept® (Pep19-2.5). Symbols: G – Glycine, C – Cysteine, K – Lysine, Y – Tyrosine, R – Arginine, F – Phenylalanine, W – Tryptophan (Gutsmann et al., 2010).

Peptide	Amino acid sequence
Aspidasept® (Pep19-2.5)	GCKKY RRFRW KFKGK FWFWG

Aspidasept® consists of a high degree of polar and positive charged amino acids with hydrophobic residues resulting in an amphipathic character (Gutsmann et al., 2010). It was initially developed as a drug against bacterial infections and inflammations. The peptide is supposed to bind and neutralize the lipid – A part of the LPS of gram-negative bacteria (Brandenburg and Schürholz, 2015). But as well an effect on gram-positive bacteria was discovered (Schürholz et al., 2013). In various trials the peptide showed an antibacterial effect against gram-negative bacteria (e.g. *Escherichia coli, Pseudomonas aeruginosa*) and gram-positive bacteria (e.g. multi-resistance *Staphylococcus aureus*) (Gutsmann et al., 2010; Pfalzgraff et al., 2016). In table 2.2 some minimal inhibitory concentrations (MIC) for Aspidasept® can be found. Aspidasept® indicates an inhibitory effect on the production of the LPS-induced tumor-necrosis-factor-α (Gutsmann et al., 2010; Heinbockel et al., 2015).

Table 2.2: Minimal inhibitory concentration (MIC) of Aspidasept®. (Kaconis, 2013)

Bacteria strain	**MIC [µg/mL]**
Escherichia coli WBB01	32
Salmonella enterica serovar Minnesota R60	128

The peptide accentuates from the others by showing an impact against sepsis (Brandenburg and Schürholz, 2015). Figure 2.3 displays a schematic view how the peptide can prevent a septic shock. Free LPS from gram-negative bacteria can bind to the toll-like receptor 4 (TLR4) of immune cells (Peri et al., 2010; Brandenburg and Schürholz, 2015). These cells respond in an over-productive manner with a cytokine release which leads to sepsis and often death. If Aspidasept® is present, free LPS are neutralized and a lower cytokine release results in a normal immune responds. Experience in rats showed that the necessary drug concentration for the treatment against sepsis is far below the critical value at which side effect are possible (Brandenburg and Schürholz, 2015).

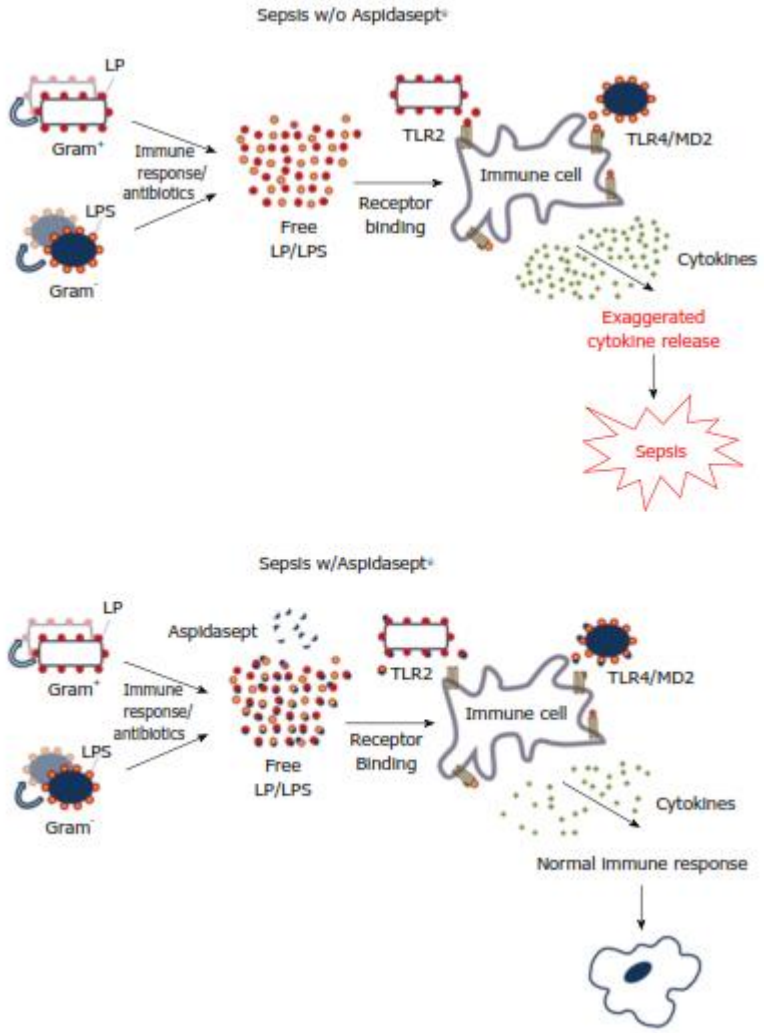


Figure 2.3: Schematic view on the immune response without and in the presence of Aspidasept®. The top drawing shows how LPS induce an exaggerated cytokine response resulting in a sepsis. Below, the effect of the LPS neutralizing peptide is shown (Brandenburg and Schürholz, 2015).

2.4 Methods for the elimination of pharmaceuticals

The sewage of households and industry is purified in waste water plants. In different stages, the sewage is cleared from dirt, nitrate, phosphoric substances and other compounds. But some pollutants, which show a negative effect on the aqueous life and human existence, cannot be removed with the old established technology of a 3-stage waste water plant (Eisenächer and Neumann, 1982). The conventional 3-stage waste water plant consists of mechanical, biological and chemical treatment methods. During the mechanical treatment gross contaminations such as stones,

leaves, dead animals, glass splinters and sand are removed. The following primary clarifier is used for the separation of undissolved materials and paper. The biological treatment consists of a two-step mechanism. During the aerobic treatment (nitrification) bacteria and protozoa (activated sludge) transform ammonium-nitrogen-compounds to nitrate-nitrogen. In the following anaerobic treatment, these compounds are transformed to gaseous nitrogen and oxygen. The activated sludge is removed from the waste water by sedimentation. In a final step chemical treatment with floatation agents and disinfectants (optional) is performed.

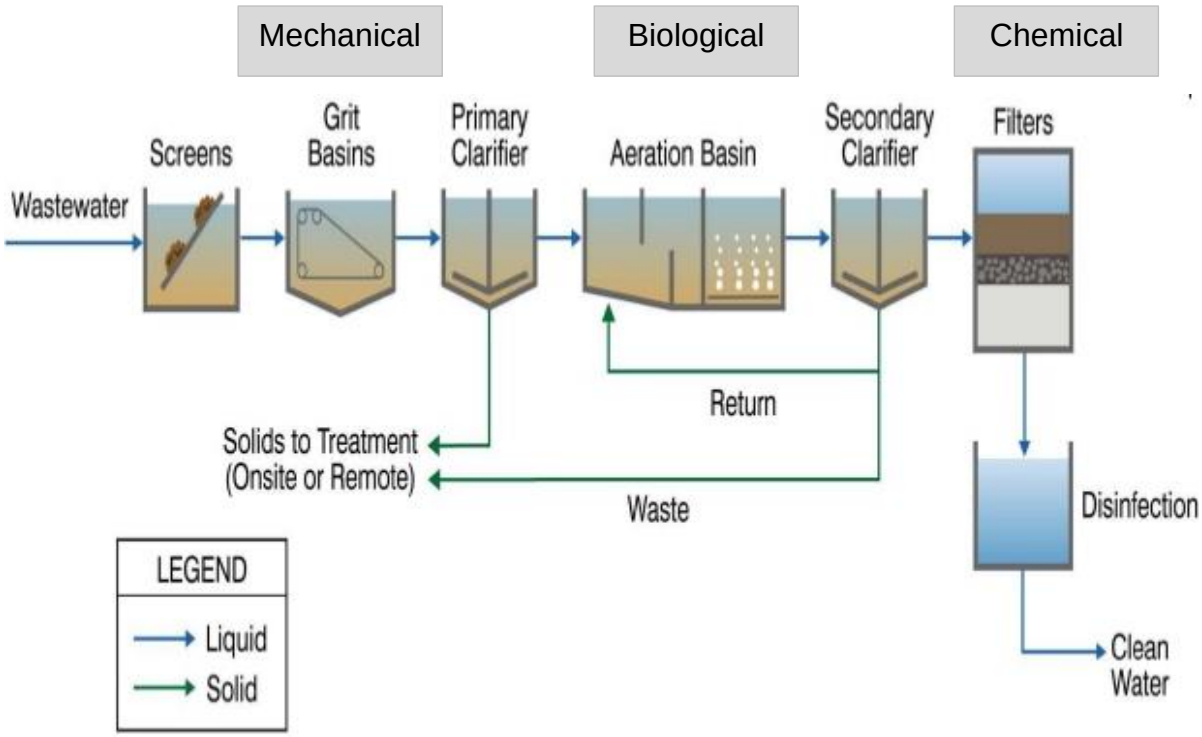


Figure 2.4: Typically 3-stage waste water treatment plant. 1st stage: mechanical treatment to remove gross soiling. 2nd stage: biological treatment for the degradation of carbon-nitrate-compounds. 3rd stage: chemically treatment with flocculating agents. The 3rd stage varies depending on the treatment plant. (Modified from Gezawa, 2014)

Despite the established methods for sewage clearance, it cannot be ensured to remove several compounds such as hormones, antibiotics and pesticides. These substances show an adverse impact on the environment. Hormones can damage the reproduction of aqueous life (Parry, 2012) and antibiotics will support the generation of multi-resistance bacteria. Thus, a 4th treatment step is necessary.

Different approaches were made and some were implemented so far. The adsorption of micro-pollutants on granulated activated carbon (Germany) or powdered activated carbon (Switzerland) is used in several plants (KOMS, 2018). But as well the ozonation, the removal with ferrate, advanced oxidation processes or a nanofiltration step are planned. Although these processes promise adequate results, they are still not implemented in many waste water treatment plant due to increasing costs.

2.4.1 Activated carbon adsorption in the waste water treatment

Adsorption is defined as accumulation and binding of a substance (adsorbing molecule) in liquid onto the surface of a second liquid or a solid material – the adsorbents (Lohrengel, 2012; Worch, 2012). The surface of the adsorbents is significant for the adsorption. The larger the surface the more substance can be adsorbed. Thus, highly porous adsorbents with a large inner surface are beneficial (Worch, 2012).

For the usage of activated carbon in an adsorption experiment, it is required to consider various parameters (Lohrengel, 2012; Worch, 2012). The increase of the adsorbing molecule leads to an increase of the adsorption. With an increase of the inner surface of the adsorbents the adsorption will increases as well. The adsorption will decrease with increasing temperature. It was observed as well that with increasing polarity of the adsorbing molecule the adsorption decreases.

Beside the simple determination of adsorption equilibriums, the examination of adsorption dynamics and adsorption kinetics are necessary to draw conclusion regarding the economic efficiency of a process (Worch, 2012). Adsorption equilibriums and adsorption kinetics are required for the characterization of the adsoption dynamics. The adsorption kinetic describes the time course of the process. This course is dependent on the transport of the adsorbing substance to the hydrodynamic interface and the transport trough the interface to the outer surface of the adsorbent. The transport to the inner surface of the adsorbents and the adsorption in itself is following. Therefore, the adsorption speed is subordinate to the diffusion processes at the outer and inner surfaces of the adsorbents. It should be

pointed out that with decrease of the adsorbents size the adsorption equilibrium is obtained faster.

The adsorption equilibrium describes the equilibrium between substance in solution and adsorbed substance. At this time point a saturation of the adsorbents takes place and maximal loading concentration can be determined. If the adsorption process is performed at a constant temperature the following isotherm can be used for the specification.

$$q_{eq} = f(c_{eq}), T = const \qquad \text{(Equation 2.1)}$$

q_{eq}	Loading of adsorbents at equilibrium	[µg/g]
c_{eq}	Concentration at equilibrium	[µg/L]
T	Temperature	[°C]

Even through different isotherms are present, during this trial the *Freundlich*-Isotherm was used for the evaluation of the obtained data. The *Freundlich*-Isotherm is often used for the description of adsorptions in liquid and on activated carbon (Benström, 2017). Even through isotherms cannot be used to determine an exact adsorption capacity; estimations can be made if only one adsorbing substance is present. The loading of the adsorbents at equilibrium q_{eq} can be determined with equation 2.2 assuming that no other adsorption processes (e.g. adsorption on experimental setup or evaporation) are present.

$$q_{eq} = \frac{V_L}{m} (c_0 - c_{eq}) \qquad \text{(Equation 2.2)}$$

V_L	Experimental volume	[L]
m	Adsorbent mass	[g]
c_0	Start concentration of adsorbing substance	[µg/L]
c_{eq}	Equilibrium concentration of adsorbing substance	[µg/L]

Using the *Freundlich*-Isotherm (equation 2.3), the loading of the adsorbents can be described. The isotherm-parameter K describes the affinity of an adsorption process; n is a measure for the curve of an isotherm (Worch, 2012). If the parameter n = 1,

the isotherm is linear and the loading of the adsorbent is proportional to the concentration of the adsorbing substance. For n < 1 the mass of the adsorbent can normally be reduced. If n > 1, the process is not beneficial, only small loadings are possible (Lohrengel, 2012).

$$q = K \cdot c^n \qquad \text{(Equation 2.3)}$$

q	Loading of the adsorbent	[µg/g]
K	Isotherm-parameter	[µg/g]^n
c	Concentration of the adsorbing substance	[µg/L]
n	Isotherm-parameter	[-]

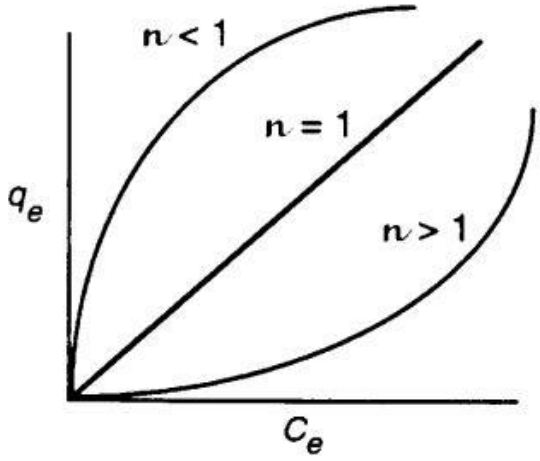


Figure 2.5: Schematic drawing of the *Freundlich*-Isotherm with different values of n. c_{eq} - loading of adsorbents at equilibrium, c_{eq} – concentration during equilibrium (modified from Arnold, 2017).

The use of activated carbon adsorption in local waste water plants has been demonstrated as suitable and economically efficient (Kårelid et al., 2017). If activated carbon should be used as a 4th sage first it has to be decided between granulated activated carbon (GAC) and powdered activated carbon (PAC). Both technologies are used in German waste water plants (GAC: e.g. Westerheim, PAC: e.g. Mannheim, Ulm). In the following the waste water treatment with GAC is characterized. In this case, the treatment takes place in packed bed adsorbers with GAC particles sizes between 0.5 and 4 mm (KOMS, 2018). After biological treatment

and secondary clarification the GAC technology is following directly (figure 2.6). For the determination of the adsorption capacity the above made descriptions can be made. Nonetheless, it has to be mentioned that the waste water has a significant impact on the adsorption of micro-pollutants (hormones and antibiotics) at activated carbon (Worch, 2012).

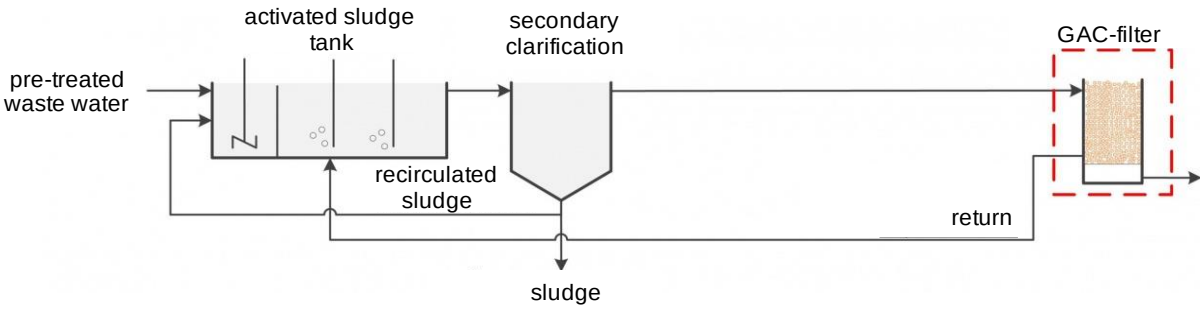


Figure 2.6: Treatment with granulated activated carbon (GAC). After biological degradation and secondary clarification, the adsorption with GAC is performed (modified from KOMS, 2018).

2.4.2 Membrane filtration

A membrane is a flat, permeable structure (Melin and Rautenbach, 2007). Whereas, only flat filter sheets were available in the past, nowadays membranes packed in various modules (e.g. wound module, or hollow fibers) are more often used. The filtration is a pressure driven, mechanical procedure which aims the clarification or separation of a mixture such as suspensions or aerosols (Melin and Rautenbach, 2007). A filter can be consists out of paper, metal, glass, synthetic material or textile fabric. In biotechnology mostly synthetic materials such as polyethersulfone, cellulose acetate or polyamide are used. The use of different materials leads to changing product membrane interactions. Polyethersulfone for example is hydrophobic which supports the adsorption of particles onto the membrane (Ohlrogge and Ebert, 2006).

During filtration not only particles larger than the membrane pores were separated because they cannot enter the pores. Retention of particles smaller than the pores will be achieved due to electrostatic interactions, particle inertia, diffusion effects or barrier effect (Melin and Rautenbach, 2007). The filtered phase is defined as

permeate. The retained phase is called retentate or filtrate. Two different filtration procedures have to be considered. The static dead-end filtration and the dynamic cross-flow filtration (figure 2.7). In case of the dead-end filtration (b) a forming filter cake leads to a blocking of the pore (fouling of the membrane) and an often high product loss (El-Safty and Hoa, 2012). To maintain a high flow the filtration has to be stopped and the membrane has to be cleaned or exchanged. During cross-flow filtration (a) no complete filter cake is formed because the feed passes the membrane with a high horizontal speed (Melin and Rautenbach, 2007). During this work a modified cross-flow filtration was used. The flow along the membrane was simulated by a magnetic stirrer.

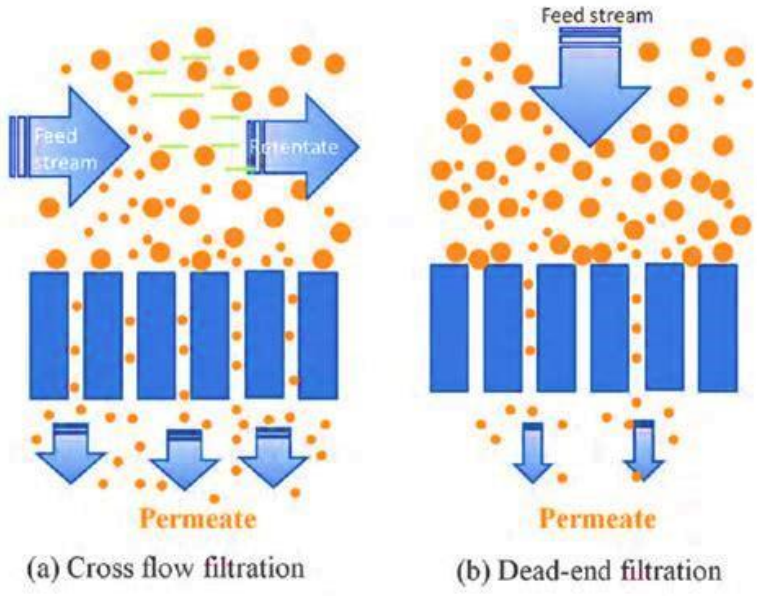


Figure 2.7: Two procedures for the membrane filtration: Cross-flow filtration and dead-end filtration. (a) The cross-flow filtration is a dynamic procedure with a horizontal flow over the membrane. (b) The dead-end filtration is static. Over the time a filter cake is forming and the membrane has to be cleaned or exchanged. (El-Safty and Hoa, 2012)

By varying the pore size of membranes the recovery of a membrane can be influenced. With the smallest pore size, reverse osmosis (RO) membranes have to be named. Particles have to be smaller than 1 nm to pass the membrane (Melin and Rautenbach, 2007). A RO membrane is able to recover nearly all in water dissolved substances. Nanofiltration membrane can be used for the separation of ions and

molecules in the molecule weight range of 200 – 2,000 Da (Basile and Charcosset, 2016). Ultrafiltration membranes usually recover particles with a molecular weight of 300 to 1,000,000 Da used for the separation of polymers, biomolecules and colloidal particles (Basile and Charcosset, 2016). The largest pore size for ultrafiltration membranes is around 0.1 µm (Melin and Rautenbach, 2007). With a pore size range between 0.1 – 1.0 µm a microfiltration is possible if macromolecules, colloids or suspended particles should be concentrated or separated (Basile and Charcosset, 2016).

To specify the membrane separation capacity, the Cut-Off is determined. The Cut-Off describes the size range were 90 % of particles with a specific molecular weight are retained (Melin and Rautenbach, 2007).

2.5 Analysis with high performance liquid chromatography

The possibility to separate mixtures with a high quantity of similar analytes is the central and required function of an efficient chromatographic separation technique (Kromidas, 2014; Meyer, 2009). A chromatography provides detailed information about the quality and quantity of a mixture or single analyte. The elution or retention time is characteristic for any substance. The area of an analyte peak is proportional to the quantity of a substance (Kromidas, 2014). Thus, a chromatography method can be used for the quantification and qualification of a single substance or a mixture of substances.

At a normal setup for the stationary phase of the chromatographic column a polar material (e.g. silica gel) is used. Consequently the mobile phase is nonpolar. A separation of mixtures is achieved by a differential adsoption of substances. In this case, nonpolar substance elutes earlier than polar substances (Meyer, 2009). Various separation mechanisms are possible, such as the attraction by van der Waals forces, ion exchange or affinity forces (Kromidas, validation guide). A second chromatographic approach is the reverse phase (RP) – stationary phase. The stationary phase consists of a nonpolar material (e.g. alkyl chains with various lengths) which is covalently bond to packing material (Ahuja, 1999). For the mobile phase a relatively polar solvent is used. The following principle applies: A nonpolar

solvent (e.g. acetonitrile) elutes faster than polar solvents (e.g. water) and a lower retention time (time from injection to detection) is achieved, this also results in the fact that the more polar a substance is the faster the elution takes place (Meyer, 2009).

Since the 1970s, the high performance liquid chromatography (HPLC) is known as a technique which promises the above made specifications. Nowadays, it is the dominating chromatographic method (Lundanes et al., 2013). Specific for the HPLC technique is the high pressure necessary to overcome the back-pressure of the small particles (less than 10 µm) of the stationary phase (Kaltenböck, 2008). A high pressure pump (up to 600 bar) is required to move the mobile phase. A typical HPLC instrument (see figure 2.8) consists of high pressure pumps with gradient unit (for the mixing of different solvents) and optional degasser, an autosampler and a column oven (Meyer, 2009). For the detection classical UV-detectors or diode array detectors (DAD) but as well fluorescence detectors can be used. The organizer, typically on top of the system, is used for the storage of solvents. The HPLC instrument is coupled to an evaluation software. A possible step for further processing of the HPLC separation is the coupling of the instrument with a mass spectrometer (LC-MS) (Kromidas, 2014).

During this study a HPLC from HITACHI with a RP-column were used. In chapter 3.6.1 the precise specifications of the used system with column and analytical software are explained.

Figure 2.8: Typical assembly of a HPLC instrument. The instrument consists of high pressure pumps with gradient unit and degasser, an autosampler, a column oven, the organizer for the storage of the mobile phase and evaluation software (modified from manufacturer specifications, HITACHI).

3 Materials and Methods

3.1 Equipment

In table 3.1, the, during this study used equipment is listed. The, for the radial diffusion assay (RDA) necessary equipment can be found in the appropriate SOP.

Table 3.1: List of used equipment

Equipment	Vendor
Ultrapure water arium® pro UV	Sartorius
Balance 770-60	Kern
Balance BP 221S	Sartorius
Balance BP 3100S	Sartorius
Balance Entris 321I-1S	Sartorius
Centrifuge minispin	Eppendorf
Conductivity meter Cond 3310	WTW
Flat filter holder for pressure filtration	MMS membrane systems
Heating Block AccuBlock Digital Dry Bath	Labnet
HPLC Primaide	Hitachi
Laboratory waste treatment system KLD4N/SR	Behr Labor-Technik
Magnetic stirrer MR Hei-Standard	Heidolph
pH-meter 766 Calimatic	Knick
Pipette Transferpette® S (10 µL/ 100 µL/ 200 µL/ 1000 µL)	Brand
Pump (vacuum)	KnF Lab Laboport
Rocking Platform	VWR
SDS-PAGE PowerPac Basic	BIO RAD
Shaked water bath with cooling unit	GFL
Shaker Reaxtop	Heidolph
Spectrometer Ultrospec 2100pro	Amersham Biosciences

3.2 Materials

In table 3.2, the used materials for the development of the chromatographic method and the performance of the SDS-PAGE are listed. As well all materials, which were required for the experiments, are listed. The, for the RDA necessary materials and chemicals can be found in the appropriate SOP.

Table 3.2: List of used materials

Materials	**Vendor**
Beakers	Schott/ KIMAX
C18 ec SPE cartridge (chromabond®)	Macherey-Nagel
Cellulose nitrate filter (0.45 µm)	Sartorius
Falcon Tubes (15 mL/ 50 mL)	VWR
Flat filter membrane: TRISEP® UA60	MICRODYN-NADIR
Flat filter membrane: XLE, NF90, NF270	DOW
Glass bottles 25 mL	Schott
Glass bottles (pressure stable) 500 mL/ 1 L	Schott
Graduated cylinders	Brand
Granulated activated carbon (Hydraffin AR 8x30)	*Donau* Carbon
HPLC glass vials (300 µL/ 1,5 mL with micro inlay)	Thermo Scientific/ VWR
Magnetic stirrer	-
Pipette tips (10 µL / 200 µL/ 1000 µL)	Brand
Reaction vessel 1.5 mL/ 2 mL	Eppendorf
Scoop	-
Syringe filters (0.45 µm)	VWR
Syringe	Henke Sass Wolf
Weighing bowls	-

3.3 Chemicals

In table 3.3, the used chemicals for the development of the chromatographic method and the performance of the SDS-PAGE are listed. The, for the RDA necessary materials and chemicals can be found in the appropriate SOP.

Table 3.3: List of used chemicals

Chemicals	Vendor
Acetate	Roth
Acetic acid	VWR
Acetonitrile	Roth
Acryl/Bisacrylamide (40 %)	Roth
APS	Roth
Boric acid	Roth
Dimethyl sulfoxide	Roth
Ethanol	Roth
Glutardialdehyd (25 %)	Merck
Glycerol	Roth
HEPES	Roth
Hydrochloric acid	Roth
Methanol	VWR
Orange G	Sigma-Aldrich
Roti Blue (5x) /Roti®-Quant Universal	Roth
Sodium azide	Roth
Sodium chloride	Roth
Sodium hydroxide	Roth
Sodium dodecyl-Sulfate	Roth
TEMED	Roth
Tricine	Roth
Trifluoroacetic acid	Alfa Aesar
Tris	Tris

3.4 Proteins and peptides

In table 3.4, the used peptides and proteins for the development of the chromatographic method, the performance of the SDS-PAGE and the membrane filtration are listed.

Table 3.4: List of used peptides and proteins

Peptide/ Protein	Vendor
Aspidasept®	Provided by Klaus Brandenburg
Bromophenol blue	Roth
DL-Tryptophan	Merck
L-Glutathione	Roth
Lysozyme	Sigma-Aldrich
Vitamin B12	Roth

3.5 Handling of Aspidasept®

To anticipate the issue of peptide stability and recovery, the handling of Aspidasept® has to be done with strict regulations. Depending on the experiment, Aspidasept® was dissolved in 0.01 % or 0.1 % trifluoroacetic acid (TFA) or 0.1 % dimethylsulfoxid (DMSO). Whereas TFA (strong acidic pH) is often used for cationic peptides, it is recommended to use an organic solvent such as DMSO for dissolving hydrophobic peptides. Since, Aspidasept® consists of both, cationic and hydrophobic amino acids the solvents show similar stabilization effects. Aspidasept® stock solutions had a concentration of 1 mM and were prepared in 0.01 % or 0.1 % TFA. After the dissolution of the peptide a heat denaturation was carried out for 10 min at 95 °C. For the activated carbon adsorption experiments, Aspidasept® was dissolved directly in 0.1 % DMSO. Diluted DMSO indicates a pH at approx. 9 which is more suitable to the sewage than acetic TFA.

This study was started with stability and recovery tests (results see chapter 4.1.5). Referring to these tests the storage conditions were defined. Stock solutions and samples with a longer storage time than 24 h or samples readily prepared for the Tricine-SDS-PAGE were frozen at -20 °C to prevent degradation over the time. Samples which were analyzed within 24 h were stored at 5 °C.

3.6 Analytical Methods

For the analysis of peptide samples, different analytical methods were established. All methods have to match the same requirement of peptide stability and recovery. The methods have to be designed in a way that precipitation and peptide changes are prevented for the time of analysis. The reversed - phase - high performance liquid chromatography (RP-HPLC) and the Tricine - sodium dodecyl sulfate poly-acrylamide gel electrophoresis (Tricine-SDS-PAGE) were used for the identification and quantification of the peptide. For the sample concentration, the solid-phase extraction (SPE) should be implemented. To test for an antimicrobial activity the radial diffusion assay (RDAI with *Escherichia coli* was performed.

3.6.1 Reversed-phase-HPLC

For the identification, quantification and verification of Aspidasept® in water samples, the reversed - phase - high performance liquid chromatography (RP-HPLC) was established. In this study, the C18-RP-column 218TP™ 5 µm from Vydac® with a RP-18 (5 µm) guard column from LiChrospher® was used. The 218TP™ column consists of a reversed – phase (hydrophobic) stationary phase of octadecyl alipathatic (C18) groups. The stationary phase is attached to the surface of 300 Å pore diameter silica particles with 5 µm diameter (Hichrom, manufacturer specifications). As shown in figure 3.1 the C18 groups are bonded to the silica beads using polyfunctional octadecylchlorosilanes. This results in a polymerization of the hydrophobic C18 phase.

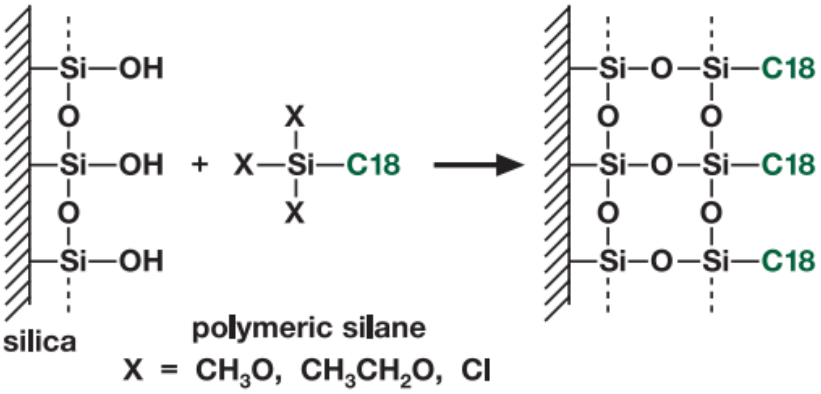


Figure 3.1: Bonding of the hydrophobic phase to silica using polymeric silane. Polymeric attachment of octadecyl alipathatic (C18) groups to the stationary silica phase of a HPLC column by Vydac® (Hichrom, manufacturer specifications)

The 218TP™ column was especially selected because it is designed for the separation of small polypeptides with a molecular weight less than 4 – 5 kDa (Hichrom, manufacturer specifications). The chromatography was performed with the Primaide HPLC from HITACHI and analyzed with the Primaide software. A 218TP™ column with the dimensions of 4.6 mm inner diameter and 250 mm length (4.2 mL column volume) was integrated.

For the HPLC a careful sample preparation was necessary to protect the column against peptide precipitation, blocking and bacteria contaminations. All samples were centrifuged for 10 min at 12.100 x g. Samples with higher contaminations, such as sewage samples, were passed through a 0.45 µm Nylon filter (sterile filtration). Shortly before injection, the samples were stirred using a Vortexer to ensure a homogeneous mixing of the sample.

Since Aspidasept® consists of characteristic hydrophobic and hydrophilic residues this property can be used for the development of an appropriate chromatographic profile. For this study, the in figure 3.2 described profile was established. Two buffers were needed. Buffer A contains a polar mobile phase with 0.1 % TFA (buffer A). Buffer B is a mixture of 70 % acetonitrile (ACN) and 0.1 % TFA (buffer B). With an increasing gradient of the hydrophobic acetonitrile, the elution of the peptides was accelerated. Before sample injection, the column was equilibrated with a mixture of buffer A and B in a ratio of 93:7 which results in a 5 % ACN solution with 0.1 % TFA. During the whole chromatographic run a flow rate of 1 mL/min was set. The column temperature was set to constant 50 °C. After sample injection the flow proceeds for 10 min with the buffer ratio of 93:7 to wash out unbound molecules. To elute the bound molecules, the ACN concentration was increased resulting in a decrease of interactions between the sample components and the hydrophobic stationary phase. Within 20 min, the ACN concentration was increased to 70 % (buffer A:B ratio 0:100). To remove remaining hydrophobic contaminations the ANC concentration was kept at 70 % for another 5 min. The run was finished with 10 min of 5 % ACN for a re-equilibration of the column. The absorbance was measured at 214 nm with a diode array detector.

To reduce permanent bound hydrophobic molecules and other contaminations the column was cleaned with 100 % methanol and 100 % acetonitrile with a reverse flow of 0.5 mL/min. For long term storage the column was rinsed with 70 % methanol.

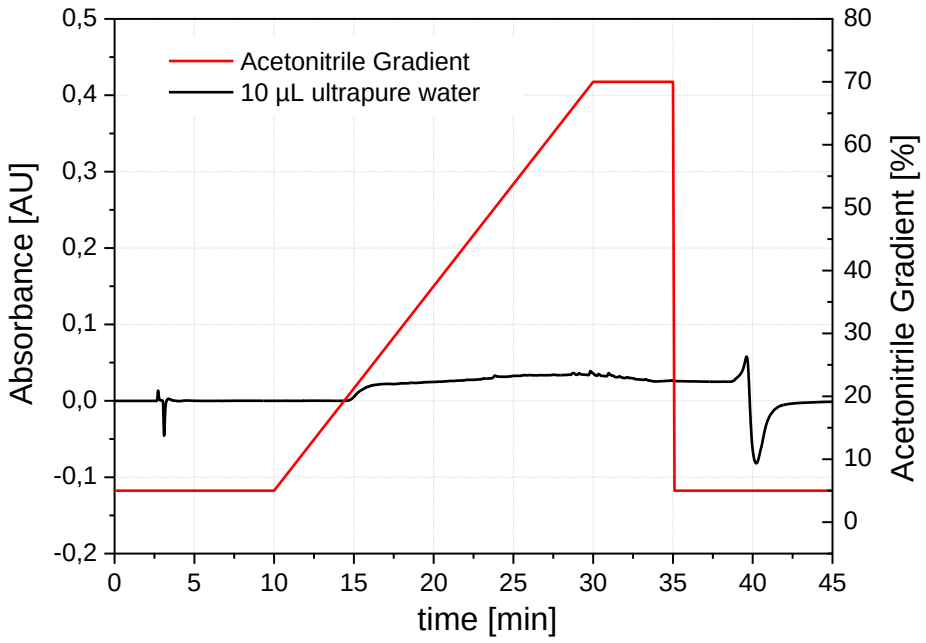


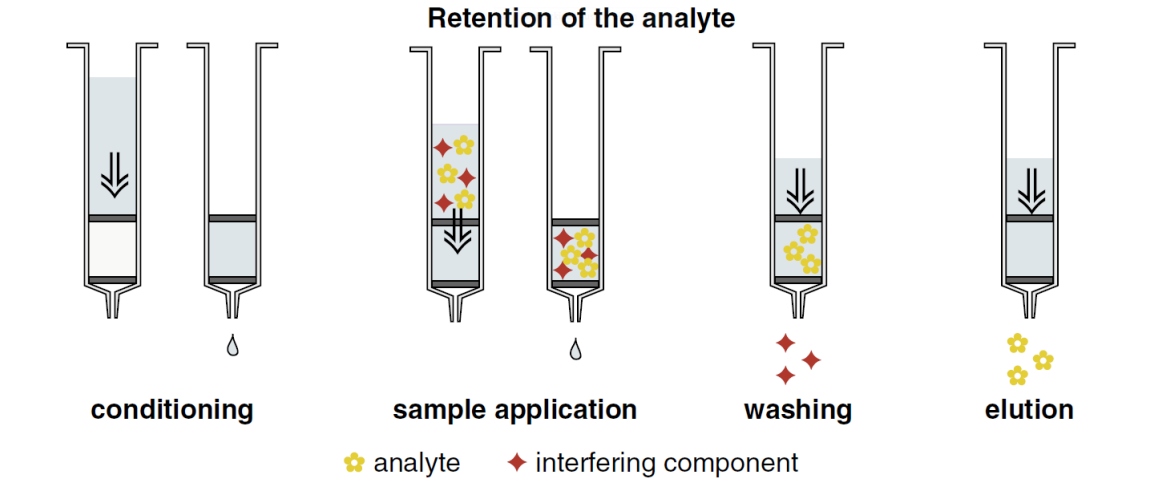
Figure 3.2: Chromatographic profile of RP-HPLC. The flow rate was set to 1 mL/min. The column temperature was 50 °C. After sample injection at time point 0 unbound molecules were washed out for 10 min with 5 % ACN. The linear gradient starts with 5 % ACN and increases to 70 % ACN within 20 min. For another 5 min the ACN concentration was kept at 70 %. The run was finished with 10 min of 5 % ACN solution for re-equilibration. Red curve: acetonitrile gradient. Black curve: Absorbance at 214 nm.

3.6.2 Solid-phase extraction

For the concentration of peptide samples, the solid-phase extraction (SPE) was implemented. Nowadays the solid-phase extraction is still the most popular sample preparation method (Hennion, 1999). The SPE is used for the concentration, purification and isolation of samples. During the SPE analytes are extracted by adsorption. SPE materials are available in various formats and with several sorbents, e.g. silica or polymers. For this work the CHROMABOND® C18 ec polypropylene cartridge was chosen. The cartridge contains an octadecyl modified silica phase with a particle size of 45 µm (specific surface 500 m²/g). All

sample preparations were performed with a 3 mL cartridge with an absorbent weight of 500 mg (specific surface 250 m²).

The conditions of the solid-phase extractions were chosen according to the manufacturer specification of Macherey-Nagel (see figure 3.3). The prepared solutions were passed though the cartridge by vacuum. During conditioning, sample application and washing a drying of the column material should be prevented. An individual sample preparation prior to the SPE was not performed.



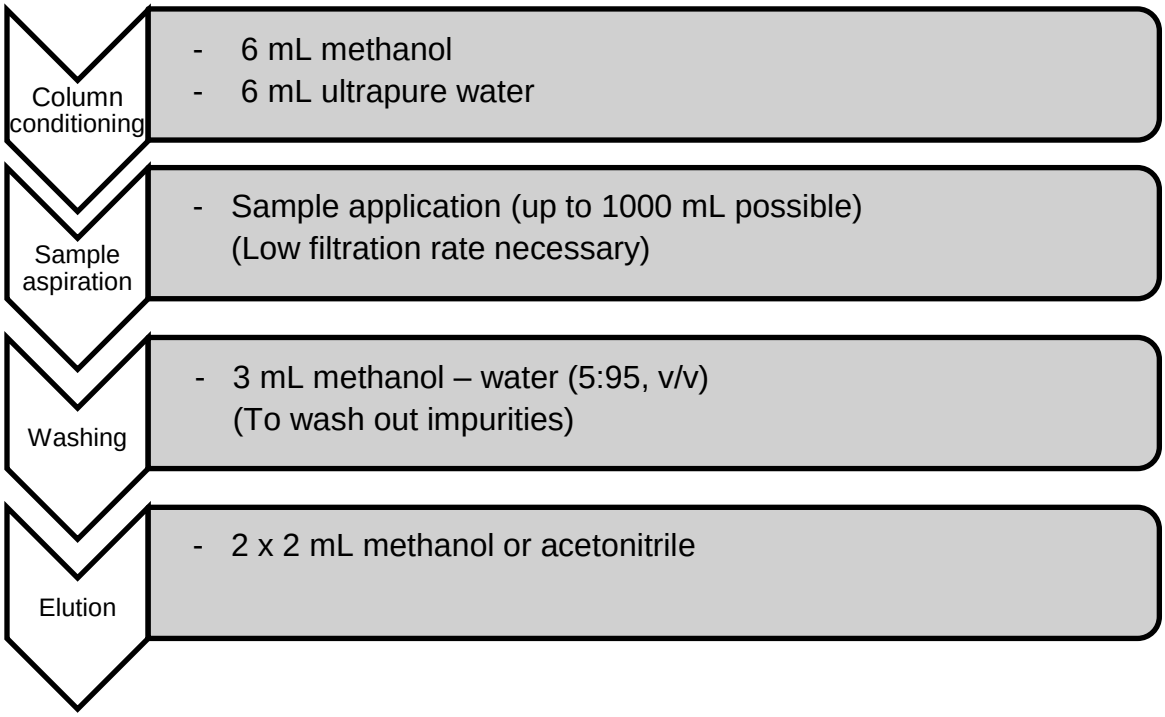


Figure 3.3: Performance of the solid-phase extraction for peptide samples using a C18 ec cartridge. Prior sample application, the column is conditioned with methanol and water. To wash out unbound molecules water is applied before elution with methanol or acetonitrile. (Modified from Macherey-Nagel, 2017)

The elution fractions were vaporized using nitrogen und resolved in 0.1 % TFA using a Vortexer. After resolving, the sample was analyzed by RP-HPLC or Tricine-SDS-PAGE.

3.6.3 Tricine-SDS-PAGE

To distinguish between proteins and peptides with similar charge and elution profiles during chromatography, the molecular weight (MW) is an elementary parameter. For the analytical determination of the molecular weight, the sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) is frequently used. Using SDS-PAGE, the separation of proteins and peptides by MW is achieved by the migration of chemically negatively charged molecules within an electrical field in a strongly cross-linked acrylamide gel. Due to the gel matrix, smaller molecules can migrate faster than larger ones and thus can be found closer to the anodic side of the gel (Westermeier and Gronau, 2005).

In prior studies a Tricine-SDS-PAGE (modified from Laemmli by Schägger and Jagow, 1987) was established for the MW separation of small peptides. As listed in table 3.5, for the preparation of the Tricine-SDS gels three different gel matrices, which vary in the acryl-/bisacrylamide concentration, were used to improve the separation. The gel chambers were assembled according to the instruction manual of Bio-Rad Laboratories. After a polymerization time of 30 minutes, the gels were used directly or stored in a close plastic bag with 100 µL water to prevent dehydration at 4 °C. Unreduced samples for the Tricine-SDS-PAGE were mixed 1:1 with 2x sample buffer (see table 3.7) and boiled at 95 °C for 5 min. For reduced samples the 2x sample buffer was mixed 10:1 with 1 M dithiothreitol (DTT). DTT is used to break disulfide bonds between the residues of two amino acids (e.g. cysteine) and prevent dimerization. The electrophoresis equipment was set up as described in the manufacturer description (BIO-RAD) and filled with cathode- and anode-buffer (see table 3.7). After loading of the gel pockets with 3 µg marker-peptides (dissolved in sample buffer), positive controls (1 µg, 0.5 µg and 0.25 µg Aspidasept® dissolved in sample buffer) and 15 µL of the sample/sample buffer preparation the Tricine-SDS-PAGE was started with 20 mA/gel (80 mA/4 gels). The electrophoresis run lasts 3 to 3.5 hours and was

stopped when the sample buffer was escaping the bottom of the gel. Afterwards the gels were fixed with gel fixation solution (see table 3.6) for 30 min on the rocker (40 rpm) and washed in aqua dem. 3 times for 5 min. During this work, the Tricine-SDS-PAGE gels were stained using Coomassie staining (see table 3.8). Finally, the gels were destained for 30 min with an ethanol mixture (see table 3.8).

Table 3.5: Composition of the SDS-PAGE for the production of two gels. The components TEMED and APS were added directly before cast to prevent premature polymerization. Starting with the separation gel, the spacer and the stacking gels were cast directly after. The composition of the gelbuffer can be found in table 3.7.

Component	Concentration	Separation gel (15 %)	Spacer gel (9 %)	Stacking gel (4 %)
Acryl-/Bisacrylamide	40 %	3.75 mL	2.25 mL	1 mL
Gelbuffer	3x	3.30 mL	3.30 mL	3.30 mL
Glycerol	87 %	2 mL	1 mL	-
Aqua dem.	100 %	0.95 mL	3.45 mL	5.70 mL
TEMED	100 %	9 µL	5 µL	5 µL
APS	40 %	12 µL	9 µL	9 µL
Cast volume / gel		3 mL	0.50 mL	1 mL

Table 3.6: Gel fixation solution of the Tricine-SDS-PAGE gels, used before staining. Glutardialdehyde is added shorty before use to prevent polymerization of the molecule (polymerized Glutardialdehyde cannot bind to the amino groups of amino acids which however is necessary for the fixation).

Component	Amount
Boric acid	12.37 g
Glutardialdehyde (25 %)	100 mL
Add 300 mL aqua dem., adjust to pH 8.5, fill up to 500 mL (for 4 gels)	

Table 3.7: Solutions for the SDS-PAGE.

Anode-buffer (10x)	
Component	**Amount**
Tris Base (2 M)	24.2 g
Adjust pH to 8.9, fill up to 100 mL, dilute to 1x buffer before use	
Cathode-buffer (10x)	
Component	**Amount**
Tris Base (1 M)	6.2568 g
Tricine (1 M)	8.98 g
SDS (1 %)	0.5 g
Adjust pH to 8.25, fill up to 50 mL, dilute to 1x buffer before use	
Gel-buffer (3x)	
Component	**Amount**
Tris Base (3 M)	36.3 g
SDS	0.3 g
NaN_3	1 mL from 1 % solution
Adjust pH to 8.44, fill up to 100 mL	
Buffer S	
Component	**Amount**
Tris Base	6.1 g
SDS	0.4024 g
NaN_3	1 mL from 1 % solution
Adjust pH to 6.8, fill up to 100 mL, use for sample-buffer	
Sample-buffer	
Component	**Amount**
Buffer S	2.5 mL
SDS	2 g
Orange G	40 mg
Glycerol (87 %)	20 mL
Fill up to 100 mL	

Table 3.8: Solutions for the Coomassie staining of the SDS-PAGE gels (4 gels)

Coomassie staining solution	
Component	**Amount**
Roti-Blue (Coomassie 5x)	100 mL
Ethanol (≥99.8 %)	100 mL
Fill up to 500 mL, Mix well to prevent colloids	
Destaining solution	
Component	**Amount**
Ethanol (≥99.8 %)	50 mL
Acetic acid (100 %)	50 mL
Fill up to 500 mL	

3.6.4 Radial diffusion assay

The radial diffusion assay (RDA) or radial activity diffusion assay is an analytical method for the proof of an antimicrobial activity of any molecule. Agar diffusion tests are established for the test of antibiotics (Bondi et al., 1947) but can also be transferred for the proof of the antimicrobial activity of peptides. The RDA was developed following Lehrer et al. (Lehrer et al., 1991)

The test was performed according to the standard operation procedure (SOP) ‘Agardiffusionstest zur Wirksamkeitsprüfung von toxischen Substanzen; speziell antimikrobiellen Peptiden’ from Jacob Brandt and Jan Demmer (version 03.06.2016). Due to an exchange of the used *Escherichia coli* strain to *E. coli WBB01* changes in the execution were necessary. In the following only the changes are described.

All solutions were prepared according to the SOP. Due to a decreased growth of *E. coli WBB01* the first shaking flask was inoculated with 100 µL (as per SOP 50 µL) of the cryo culture as early as possible at day 1. In the morning of day 2 the second shaking flask was inoculated with 300 µL (as per SOP 200 µL) and incubated for at least 3.5 h (as per SOP 2.5 h). The agar was cooled down to 42 °C (as per SOP 48 °C) to prevent prematurely cell death during casting of

E. coli inoculated agar. The agar was seeded with 4 x 10^6 colony forming units. After the application of samples and controls, the second agar layer was casted at 42 °C. The plate was incubated at 30 °C for approx. 40 h (as per SOP 18 h) to obtain a dense bacterial lawn and evaluated using the software ImageJ.

3.7 Peptide elimination on the model waste water treatment plant

Beside the characterization of Aspidasept® and the development of suitable analytic methods, the aim of this study was the investigation of the peptide behavior and elimination on various new steps of the waste water treatment. For this purpose small scale models of each treatment step were adjusted and used to simulate the clearance of the peptide. In the following chapters the methods for peptide elimination on the model waste water treatment plant are described.

3.7.1 Activated carbon adsorption

3.7.1.1 Activated carbon

For all adsorption trials the granulated activated carbon (GAC) *Hydraffin AR 8x30* (company: *Donau Carbon*) was used. In table 3.9 the characteristics based on manufacturer specifications are listed. Prior starting the adsorption studies the granulated activated carbon was cleaned according to Worch (2012). To remove proportions of fines and further impurities the GAC was washed with demineralized water. Following, the GAC was dried at 110 °C for 24 h. During the storage of the cleaned and dried GAC it is required to protect it from moisture and oxygen absorption.

Table 3.9: Specifications of the granulated activated carbon *Hydraffin AR 8x30*. (Manufacturer specification, *Donau* Carbon, 2017)

Specification of *Hydraffin AR 8x30*	
Granulation size (mesh) [0.60-2.36mm]	8 x 30
Bulk density [kg/m³]	500 ± 30
Water content [wt. %] (during packaging)	< 5
Iodine adsorption [mg/g]	> 950
Hardness [wt. %]	> 90
Bulk density [kg/m³] (after classification of backwash)	App. 450
Inner Surface [m²/g] (BET-method)	App. 1100
Ash content [wt. %]	< 50

3.7.1.2 Adsorption kinetic on granulated activated carbon

To determine the time to an adsorption equilibrium of a known Aspidasept® concentration on a known GAC mass adsorption kinetics were performed. For the kinetic trials Aspidasept® was dissolved in 0.1 % DMSO and added to a constant volume (V_L) of deminerized water (end concentration of DMSO 0.01 %). The solution was temperated to 10 °C or rather 20 °C in a shaking water bath (100 rpm). The trials were performed in 25 mL glass bottles. To the peptide solution, a known amount of GAC was added. The GAC amount varies during the experiments. To eliminate influences of the test matrix negative and positive controls were performed. For each positive control and each peptide sample 1 mg Aspidasept® were weigh in (c_0 = 100 mg/L). Table 3.10 shows the composition of the experiment set up.

Table 3.10: Composition of the experiment set up of the adsorption trials. ✓ Component added, x Component absent.

Experiment	Volume V_L	Test matrix	Aspidasept®	GAC
Negative control	10 mL	dem. water with 0.01 % DMSO	x	✓
Positive control			✓	x
Peptide sample			✓	✓

For the adsorption kinetics, five different GAC concentrations were tested. In table 3.11, the GAC concentrations are listed.

Table 3.11: GAC concentrations prepared for the adsorption kinetic trials.

GAC concentration [g/L]	GAC mass [g] / 10 mL
5	0.05
7.5	0.075
10	0.1
20	0.2
50	0.5

During the experiments samples (100 µL) at different time points were taken and centrifuged for 10 min at 12.100 x g. After centrifugation 50 µL of the supernatant were taken and analyzed using RP-HPLC and Tricine-SDS-PAGE. For the analysis 10 µL sample volume were applied to the RP-HPLC and 7.5 µL to the Tricine-SDS-PAGE. The sampling times are shown in table 3.12. During the experiments, the temperature was measured to ensure constant test conditions.

Table 3.12: Sampling times of the adsorption kinetic trials. ✓ Sample taken, x No sampling.

Time	Peptide sample	Negative control	Positive control
0 min	✓	✓	✓
5 min	✓	x	x
15 min	✓	x	x
22 min	✓	x	x
40 min	✓	x	x
60 min	✓	x	x
120 min	✓	✓	✓
6 h	✓	x	x
24 h	✓	✓	✓
30 h	✓	x	x
48 h	✓	✓	✓

The received results of the Aspidasept® concentration were presented in a c/c_0 – *t*-diagram. Using the obtained data, the time that is needed to reach the adsorption equilibrium can be estimated.

3.7.1.3 Adsorption equilibrium on granulated activated carbon

The adsorption equilibrium provides insight into adsorption behavior of Aspidasept® and the adsorption loading capacity of the granulated activated carbon. To determine the adsorption equilibrium, the 48 h values of the adsorption kinetics were used. Here an equilibrium between peptide in solution and adsorbed peptide can be assumed. The equilibrium loading conditions q_{eq} of the activated carbon can be calculated by equation 3.1 and plotted in a q_{eq} – *c*-diagram. Using these results, the *Freundlich*-isotherm can be determined.

$$q_{eq} = \frac{V_L}{m_{GAC}} \cdot (c_0 - c_{eq}) \qquad \text{(Equation 3.1)}$$

q_{eq}	Loading of GAC during equilibrium	[µg/g]
c_0	Start concentration of Aspidasept®	[µg/L]
c	End concentration of Aspidasept®	[µg/L]
m_{GAC}	Granulated activated carbon mass	[g]
V_L	Test volume	[L]

3.7.2 Membrane filtration

3.7.2.1 Membrane for peptide filtration

For the peptide filtration trials, a nano-/ultrafiltration membrane was chosen. Before starting the filtration trials, the adsorption of Aspidasept® on various membranes were analyzed. For this purpose, various flat membranes of the company *DOW Filmtec* were used. A membrane for reverse osmosis (XLE) and two membranes for nano-filtration (NF-90 and NF-270) were tested. All membranes are made of polyamide but with different pore sizes. From small to large: XLE → NF90 → NF270.

With the largest pores distribution, the membrane TRISEP® UA60 of the company *MICRODYN-NADIR* was chosen for the peptide filtration. With a Cut-Off in the range of 1000 Da, it belongs to the range between ‘tight’ ultra-filtration membranes and ‘open’ nano-filtration membranes. It is based on a piperazine-based thin-film composite membrane. With a NaCl rejection of 10 % and an $MgSO_4$ rejection between 70 – 90 % a peptide recovery of nearly 100 % should be achieved (manufacturer specification, *MICRODYN-NADIR).* The specifications can be found in table 3.13.

Table 3.13: Specifications of the used reverse osmosis and nano membranes. All membranes are available in spiral-wounded modules and flat sheets. (Manufacturer specifications, DOW & MICRODYN-NADIR)

Membrane	**XLE**	**NF90**	**NF270**	**TRISEP® UA60**
Vendor	DOW	DOW	DOW	MICRODYN-NADIR
Specification	Reverse Osmosis	Reverse Osmosis	Reverse Osmosis	Nano-(loose)/ Ultrafiltration(tight)
Cut-Off	~ 100 Da	200 - 400 Da	200 - 400 Da	Approx. 1000 Da
Material	polyamide	polyamide	polyamide	Piperazine amide
Polarity	polar	polar	polar	polar
Commercially available	✓	✓	✓	✓
Commercial usage	water treatment	drinking Water/ industrial water demineralization	local drinking water	water treatment

3.7.2.2 Membrane adsorption and buffer recovery of Aspidasept®

Before starting the peptide filtration trials the short time recovery of Aspidasept® in presence of various buffer salts and pH values and the adsorption affinity of the peptide and different membranes was essential to select appropriate filtration conditions for later experiments.

The pretests were performed in small-scale glass vials in a test volume (V_L) of 1 mL. To assess the impact of buffer and pH positive controls without membrane were prepared. For this purpose, six different buffer conditions (see table 3.14) were evaluated. For all positive controls 10 µL of 1 µg/µL Aspidasept® (dissolved in 0.01 % TFA) were added to 990 µL buffer. After 0 min, 30 min, 1 h and 3 h samples were taken.

Table 3.14: Buffer compositions used for the stability pretests of Aspidasept®. All buffers were filtrated through a 0.45 µm syringe filter.

Buffer salt / component	Molarity / concentration	pH	NaCl molarity
Trifluoroacetic acid	0.1 %	-	-
Dem. H_2O	-	-	-
Acetate	10 mM	5.5	50 mM
HEPES	10 mM	7.5	50 mM
Tris	10 mM	7.0	50 mM
Tris	10 mM	9.0	50 mM

To exclude the influence of possible membrane extractables on the sample analysis, negative controls with buffer and membrane without peptide were prepared. Therefore, 1 x 1 cm pieces of the membrane were stored for 3 h in 0.1 % TFA respectively Tris-buffer pH 7 (for composition see table 3.14).

To examine the adsorption of Aspidasept® to nano- and ultrafiltration membranes, four different flat membranes were used (for specifications see table 3.13). Such as for the negative control 1 x 1 cm pieces were added to 990 µL 0.1 % TFA respectively Tris-buffer pH 7 and 10 µL 1 µg/µL Aspidasept® (dissolved in 0.01 % TFA). Samples were taken at time point zero and after 30 min, 1 h and 3 h. For the analysis of all control and adsorption samples, 99.5 µL sample volume were applied onto the RP-HPLC. All experiments were realized at room temperature.

3.7.2.3 Cut-Off determination of membrane UA60

To confirm the Cut-Off, specified by the manufacturer, experiments were performed with various substances. For each filtration, 2 mg substance was dissolved in 200 mL Tris buffer (10 mM Tris, 50 mM NaCl, pH 7). Table 3.15 lists the used substances with corresponding molecular weight. The solution were filled into the filtration cell and 1 mL feed sample were taken for the analysis. The filtration was started by applying overpressure. Each filtration was performed at 8 bar with a magnetic stirrer speed of 200 rpm. The filtration was stopped after 100 mL permeate were reached (measured by balance). For analysis, 1 mL

permeate and 1 mL retentate were sampled. All samples were analyzed using a UV/VIS-Spectrometer at different wavelength (see table 3.15).

Table 3.15: Substances used for the Cut-Off determination of membrane UA60.
The molecular weight and the used wavelength are given.

Substance	Molecular Weight [Da]	Wavelength [nm]
Lysozyme	14300	280
Vitamin B12	1355	361
Bromophenol blue	670	590
L-Glutathione	307	219
DL-Tryptophan	204	280

After each filtration for the Cut-Off determination, a reference filtration with 200 mL dem. water (100 mL permeate) was carried out. The reference filtrations were executed at 8 bar with a magnetic stirrer speed of 100 rpm. Reference test were necessary to monitor the fouling (clogging of the membrane) by molecules used for the Cut-Off determination. With increasing clogging, the flux decreases and the filtration time increases.

The recovery in % of each substance can be calculated from the measured absorbance using equation 3.2.

$$R = \left(1 - \frac{Ab_{per}}{\frac{Ab_{feed} + Ab_{ret}}{2}}\right) \cdot 100 \qquad \text{(Equation 3.2)}$$

R	Recovery	[%]
Ab_{per}	Absorbance of permeate	[AU]
Ab_{feed}	Absorbance of feed	[AU]
Ab_{ret}	Absorbance of retentate	[AU]

3.7.2.4 Aspidasept® membrane filtration

After verifying the Cut-Off, the membrane was used to investigate the filtration behavior of Aspidasept®. For this purpose a filtration series with increasing pressure was performed. First, 2 mg Aspidasept® was dissolved in 1 mL 0.1 % TFA and diluted with Tris-buffer (20 mM Tris, 50 mM NaCl, pH 7) to 200 mL (end concentration 10 mg/L). To decrease the amount of peptide, necessary for the filtrations it was planned to perform the series with one peptide solution. Due to the rapidly decrease of Aspidasept® during the first filtration run, a second peptide solution was required.

Before starting each filtration, 1 mL feed sample was taken. Each filtration was started with 200 mL peptide solution with a magnetic stirrer speed of 200 rpm and different pressures. The filtration was stopped when 50 mL permeate were reached. Samples of permeate and retentate were taken for the analysis. The filtration series was started with pressures of 8 and 10 bar. After the filtration at 10 bar, the second solution was used for the last three filtration runs at 10, 12 and 16 bar. Each filtration run was repeated with a second UA60 membrane. Between every peptide run, a reference filtration with dem. water was performed to observe the fouling of the membrane.

3.7.3 Biological treatment

The elimination of substances by biological reduction is probable the most well-known method of waste water treatment. Although the biological treatment is an established method for the waste water treatment is was necessary to examine the impact of an antimicrobial peptide on the bacteria and protozoa used in this step. For the degradation, trials of Aspidasept® two experiments were made. Both experiments were performed in small scale because it was not possible to use the laboratory waste treatment system (KLD4N/SR) of the company *Behr Labor-Technik*. However, the waste water used for the Aspidasept® clarification was gathered of the laboratory waste treatment system (KLD4N/SR). In this case two different waste waters can be named. The first one is the synthetic waste water.

The composition can be found in table 3.16 and 3.17. The second one is the clarified waste water.

Table 3.16: Composition of the trace element in the synthetic waste water. The medium is cooled at 4 °C to decrease degradation.

Compound	**c synthetic waste water [mg/L]**
Calcium chloride ($CaCl_2$)	5,000
Sodium hydrogen carbonate ($NaHCO_3$)	25,000
Ferric(II)-sulfate-hepta hydrate ($FeSO_4 \cdot 7\ H_2O$)	10,000
Copper(II)-chloride-dihydrate ($CuCl_2 \cdot 2\ H_2O$)	0,480
Cobalt(II) chloride hexahydrate ($CoCl_2 \cdot 6\ H_2O$)	0,050
Zinc chloride ($ZnCl_2$)	0,180
Manganese(II) Sulfate Monohydrate ($MnSO_4 \cdot H_2O$)	0,100
Sodium molybdate (Na_2MoO_4)	0,170
Chromium(III)-nitrate-nonahydrate ($Cr(NO_3)_3 \cdot 9\ H_2O$)	0,680
Nickel(II) sulfate hexahydrate ($NiSO_4 \cdot 6\ H_2O$)	0,300

Table 3.17: Composition of the medium in the synthetic waste water. The medium is cooled at 4 °C to decrease degradation.

Compound	**c synthetic waste water [g/L]**
Peptone	0,160
Meat extract	0,110
Urea	0,030
NaCl	0,007
$CaCl_2 \cdot 2\ H_2O$	0,004
$MgSO_4 \cdot 7\ H_2O$	0,002
K_2HPO_4	0,028

As it must be assumed that Aspidasept® will adsorbed at particles present in the waste water, two experiments were planned. For the first experiment, almost particle free waste water was used obtained by centrifugation to investigate the recovery in the environmental conditions of waste water. The second trial was performed with untreated waste water to observe the adsorption and elimination of the peptide on the particles of the waste water.

3.7.3.1 Recovery tests in particle free waste water

The recovery test was performed with the feed solution of the laboratory waste treatment system (KLD4N/SR) and the cleared waste water. To obtain particle free waste water a centrifugation step at 12.100 x g for 10 min was performed. The supernatant was used for the experiments. For a 1 µg/7.5 µL peptide concentration 950 µL particle free waste water and 50 µL 1 mM Aspidasept® were mixed in a 1.5 mL glass vial. The experiment was performed at 4 °C. Samples were taken at the time points: 0 min, 30 min, 1.5 h, 3.25 h, 20.75 h, 27.25 h, 48 h, 72 h and 6 days. The samples were analyzed by RP-HPLC.

3.7.3.2 Elimination in waste water

The peptide elimination was performed in waste water coming from the denitrification step of the laboratory waste treatment system (KLD4N/SR). Using a 1 mM Aspidasept® solution waste water samples with various peptide concentrations (0, 1, 5, 10, 25, 50, 100 µg/mL) were prepared. The solutions were incubated overnight for 21 h in glass vials at room temperature. After incubation the samples were centrifuged (10 min, 12.100 x g). The supernatant was analyzed using the RP-HPLC. The pellets were resuspended in 100 µL SDS-PAGE sample puffer and analyzed by Tricine-SDS-PAGE.

4 Results

In the following chapters, the results of the peptide characterization by various analytical procedures are described. Based on new implemented methods for peptide analysis, the elimination studies with different waste water treatment models are displayed. The adsorption at granulated activated carbon, membrane filtration and the biological treatment were examined.

4.1 Characterization of the antiseptic peptide Aspidasept®

To establish a strategy for the elimination of Aspidasept® on a model waste water treatment plant the antiseptic peptide was characterized using reversed-phase-HPLC, solid-phase extraction, Tricine-SDS-PAGE and radial activity diffusion assay. Whereas the RP-HPLC and the Tricine-SDS-PAGE was primary used for the identification and quantity determination of the peptide, the solid-phase extraction should be developed for the concentration of larger sample volumes. For a proof of antimicrobial activity, the radial activity diffusion assay was implemented. All performed experiments were performed according to the prior described methods.

4.1.1 Verification of Aspidasept® by RP-HPLC

For the analysis of particle free peptide samples the RP-HPLC were established. First trails showed that 1 µL pure Aspidasept® dissolved in 0.01 % TFA results in two clearly baseline separated peaks. The first one elutes with a retention time of 23.027 min, which corresponds to an acetonitrile concentration of 47.5 %. A second peak is detected after 23.980 min (acetonitrile concentration of 51 %). To gather more information about the unexpected second peak a sample with dithiothreitol (DTT) reduced peptide was applied. With a final DTT concentration of 3.33 mM 1 µL was applied resulting in a single peak at 23.013 min. The retention time is complying with the first peak of the untreated peptide leading to the assumption that Aspidasept® forms dimers in aqueous solutions. As well, it can be presumed that, the monomer experience less retention on the RP-HPLC column and elutes first.

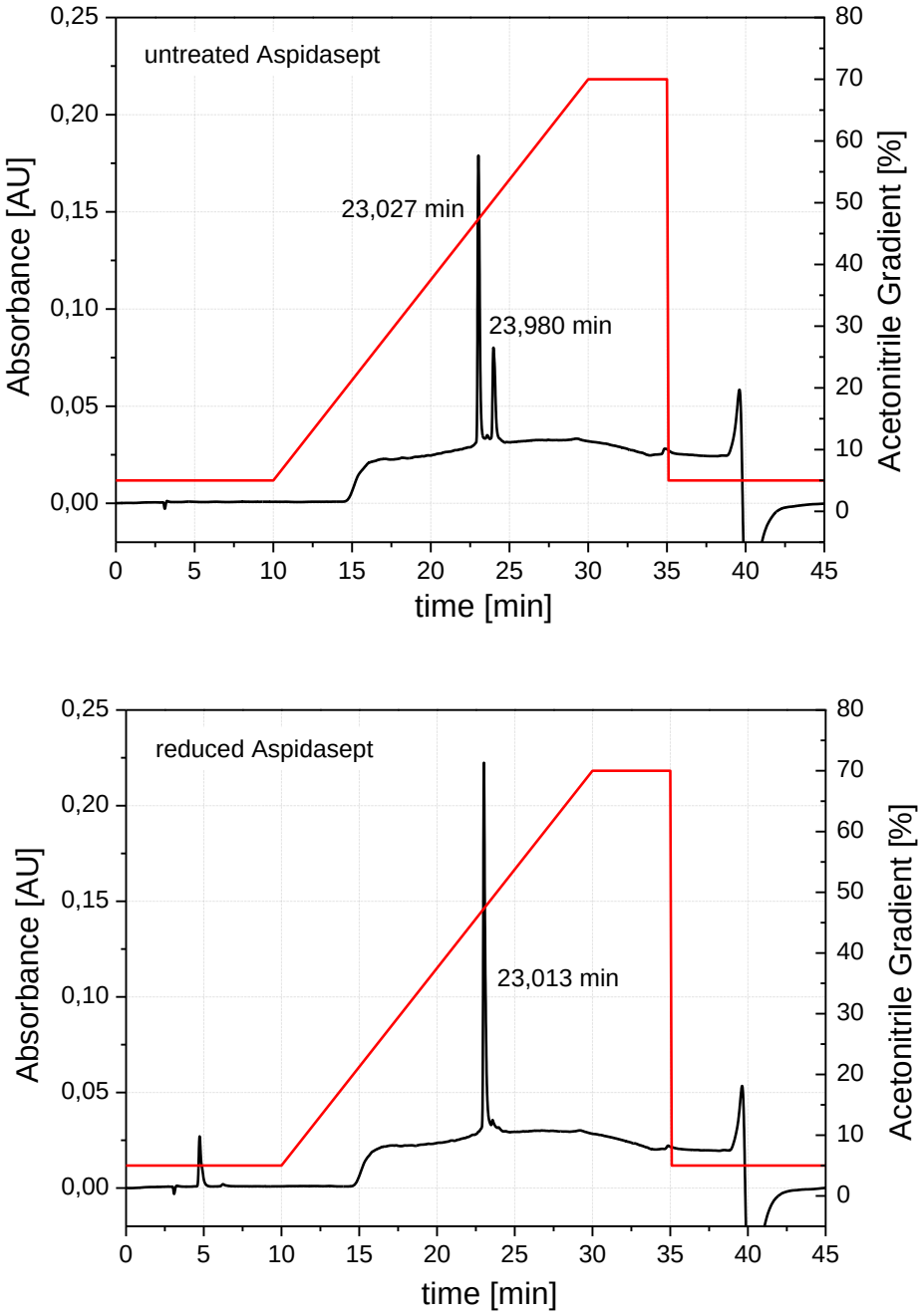


Figure 4.1: Characterization of Aspidasept® via RP-HPLC. (A) 1 µg Aspidasept® applied on the RP-HPLC (sample volume 1 µL). A linear gradient of acetonitrile from 5 % to 70 % was performed (red curve). Two peaks at a retention time of approx. 23 min and 24 min (47.5 % and 51 % Acetonitrile) were detected (black curve). (B) 1 µg with DTT reduced Aspidasept® applied on the RP-HPLC (sample volume 1 µL, DTT molarity 3.33 mM) results in a single peak with a retention time of approx. 23 min (47.5 % Acetonitrile) (black curve). A linear gradient of acetonitrile from 5 % to 70 % was performed (red curve).

Since the RP-HPLC was the most important analytical method, it was desirable to determine the accuracy of the system. An evaluation with four different peptide concentrations and injection volumes were performed. The samples 1 µg/10 µL, 1 µg/50 µL, 5 µg/10 µL and 5 µg/50 µL were injected 10 times. All samples series were repeated twice with a new sample solution. Figure 4.2 shows the results of both injection series. Whereby, the data of the injections of 1 µg results in equal values between the two series, the injection with 5 µg only shows similar values within a series. In comparison to the first injection series, the second series reached unlikely higher values.

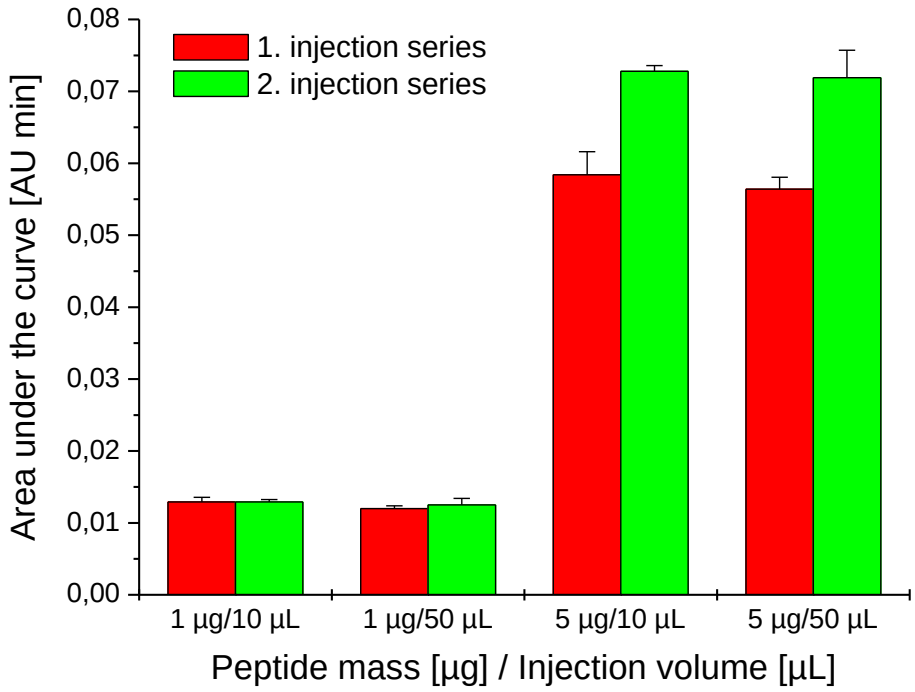


Figure 4.2: HPLC evaluation with injection series of 4 peptide concentration and injection volumes. The samples 1 µg/10 µL, 1 µg/50 µL, 5 µg/10 µL and 5 µg/50 µL were injected 10 times per series. For the complete series is n = 2.

Using the obtained mean of the integrated RP-HPLC results and the standard deviation, the coefficient of variation can be calculated (see table 4.1). For the used sample preparations values between 1.0 and 7.3 % were obtained. No significant volume dependent differences in the accuracy of the analytic can be recognized.

Table 4.1: Coefficient of variation of the RP-HPLC evaluation.

Experiment	Deviation [%] / series 1	Deviation [%] / series 2
1 µg/10 µL	5.11	2.65
1 µg/50 µL	3.12	7.30
5 µg/10 µL	5.50	1.06
5 µg/50 µL	2.95	5.29

4.1.2 Reference curve and level of detection on the RP-HPLC

To estimate the amount of peptide during the experiments by analyzing the area under the curve, a reference curve was necessary. Due to an absence of data for Aspidasept® on RP-HPLC, the peptide was applied without reduction first (see figure 4.3). The samples were untreated to obtain the LOD for the unreduced peptide. However, since the ratio between monomer and dimer is dependent on the storage conditions and the storage time, a second approach (reduced peptide) was made as reference for the quantification.

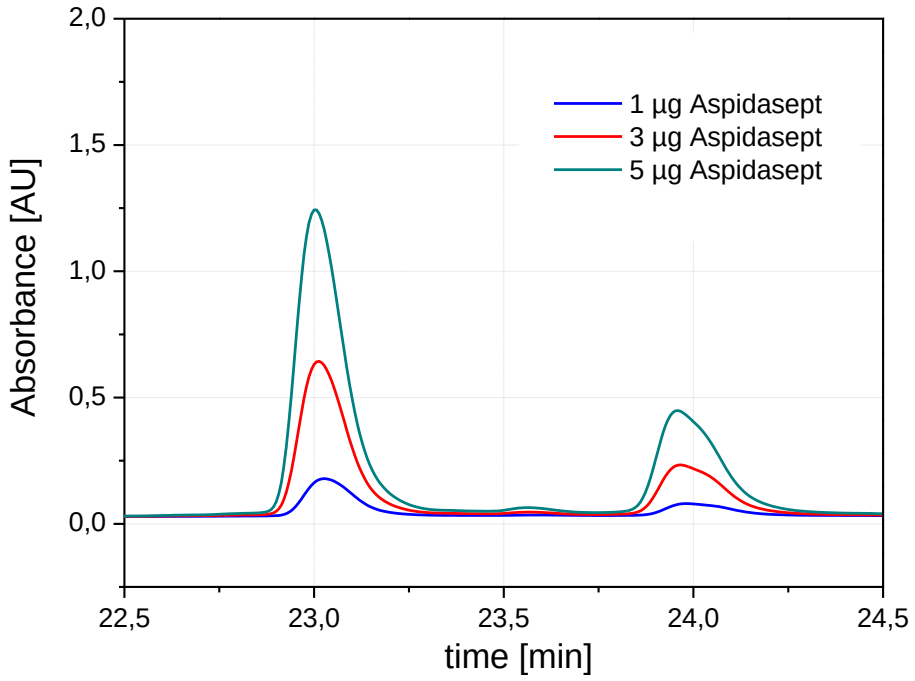


Figure 4.3: Level of detection chromatograms of Aspidasept® on the RP-HPLC. 1 µg, 3 µg and 5 µg Aspidasept were applied onto the column. The peptide elutes in two clearly separated peaks at 23 min and 24 min.

Thus, the reference curve was created using reduced peptide samples from 0.1 µg to 6 µg (see figure 4.4). The experiment showed that an increasing peptide amount results in increasing peaks. Independent from the sample amount or volume the peptide is characterized by the same retention time (23 min, 47.5 % acetonitrile) each run which indicates the RP-HPLC as an appropriate and reproducible analytical method. It should be noted that sample amounts larger than 10 µg cannot be detected properly due to the detector limit.

By integrating the area under the curve with the Origin software the reference curve was set. Using the following equation and the area under the curve of each sample the peptide amount can easily calculated.

$$peptide\ [\mu g] = \frac{area\ under\ the\ curve\ (x) - intersection}{slope} \qquad \text{(Equation 4.1)}$$

$$peptide\ [\mu g] = \frac{x}{0.03887}$$

As it was proved that with the application of buffer without peptide no peak will detected it can be assumed that the reference curve passes through zero. Thus, the intersection was neglected during the evaluation of the RP-HPLC chromatogram.

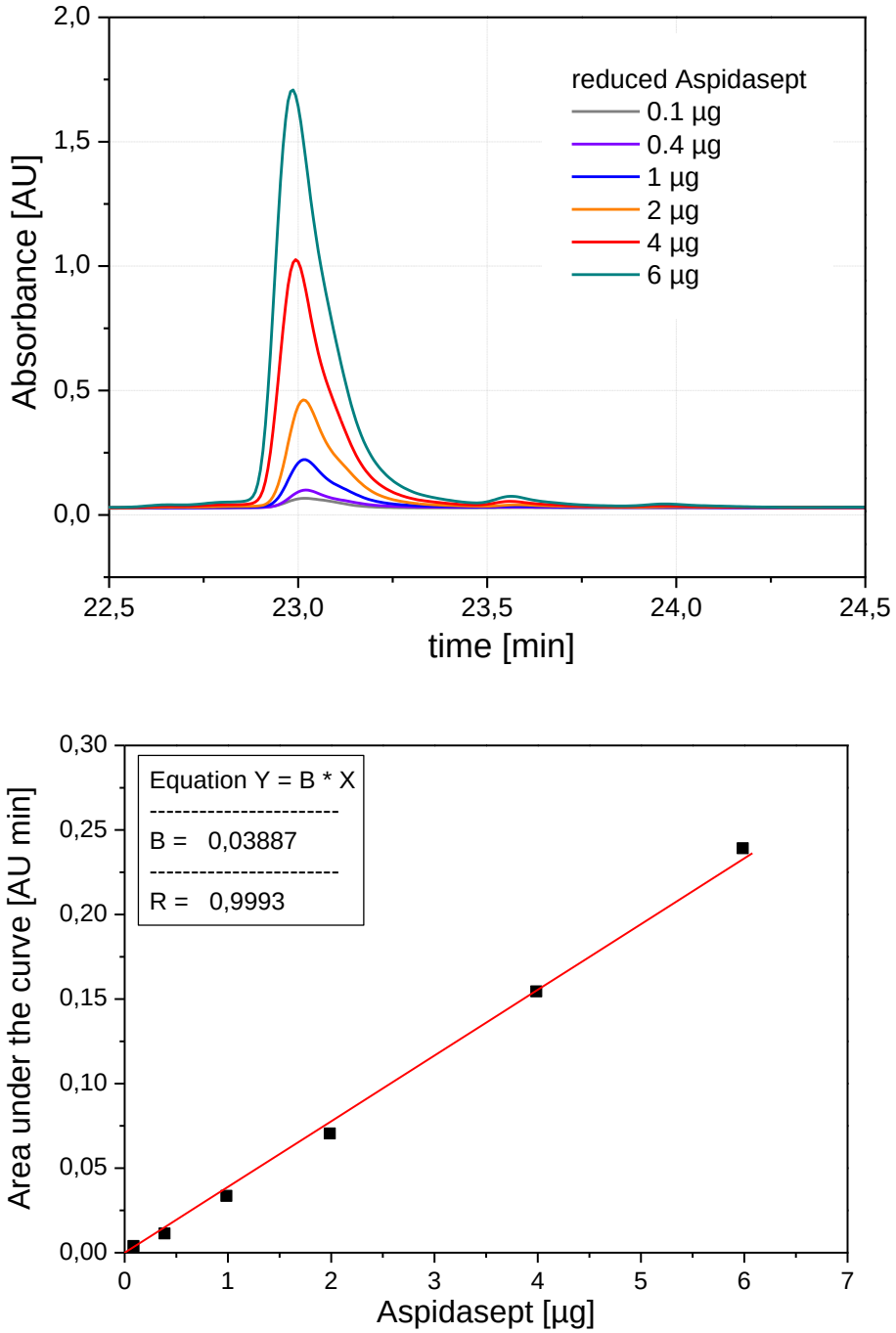


Figure 4.4: RP-HPLC-Chromatogram of reduced Aspidasept® and resulting reference curve. (A) Level of detection for reduced Aspidasept® samples (DTT concentration 3.33 mM). Sample amounts from 0.1 µg to 6 µg were applied (sample concentration 1 µg peptide/ 1 µL volume). The peptide elutes in a single peak at 23 min. (B) Reference curve created from the results of reduced Aspidasept® samples by integration of the area under the curve.

4.1.3 Verification of Aspidasept® by Tricine-SDS-PAGE

For the verification of Aspidasept®, a Tricine-SDS-PAGE was performed to analyze the molecular weight. The peptide was applied unreduced and with DTT reduced (see figure 4.5). The first lane shows the standard peptide marker with a molecular weight range of 2.5 kDa to 17.0 kDa. Lane 2 to 4 shows the peptide Aspidasept® without further treatment in sample amounts of 1 µg, 0.5 µg and 0.35 µg. In all concentrations three peptide bands can be seen with molecular weights of 3.3 kDa, 3.8 kDa and 6.0 kDa. Whereby the bands with lower molecular weight seem to get fainter, the band with the highest molecular weight increases its intensity with decreasing peptide mass. In the last three lanes reduced peptide was applied. An intense peptide band is detected at a molecular weight of 3.8 kDa. Beneath the intense band a fainter band at a molecular weight of 3.3 kDa can be seen.

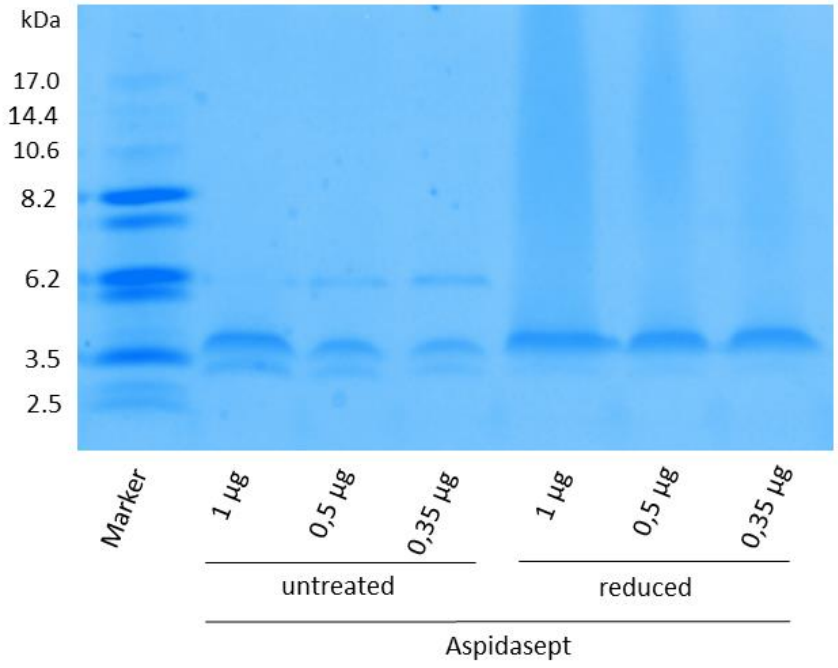


Figure 4.5: Tricine-SDS-PAGE with unreduced and reduced Aspidasept®. The first lane contains the marker peptide marker with standard molecular weight. Lanes 2 to 4 show the unreduced peptide in sample amounts of 1 µg, 0.5 µg and 0.35 µg. Lanes 5 to 7 show the with 3.33 mM DTT reduced peptide in sample amounts of 1 µg, 0.5 µg and 0.35 µg.

The calculated molecular weight of the peptide Aspidasept® is 2.7 kDa. Since peptides can show a different behavior compared to proteins on the SDS-PAGE the standard peptide marker was used only as reference point. Nonetheless, it can be assumed that due to its lack in the reduced samples, the peptide band with the molecular weight of 6.0 kDa (unreduced peptide) represents the dimer of Aspidasept®. Because the band with a molecular weight of 3.8 kDa gains intensity after reduction, it can be presumed as the peptide monomer. The band with the lowest molecular weight is probably peptide with different conformation or degraded peptide.

4.1.4 Reference curve and level of detection on the Tricine-SDS-PAGE

For some Aspidasept® samples it was assumed that the peptide adsorbs to particles of the sample matrix. For these samples only the analysis by Tricine-SDS-PAGE was possible to protect the HPLC column. A reference curve is necessary for the quantification. Figure 4.6 displays an exemplary Tricine-SDS-PAGE for a reference curve. Because the intensity of the peptide bands can vary strongly from gel to gel a reference curve on every gel is necessary. Figure 4.6 shows 0.1 µg to 1 µg peptide. If necessary, smaller peptide amounts can be included in the reference curve of the SDS-PAGE. The level of detection (LOD) is between 0.025 and 0.05 µg. By evaluating the intensity of the peptide bands the equation of the reference curve can be determined.

$$peptide\ [\mu g] = intersection + slope \cdot volume\ intensity\ (x) \qquad \text{(Equation 4.2)}$$

$$peptide\ [\mu g] = 0.0023 \cdot x - 1.29621$$

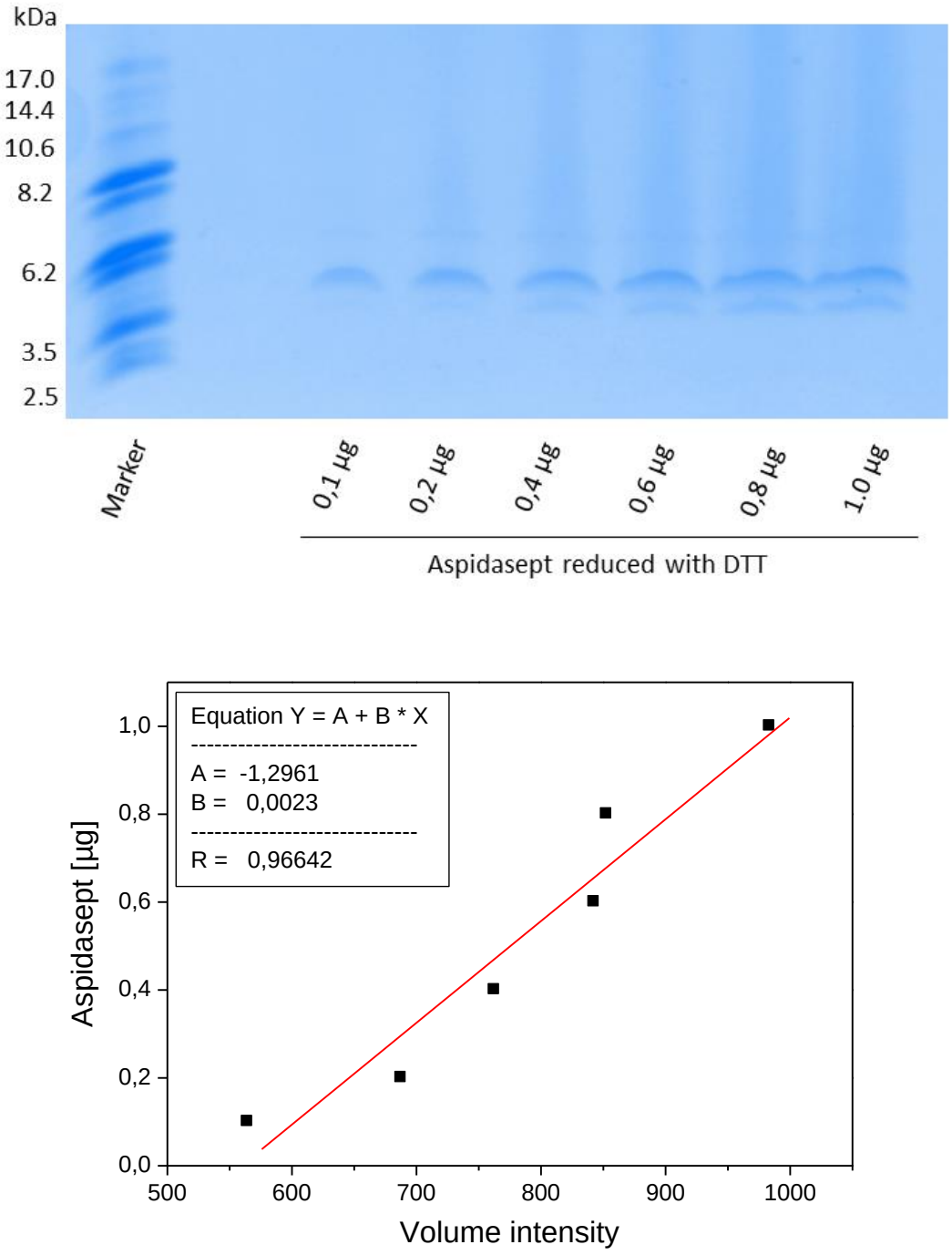


Figure 4.6: Tricine-SDS-PAGE of reduced Aspidasept® and reference curve. (A) Level of detection for reduced Aspidasept® samples. Sample amounts from 0.1 µg to 1 µg were applied (sample concentration 1 µg peptide/10 µL volume). (B) Reference curve created from the results of reduced Aspidasept® samples by evaluating the intensity of the peptide bands.

4.1.5 Recovery of Aspidasept®

Before starting the experiments with Aspidasept® for this study, it was necessary to test the recovery of the peptide under defined conditions. The peptide was solved in 0.01 % TFA and diluted to a 1 µg peptide / 10 µL 0.001 % TFA solution. During the experiments, the peptide preparation was stored at room temperature (approx. 22 °C), 4 °C or -20 °C. The analysis was performed using RP-HPLC.

The series at 4 °C showed only a slight decrease in the peptide concentration (see figure 4.7). After 168 h (7 days) still 97 % peptide was available. During the storage at room temperature, the recovery decreased to 78 % in 168 h (7 days). Samples, which were frozen and thawed after 1 day, showed an increase of dimers. Whereas a sample that is applied immediately after dissolving has a monomer/ dimer ratio of 3/1, the frozen sample is characterized by a ratio of 1/1. However, the same total peptide mass was detected independent from storage procedure.

Based on the results of the recovery experiments it was defined that peptide standard solutions (1 mM in 0.01 % TFA) were stored at -20 °C to avoid peptide loss. Samples with lower molarity were stored at 4 °C.

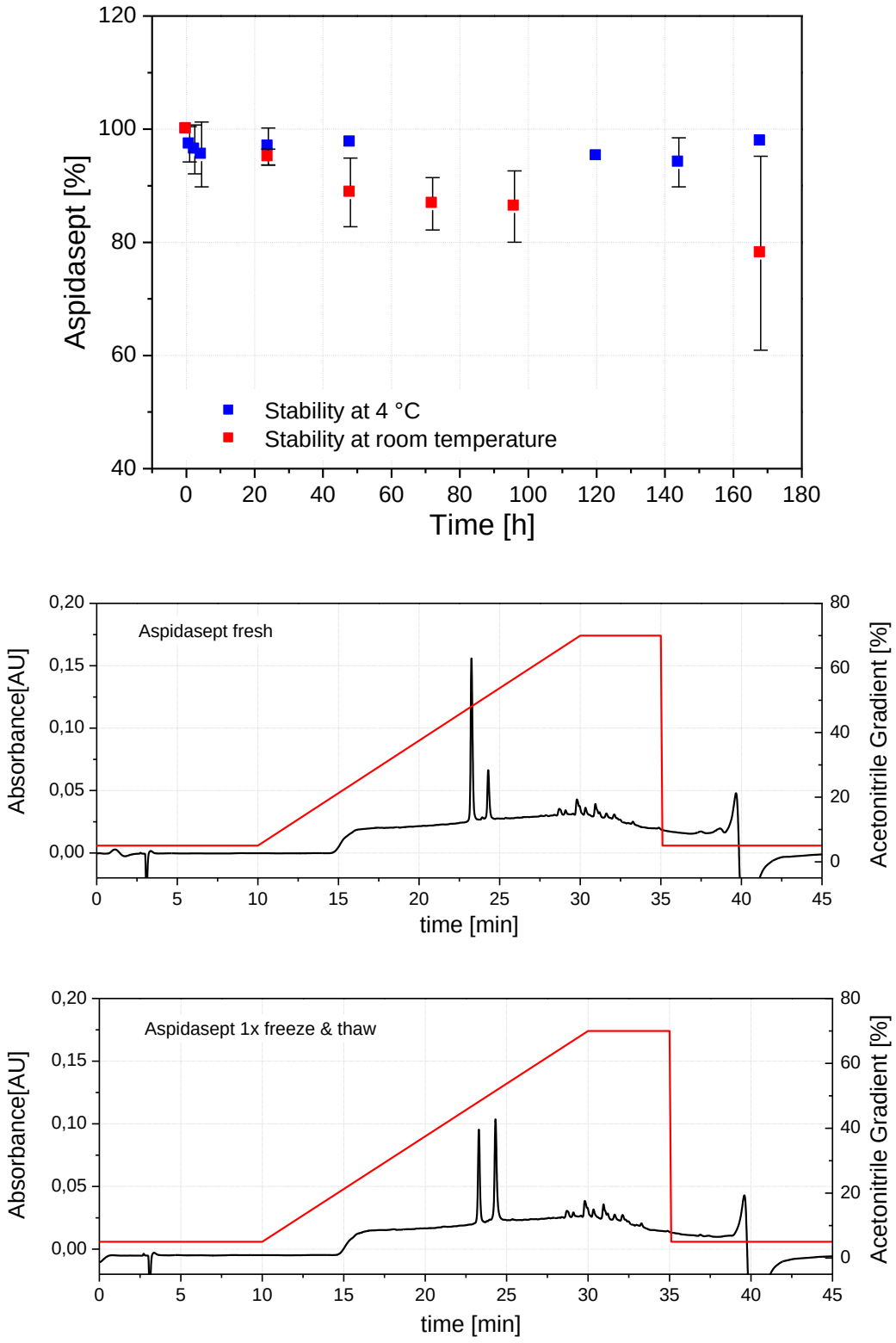


Figure 4.7: Stability of Aspidasept® at different storage conditions. (A) Comparison of the stability at room temperature (orange curve) and 4 °C (blue curve). (B1) Monomer/ dimer ratio of 3/1 if applied directly after dissolving. (B2) Monomer/ dimer ratio of 1/1 if applied after freeze & thaw.

4.1.6 Verification of antimicrobial activity by radial diffusion assay

To assess the antimicrobial activity of the peptide Aspidasept® a radial diffusion assay (RDA) was performed. Figure 4.8 shows an exemplary result of the performed RDA. As positive controls Melittin, an antibiotic peptide found in bee`s venom, and synthetic ALK-OH, an antimicrobial peptide were chosen. Both peptides showed an inhibiting effect on the microbial growth at previous tests. For a negative control dem. water was applied. To examine the impact of TFA, used for the dissolving and storage of Aspidasept®, two different concentrations were tested.

Positive and negative controls showed the expected results. Melittin, at position 1 (50 µg/mL) and 2 (10 µg/mL) at the shown agar plate both displayed an inhibitory effect. Dem. water (position 3) showed no impact on the bacterial growth. Whereas 1% TFA (position 6) leaded to an inhibition zone, 0.01 % TFA (position 5) had no effect. The inhibition of 1 % TFA can be classified as unproblematic. During this study Aspidasept® was dissolved only in 0.01 % or 0.1 % TFA. The synthetic peptide ALK-OH (40 µg/mL, position 4) displayed a similar positive inhibition effect as Melittin (50 µg/mL).

However, Aspidasept®, among high tested concentrations, showed no growth inhibition. At position 8 (5 µg/mL, lowest concentration) and at position (500 µg/mL, highest concentration) exemplary results for Aspidasept® are displayed. But more concentrations were tested (all concentrations were tested twice on different agar plates, results shown in appendix C). Nevertheless, still the highest applied concentration had no negative effect on the bacterial growth.

Figure 4.8: Radial diffusion assay with Aspidasept® and synthetic ALK-OH. Substances were applied at the positions 1 to 8: 1) Melittin [50 µg/mL], 2) Melittin [10 µg/mL], 3) dem. H_2O, 4) synthetic ALK-OH [40 µg/mL], 5) 0.01 % TFA, 6) 1 % TFA, 7) Aspidasept® [500 µg/mL], 8) Aspidasept® [5 µg/mL].

4.1.7 Sample concentration by solid-phase extraction

The solid-phase extraction should be developed for the concentration of larger sample volumes. A sample concentration is often necessary due to the limitations of the analytical methods, such as RP-HPLC and Tricine-SDS-PAGE. As described in chapter 3.6.2 the SPE was performed with different sample volumes and concentrations.

Until the end of the study it was not possible to obtain the desired results. The elution fraction of the SPE only contains a small part of the applied Aspidasept®. The Aspidasept® amount varies during the different experiments from 0 to only 10 %. The waste of the sample application and washing steps were analyzed as well to exclude a low binding of the peptide to the cartridge material. However, only very small peptide amounts (3 – 10 % of the applied peptide amount) were found in these samples.

To investigate in the low recovery of Aspidasept® during the SPE procedure, the sample evaporation with nitrogen was examined. Five samples (10 µg/mL) in acetonitrile (100 %) were prepared, evaporated under nitrogen and dissolved in 200 µL 0.1 % TFA. RP-HPLC analysis showed that only a peptide recovery of 76.5 % was obtained. This results in an approx. 25 % peptide loss during the evaporation step.

Nevertheless, these results do not explain the low Aspidasept® amount in the elution fraction of the SPE. An explanation for a possible loss is an uncompleted elution if acetonitrile is applied or an irreversible adsorption of the peptide onto the polypropylene material of the cartridge.

4.2 Elimination of Aspidasept® by activated carbon adsorption

The adsoption on activated carbon is one of the newest approaches for the elimination of pharmaceuticals. During this trial the adsorption of Aspidasept® on defined amounts of activated carbon at 10 °C or 20 °C was investigated. Using the results of the 10 °C experiments the *Freundlich*-Isotherm was determined.

4.2.1 Adsorption in demineralized water at 10 °C

In a first approach, the kinetic (time course of the adsorption) was evaluated. Keeping the initial peptide concentration c_0 constant, a granulated activated carbon (GAC) variation with five different concentrations was performed. The time dependent adsorption of the peptide in solution was observed for 48 h. Figure 4.9 displays the adsorption kinetics at 10 °C. For any GAC concentration the concentration equilibrium c_{eq} between peptide in solution and adsorbed peptide were reached after 24 h. In case of the highest GAC amount (0.5 g) the adsorption equilibrium was attained after 5 h.

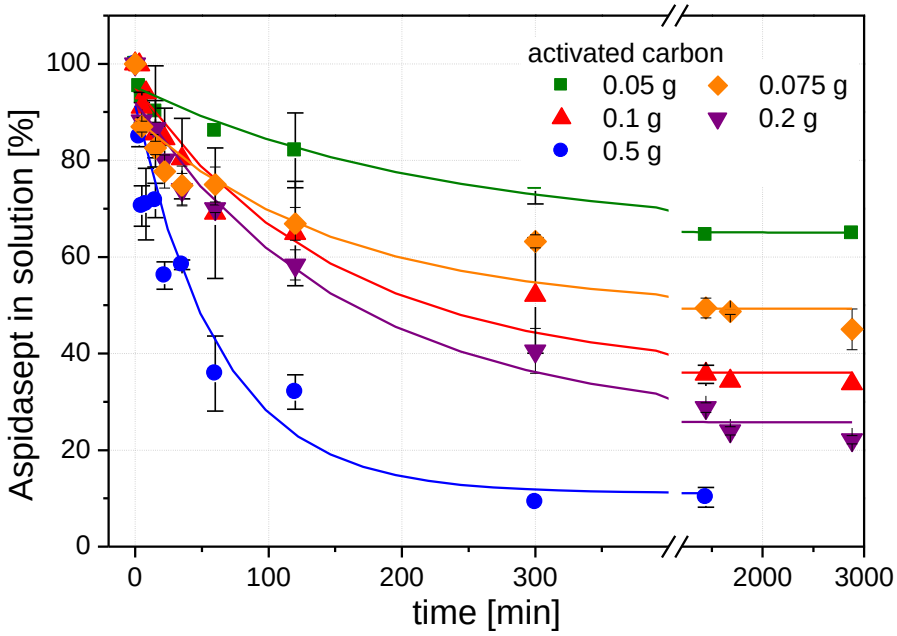


Figure 4.9: Activated carbon adsorption of Aspidasept® on varying amounts of activated carbon at 10 °C. The adsorption was observed for 48 h. The activated carbon quantities 0.05, 0.075, 0.1, 0.2 and 0.5 g were tested (n = 2).

Using the end concentrations of the kinetic experiments, the adsorption equilibriums were determined and thereof the resulting *Freundlich*-Isotherm (see equation 2.3) was calculated. In table 4.2 the results of the GAC loading calculations are listed. For the calculation equation 2.2 was used.

Table 4.2: Equilibrium concentration and calculated GAC loading.

m_{GAC} [g]	c_{eq} [g/L]	q_{eq} [µg/g_{GAC}]
0.05	0.065	7.028
0.075	0.045	7.332
0.1	0.034	6.624
0.2	0.022	3.892
0.5	0.010	1.798

In double logarithmic q_{eq}/c_{eq} – diagram the linear regression can be used for the parameter calculation of the *Freundlich*-Isotherm. For the calculation of the

Freundlich-Isotherm the experiments with the lowest GAC concentration was excluded. These experiments were performed with a too low concentration. Figure 4.10 displays the double logarithmic q_{eq}/c_{eq} – diagram with calculated linear regression.

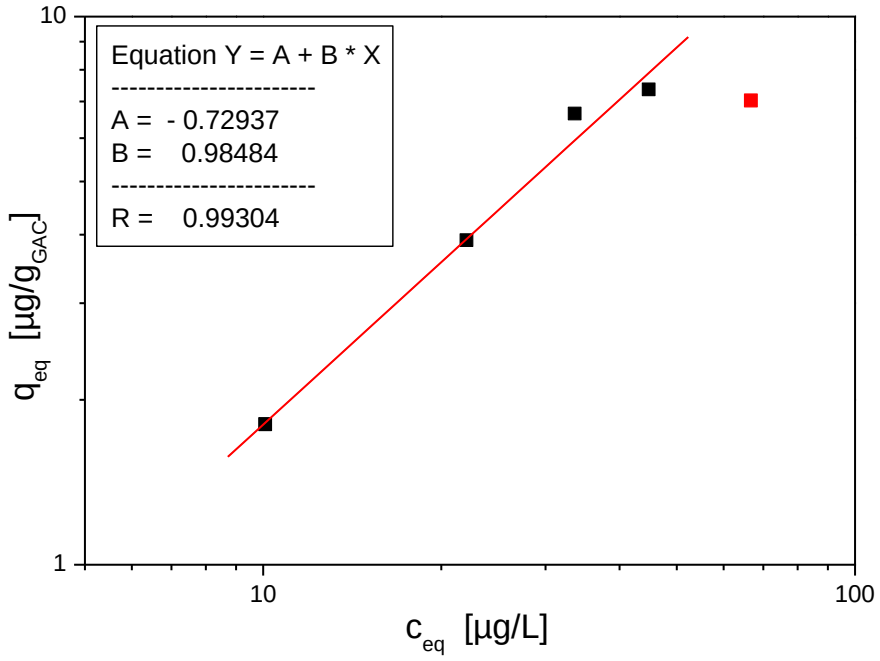


Figure 4.10: Double logarithmic q_{eq}/c_{eq} – diagram with calculated linear regression. The equation of the linear regression can be used for the determination of the *Freundlich*-Isotherm. The red data is excluded from the calculation for the *Freundlich*-Isotherm.

The equation for the *Freundlich*-Isotherm was calculated using equation 2.3. Whereas the parameter n is equal the slope of the linear regression, the parameter K can be calculated with the values of any experiment. For the *Freundlich*-Isotherm the mean of the K-values were used. For the performed experiment series a rather linear *Freundlich*-Isotherm was determined (n ~ 1).

Linear regression: $y = -0.729 + 0.985 \cdot x$ (Equation 4.3)

Freundlich-Isotherm: $q = 0.187 \cdot c^{0.985}$ (Equation 4.4)

4.2.2 Adsorption in demineralized water at 20 °C

To examine the impact of the temperature of the peptide adsorption on granulated activated carbon the 10 °C values were compared with the data of the 20 °C experiments. The experiments at 20 °C were performed with 0.1 g and 0.5 g activated carbon. Figure 4.11 shows the comparison between the obtained data. In both cases, the exponential curves located closely together. For both temperatures equal adsorption equilibriums were determined. The experiments with 0.1 g activated carbon, results in approx. 34 % peptide in solution after 48 h. For the tests with 0.5 g the equilibrium was 10 %. Although the equilibrium seems to be temperature independent, the speed of adsorption indicates minor differences. For both activated carbon concentrations a slightly faster adsorption at a temperature of 20 °C was obtained.

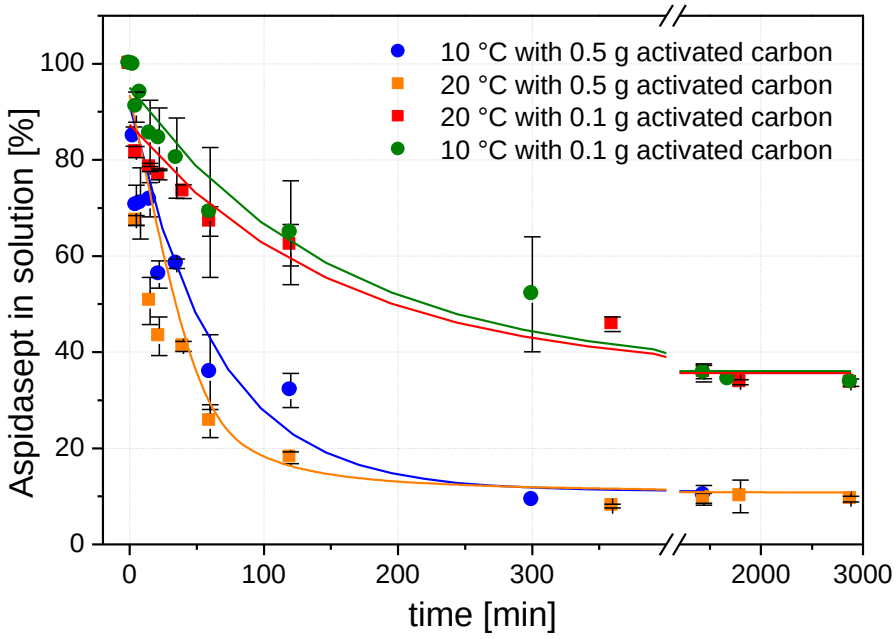


Figure 4.11: Comparison of activated carbon adsorption at 10 °C and 20 °C with 0.1 g and 0.5 g activated carbon. The adsorption was observed for 48 h. No significant differences in terms of adsorption speed and equilibrium between the temperatures were determined.

4.3 Membrane filtration

The next chapter deals with the Aspidasept® filtration. Starting with the test of recovery in different buffers and the adsorption of the peptide on various membranes the filtration was planned. Prior filtration the Cut-Off of the membrane UA60 was determined. The peptide filtration was than performed with pressure variances. The filtration trials were performed with the help of Lukas Cordts (Bachelor Thesis, 2018).

4.3.1 Membrane adsorption and buffer recovery of Aspidasept®

Before starting the membrane filtration trails it is required to investigate the recovery of Aspidasept® under different buffer conditions and the adsorption behavior of the peptide in presence of various membranes. Figure 4.12 displays the results of the recovery test in selected buffers. For each tested buffer the composition is listed in chapter 3.7.2.2. It can be seen, that for each buffer the peptide in solution has not decreased fewer than 20 % within the sample period of 3 h. No significant differences in recovery results from the used buffers. Only 0.1 % TFA showed an increased recovery in comparison to physiological buffer systems.

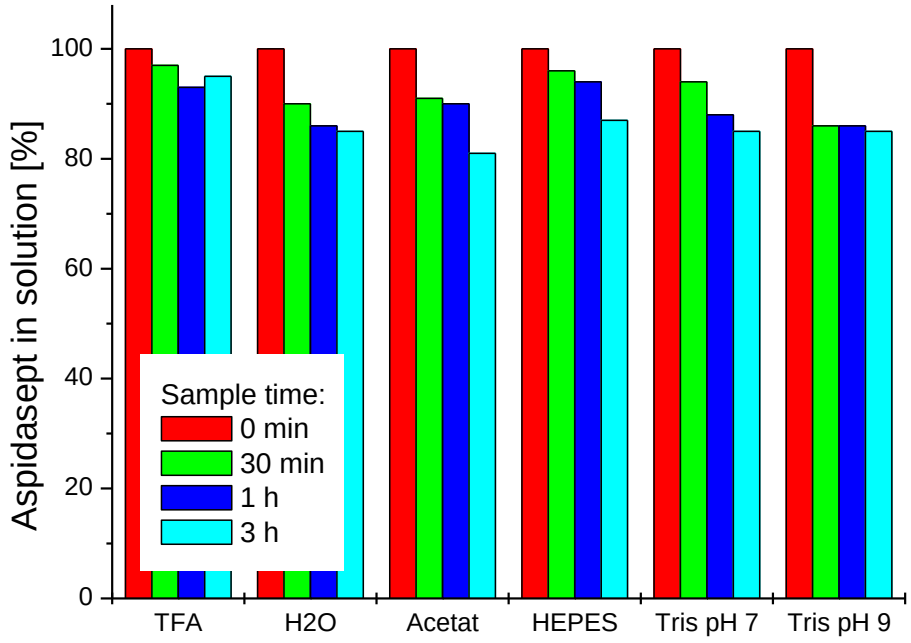


Figure 4.12: Aspidasept® recovery in the presence of different buffers and salt. The peptide recovery of 0.1 % TFA, dem. H_2O and the buffers acetate, HEPES and Tris (pH 7 and pH 9) were tested over 3 h. Samples were taken after 0 min, 30 min, 1 h and 3 h. After 3 h in all buffer systems a peptide concentration of over 80 % was left.

Subsequently it was essential to verify the adsorption of the peptide at membranes used for the filtration. Although only the membrane UA60 was used for the filtration, the membranes XLE, NF90 and NF270 were tested as well to examine the influence of the pore size distribution on the adsorption behavior. The test series with all four membranes was performed in 0.1 % TFA. As buffer system Tris with a pH of 7 was chosen but only tested with the membrane UA60.

Figure 4.13 shows the results of the membrane adsorption within 3 h. For each membrane a strong decrease of the peptide concentration during 3 h was obtained. The results for the adsorption of the XLE and NF90 membranes in 0.1 % TFA give values around 90 % Aspidasept® in solution (10 % loss). The prior performed recovery tests showed a peptide concentration of 95 % after 3 h in 0.1 % TFA without membrane. Thus, only a small amount of peptide is adsorbed to the XLE and NF90 membranes. For the NF270 (65 % in solution) and UA60 (56 % in solution) a much higher adsorption rate is observed. Thus, it must be assumed, that with surpassing of a certain pore size the adsorption of the peptide takes place not only on the surface of the membrane but as well in the pores of the membrane. If changing 0.1 % TFA to Tris (pH 7) a similar adsorption result for the UA60 membrane is achieved. Even tough 47 % of the peptide is left in solution it has to be mentioned that the peptide recovery in Tris (pH 7) is significantly lower than in 0.1 % TFA.

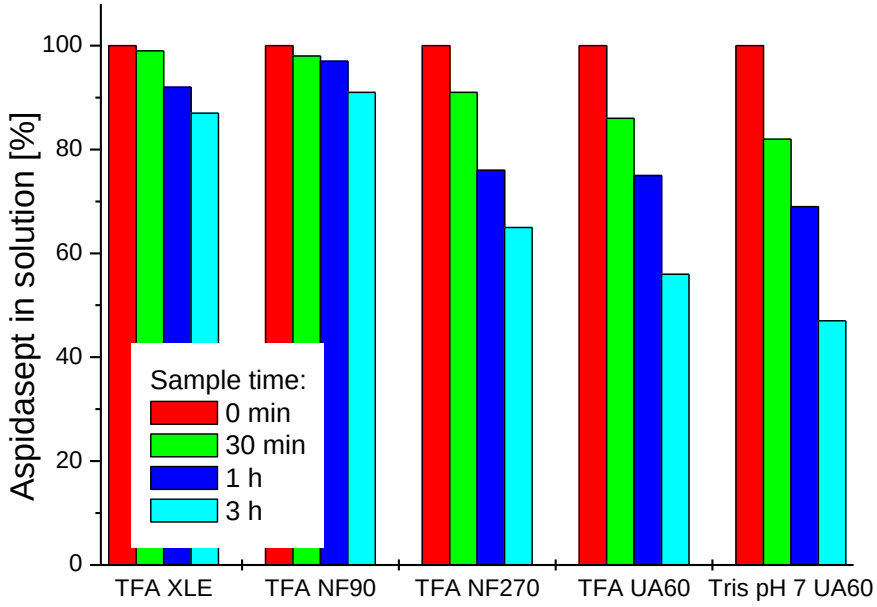


Figure 4.13: Aspidasept® adsorption on the membranes XLE, NF90, NF270 and UA60 in 0.1 % TFA and Tris (pH 7). Samples were taken after 0 min, 30 min, 1 h and 3 h. After 3 h an extremely high decrease of the Aspidasept® concentration in solution was determined. With increase of the pore sizes of the membranes the decrease of the peptide concentration enlarged.

To exclude the impact of the membrane to the RP-HPLC analysis negative controls with all membranes in all buffers were performed. Since the results showed that the RP-HPLC analysis showed no membrane extractables (data not shown) it was assumed that the membrane sample matrix has no influences on the RP-HPLC measurement.

4.3.2 Cut-Off determination of UA60 membrane

The Cut-Off of the membrane UA60 was determined to confirm the manufacturer specifications which were declared at a range of 1000 Da. Using the substances DL-Tryptophan, L-Glutathione, Bromophenol blue, Vitamin B12 and Lysozyme (molecular weight 204 – 14300 Da, see table 3.15) the Cut-Off at a recovery rate of 90 % was calculated. With the help of these substances a Cut-Off of approx. 670 Da was estimated. This is lower than expected. Different options are possible for the deviations. On one side is the manufacturer specification only range specifications,

on the other hand substances with different structure were used. These structures could affect the interaction of substance and membrane in different ways which is why the recovery is not only influenced by the molecular weight but as well from the structure of the individual substance.

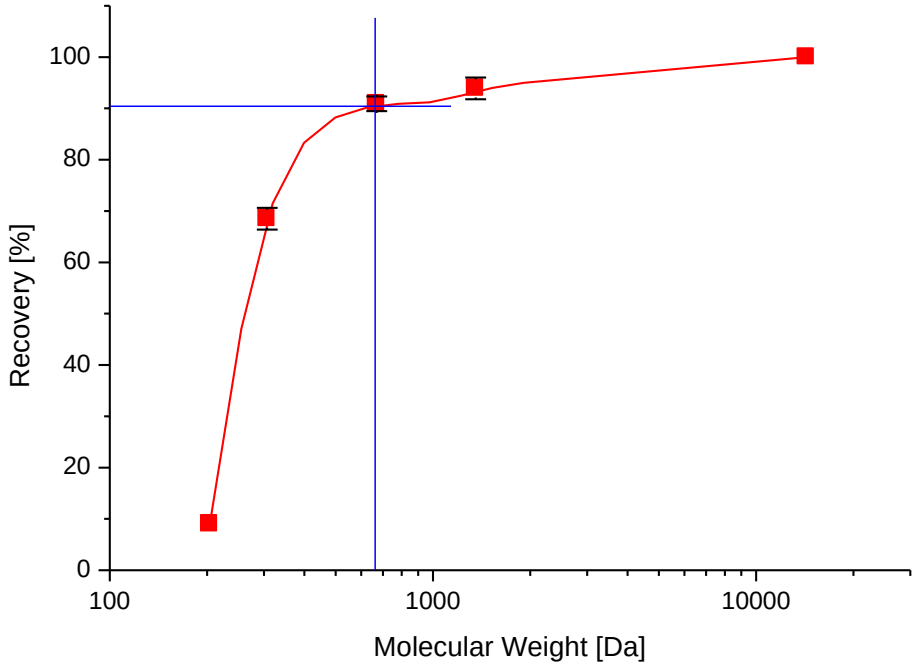


Figure 4.14: Cut-Off determination of membrane UA60. The Cut-Off determination was performed with five substances with different molecular weight (204 – 14300 Da, for specific information about the used substances and the molecular weight see table 3.15). The Cut-Off was calculated at a recovery of 90 %. This equals a molecular weight of approx. 670 Da. All filtrations were performed twice, n = 2.

4.3.3 Aspidasept® filtration with UA60 membrane

The peptide filtration was performed with the UA60 membrane and an Aspidasept® solution in Tris buffer to obtain a physiological environment. Because the calibration curve for peptide in 0.01 % TFA was not suitable for peptide dissolved in Tris buffer a new calibration curve in Tris buffer was necessary. In Tris buffer the obtained values are significant lower than in 0.01 % TFA, mostly due to a low solubility and a fast precipitation. Figure 4.15 displays the calibration curve for Aspidasept® in Tris buffer.

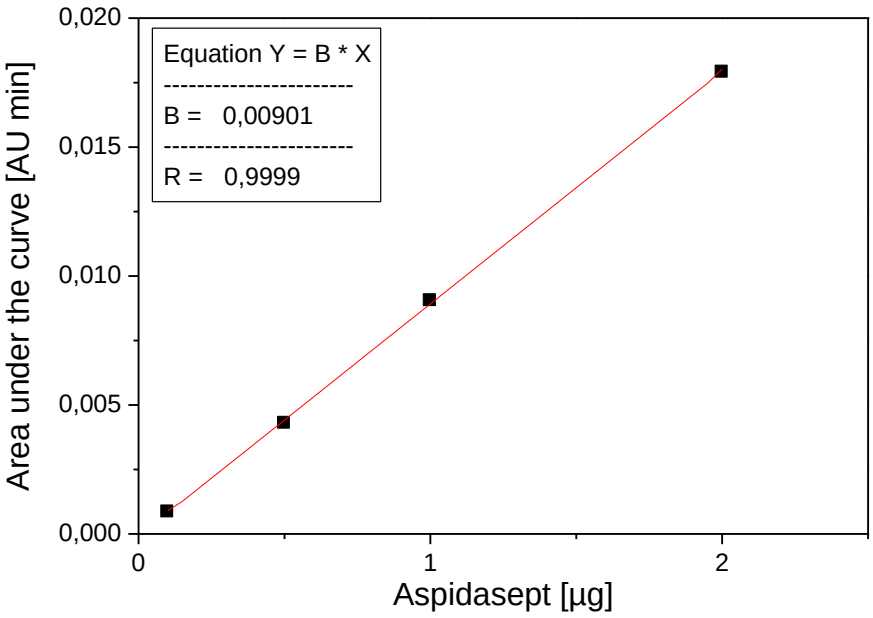


Figure 4.15: Calibration curve of Aspidasept® dissolved in Tris buffer determined by RP-HPLC analysis. The samples were reduced using 3.33 mM DTT. The reference curve was obtained by calculating the area under the curve.

The peptide filtration experiments were carried out according to chapter 3.7.2.4. The samples were analyzed by RP-HPLC and calculated by the help of the Tris reference curve. To obtain reliable results all experiments were repeated with a second UA60 membrane sheet (see table 4.3 and 4.4). It was necessary to prepare a second peptide solution after two experiments because the recovery in Tris buffer was lower than expected.

Table 4.3: Pressure variation peptide filtration with membrane 1. The pressures 8 – 16 bars were tested. Between the 10 bar experiments new peptide solution were prepared. The area under the curve (RP-HPLC) of the Feed, Retentate and Permeate and the total amount of peptide (mg) are listed. The unit of the area under the curve is AU min. The total recovery is calculated by the recovery in the retentate and permeate.

Pressure	Feed		Retentate			Permeate		Recovery
bar	AU min	mg	AU min	mg	%	AU min	%	%
8	0.0077	1.7	0.0054	0.9	52.7	0.000	0	52.7
10	0.0036	0.8	0.0041	0.7	85.7	0.000	0	85.7
10	0.0113	2.5	0.0085	1.4	56.2	0.000	0	56.2
12	0.0057	1.3	0.0038	0.6	49.1	0.000	0	49.1
16	0.0021	0.5	0.0020	0.3	71.0	0.000	0	71.0

Table 4.4: Pressure variation peptide filtration with membrane 2. The pressures 8 – 16 bars were tested. Between the 10 bar experiments new peptide solution were prepared. The area under the curve (RP-HPLC) of the Feed, Retentate and Permeate and the total amount of peptide (mg) are listed. The unit of the area under the curve is AU min. The total recovery is calculated by the recovery in the retentate and permeate.

Pressure	Feed		Retentate			Permeate		Recovery
bar	AU min	mg	AU min	mg	%	AU min	%	%
8	0.0046	1.0	0.0037	0.6	60.7	0.000	0	60.7
10	0.0023	0.5	0.0026	0.4	84.5	0.000	0	84.5
10	0.0092	2.0	0.0077	1.3	63.0	0.000	0	63.0
12	0.0036	0.8	0.0021	0.3	42.6	0.000	0	42.6
16	0.0006	0.1	0.0005	0.1	72.8	0.000	0	72.8

Figure 4.16 displays the means of the performed experiments with two UA60 membranes. The trials with pressure variation showed no tendency in recovery. In all experiments, the RP-HPLC analysis showed an absence of Aspidasept® in the permeate. Thus, it can be assumed that the chosen membrane was too tight for the peptide to pass. However, the obtained values of the retentate showed that it was not possible to retrieve the peptide concentration of the feed. A high peptide loss was calculated. Only recovery rates from approx. 40 to 85 % were achieved. The

peptide loss probable can be explained by a high adsorption on the membrane or the filtration equipment.

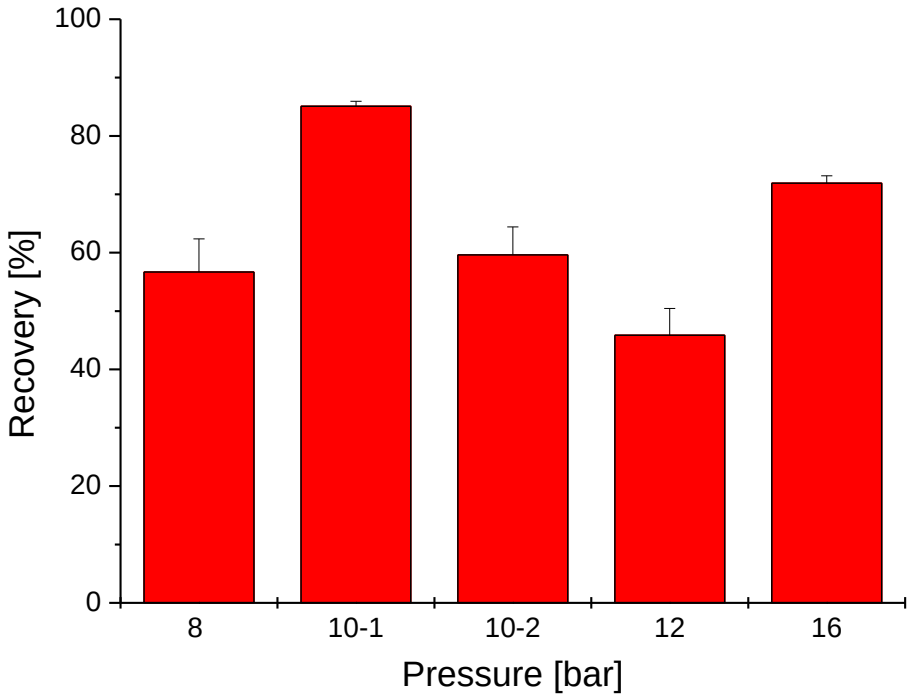


Figure 4.16: Recovery of the peptide filtration with pressure variation. The mean was calculated with the data from table 4.3 and 4.4. The tests were performed from the lowest pressure to the highest. Between the two 10 bar experiments a new test solution was prepared. n = 2.

During the filtration study reference filtrations with water and constant pressure were performed to observe possible blocking of the membrane. Figure 4.17 displays the obtained data. The first reference filtration was performed prior the first filtration with peptide. A permeate flux of 133 L/(h·m^2) was obtained. Reference number 6 corresponds to the filtration with water after the last peptide filtration at 16 bar. The permeate flux decreased to 90 L/(h·m^2). A reduction of the permeate flux of 32 % were caused by the five peptide filtration steps.

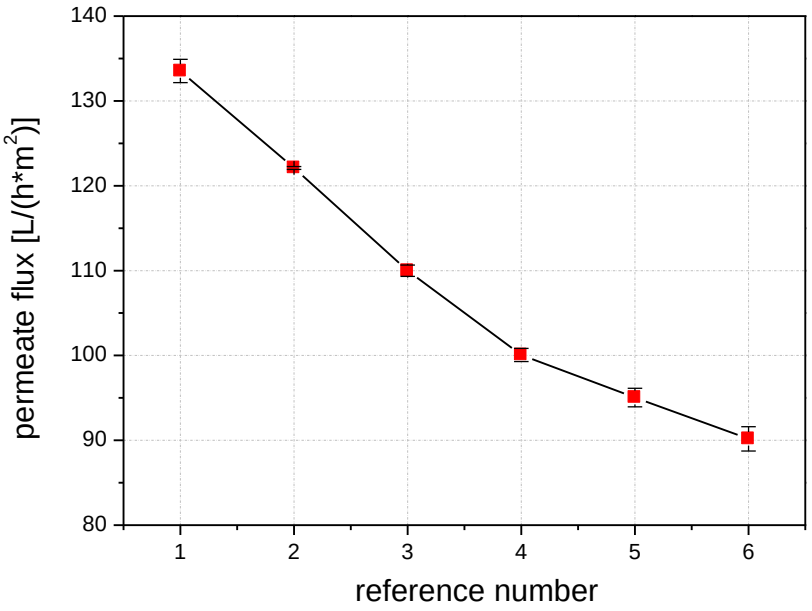


Figure 4.17: Permeate flux of the reference filtrations with water and constant pressure. All reference filtrations were performed at 10 bar with a magnetic stirrer speed of 100 rpm. The reference number 1 corresponds to the filtration with water prior the first peptide filtration. Reference number 2 corresponds to the filtration with water after the peptide filtration at 8 bar. etc.

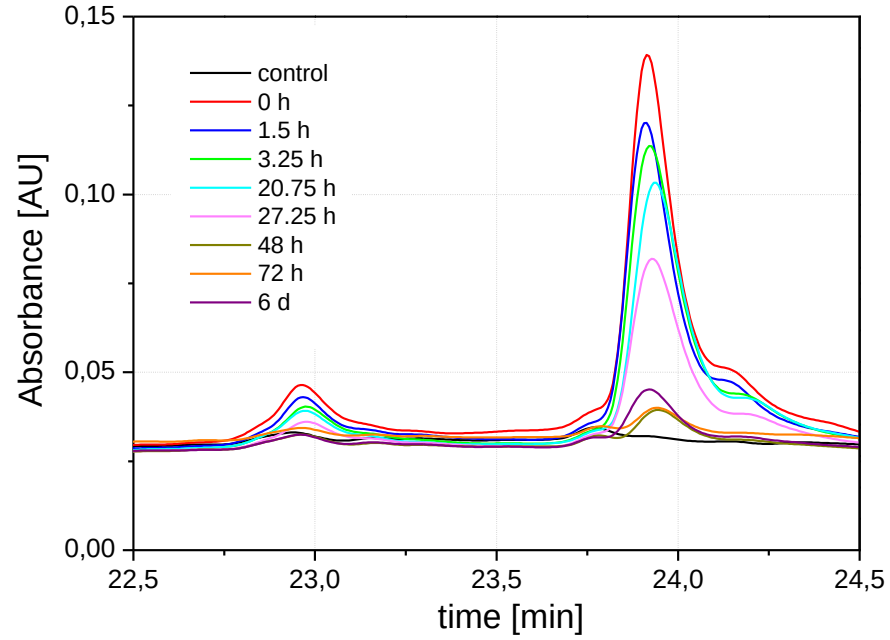
4.4 Biological treatment

Besides activated carbon adsorption and membrane filtration, it was tested in which dimensions the biological environment influences the peptide behavior and stability. Before starting the tests in waste water the stability was examined in particle free water. Due to a lack of time and instabilities of the model laboratory waste treatment system it was not possible to investigate the peptide behavior and the microbial reaction in a waste water elimination system.

4.4.1 Recovery test in particle free waste water

The recovery of Aspidasept® was tested in particle free synthetic waste water and cleared waste water. In both approaches the peptide concentration was measured for 6 days (see figure 4.18). After approx. 24 h only the half of the initially concentration was left. In the following 24 h (48 h after start) the concentration decreased further to 10 % (synthetic waste water) and 20 % cleared waste water.

The experiment showed that the recovery of Aspidasept® in particle free waste water is significant lower as in dem. water (approx. 80 % after 7 days). Here, it should be considered, that with centrifugation probable not all particles were removed which support the adsorption and precipitation of the peptide.



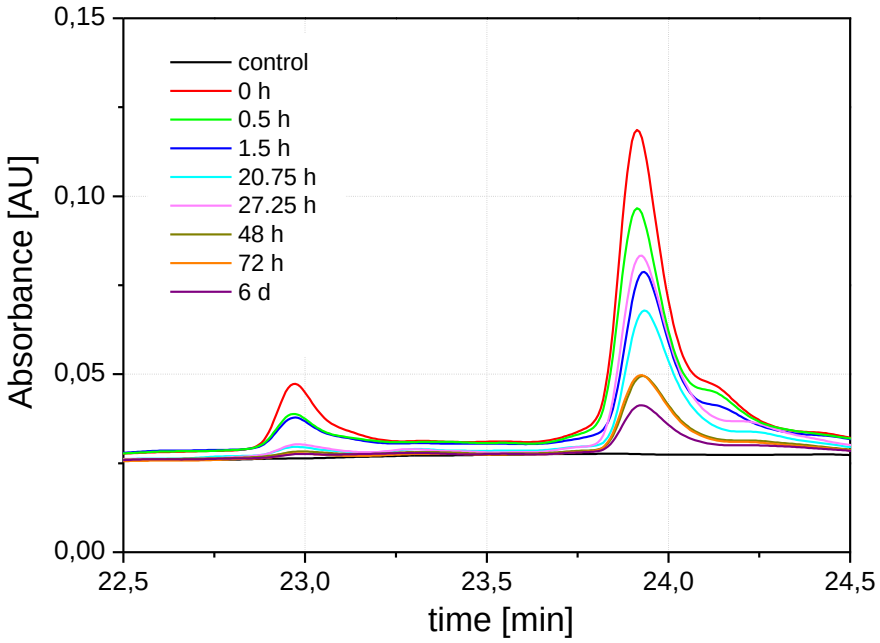


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Figure 4.18: Aspidasept® recovery in particle free waste water over the time period of 6 days. A) Recovery in synthetic untreated waste water. B) Recovery in cleared waste water (by laboratory waste water treatment). All samples were analyzed by RP-HPLC. The control corresponds to an Aspidasept® concentration of zero.

4.4.2 Elimination in waste water

For this trial untreated waste water was mixed with Aspidasept®. After a defined incubation time solid components were centrifuged and analyzed by SDS-PAGE. The cleaned supernatant was applied on the RP-HPLC. The analysis with RP-HPLC showed the absence of the peptide in the supernatant. For all peptide concentrations (up to 100 µg/mL) the RP-HPLC was not able to detect the peptide. The analysis with SDS-PAGE showed different result. Here it was possible to prove the presence of Aspidasept®. Table 4.5 shows the results of the SDS-PAGE with the recovery rates of the peptide. For the quantities 0, 1, 5 µg no peptide band was observed. It has to be assumed that the quantity is below the detection limit. The results were excluded in the result table. The original gels can be found in figure 4.19.

Table 4.5: SDS-PAGE results of the peptide elimination in waste water. The results of Gel 3-6 are evaluated. The total quantity is the amount of recovered peptide after the including of all concentration and dilution steps.

Sample [µg]	Gel no./ lane no.	Band intensity	Quantity on gel [µg]	Total quantity [µg]	Recovery [%]
10	3/ 8	1,522,158	2.44	5.94	59.4
10	4/ 8	1,951,554	2.97	4.88	48.8
25	3/ 10	1,379,730	2.15	21.5	86.0
25	4/ 10	1,367,718	1.72	17.2	68.8
50	5/ 8	765,700	0.71	35.5	71.0
50	6/ 8	504,465	0.40	20.0	40.0
100	5/ 10	771,290	0.72	72.0	72.0
100	6/ 10	595,140	0.71	71.0	71.0

Recoveries between 40 % and 86 % were obtained. Samples applied on different gels show partly strong variances. With increasing start quantity, the recovery, seems to be higher.

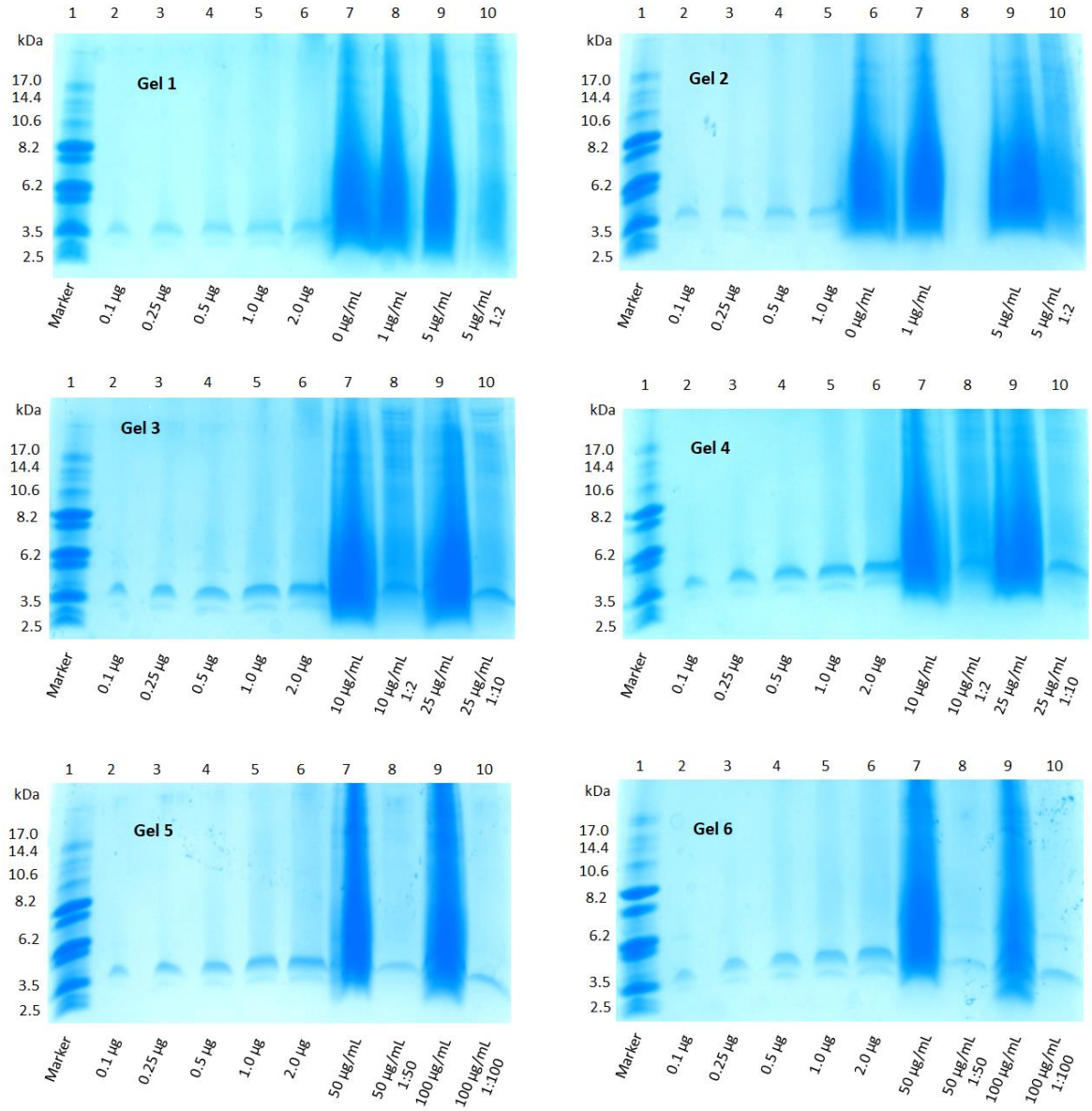

Figure 4.19: Peptide elimination in waste water. Various peptide quantities were added to waste water. After centrifugation the particle containing solution was applied onto the SDS-PAGE. The marker is applied on lane 1. Lanes 2 to 6 are used for the reference curve required for quantification. The lanes 7 to 10 contain the analyzed samples.

5 Discussion

An aim of this study was the analytical characterization of the antiseptic peptide Aspidasept® in terms of general behavior, stability and antimicrobial activity. For that purpose, methods for RP-high performance liquid chromatography and SDS-PAGE were established. Furthermore, a radial diffusion assay was used to examine the antimicrobial activity against *Escherichia coli* and the solid phase extraction technology was tested for the sample preparation and concentration.

Both, RP-HPLC and SDS-PAGE demonstrated to be appropriate for the analyses of peptide samples. Whereas the RP-HPLC needs clean (achieved by centrifugation or 0.45 µm filtration) samples, the SDS-PAGE showed less vulnerability and thus can be used for the analysis of waste water samples. However, the analytical accuracy of the methods is different. For the RP-HPLC a coefficient of variation of 1.0 to 7.3 % was obtained. The SDS-PAGE showed a much lower accuracy not only between two gels but as well between the same samples at the same gel. In literature a coefficient of variation for the HPLC of < 1 % is specified as accurate (Kromidas, 2014). However, the coefficient of variation is depending on the used column, the chromatographic conditions and the substance which is to be analyzed. But as literature is giving a higher coefficient of variation for research methods, the obtained results were accepted as sufficiently accurate for the desired purpose. Thus, if possible, the sample analysis should be performed by RP-HPLC to minimize the analytic error. Only in cases where it is necessary to examine untreated samples with sewage particles or other impurities an SDS-PAGE should be chose.

Still both methods showed similar results regarding quality and stability. As well the quantification with a standard reference curve is for both methods possible even though it is more precisely if the RP-HPLC is used. If Aspidasept® is examined chemically untreated two peaks (in RP-HPLC) respectively two bands (SDS-PAGE) are obtained. The same sample reduced with DTT prior analysis, results in a single peak (in RP-HPLC) or rather a single band (SDS-PAGE). Thus, it appears that a dimerization of the peptide is responsible for the second signal. As DTT is reducing disulfides (Cleland, 1964), only the peptide monomer is detected. Thus, it can be

assumed that the dimerization bridge is formed between the amino acid cysteine at position 2 of the Aspidasept® sequence. Cysteine is based on a sulfhydryl connection in the side chain. This connection is oxidized in aquatic solution in the presence of atmospheric oxygen. As well the formation of dimers seems to be a time and temperature dependent process. Freshly prepared peptide showed a significantly lower dimer ratio than a peptide solution which is frozen and thawed after a few days. During the freeze & thaw process the peptide is exposed to oxygen. As a result, the dimerization process will have more time to develop disulfide bridges. But not only has the exposure to oxygen an impact on the peptide behavior. Tests at different temperatures showed a temperature dependent recovery of the peptide. At room temperature (between 20 °C and 23 °C) only a recovery of approx. 80 % was obtained over a period of 7 days. At 4 °C almost 100 % of the peptide could be recovered after 7 days. Even if this test results in acceptable results for this trial, more tests should be performed to increase the recovery. The application of different chemical stabilizers could enhance the recovery. As well, the dimerization process should be examined further.

Although only two peaks in the RP-HPLC (one in reduced samples) are detected, the SDS-PAGE showed three bands (two in reduced samples). Two bands can be explained by the prior discussed formation of dimers. The third band, with a smaller molecular weight as the monomer is independent of the sample treatment and increases with increase of the applied quantity of sample. However, the intensity of the band is in any case significantly below the bands for the monomer or dimer. Even though it is possible that these faint bands correspond to degradation products of the peptide, it is more likely that the bands indicate a peptide with a different conformation. The RP-HPLC supports this assumption as well. Degradation products would show a different retention in the RP-HPLC. Whereat, peptides with different conformations could have the same retention time. As a result of this it was decided that the faint bands were included into the calculations for the quantifications.

The test for antimicrobial activity with the radial diffusion assay showed no growth inhibitory effect of Aspidasept® on the used *Escherichia coli* strain. It has to be assumed, that the peptide has no antimicrobial activity or that the test is not suitable

for the proof of an antimicrobial activity. For similar peptides it was possible to prove the antimicrobial activity with the help of the assay. Thus, the test is definitely appropriate for the evidence of the inhibition of bacterial growth by peptides. Nevertheless, in various trials it was possible to detect an antibacterial effect against gram-negative bacteria such as *Escherichia coli* (e.g. Gutsmann et al., 2010). Still in this study a different test approach, microdilution susceptibility assay was used. The microdilution susceptibility assay is a common method for the first assessment of bacterial inhibition (Otvos and Cudic, 2007) and is based on the bacterial growth in broths in the presence of peptides or antibiotics. This assay has the benefits of a homogenous bacteria-peptide-solution and eliminated the restrictions of peptide diffusion inside solid agar. For that reason, it should be considered to perform a microdilution susceptibility assay if an antibacterial activity should be proved. As well it should be mentioned, that the radial diffusion assay was performed with an old charge of the peptide which could have an influence on the activity. Moreover, it the activity was only examined with one *Escherichia coli* strain. To assess the impracticality of the radial diffusion assay for Aspidasept® a control with different bacteria strains should be considered.

The behavior and elimination of the peptide in different waste water treatment methods was examined afterwards. First, the adsorption on granulated activated carbon was investigated. With various amounts of GAC and two different temperatures the adsorption equilibrium was determined and the kinetics were examined. The investigation of the adsorption equilibrium confirmed the expected results. With increasing amount of GAC the equilibrium was reached in a shorter period of time. Using the in figure 4.9 displayed data a loading saturation can be seen. The obtained data converge to a maximum (last three data point, inclusive excluded data point, displayed in figure 4.9). With increasing amount of peptide still no increase of the loading of the adsorbent is achieved. To confirm this presumption, further experiments should be made.

The obtained *Freundlich*-Isotherm (equation 4.4) can provide information about the adsorption capability of the substance. The received n for the *Freundlich*-Isotherm has a value of approx. 1. This means that the loading of the adsorbents changes proportional to the concentration of the substance which is adsorbed (Lohrengel,

2012). Even though a value below 1 is favorable for the parameter n (the used amount of adsorbent can be decreased), a value higher than 1 would be not economically (Lohrengel, 2012). The second parameter K which is used for the definition of the *Freundlich*-Isotherm describes the how proficiently a substance is adsorbing (Worch, 2012). The higher the value is, the higher the loading of the adsorbent is. During this study a value of K of 0.187 was obtained. In comparison to values found in literature, this value is low for the adsorption on activated carbon. Still these values were obtained for the adsorption of antibiotic and thus not really comparable. Until now two independent studies were performed at the university investigating the adsorption of different antibiotics. Lichtenberg (2017) observed the adsorption of Sulfamethoxazole (SMX). During the study K values between 3 - 10 $[\mu g_{SMX}/g_{GAC}]^n$ with n ~ 0.4 – 0.54 was obtained (Lichtenberg, 2017). The second study is examining the adsorption of Trimethoprim (TMP). Romanow (2017) achieved as isotherm parameters K ~ 1.4 – 2.4 $[\mu g_{TMP}/g_{GAC}]^n$ and n ~ 0.6 – 0.96. For the adsorption of antibiotics disparately higher values for K were achieved. Thus it has to be mentioned that for antibiotics a higher adsorbent loading capacity is obtained which makes it more economically than the adsorption of Aspidasept®.

The temperature variation from 10 °C to 20 °C showed no changes in terms of the adsorption equilibrium. Still it can be seen, that the equilibrium at 20 °C was reached faster for both examined GAC concentrations than for 10 °C. This, however, is in counter to the common information found in literature. Even though it is known that the equilibrium is influenced by the concentration of substance which is adsorbed and the temperature, literature specifies that with increasing temperature the adsorption decreases (Worch, 2012). Since it cannot be assumed that the obtained data are not appropriate (all test were performed twice) or the peptide behaves different to the common scenarios. Reasons for that could be the characteristic structure of a peptide different from those in the literature. To obtain clarification how the adsorption process is affected by the temperature further investigations are necessary.

However, not only the temperature can have an impact on the adsorption capacity. The pH as well as the ionic strength of the adsorption matrix can show an influence on the adsorption capability of activated carbons (Al-Degs et al., 2008). During the

adsorption the peptide was dissolved in 0.1 % DMSO. After dilution with dem. water an almost neutral pH was obtained. By reducing the pH to an acidic environment (e.g. use of TFA) the adsorption capacity could be increased (Al-Degs et al., 2008). However, in this case, a greater variance from the normal environmental conditions would be obtained (waste water shows an almost neutral pH). An increase ionic strength (addition of buffer salt) would help to reduce the peptide in solution. But most probably the reduction would be obtained not by increasing the adsorption capability, but rather by increasing the precipitation. This indicates the results of the recovery tests in various buffer salts (see chapter 4.3.1). In these trials the precipitation is supported by the addition of buffers and NaCl.

The purification of waste water using activated carbon is a frequently investigated process. Various studies examined the adsorption possibility of different amino acids and toxic peptides. A research group studied the adsorption of the toxic compounds cylindrospermopsin and microcystin (cyclic heptapeptide) from cyanobacteria on powdered activated carbon (PAC) under simulated conditions of a waste water plant (Ho et al., 2011). During this study it was possible to remove the toxic substances by PAC. Here it became evident that the adsorption capacity increased with decreasing particle diameter. A second study describes the adsorption of single amino acids on activated carbon (Cermakova et al., 2017). They discovered a strong impact on the adsorption of different amino acids which is dependent on electrostatic and hydrophobic interactions. With increasing ionic strength of the solution the adsorption decreased. With increasing hydrophobicity of the amino acid the adsorption decreased as well. Both studies provide guidance how the environmental conditions of the adsorption process influences the adsorption and which steps could be made to increase the process. Instead of GAC which was used during this thesis, the adsorption on PAC should be observed as well.

Summarizing it can be seen that the adsorption on activated carbon showed not only expected results. Nonetheless, the adsorption of Aspidasept® on granulated activated carbon is readily possible and suitable for the waste water treatment. Still, a lot of research is necessary to completely understand the influences of the adsorption and to develop the most economical way of peptide elimination. Furthermore, only adsorption tests in dem. water were performed. If the trails would

be repeated in a synthetic waste water matrix, compounds of the matrix could influence the adsorption of the peptide. Moreover, components, such as antibiotics, found in the non-synthetic waste water of local sewage plants will have an impact of the adsorption of the target compound (Worch, 2012). In addition, it is not possible to use the *Freundlich*-isotherm or other common isotherms if a multi-component is present (Benström, 2017).

A more or less recent sewage treatment method for the elimination of pharmaceuticals from waste water is the nanofiltration. Before starting the filtration trials, the peptide stability and the peptide adsorption at the membrane was observed. The recovery test in various solutions showed similar trends for the different tested buffers. After 3 h the Aspidasept® concentration decreased to approx. 80 %. The used buffers consist of similar components. The buffers salt in a low concentration and NaCl in a higher concentration which is equal for each buffer. Thus, it can be assumed, that the ionic strength of each buffer is very similar. The ionic strength as well as the pH has an impact on the stability of peptides (Léonil, 1994). Since no significant differences between the buffers in terms of recovery were observed, the Tris buffer and 0.1 % TFA were used for further membrane adsorption tests. These tests showed that with increasing pore size the adsorption at the membrane increases. The peptide seems to be small enough to diffuse into the pores. With increasing pore size an easier entrance into the pore is guaranteed which is why the adsorption increases. This adsorption can only be prevented by using a membrane which does not support the interaction with the peptide. Still more investigations should be made to examine if at a specific pore size the membrane adsorption reaches a maximum.

To specify the used membrane, the Cut-Off was determined. The Cut-Off is used for the determination of the recovery of different molecular weights (Melin and Rautenbach, 2007). According to manufacturer specification the Cut-Off should be in the range of 1000 Da. During this study only a cut-off of approx. 670 Da was determined. For this deviation various reasons could be named. If determining the Cut-Off it is necessary to prevent the formation of surface layer (Melin and Rautenbach, 2007). This layer would increase the recovery of the substances used for the trials (Melin and Rautenbach, 2007). During this study a larger volume than

required was filtrated for the Cut-Off determination. Thus, a formation of a surface layer is likely. For some substances (e.g. Bromophenol blue, Vitamin B12) a surface layer even could be observed. On the assumption that these surface layers change the filtration behavior of the membrane a too low Cut-Off was determined. But as well other reasons could be assumed. For the determination of the Cut-Off significantly different substances were used. These substances show different interactions with the membrane which will influence the filtration behavior. In considerations of the named factors the obtained Cut-Off can only be seen as an approximate value. Nevertheless, the membrane was used for first filtration trials of Aspidasept®.

The filtration with the membrane UA60 showed no Aspidasept® in the permeate at any time. Still it was not possible to recover 100 % peptide in the retentate. Depending on the applied pressure, the recovery of the peptide fluctuates. No clear trend can be determined. A blocking of the membrane however can be recognized. The reference filtrations with water performed prior and after each peptide filtration, show a strong decrease of the flux. Thus, it must be assumed, that the peptide is irreversible interacting with the membrane. The low recovery is probably favored by the low stability of the peptide in Tris buffer which leads to precipitation and further blocking of the membrane. As well interactions of the peptide with the stainless steel filtration housing and the magnetic stirrer cannot be excluded. Further filtration studies using membrane UA60 should not be implemented. If the nanofiltration of Aspidasept® should be examined further a different membrane should be chosen. To exclude the interactions of the peptide with the membrane a coating with a protein such as bovine serum albumin or e.g. with cross-linked poly (ethylene glycol) diacrylate should be considered (Ju et al., 2009). Coatings like that could prevent the fouling of the membrane by the peptide. Furthermore, it is necessary to spend time for the establishment of a different buffer. If Tris buffer is continuously used, the recovery issue will remain.

The last part of this study was the examination of Aspidasept® behavior in differently treated waste water samples. Peptide samples were added to synthetic waste water and cleared waste water (both purified by centrifugation). The course of the peptide concentration over 6 days showed a strong decrease. With only 50 % of the initial

concentration a significant lower amount is left than in the recovery studies in dem. water. This is confirmed by the further course of the study (only 10 % left after 6 days). In cleared waste water a slightly higher recovery is detected. The low obtained recovery rate in both experimental designs can be explained by remaining particles which were not removed during the pretreatment of the waste water. The particles have an effect as adsorbent and the peptide will be adsorbed over the time which leads to a reduction of measurable Aspidasept® in solution. But a second reason has to be discussed as well. The waste water samples were not filtrated or treated in a similar way to obtain a sterile environment. As well it was necessary to open the sample vial for the sampling. Thus, additional germs could enter the vial. Until now it is not known if microbial contaminations have an impact on the peptide stability. Possible effects were degradation or a supported precipitation of the peptide. Unfortunately it is not feasible to detect these processes using the available methods. For the investigation of these processes a mass spectrometer is required. Further studies shall examine degradation process to distinguish them from simple adsorption.

To examine the influence of particles on Aspidasept® untreated waste water was used. After the incubation period is was not possible to detect peptide in solution. The waste water particles show a peptide adsorption. Recoveries from 40 % to 86 % were obtained. Not only between two samples with equal initial peptide concentration but also over the whole experimental setup large deviations appeared. Many reasons could be named trying to explain this variability. Even though the used waste water was homogenized before splitting up into the single experiments is cannot be excluded that in some sample vials more particles are present. As well is the analysis with SDS-PAGE not that reliable than with RP-HPLC. The evaluation of the SDS-PAGE is more error-prone. Moreover, a higher recovery with increasing initial peptide amount can be noticed. To examine if the initial peptide amount have an effect on the recovery it is necessary to perform more experiments with varying peptide concentrations. Still it cannot be excluded that the peptide is adsorbing onto the equipment used during this study which would lead to a lower recovery.

During this study it was only possible to start the investigation of the peptide behavior in synthetic waste water. Though the first obtained results showed that the

peptide tend to adsorb on the particles it is not known until now if a degradation process takes place. As well it could be interesting to explore the behavior of the microbes and protozoa in the presence of Aspidasept®.

6 Conclusion and Outlook

During this study a characterization of the peptide Aspidasept® was successfully performed. Analytical methods were established and the formation of dimers was observed. Still it was not possible to prove the antimicrobial activity of the peptide. The handling of the peptide provides some great challenges. In the presence of buffer salts low recovery rates were received. First results in terms of peptide elimination on different stages of waste water plants were obtained. The results of the adsorption study on activated carbon indicate the good feasibility of the method for the purification of peptide contaminated waste water. The membrane filtration with a 1000 Da flat filter results in a complete peptide retention but as well in a low recovery due to precipitation and adsorption of the peptide. First experiment in synthetic and clarified waste water showed an adsorption of the peptide on waste water particles and thus purification is achieved.

Even though good results were obtained during this study a lot of work has to be performed to fully understand the behavior of Aspidasept® in different environmental conditions. It has to be determined in which case peptide precipitation, adsorption or degradation takes place. To prove the antimicrobial activity and to find a process for peptide concentrations more tests are necessary.

Further investigations have to be made to determine the economic efficiency of the use of activated carbon and membrane filtration in the waste water treatment. After the examination of the adsorption under optimal conditions in dem. water, the adsorption in clarified waste water should be observed. As well more experiments should be performed to determine the temperature dependency of the adsorption process. To increase the adsorption capacity powdered activated carbon should be evaluated. It is characterized by a larger surface and a higher capacity can be obtained.

For the filtration trials of Aspidasept® a different membrane is necessary. With the application of a membrane with larger Cut-Off a higher flow rate at a lower pressure can be achieved which increase the economic efficiency of the process. Possible is the use of the SBNF flat membrane with a Cut-Off of 2000 Da from MICRODYN-

NADIR made out of cellulose acetate. Of course it is required to examine the recovery of the peptide with the suggested membrane first. As well it is necessary to reduce the fouling effect of the peptide on the membrane to increase the recovery in the retentate. The above mentioned membrane could provide this. It is made out of cellulose acetate which is a hydrophilic polymer. This shows a low fouling effect in the presence of hydrophobic elements. A coating of the membrane with protein could as well decrease the membrane fouling.

To gather further information about the influence of Aspidasept® on the bacteria and protozoa used in the biological treatment long-term studies in the laboratory waste water treatment plant should be performed. In this case not only the recovery and stability of the peptide but as well the impact of the peptide on the bacterial functioning is of high relevance. For this, the established methods are not sufficient. To determine the stability of the peptide mass spectroscopy is necessary. The impact on the bacteria and protozoa can be observed via microscopy.

But even though there is a lot of research necessary it was possible to achieve great results in the, during this work, developed methods.

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Appendix

A Figure index

B Table index

C Radial diffusion assay – Results

In the following figures the agar plates of the radial diffusion assay are displayed. All samples were applied twice on different plates. Three plates were used. The third one is displayed in the result chapter. As shown in the results: Aspidasept® showed no growth inhibition.

Figure C.1: Radial diffusion assay with Aspidasept® and synthetic ALK-OH. Substances were applied at the positions 1 to 8: 1) Melittin [50 µg/mL], 2) Melittin [10 µg/mL], 3) dem. H_2O, 4) Aspidasept® [500 µg/mL], 5) Aspidasept® [200 µg/mL], 6) Aspidasept® [100 µg/mL], 7) Aspidasept® [70 µg/mL], 8) Aspidasept® [50 µg/mL], 9) Aspidasept® [10 µg/mL].

Figure C.2: Radial diffusion assay with Aspidasept® and synthetic ALK-OH. Substances were applied at the positions 1 to 8: 1) ALK-OH [40 µg/mL], 2) 0.01 % TFA, 3) 1 % TFA, 4) Aspidasept® [5 µg/mL], 5) Aspidasept® [10 µg/mL], 6) Aspidasept® [50 µg/mL], 7) Aspidasept® [70 µg/mL], 8) Aspidasept® [100 µg/mL], 9) Aspidasept® [200 µg/mL].

Eidesstattliche Erklärung

Hiermit versichere ich, dass ich die vorliegende Master Thesis mit dem Titel „Establishment of RP-HPLC and SDS-PAGE analytics of an antiseptic peptide to investigate its elimination on a model waste water plant“ ohne fremde Hilfe selbstständig verfasst und nur die angegebenen Quellen und Hilfsmittel verwendet habe. Wörtlich oder dem Sinn nach aus anderen Werken entnommene Stellen sind unter Angabe der Quellen kenntlich gemacht.

Ettlingen, 12.03.2018 _____

Silke Allers