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„Enzymatische Metabolisierung des MCHR-1 Antagonisten SNAP-7941 zur SNAP-Säure und Auswertung durch Hochleistungsflüssigkeitschromatographie (HPLC)“

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List of Abbreviations

A	Absorbance
Arg	Arginine
Asp	Asparagine
AUC	Area under the curve
b	Thickness of the cuvette in cm
c	Concentration
E	Enzyme
EP	Enzyme/product complex
ES	Enzyme/substrate complex
g	Gravitational- force
GPcR SLC-1	G-protein coupled receptor somatostatin receptor-like protein-1
h	Height
hERG	Human Ether-a-go-go Related Gene
HOMO	Highest occupied molecular orbital
HPLC	High performance liquid chromatography
I_0	Intensity before the light meets the sample
I_t	Intensity of the light after going through the sample
k	Retention factor
k_1	Rate constant of the association of E and S
k_2	Rate constant of the dissociation of ES to E and P
K_2HPO_4	Potassium phosphate dibasic
k_3	Rate constant of the reverse reaction
k_4	Rate constant of the dissociation of EP to E + P
k_{cat}	Turnover number
KH_2PO_4	Potassium phosphate monobasic
K_m	Michaelis-Menten constant
LUMO	Lowest unoccupied molecular orbital
M	Mol
MCH	Melanin Concentrating Hormone
MCHR-1	Melanin Concentrating Hormone Receptor 1

MCHR-2	Melanin Concentrating Hormone Receptor 2
mM	Millimol
MSH	Melanocyte-stimulating hormone
Na_2CO_3	Sodium carbonate
P	Product
p-NP	p-Nitrophenole
p-NPA	p-Nitrophenylacetate
PET	Positron emission tomography
R	Resolution
R^2	Coefficient of determination
RP	Reverse phase
rpm	Revolutions per minute
S	Substrate
t	Time
t_0	Dead time
TCA	Trichloroacetic acid
t_R	Retention time
UV/VIS	Ultraviolet–visible
v (t)	Velocity
w	Baseline width
α	Measure for quality of separation
ε	Molar extinction coefficient

Table 1: List of Abbreviations

Table of Reagents

4-Nitrophenol ReagentPlus® ≥ 99%, CAS [100-02-7]
Acetic acid 99.8-100.5%, CAS [64-19-7]
Acetonitrile CHROMASOLV® Plus, for HPLC ≥ 99%, CAS [75-05-8]
Ammonium acetate ≥98%, CAS [631-61-8]
Calcium acetate monohydrate, ACS reagent, ≥99.0%, CAS [5743-26-0]
Calcium chloride dihydrate zur Analyse, CAS [10035-04-8]
Casein from bovine milk, Bioreagent, suitable for insect cell culture, CAS [9000-71-9]
Ethanol EMPROVE®, absolute suitable for use as excipient CAS [64-17-5]
Folin's Ciocalteu's phenol reagent, Lithium sulfate (≥10 % - < 20%), CAS [10377-48-7] Sodium tungstate (≥ 1 % - < 10%), CAS [13472-45-2] Phosphoric acid (≥ 5% - < 10%), CAS [7664-38-2] Hydrochloric acid (≥1% - < 5%), CAS [7647-01-0]
Glyceryl trioleate ≥ 99%, CAS [122-32-7]
L-Tyrosine, reagent grade, ≥98% (HPLC), CAS [60-18-4]
Magnesium chloride hexahydrate, CAS [7791-18-6]
p-Nitrophenyl acetate, esterase substrate, CAS [830-03-5]
ortho-Phosphoric acid 85%, pro analysis, CAS [7664-38-2]
Potassium phosphate dibasic, ACS reagent, ≥98%, CAS [7758-11-4]
Potassium phosphate monobasic, ACS reagent, ≥99.0%, CAS [7778-77-0]
Sodium acetate trihydrate, for analysis indifferent to potassium permanganate, CAS [6131-90-4]
Sodium carbonate, water free for Analyse EMSURE® ISO, CAS [497-19-8]
Sodium hydrogencarbonate, ACS reagent, ≥99.7%, [CAS Number 144-55-8]

Sodium hydroxide pellets > 98%, CAS [1310-73-2]
Sodium phosphate dibasic dihydrate puriss. pa., Reag. Ph. Eur.,98.5-101.0% (calc. to the dried substance), CAS [10028-24-7]
Sodium phosphate monobasic monohydrate, puriss. pa. ACS reagent, ≥ 99,0%, CAS [13472-35-0]
Trichloroacetic acid ≥ 99.0% CAS [76-03-9]
All reagents were purchased from the companies Merck, Darmstadt, Germany; Fluka and Sigma-Aldrich, Vienna, Austria.

Table 2: Reagents

Produced reagents

20mM phosphate buffer, pH 7.4

20mM NaH_2PO_4 (1.85g/l) and 20mM Na_2HPO_4 (2.18g/l) in aqua purificata.

50mM phosphate buffer, pH 7.5

50mM (11.4mg/ml) potassium phosphate dibasic trihydrate in aqua purificata, the pH was adjusted with 1M hydrochloric acid.

6.5% casein-solution

6.5mg/ml casein were dispersed in the 50mM phosphate buffer, pH 7.5, stirred and heated gently up to 80-85°C, till the solution was homogenous (about 10 minutes).

0.5M sodium carbonate solution

53mg/ml sodium carbonate were dissolved in aqua purificata.

Enzyme-diluent solution

5.19mM (0.8203g/l) calcium acetate monohydrate and 6.58mM (0.89g/l) sodium acetate trihydrate were dissolved in aqua purificata.

Folin and Ciocalteus`s Phenol Reagent

The Folin and Ciocalteus`s Phenol Reagent received from Merck was diluted 1:100 to lighten the reagent's colour.

110mM trichloroaceticacid

18g/l trichloroaceticacid were diluted in distilled water to a concentration of 110mM.

Used tools

High Pressure Liquid Chromatographie (HPLC) device

Agilent Technologies 1200 Series equipped with

- Quaternary pump with degasser unit
- Variable wavelength UV-Detector
- Manual injection valve

Software: Raytest GINA star, Version 5.9

UV/VIS - Spectrophotometer

Hitachi U-2000

Thermo-Incubator

Eppendorf (Hamburg, DE)
Thermomixer compact

Refrigerated Centrifuge
Hettich Lab Technology (Tuttlingen, DE)
Universal 30 RF

List of used enzymes

All enzymes, except the Butyrylcholinesterase from equine serum (Sigma-Aldrich), were generously given to us by the Institute of Organic Chemistry, University of Vienna.

- Acid Protease II Amano NWT 02510
- Amano Lipase PS Burkholderia cepacia
- Butyrylcholinesterase from equine serum
- Chirazym P-Z, 2,5U/mg PPA 5996
- Lipase Candida cylindracea 85000U/g (PPH phar)
- Lipase Type II Porcine Pancreas / Sigma L-3126
- Papain v. Carica Papaya / Sigma 76220
- Proleather Amano PRS 12516C (1997)
- Protease A Amano 7.1.1997
- Protease M Amano PRT 04513M
- Protease N Amano PRT 03530N
- Protease S Amano PRT 07515
- Prozyme 6 Amano PZT 01507 (1997)

Synthesis of SNAP-7941 and Precursor

SNAP-7941 and SNAP-acid were synthesised in cooperation with the Department of Pharmaceutical Chemistry, Division of Drug Synthesis, University of Vienna.

1. Introduction

1.1 MCH – System

1.1.1 History

In 1931, the hypothesis was proposed that two antagonistic peptides are responsible for the fish skin's adaptability to light and dark. Soon the hormone responsible for darkening the skin could be identified and was called melanocyte-stimulating hormone (MSH). The peptide responsible for lightening the skin due to the aggregation of melanophores, melanin concentrating hormone (MCH), was discovered in salmon in 1983. (1) After some research, a homologue cyclic 19-amino acid neuropeptide could be isolated and cloned from mammals. (2)

In search of a ligand for the GPcR SLC-1 (G-protein coupled receptor somatostatin receptor-like protein-1) it was discovered that MCH binds this receptor specifically and activates it. Therefore, the receptor was renamed MCH-R1. (3) A short time later another MCH-Receptor was discovered and called MCH-R2. Both of them are mainly located in the brain, hypophysis and in fat tissue. (4)

1.1.2 Melanin concentrating hormone

The original purpose of MCH is the ability of fish and amphibians to adapt to light and dark environments. In mammals however, MCH affects the ingestion, energy usage, sleep and high levels of melanocyte-stimulating hormone can be associated with depression. MCH can also be seen as neurotransmitter because it is expressed in the lateral hypothalamus and zona incerta and is taken up by neurones. MCH positive neurones are usually inactive and only start operating if they are activated by synaptic contacts or an enhanced concentration of glucose.

Activated, they distribute MCH into the synaptic gap. (5) Rats overexpressing MCH tend to be overweight and eat more than their conspecifics with normal expression of MCH. Also, ingestion could be induced by injecting MCH intracerebraly into rats. (6)

1.1.3 MCH-Receptor

MCH-R1 is G-protein coupled, has seven transmembrane domains and shows different expression forms. The similarity between the receptor of humans, mice and rats is over 90%. (7) MCH-R1 is mainly located in the brain and the highest concentration is found in the piriform cortex as well as the olfactory tubercle. Due to this location it is assumed that MCH also plays a role in smelling and reinforcement mechanisms which play an important role for ingestion. (2)

Essential for MCH-R1 binding MCH is an aspartic acid retain (Asp^{123}) on the third domain. In return, an arginine (Arg^{11}) is necessary for MCH binding the receptor. It is assumed that these two amino acids form an ion pair which is needed for activating MCH-R1. (8) The affinity of MCH to MCH-R1 lies in the nano molecular area. (9) To link MCH-R1 on the extracellular N-terminus N-glycosydically, like most g-protein coupled receptors, an asparagine residue is needed. (10) For functioning of the receptor, the C-terminus is also essential. It seems that in mice brains, periplakin, an actin and intermediate filament-binding protein, is distributed which can inhibit signal transduction by interacting with the C-terminus. (11) The receptor binds to G_i, G_0 and G_q and launches a signalling cascade.

A high density of MCH-R1 is found in the frontal cortex and in the basolateral amygdala and hippocampus. (12) Therefore, it is shown that MCH-R1 derives an important anatomic foundation for cognitive processing and mice lacking the receptor have poor memory and learning achievement. (13) However, they present an antidepressant and anxiolytic phenotype. (14)

MCH-R2 was discovered in the brain of humans and rhesus monkeys. In contrast to MCH-R1, this receptor does not subsists in rodents. (15)
The physiological part of MCH-R2 is not yet clarified, but it is assumed that MCH-R2 plays a role in cognitive and emotional aspects of ingestion. (16)

1.1.4 MCH-R1 antagonists

MCH-R1 antagonists have anxiolytic and antidepressant effects. (17) Besides, it could be demonstrated that MCH-R1 antagonists cause a reduced ingestion and loss of weight. However, in animal experiments it has been determined that some MCH-R1 antagonists might lead to paradoxical effects, with regard to the anxiolytic and antidepressant effects. This impact was spotted, for instance, with SNAP-7941. (18) These various results reflect the diversity of factors which have to be minded in experiments. Not only the study criteria but also the receptor difference in diverse populations have to be noted. (19)

An important question for the use of MCH-R1 antagonists is, if they have the same negative effects on memory capacity and cognition as standard antidepressants and anxiolytic drugs. (20)

Over the last years, different MCH-R1 antagonist were presented, however, many of these substances could not be utilised due to a significant affinity to hERG (human Ether-a-go-go Related Gene) (21) An interference with hERG might cause long QT-syndrome, which might lead to torsades de pointes and furthermore to a sudden cardiac death. (22) Nevertheless, some of them are tested in clinical studies for obesity therapy. (23)

PET-tracer/ SNAP-7941

To accomplish transmission of preclinical to clinical data, the design and analysis of a positron emission tomography (PET) tracer is necessary. In vivo PET studies

indicate a strong involvement of MCH-R1 in energy balance and diabetes. Furthermore, they might lead to a deeper understanding of endocrine hormones and pharmacological interactions. Moreover, a MCH-R1 PET tracer is important for the selection of the right dose range of MCH-R1 antagonists. (24)

PET is a technology, which is able to do a non invasive in vivo quantification and visualisation of receptor systems, enzymes and carriers. Most PET tracers are antagonists to prevent the signalling cascade, which agonists would trigger. (25) SNAP-7941 is a competitive MCH-R1 antagonist with high affinity to the receptor. (17) On base of this molecule, [^{11}C]SNAP-7941, the first PET tracer for MCH-R1 was developed whose precursor is called SNAP-acid. (26)

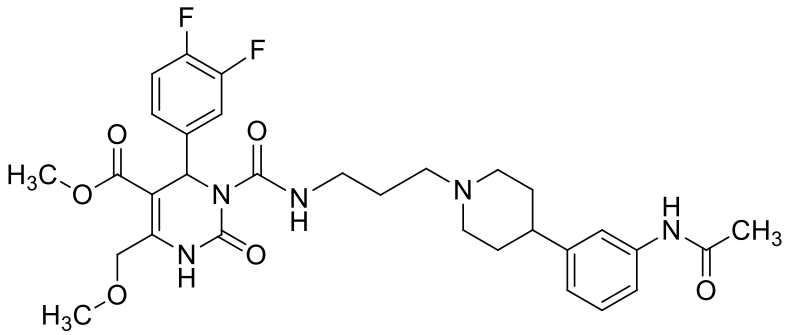

Figure 1: SNAP-7941 (methyl-3-{[(3-{4-[3- (acetylamino)phenyl]-1-piperidinyl}propyl)amino]carbonyl}-4- (3,4-difluorophenyl)-6-(methoxymethyl)-2-oxo-1,2,3,4-tetra- hydro-5-pyrimidinecarboxylate hydrochloride)

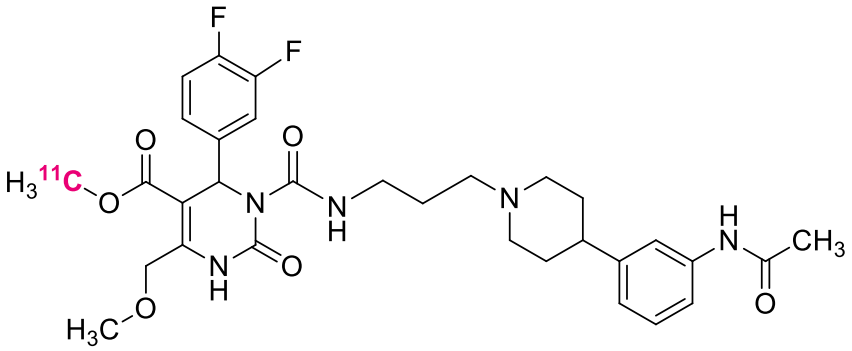

Figure 2: [^{11}C]SNAP-7941

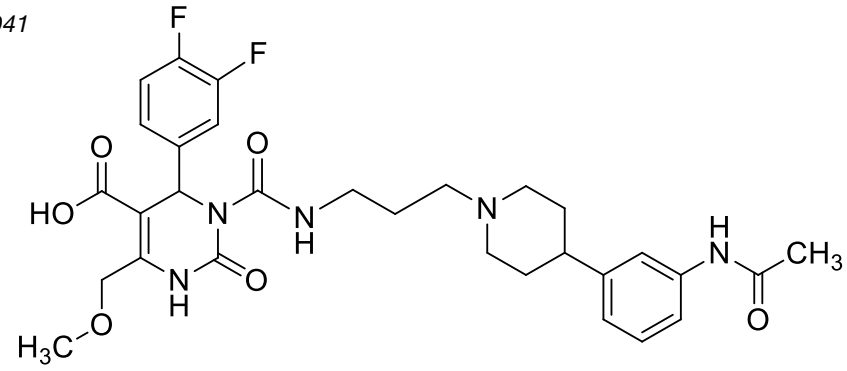

Figure 3: SNAP-acid

1.2 Enzymes

Enzymes are organic catalysts which assist biological reactions in a living organism. They have some qualities that differ from common chemical catalysts, for instance their high specificity despite mild reaction conditions. Furthermore, they are extremely efficient and versatilely regulable. (27)

Enzyme's active site

The active site is an enzyme's part which is responsible for the interaction with the substrate. Often the active site is located in a protein pocket and builds a highly organized micro-environment having its own environmental conditions for instance hydrophobe instead of hydrophile as in the cellular environment.
If a substrate reaches the active site due to diffusion or electrostatic attraction, a conformational change is induced so that the transient state of the substrate is stabilised. This mechanism is called „induced fit". Only molecules which are high complementary to the enzyme's active site, can be stabilised by it. It functions like a key and lock mechanism. (28)

1.2.1 Kinetics of an enzymatic reaction

Orders of a reaction

Reactions of first order

A reaction is called reaction of first order if the reaction velocity is depending only on the concentration of the one reacting educt.

$$A \longrightarrow B + C$$

Equation 1: Reaction of a decay process

A symbolises the educt and B and C are the created products. The reaction speed is directly constringent on the concentration of the disintegrating molecule. Examples are for instance radioactive decay or enzyme catalysed effects.

$$v\,(t) = -\frac{\Delta c_A\,(t)}{\Delta t} = k * [A]$$

Equation 2: Rate law of a reaction of first order (28)

v (t) is the speed of the enzymatic reaction

$\Delta\, c_A\,(t)$ is the variation of the concentration of the educt in time

Δ t is the variation of time

k is the rate constant

Through integration it results:

$$\ln c\,[A] = -k * \mathrm{t} + \ln c_0\,[A]$$

Equation 3: Integrated rate law of reaction of first order

$\ln c_0\,[A]$ is the integration rate which is the initial concentration of A at t = 0. If $\ln c\,[A]$ is plotted against time a line is created having the gradient –k. From the gradient k the reaction constant can be determined.

$$ln\frac{c\,[A]}{c_0\,[A]} = -k * t$$

Equation 4: Transformation of Equation 3

$$c\,[A] = c_0\,[A] * e^{-kt}$$

Equation 5: Transformation of Equation 4

Equation 5 leads to time dependency of the concentration of A. (29)

Reaction of second order

In a reaction of second order the reaction velocity is depending on two different substrate concentrations. (29)

$$A + B \longrightarrow P$$

Equation 6: Scheme of reaction of second order

Speed of the enzymatic reaction of second order: (30)

$$v = -\frac{\Delta\,[A]}{\Delta t} = -\frac{\Delta[B]}{\Delta t} = k * c[A] * c[B]$$

Equation 7: Rate law of a reaction of second order

The speed of a bimolecular reaction depends linear on the concentration of both, A and B. (28)

It may be that the concentrations are equal $[A] = [B]$. If this case occurs, the equation can be reduced to:

$$2\,A \longrightarrow P$$

Equation 8: Schema of a reaction of second order

If there is only one educt concentration the speed of the enzymatic reaction can be described in the following equation.

$$v\,[A] = \frac{\Delta\,c\,[A]}{\Delta\,t} = k * \,c^2\,[A]$$

Equation 9: Rate law of a reaction of second order (only one educt concentration)

Through integration it results:

$$\frac{1}{c\,[A]} = k * t + \frac{1}{c_0\,[A]}$$

Equation 10: Integration of Equation 9

$c_0\,[A]$ is the initial concentration of A at t = 0. Afterwards, the concentration's reciprocal value $\frac{1}{c\,[A]}$ is proportional to time. (29)

Michaelis-Menten Kinetics

The Michaelis-Menten-equation is a mathematical model, which describes the enzyme kinetic approximately. The Michaelis-Menten equation is the basic of enzyme kinetic.

First the free enzyme binds the substrate reversely and builds an enzyme-substrate-complex. Thereupon the substrate is converted and the complex decays into free enzyme and reaction product. (27)

$$E + S \underset{k_3}{\overset{k_1}{\rightleftarrows}} ES \overset{k_2}{\rightarrow} EP \overset{k_4}{\rightarrow} E + P$$

Equation 11: Enzyme/Substrate relation

E: Enzyme

S: Substrate

ES: Enzyme/substrate complex

EP: Enzyme/product complex

P: Product

k_1: Rate constant of the association of E and S.

k_2: Rate constant of the dissociation of ES to EP.

k_3: Rate constant of the reverse reaction.

$k_{4:}$ Rate constant of the dissociation of EP to E + P.

The complex ES, consisting of the enzyme and its substrate, is built due to different interactions. (Van-der-Waals-forces, hydrophobic interactions or hydrogen bonds)

It can dissociate with k_2 to enzyme and product and do the revers reaction ($ES \rightarrow E + S$) with k_3. The formation of the complex is a bimolecular reaction of second order whereas the process of disintegration is a reaction of first order. (28)

Since the initial speed is measured, the reverse reaction $E + P \rightarrow ES$ can be neglected because of the low product concentration at the beginning of the reaction.

The assumption exists that in an enzymatic reaction, after a short pre-steady-state-period there is a steady-state.

$$k_1 [E][S] = k_2 [ES] + k_3 [ES]$$

Equation 12: Steady-state

The Michaelis-Menten constant (K_m) condenses k_1, k_2 and k_3 in one equation.

$$K_m = \frac{k_2 + k_3}{k_1}$$

Equation 13: Michaelis- Menten constant

Michaelis-Menten equation:

$$v_0 = \frac{v_{max} [S]}{(K_m + [S])}$$

Equation 14: Michaelis-Menten equation

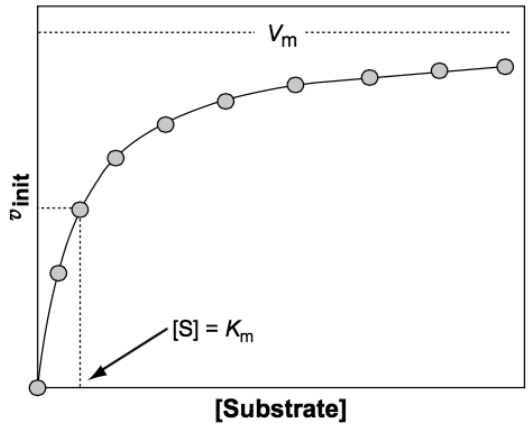

Figure 4: Michaelis-Menten graph (31)

In Figure 4 the initial velocity is plotted against the substrate concentration. The velocity approaches asymptotically against the maximal velocity, which is contingent on the substrate.

Michaelis-Menten constant complies with the substrate concentration which is needed for the enzyme working with half maximal speed. Another often used parameter to characterize an enzymatic reaction is k_2 or k_{cat}, if there are other rate constants that should be observed. k_{cat} is also called turnover number, this constant shows how many substrate molecules can be transformed by one enzyme molecule. To estimate the efficiency of an enzyme, k_m and k_{cat} have to be regarded. (27)

1.3 HPLC - High performance liquid chromatography

High performance liquid chromatography (HPLC) is a separation method for chemical compounds that can be used for analytical and preparative purposes. The compounds are dissolved in a liquid solvent (mobile phase) and pumped through a column filled with a stationary absorbent phase (stationary phase). Separation of the compounds is achieved via different interactions of the compounds with the stationary phase, resulting in different retention times of the compounds. There are different separation principles, for example the principle of absorption. Here, separation is done due to different dispersion of substances between mobile and solid phase. (32)

Components of an HPLC

- Mobile-phase reservoir
- Degasser
- Mixer and pump system
- Column
- Detector

1.3.1 Mobile-phase

High purity of eluents is a condition for proficient analysis. The mobile phase which is chosen does not lead to any interference signals in the detector, dissolved gases should be avoided as well because they might appear as a peak in the chromatogram. The eluent can be degassed, for instance, via ultrasonic bath. The eluting power of an eluent differs in hydrophilicity or lipophilicity. Solvents are sorted in the eluotropic range by increasing hydrophilicity in the case of normal-phase chromatography. If a reversed-phase chromatography is used, the eluting power is inversely proportional to polarity. If several eluents equal in selectivity and polarity are appropriate, the one lowest in viscosity is used, to keep the pump pressure as little as possible.

Mobile-phase consists of one to four different fluids. All used components are completely mixable to ensure a reproducible separation of compounds. (32)

Isocratic elution

In an isocratic setup, composition of the mobile phase stays the same during the separation. A pre-mix, where eluents are mixed in the needed ratio before starting the separation, can be used. The advantage of this method is that a clear baseline can be received and the drifting of the line can be kept to a minimum. Disadvantageous is the lack of flexibility in changing the mobile phase during separation. This could be necessary due to separation problems or trying to shorten the analysis's time. If another ratio is needed, a new pre-mix has to be prepared. (33)

Gradient elution

To obtain an optimal separation, an isocratic mixture can be insufficient and a modification of the mixing ratio during the analysis can be necessary. For this purpose, a gradient mixer is used.
The simplest type is the step-gradient, the volume ratio of eluent's components is changed in defined time-intervals. There are two kinds of continuous gradients, low-pressure-gradient and high-pressure-gradient. For low-pressure-gradient only one HPLC-pump is needed. Different solvents are pumped through the system into a mixing chamber and the volume of the solvents is regulated due to switching valves. If a high-pressure-gradient is used, every component needs its own HPLC-pump. (32)

1.3.2 Column/Solid-phase

The column is usually made of steel or, in analytical setups, inert plastic. It is up to 30cm long and has a diameter of up to 10mm. At the end, the column has frits and if a sample having a high amount of impurities (e.g. protein rich samples) is analysed, a pre-column should be used to avoid a contamination of the main column. The solid-phase is packed into the column, segregation-materials differ in their grain-size, grain-size-distribution, pore-size, surface-quality, hydrophilicity and lipophilicity. The particle-diameter is 1.5 to 15µm. The smaller the particle-diameter the better is the separation-efficiency. To ensure a reproducible separation and a good separating-effect the particle-diameter is evenly distributed. Materials having a large specific surface are appropriate, also a highly porous surface is a condition for a good separation. Also they have to withstand high pressures in the HPLC-system, maintaining their shape and position. Often used polar materials are silica-gel and aluminium-oxide. For reversed-phase chromatography, usually chemical modified material is used, for instance silica-gel with attached apolar alkyl-groups on the former polar ending hydroxy-groups. For derivatisation frequently used materials are octylsilane and octadecylsilane, however, octadecylsilane is more common in use.

A difficulty with this derivatisation is, the alkyl-groups needing a lot more space than the silano-groups and the biggest part of the silano-groups is not on the external surface, but inside the pores of the silica-gel, so only around 50 percent of the silano-groups can be transformed. Most of the time, in reversed-phase chromatography, an apolar surface is needed, so a second derivatisation-step is done. This step is called endcapping. A smaller molecule like trimethylechlor-silane is attached to the free silano-groups. Due to the minor size, also silano-groups inside the pores can be reached, so the silica-gel becomes more apolar. (32), (33)

1.3.3 Detector

A wide variety of detectors can be used with the HPLC, such as UV/VIS-detector, fluorescence-detector, radio-detector, refractive-index-detector or mass-spectrometry. (32)
For this work, only the UV/VIS-detector was used, therefore it is described in the next paragraph.

UV/VIS-Detector

Light absorption of different molecules within the eluate is measured in the ultraviolet or in the visible part of the light spectrum. The detector includes a lamp, a mirror system and a photocell. For visible light spectrum a range of approximately 400 to 700nm, a tungsten lamp, or, for the ultraviolet spectrum 190 to approximately 400nm, a deuterium lamp is used.
The solvent should have no inherent absorption at the used wave-length or contain absorbing impurities, otherwise additional signals might occur in the chromatogramm. The eluate flows through a cuvette which is penetrated by a ray of light. The beam is formed through light which is bundled by a prism and falls through a gap on the cuvette. The concentration of the sample can be calculated via Lambert–Beer's law. (See paragraph 1.4.1)

UV/VIS-detectors can either be a single- or a multi-wave-length detector. However, there is also a difference between fixed- and variable-wave-length-detectors. (33)

1.3.4 Evaluation of a chromatogram

The evaluation of a chromatogram can be complex. There are different elements which have to be taken into account.

First of all, a peak should look like a gaussian curve. If fronting - the peak is long drawn on its beginning - or tailing - the peak is long drawn at the end - occurs, it is sometimes difficult to find the end or the beginning of the peak, which implicates that the AUC (area under the curve) might not be calculated accurately. Fronting and tailing appear often in cases of elevated concentration of the substance. Furthermore, peaks should be separated on the baseline, to have no complications to distinguish them. (34)

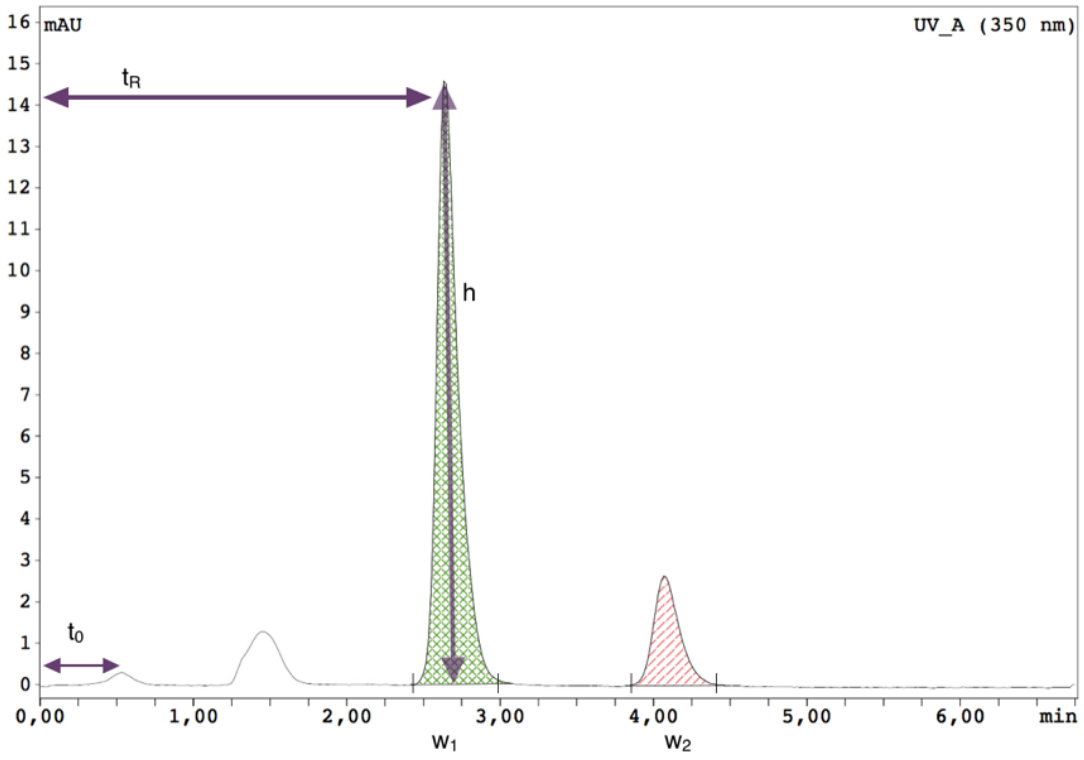

Figure 5: Evaluation of a chromatogram

h: Height of a peak, however, essential for comparing concentrations is not the peak's height, but the area under the curve.

t_0: Dead time, the time which is needed for a compound to be detected by having no interaction to the mobile and the stationary phase.

t_R: Retention time, the time which passes between injecting the substance and detecting it.

Retention time is affected by flow, length of the column and the solvent. The slower the flow and the longer the column are set, the longer the retention time will become. For this reason, the retention factor (k) should be used for a characteristic analysation. The optimum is if k is located between 1 and 10. A separation of the compound can be achieved due to a variation of the factor k of the different components.

$$k = \frac{t_R - t_0}{t_0}$$

Equation 15: Retention factor

The separating factor α is the proportion of two k factors. α is a measure for the quality of a separation. To achieve a separation of two components $\alpha > 1$. For the calculation of α the baseline wide is not considered because α takes only the retention times (means top of the peaks) into account, so even tough peaks have different separation factors, they might overleap. That is the reason for calculating the resolution R, here the baseline width w is considered.

$$R = \frac{t_{R2} - t_{R1}}{w_1 + w_2}$$

Equation 16: Resolution

To gain a separation at the baseline at least R = 1.5 is necessary. (35)

1.4 UV/VIS-Spectroscopy

In UV/VIS-Spectroscopy, the energy of visible and ultraviolet light is partly absorbed by most molecules. Through a graphical presentation of absorption against wavelength an absorption spectrum is created. (32)

Components of an UV/VIS-Photometer

- Deuterium lamp for the UV region (190-400nm)
- Tungsten lamp for the visible region (400-900nm)
- Monochromator to split the light into the different wavelengths

The beam goes from the light source through a slit, gets dispersed at the monochromator (prisma + slit component), falls through another slit through the sample on the detector. (33), (36)

Chromophore

Chromophore is the part of a molecule, which is responsible for absorption of visible and ultraviolet light, due to its containing of energisable π- and n-electrons. Electrons can switch between binding σ- and π- orbitals to the respective anti-binding σ*- and π*- orbitals. They can also shift between a not-binding n-orbital and anti-binding σ*- and π*- orbitals. The transition is between the highest occupied molecular orbital (HOMO) and the lowest unoccupied molecular orbital (LUMO). (32)

Examples for chromophores

- Alkenes/Alkines
- Polyenes
- Aromates
- Carbonylic groups
- Compounds containing a double bound nitrogen

1.4.1 Lambert–Beer's law

The Lambert-Beer's law describes the attenuation of light's intensity after passing a medium containing an absorbing compound depending on the concentration of the absorbing compound and the thickness.

$$A = \log \frac{I_0}{I_t} = \varepsilon * b * c$$

Equation 17: Lambert-Beer's law

A: Absorbance
I_0: Intensity before the light meets the sample
I_t: Intensity of the light after going through the sample
ε: molar extinction coefficient
b: thickness of the cuvette in cm (usually 1cm)
c: concentration of the analyte in M/L

The law applies to mediums that are
- homogenous distributed
- clear (have a negligible multiple scattering)
- low concentrated (if to high, interactions lead to diversion)

Furthermore, the variation of the absorption coefficient within the measured range has to be negligible and the own emission has to be negligible too. (36)

2. Experimental Part

2.1 SNAP-7941 and SNAP-acid regression line

2.1.1 Experimental setup

A stock solution of 59.75mM (10.0mg/ml) SNAP-7941 and 58.35mM (10.0mg/ml) SNAP-acid in ethanol was prepared. The stock solution was diluted in 20mM phosphate buffer pH 7.4 in different concentrations, as shown in the table below. For better overview some data is indicated in mg/ml.

	µg/mL						
SNAP-7941	10.0	7.5	5.0	2.5	1.0	0.5	0.1
SNAP-acid	10.0	7.5	5.0	2.5	1.0	0.5	0.1

Table 3: Concentrations of SNAP-7941/SNAP-acid regression lines

For obtaining respective concentrations, all compounds were pipetted into reaction-vials as duplicate, vortexed and injected into the HPLC as triplicate. (33) A regression line for SNAP-7941 and its expected main-metabolite SNAP-acid was done.

	HPLC-Conditions
Column	Chromolith® performance RP-18e 100x4.6mm, 5µm
Mobile phase	buffer/ acetonitrile 70:30 buffer: 32.43mM ammonium acetate in water/ acetic acid 97.5/ 2.5 (v/v), pH 3.57
Flow	1.0ml/min
Loop volume	20µl
UV-Detector	254nm

Table 4: HPLC-Conditions for SNAP-7941/SNAP-acid regression lines

2.1.2 Results

SNAP-7941 and SNAP-acid chromatogram

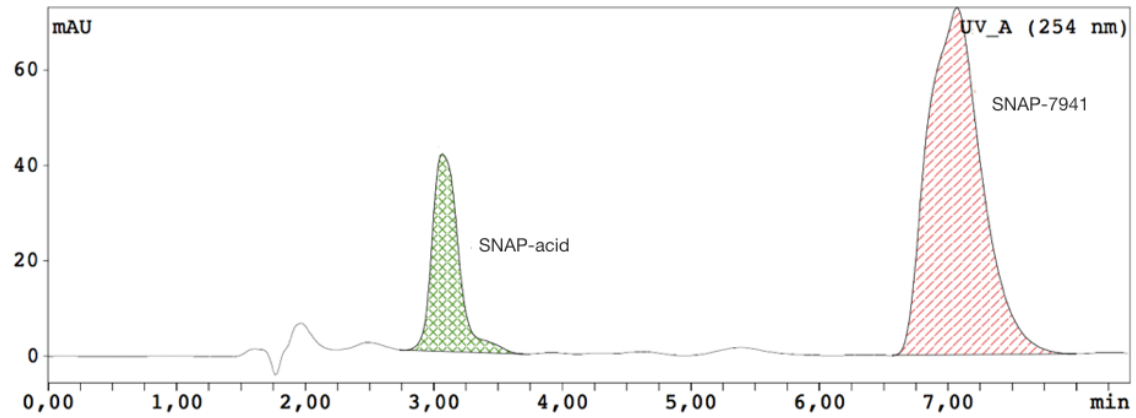

Figure 6: SNAP-7941 and SNAP-acid chromatogram (10µg/ml)

In this chromatogram (Figure 6), two peaks can be seen, they belong to SNAP-7941 and SNAP-acid. SNAP-7941 (10µg/ml) was spiked with SNAP-acid (10µg/ml), to gain a better comparability between the initial substance SNAP-7941 and the expected metabolite SNAP-acid.

SNAP-7941 has a retention time at around 7.0 minutes, in this diagram the peak is coloured red. SNAP-acid has a retention time at around 3.0 minutes, in this diagram the peak is coloured green.

The different height and AUC of the two peaks could be explained due to different light absorbance at a wave length of 254nm.

SNAP-7941 regression line

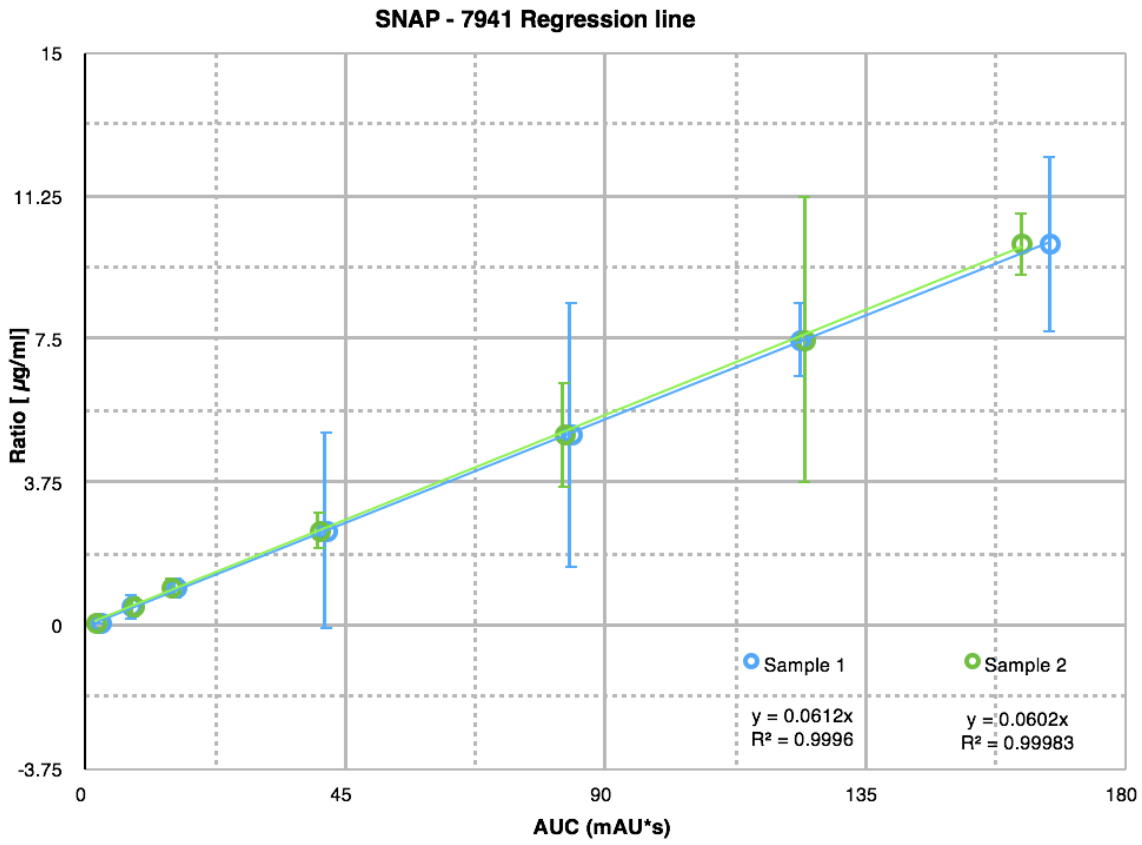

Diagram 1: SNAP-7941 regression line

In the diagram (Diagram 1), the AUC mean value, of each of the two measured samples, is drawn against the used concentration. The error bars reflect the standard error.

The first equation and R^2-value belong to Sample 1, the second equation and R^2-value to Sample 2.

The standard deviation and the individual measurements, for each sample, are indicated in the annex. (Table 17 and Table 18)

SNAP-acid regression line

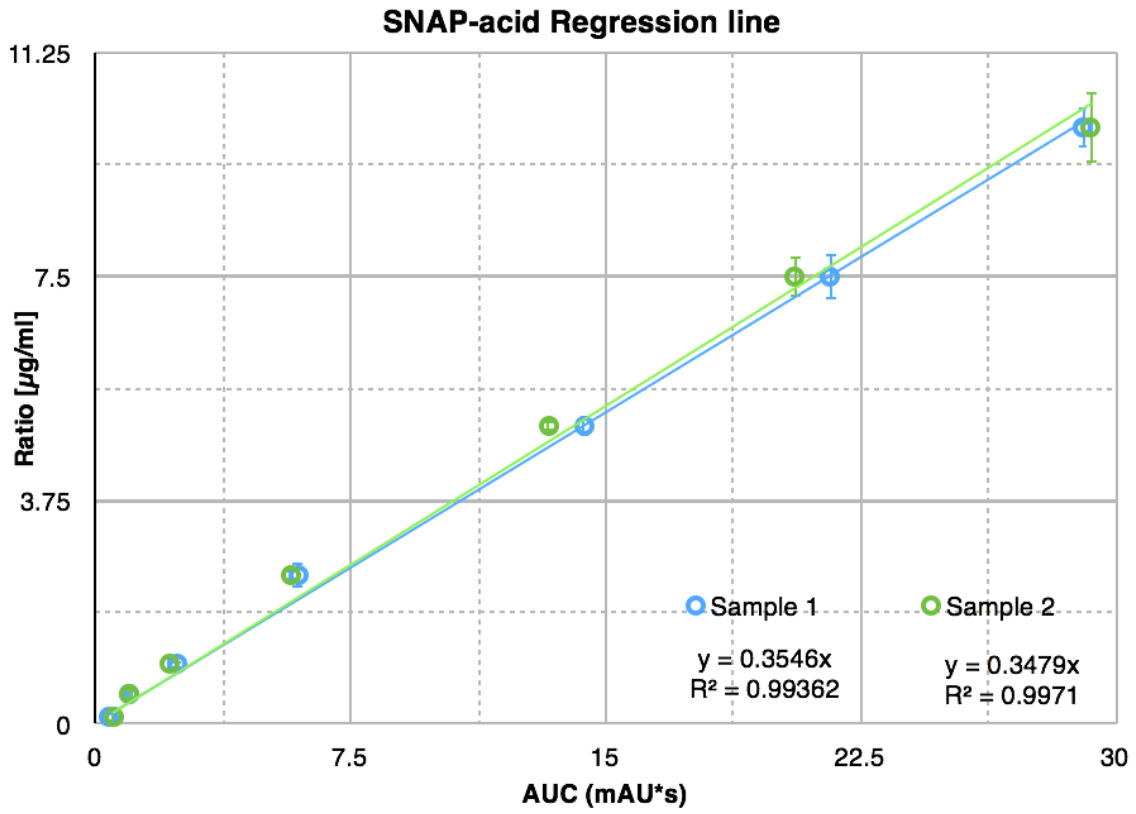

Diagram 2: SNAP-acid regression line

In the diagram (Diagram 2) the AUC mean value, of each of the two measured samples, is drawn against the used concentration. The error bars reflect the standard error.

The first equation and R^2-value belong to Sample 1, the second equation and R^2-value to Sample 2. The standard deviation and the individual measurements, for each sample, are indicated in the annex. (Table 19 and Table 20)

2.2 Proteases

2.2.1 Experimental setup

Pretests to verify the functionality of selected Proteases

The used activity of selected proteases was tested as described in (37)

Used reagents

- 50mM phosphate buffer, pH 7.5
- 0.65% casein solution
- 2M sodium carbonate solution
- Enzyme diluent solution
  - 5.19mM calcium acetate
  - 6.58mM sodium acetate
- Folin and Ciocalteu's Phenol Reagens 1:100
- 110mM trichloracetic acid
- 1.1mM L-tyrosine solution

Standard regression

A regressionline was done with L-tyrosine. 1.1mM (0.2mg/ml) L-tyrosine was dissolved in distilled water, while heated gently, up to 40°C. Out of this stock solution 6 different dilutions were prepared. Into each vial distilled water was added to gain a volume of 2ml, as shown in the table below.

	Dilution (mL)					
L-tyrosine	0.01	0.05	0.10	0.20	0.40	0.50
Aqua dest.	1.99	1.95	1.90	1.80	1.60	1.50
Fiinal volume	2.00	2.00	2.00	2.00	2.00	2.00

Table 5: Concentration of L-tyrosine regression line

5ml Na_2CO_3-solution and 1ml Folin's Ciocalteu's phenol reagent were added to dilution vials. In case of a pH drop, the addition of Na_2CO_3-solution was required. The vials were incubated for 30 minutes at 37°C in a heating device.

In difference to Sigma's non-specific protease activity assay (37) the samples were not filtrated but centrifuged for 10 minutes at *1649g* (4000rpm). The more L-tyrosine the sample contained, the darker blue the colour became. Samples were measured via UV/VIS spectrophotometer at 660nm.

Testing the protease

For each tested protease, four vials were needed. The first vial was used as a blank, in the others different amounts of protease were prepared.
Before the experiment was started, the enzyme was weight out and dissolved in the enzyme diluent solution to gain a concentration of 1mg/ml. Dilutions were prepared, as shown in the table below.

	Stock solution	Dilution 1	Dilution 2
Enzyme (mg/ml)	1.0000	0.0100	0.0001

Table 6: Concentration protease activity

5ml 0.65% casein solution, tempered at 37°C were inserted into all reaction-vials. Afterwards, 1ml of the three different concentrations of the prepared enzyme mixtures were pipetted into three vials containing the casein solutions. In the blank vial, to obtain the same volume as in the other vials, 1ml enzyme diluent solution was added. At points in time of 0, 10 and 20 minutes 1.5ml of each vial were pipetted into vials containing 5ml of 110M TCA in order to stop the enzymatic reaction. Samples were centrifuged for 10 minutes at 1649*g* (4000rpm). Subsequently, 2ml supernatant of each vial were pipetted into vials containing 5ml Na_2CO_3-solution. The Na_2CO_3-solution was used to stabilise the pH in an alkaline range. 1ml of the diluted Folin and Ciocalteas`s Phenol Reagens was added to each sample. After an incubation of 30 minutes at 37°C the samples were measured in the UV/VIS spectrophotometer at 660nm.

Enzymatic conversion of SNAP-7941 via Proteases

To receive a solution of 1mg/ml, the enzymes were diluted in 20mM phosphate buffer, pH 7.4. SNAP-7941 was dissolved in ethanol, obtaining a concentration of 10mg/ml. Three different enzyme/SNAP rates were prepared, as shown in the table below.

Ratio	1/100	1/10	1/1
Enzyme solution (µl)	990	100	10
SNAP solution (µl)	10	10	10
Buffer (µl)	0	890	980

Table 7: Concentrations of enzymatic conversion of SNAP-7941 via proteases

The different preparations were pipetted into reaction-vials, vortexed, tempered to 37°C and shook gently at 650rpm in an incubator device. The reaction was stopped after defined points in time (0, 1, 2, 3, 24 hours) by pipetting an aliquot of 100µl of the incubating mixture to the same amount of acetonitrile and mixing it thoroughly. Mixtures were centrifuged at 1649*g* (4000rpm) for 5 minutes to separate precipitated proteins. The supernatant was injected into HPLC. (38)

	HPLC-Conditions
Column	Chromolith® performance RP-18e 100x4.6mm, 5µm
Mobile phase	buffer/ acetonitrile 70:30 buffer: 32.43mM ammonium acetate in water/ acetic acid 97.5/ 2.5 (v/v), pH 3.57
Flow	1.0ml/min
Loop volume	20µl
UV-Detector	254nm

Table 8: HPLC-Conditions of Enzymatic conversion of SNAP-7941 via proteases

2.2.2 Results

Pretests to verify the functionality of selected Proteases

For a better comparison, of the increasing L-trypsin concentrations, which were achieved using different concentrations of each protease (0.0001, 0.01 and 1mg/ml) they are expressed in one diagram.

The error bars reflect the standard error. The standard deviation and the individual measurements, for each sample, are indicated in the annex.
(Table 21 to Table 29)

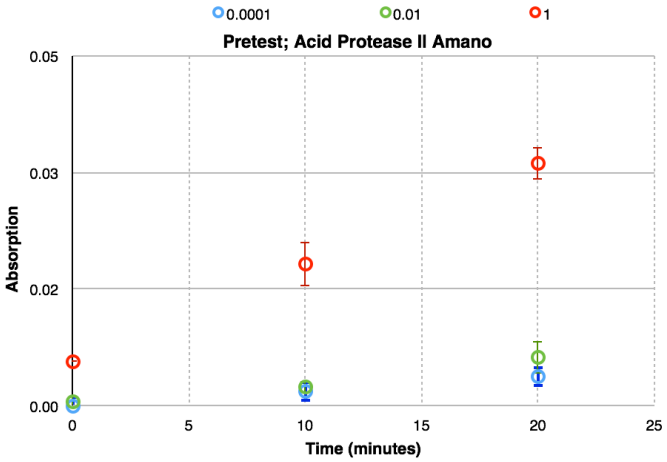

Diagram 3: Pretest; Acid Protease II Amano

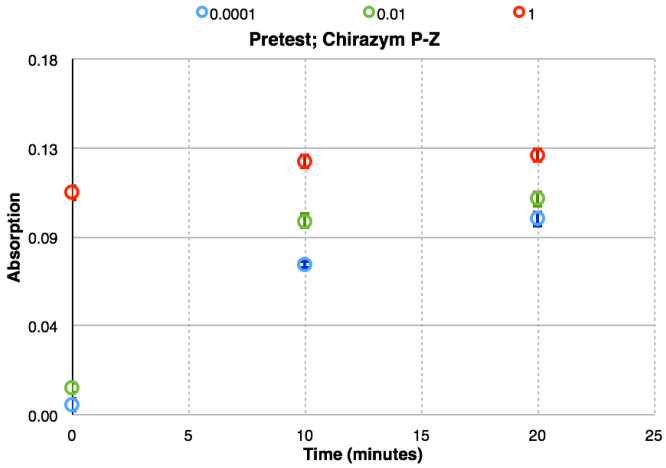

Diagram 4: Pretest; Chirazym P-Z

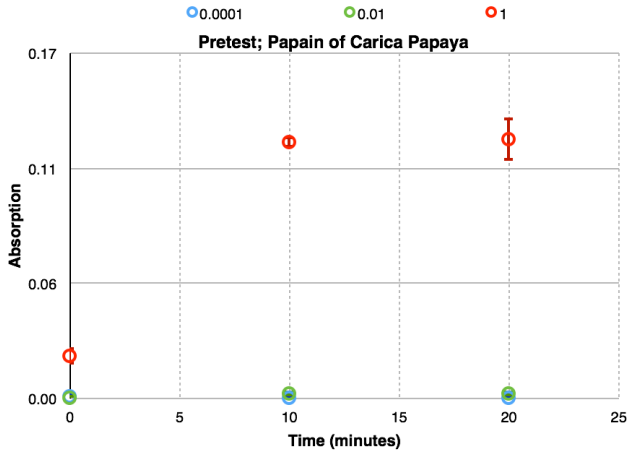

Diagram 5: Pretest; Papain of Carica Papaya

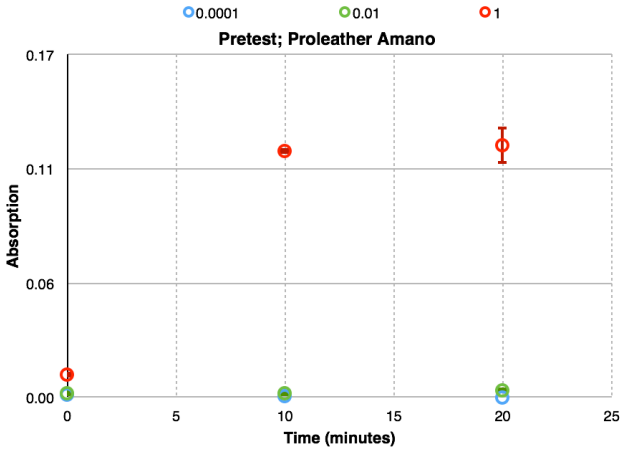

Diagram 6: Pretest; Proleather Amano

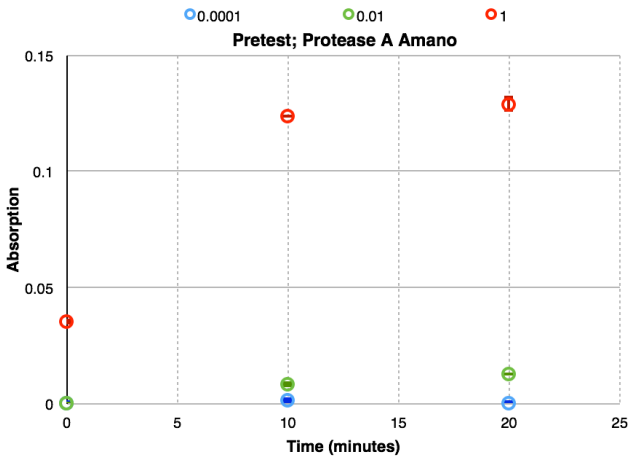

Diagram 7: Pretest; Protease A Amano

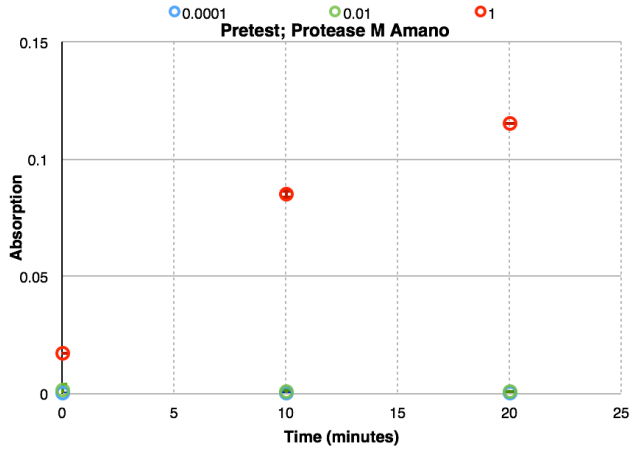

Diagram 8: Pretest; Protease M Amano

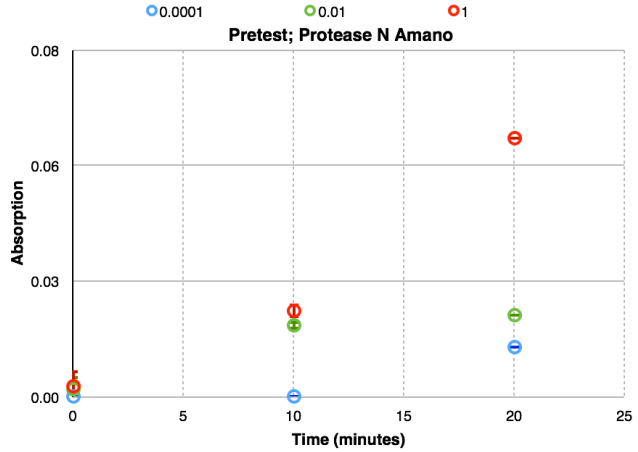

Diagram 9: Pretest; Protease N Amano

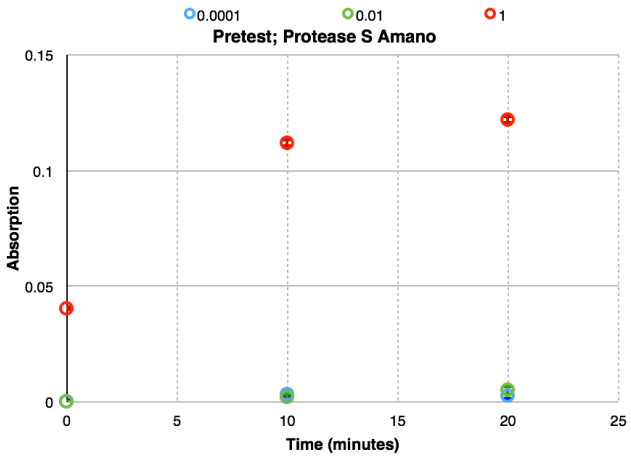

Diagram 10: Pretest; Protease S Amano

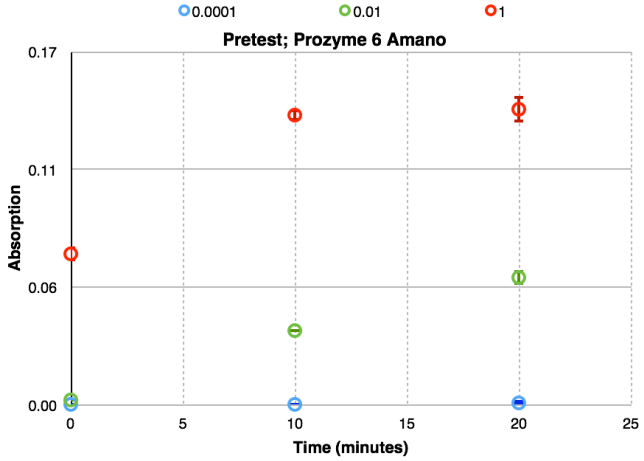

Diagram 11: Pretest; Prozyme 6 Amano

As it is shown in the diagrams (Diagram 3 to Diagram 11), for all used proteases, the cleavage of casein to L-Trypsin worked best with the highest tested amount of protease (1mg/ml). Furthermore, it can be seen a longer incubation time led to a higher L-trypsin concentration.

After the pretest, it was determined that all used Proteases are active. It was decided to use all of them for the enzymatic conversion experiments of SNAP-7941.

The data that was taken from each protease triplicate is shown in one diagram. The used numerical value is the mean value of each sample triple injection into the HPLC.

The error bars reflect the standard error. The standard deviation and the individual measurements, for each sample, are indicated in the annex.

(Table 30 to Table 38)

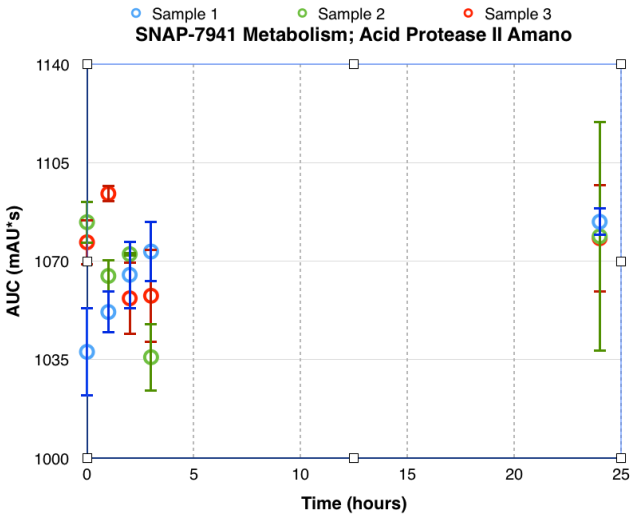

Diagram 12: SNAP-7941 Metabolism; Acid Protease II Amano

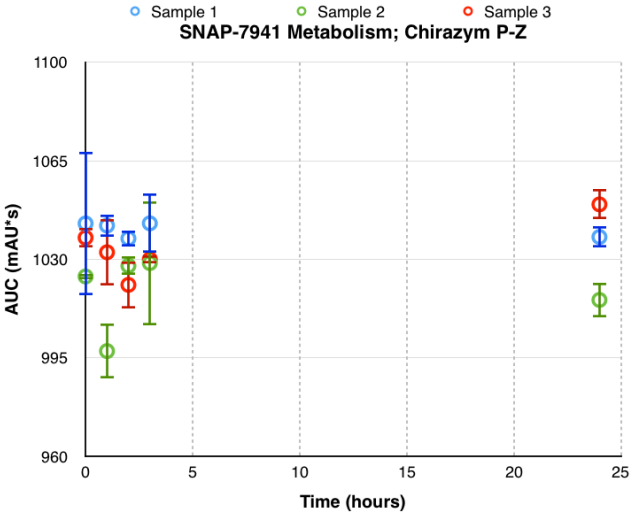

Diagram 13: SNAP-7941 Metabolism; Chirazym P-Z

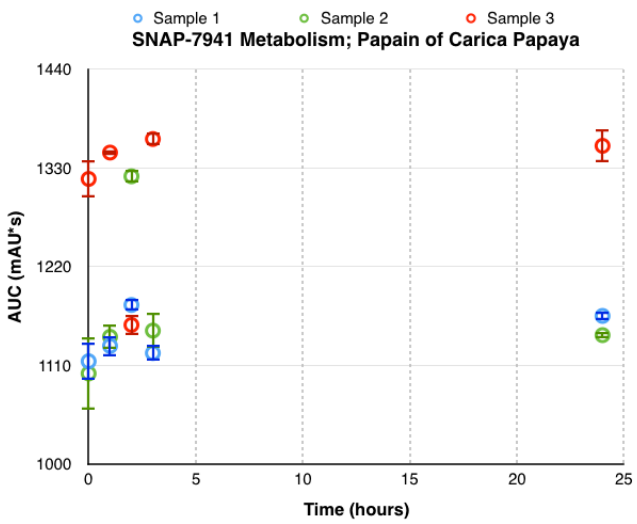

Diagram 14: SNAP-7941 Metabolism; Papain of Carica Papaya

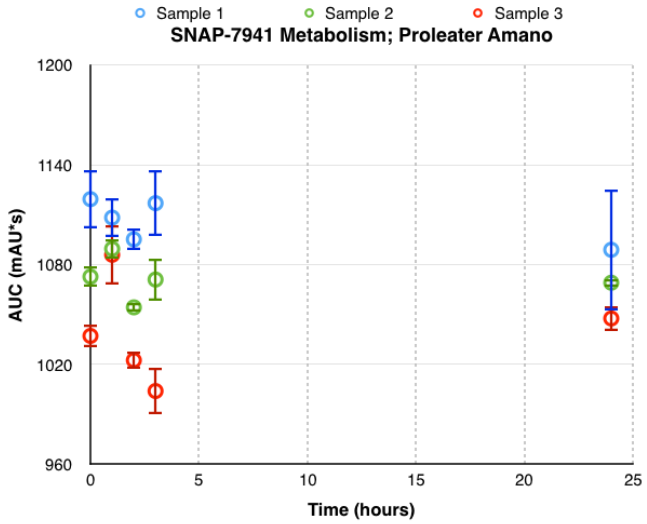

Diagram 15: SNAP-7941 Metabolism; Proleater Amano

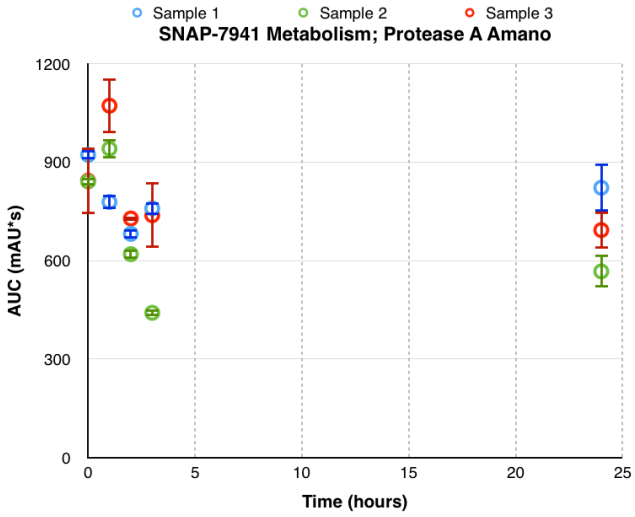

Diagram 16: SNAP-7941 Metabolism; Protease A Amano

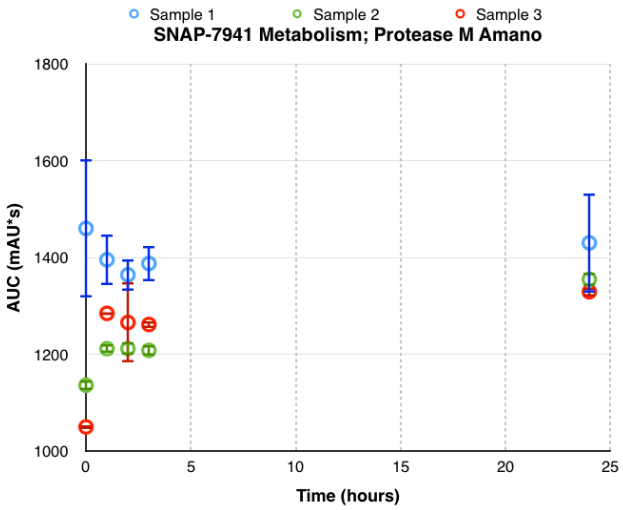

Diagram 17: SNAP-7941 Metabolism; Protease M Amano

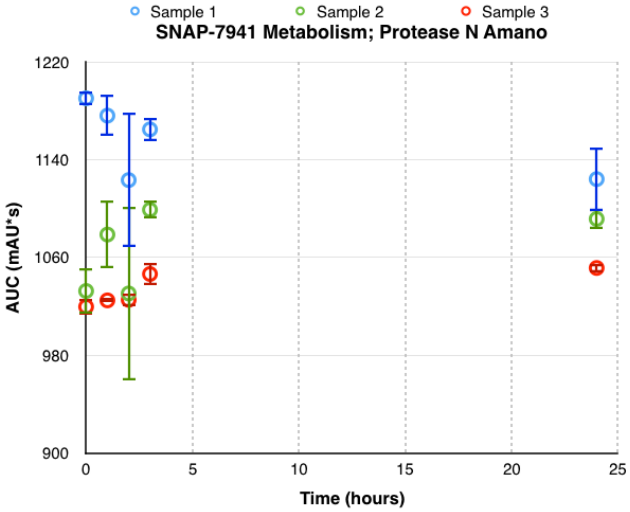

Diagram 18: SNAP-7941 Metabolism; Protease N Amano

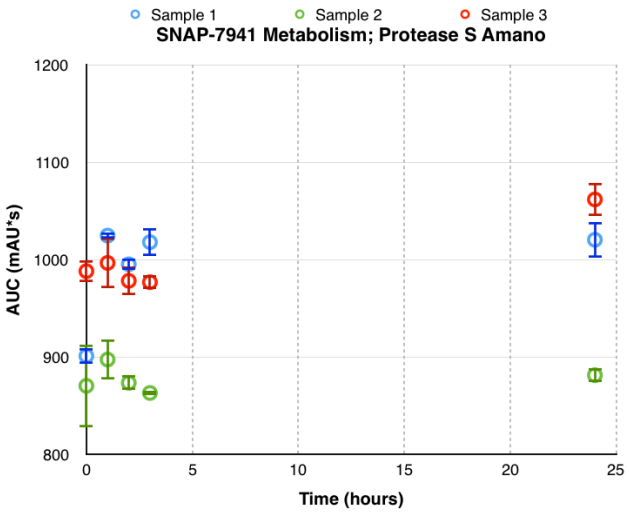

Diagram 19: SNAP-7941 Metabolism; Protease S Amano

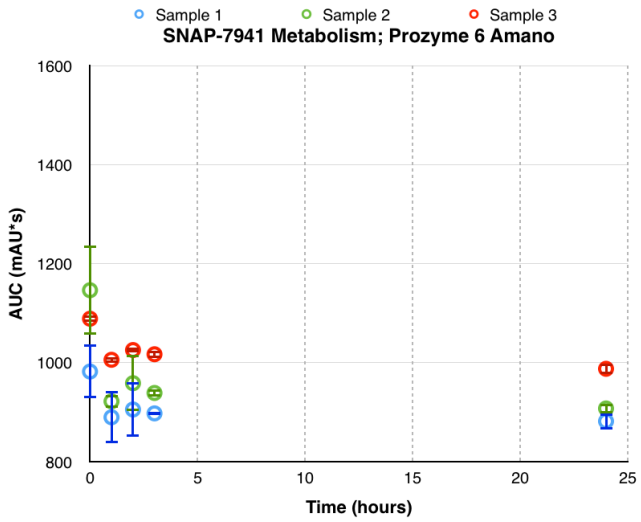

Diagram 20: SNAP-7941 Metabolism; Prozyme 6 Amano

The diagrams (Diagram 12 to Diagram 20) show the measured area under the curve of SNAP-7941 after incubating with the protease in different points in time. All of them show a straight line between the different points in sample taking, so it can be assumed that there was no decrease of SNAP-7941, also no peak of the SNAP-acid could be seen in the chromatogram.

Figure 7 is an example of the chromatograms, which were obtained.

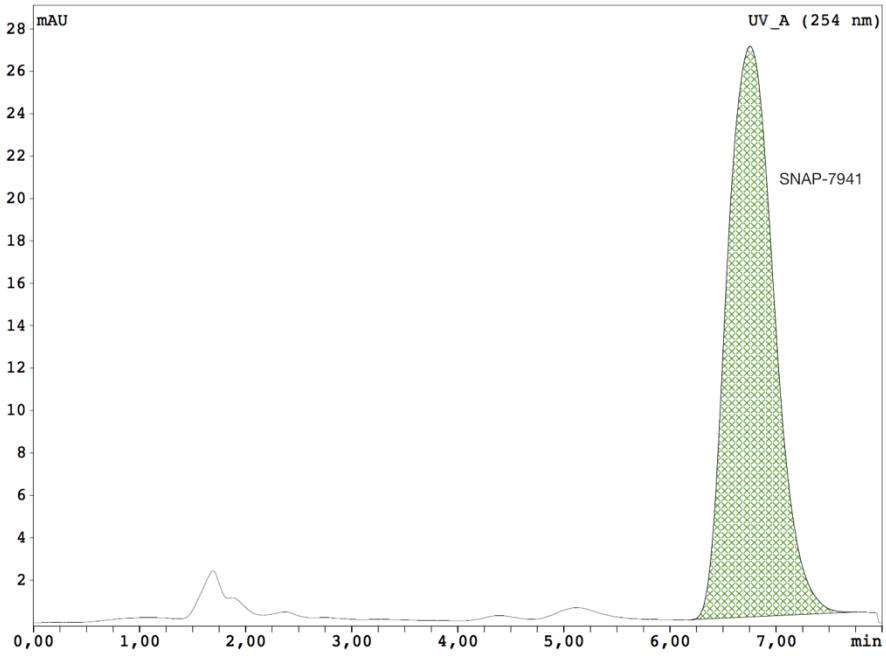

Figure 7: Chromatogram of Protease A Amano after one hour incubation time

The chromatogram (Figure 7) shows the SNAP-7941 peak at a retention time of 7 minutes. Due to the reference chromatogram (Figure 6) a retention time of 6.5 minutes for SNAP-7941 can be indicated.
However, in case of the SNAP-acid, the peak should appear in the chromatogram at a time of 3 minutes. Since there is no such peak in the received chromatogram, the production of SNAP-acid can be excluded.

2.3 Esterases

2.3.1 Experimental setup

Pretests to verify the functionality of selected Esterase

To test the butyrylcholinesterase's activity, p-nitrophenylacetate (p-NPA), was used as a substrate. It is cleaved, due to the use of esterase, to p-nitrophenole (p-NP). (39)

Testing the standard

First, p-NPA and p-NP were diluted in 20mM phosphate-buffer pH 7.4 and injected into the HPLC, to obtain each compound's retention time.

	HPLC-Conditions
Column	Merck LiCrospher® 100 RP-18 125x4mm, 5µm
Mobile phase	acetonitrile/ phosphate buffer pH 2.3 50:50
Flow	1.5ml/min
Loop volume	20µl
Detector	UV/VIS - spectrophotometer; 350nm

Table 9: HPLC-Conditions of Pretests to verify the functionality of selected Esterase

Testing the esterase

To test the esterase activity, a concentration of 5mg/ml butyrylcholinesterase in 20mM phosphate-buffer was created. (40)
The p-NPA was diluted in acetonitrile to a concentration of 50mM. Three samples were prepared, each of them contained 30µl enzyme-solution, 10µl p-NPA solution and 460µl phosphate-buffer in a reaction-vial, to receive a final volume of 500µl. The samples were vortexed, tempered at 37°C and shook gently at 650rpm.

At 0, 0.5, 1, 2.5 and 5 minutes, 30µl of each of the three vials were retrieved and the reaction was stopped with 150µl acetonitrile and centrifuged at 1649*g* (4000rpm). Two blank solutions were prepared without adding an enzyme. The supernatant was injected into HPLC.

For HPLC-conditions see Table 9.

Enzymatic conversion of SNAP-7941 via Esterase

To receive a solution of 1mg/ml, the enzymes were diluted in 20mM phosphate buffer pH 7.4. SNAP-7941 was dissolved in ethanol, obtaining a concentration of 10mg/ml. Three different enzyme/SNAP rates were prepared as shown in the table below.

Ratio	Enzyme solution (µl)	SNAP-7941 solution (µl)	Buffer (µl)
1/100	990	10	0
1/10	100	10	890
1/1	10	10	980

Table 10: Concentrations of SNAP-7941 and enzyme-solution in conversion of SNAP-7941 via Esterase

The different preparations were pipetted into reaction-vials, vortexed, tempered at 37°C and shook gently at 650rpm in an incubator device. The reaction was stopped after defined points in time (0, 1, 2, 3 and 24 hours), by pipetting an aliquot of 100µl of the incubating mixture to the same amount of acetonitrile and mixing it thoroughly. Mixtures were centrifuged at 1649*g* (4000rpm) for 5 minutes to separate precipitated proteins. The supernatant was injected into HPLC. (38)

	HPLC-Conditions
Column	Chromolith® performance RP-18e 100x4.6mm, 5*µ*m
Mobile phase	Buffer/ Acetonitrile 70:30 Buffer: 32.43mM ammonium acetate in water/ acetic acid 97.5/ 2.5 (v/v), pH 3.57
Flow	1.0ml/min
Loop volume	20*µ*l
Detector	UV at 254nm

Table 11: HPLC-Conditions of Enzymatic conversion of SNAP-7941 via Esterase

2.3.2 Results

Pretests to verify the functionality of selected Esterase

For a better comparison, three measurements are expressed in one diagram. The green spots mark the points of p-NP, the blue ones the points for p-NPA.

The error bars reflect the standard error. The standard deviation and the individual measurements, for each sample, are indicated in the annex.
(Table 39 and Table 40)

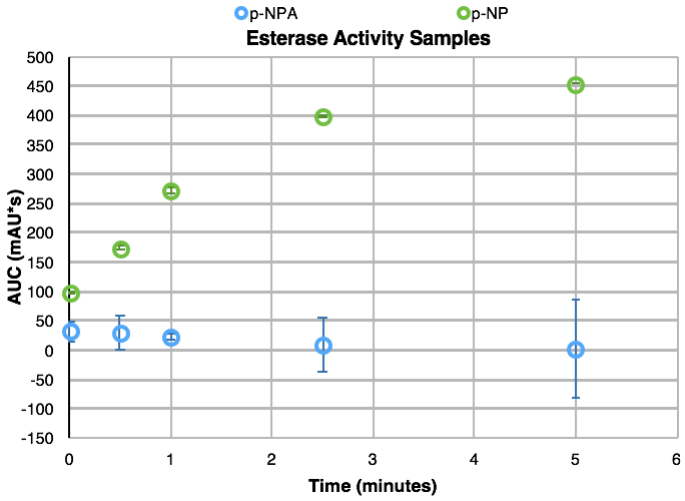

Diagram 21: Esterase Activity Samples

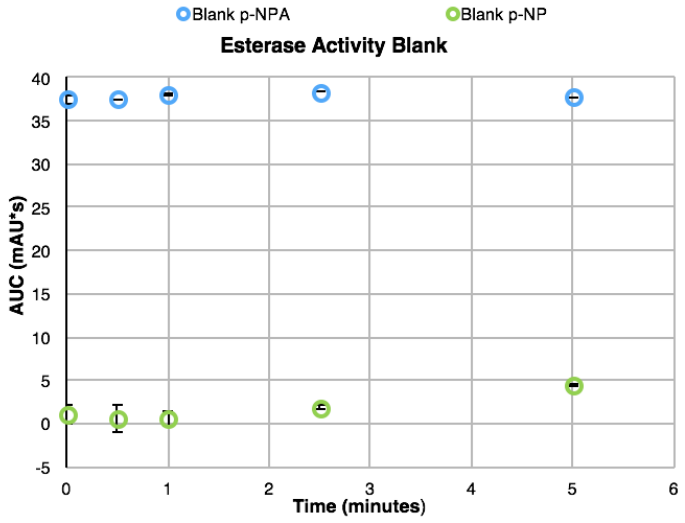

Diagram 22: Esterase Activity Blank Samples

Diagram 21 shows that the p-NP concentration (green line) is increasing and the p-NPA concentration is decreasing.

By comparison to the blank sample (Diagram 22) it can be seen that p-NPA concentration is much higher than the p-NP concentration. Both of them stay the same level, except for a small increasing of p-NP and decreasing of p-NPA which can be lead to a decomposition of p-NPA to p-NP at 20°C and atmospheric conditions.

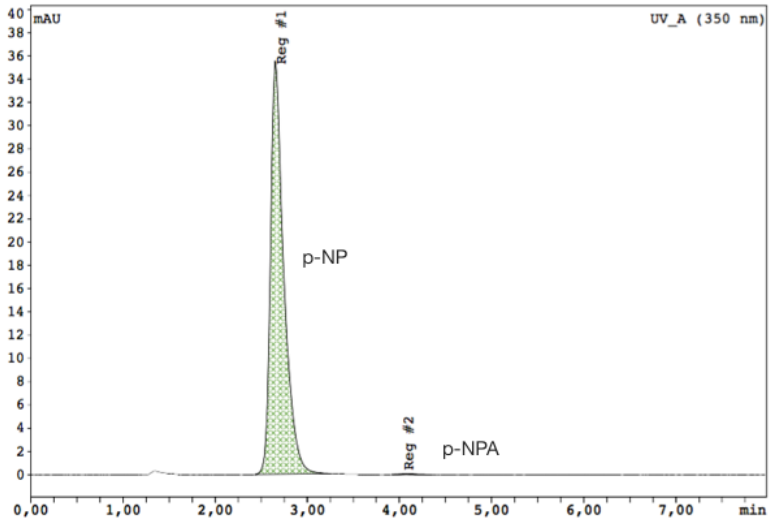

Figure 8: Protease activity; sample; after 5 minutes

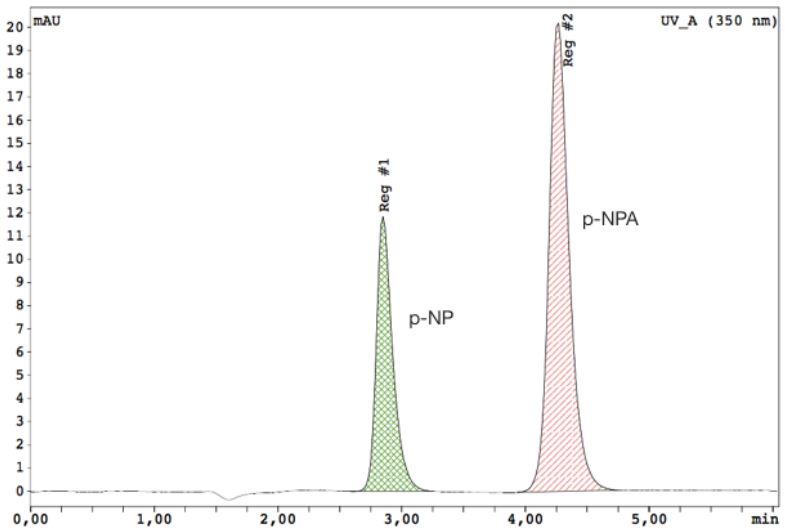

Figure 9: Esterase activity; sample without esterase; after 5 minutes

Figure 8 shows the chromatogram of the pretest containing butyrylcholinesterase after 5 minutes of incubation. The chromatogram demonstrates that nearly all p-NPA was metabolised to p-NP via the used esterase.

Figure 9 shows a chromatogram of the blank after 5 minutes. The p-NPA peak (red marked peak) has a retention time of 4.07 minutes. This peak has a higher AUC than the p-NP peak (green marked peak), which's retention time is 2.8 minutes.

The data, which has been taken taken from each esterase sample.
The error bars reflect the standard error. The standard deviation and the individual measurements, for each sample, are indicated in the annex. (Table 41)

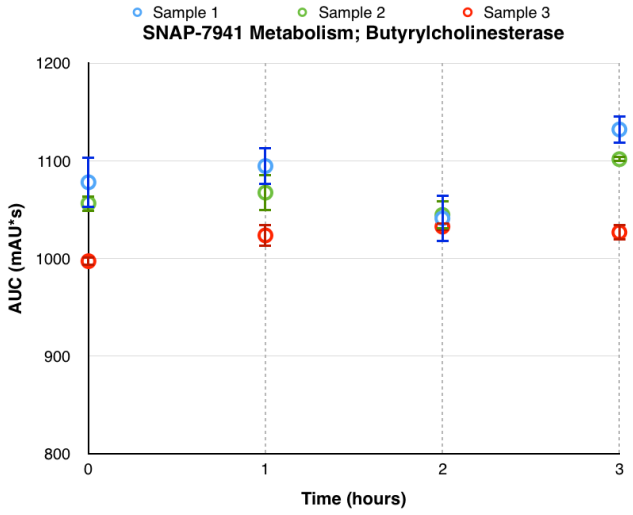

Diagram 23: SNAP-7941 Metabolism; Butyrylcholinesterase

Diagram 23 shows the measured Area under the curve of SNAP-7941 after incubating with the butyrylcholinesterase in different points in time. All samples show a straight, so it can be assumed that there was no decrease of SNAP-7941, also no peak of the SNAP-acid could be seen in the chromatogram.

2.4 Lipases

2.4.1 Experimental setup

Pretests to verify the functionality of selected Lipases

Triolein is cleaved by lipases to oleic acid, triglycerole and mono- and diglycerides. Triolein showed no peak in the HPLC-spectrum, so a wavelength-check was done in the UV/VIS spectrophotometer. Since there was no wavelength found which could be used at the HPLC-analysis, the HPLC parameters, such as retention time, were taken only of the oleic acid. The constitution of the used mobile phase is shown in the table below.

	HPLC-Conditions
Column	LiCrospher® 100 RP-18 125x4mm, 5µm
Mobile phase	water, acetonitrile
Flow	1.0ml/min
Loop volume	20µl
UV-Detector	205nm

Table 12: HPLC-Conditions of Pretests to verify the functionality of selected Lipases

Used Gradient		
Minute	Water (%)	Acetonitrile (%)
0	30	70
20	0	100
25	30	70

Table 13: Used Gradient in Pretests to verify the functionality of selected Lipases

Testing the lipase

Due to solubility problems of triolein using common solvents, isooctane was used instead. (41)

The lipase was dissolved in 250µl 20mM phosphate buffer, pH 7.4 instead of the 50mM phosphate buffer, described in the paper, because of better comparability to the other experiments with SNAP-7941. The ratio of lipase to triolein was calculated 1:4.43. (42)

Lipase was scaled and afterwards the amount of triolein, which should be added, was calculated. Triolein was diluted in isooctane to gain a total volume of 1ml.

Reaction was started by combining the organic and the water phase. The vial was shaken at 650rpm at 37°C in an incubator device. At 0, 1 and 3 hours, an aliquot of the lipophilic phase was pipetted out and injected into HPLC.

The mobile phase was set up as published by M. Holčapec (43) with some modifications. The 2-propanol-hexane was left out, due to better agreement with the used HPLC-conditions. The constitution of the used mobile phase is shown in the table below.

	HPLC-Conditions
Column	LiCrospher® 100 RP-18 125x4mm, 5µm
Mobile phase	Water, Acetonitrile
Flow	1.0ml/min
Loop volume	20µl
UV-Detector	205nm

Table 14: HPLC-Conditions of Pretests to verify the functionality of selected Lipases

Used Gradient		
Minute	Water (%)	Acetonitrile (%)
0	30	70
20	0	100
25	30	70

Table 15: Used Gradient in Pretests to verify the functionality of selected Lipases

Enzymatic conversion of SNAP-7941 via Lipases

A 1.0mg/ml solution in 20mM phosphate buffer pH 7.4 of any of the lipases was created. SNAP-7941 was diluted in ethanol to a concentration of 10mg/ml. The proportion of lipase to SNAP-7941 was 1: 2.21. (42)

The different components were pipetted into a reaction-vial, vortexed, tempered at 37°C and shook gently at 650rpm in an incubator device. The reaction was stopped at defined points in time (0, 1, 2, 3 and 24 hours) through pipetting an aliquot of 100µl into a vial containing 100µl acetonitrile.
The samples were centrifuged at 1649*g* (4000rpm) for 5 minutes to separate the precipitated from the liquid. The supernatant was injected into HPLC. (38)

	HPLC-Conditions
Column	Chromolith® performance RP-18e 100x4.6mm
Mobile phase	Buffer/ Acetonitrile 70:30 Buffer: 32.43mM ammonium acetate in water/ acetic acid 97.5/ 2.5 (v/v), pH 3.57
Flow	1.0ml/min
Loop volume	20µl
UV-Detector	254nm

Table 16: HPLC-Conditions of Enzymatic conversion of SNAP-7941 via Lipases

2.4.2 Results

Pretests to verify the functionality of selected Lipases

For a better comparison, the measurements are expresses in one diagram. The mean value of sample 1 expressed in blue, sample 2 in green.

The individual measurements, for each sample, are indicated in the annex. (Table 42 to Table 44)

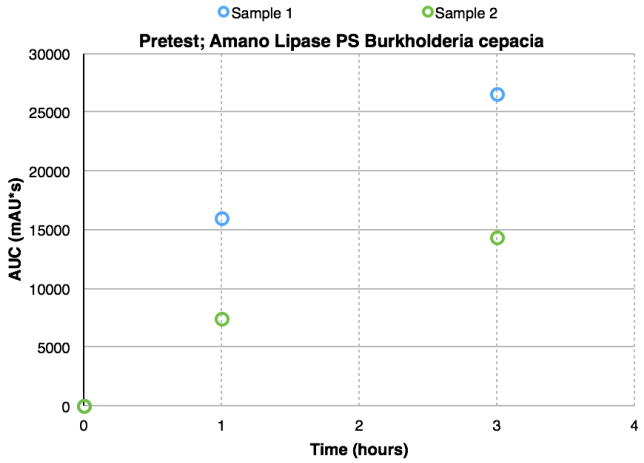

Diagram 24: Pretest; Amano Lipase PS Burkholderia cepacia

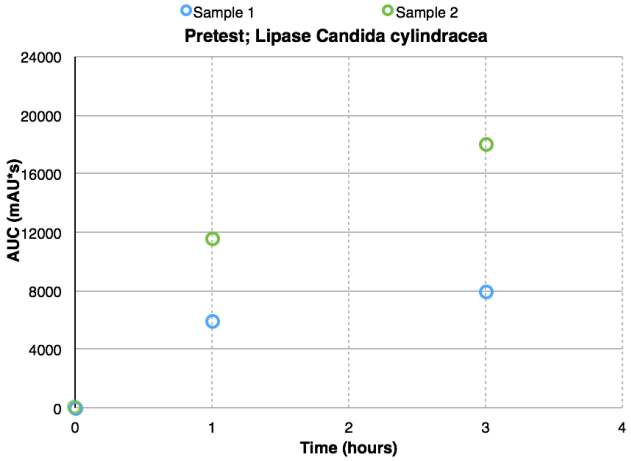

Diagram 25: Pretest; Lipase Candida cylindracea

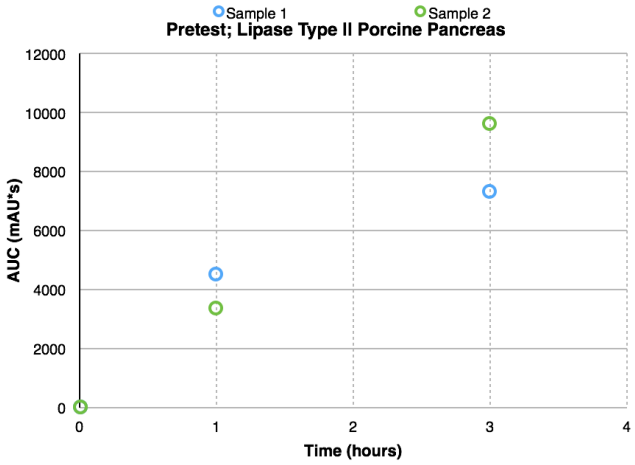

Diagram 26: Pretest; Lipase Type II Porcine Pancreas

Diagram 24 to Diagram 26 show that the concentration of the area under de curve of the oleic acid is increasing with a longer incubation time, which leads to the conclusion that all tested lipases are working.

Figure 10 is an example of the chromatograms which were received. The peak of oleic acid is shown at the time of 12.1 minutes.

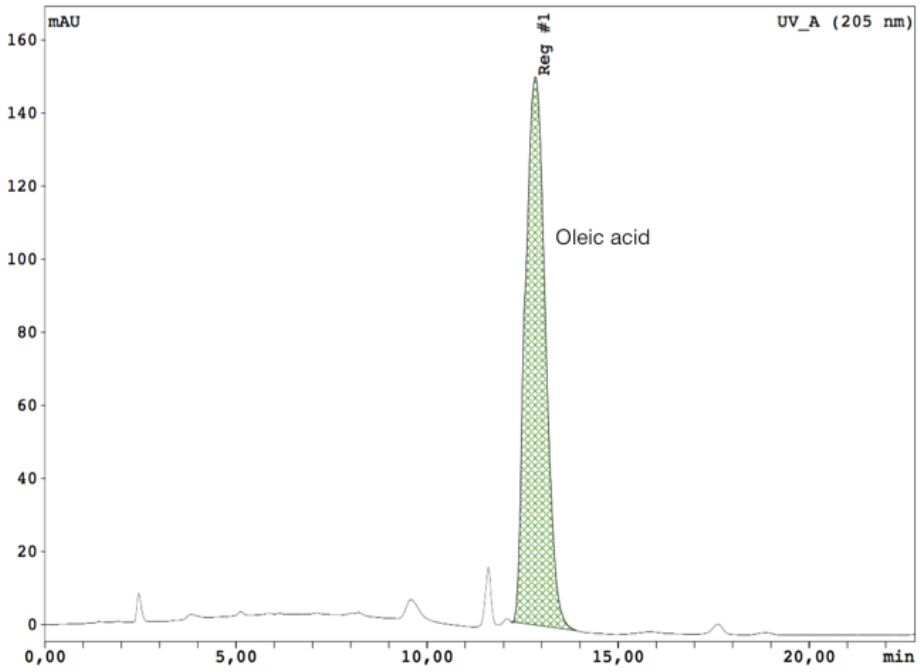

Figure 10:Pretest; Lipase Candida cylindracea; Sample 1 after two hours incubation time

The data that was taken from each lipase triplicate is shown in one diagram. The used numerical value is the mean value of each samples triple injection into the HPLC.

The error bars reflect the standard error. The standard deviation and the individual measurement, for each sample, are indicated in the annex. (Table 45 to Table 47)

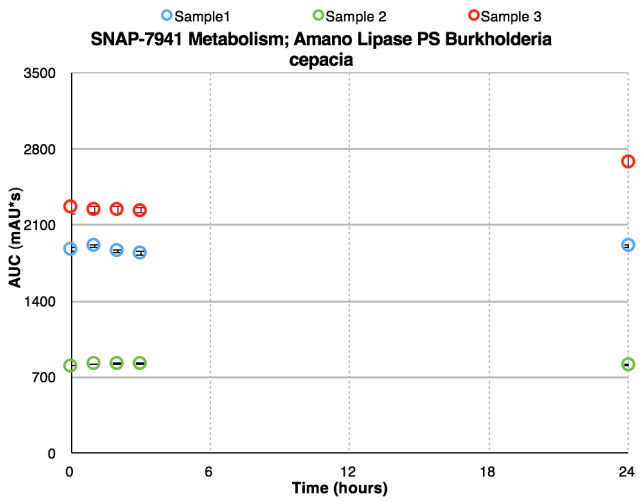

Diagram 27: SNAP-7941 Metabolism; Amano Lipase PS Burkholderia cepacia

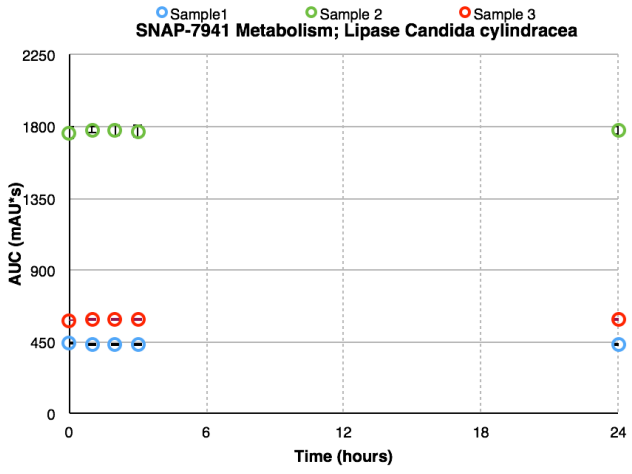

Diagram 28: SNAP-7941 Metabolism; Lipase Candida cylindracea

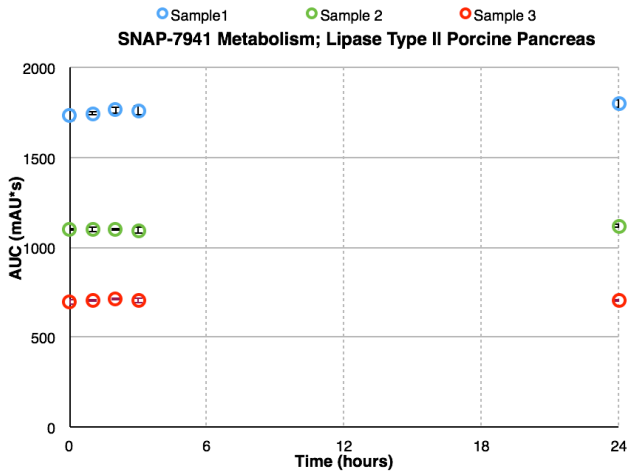

Diagram 29: SNAP-7941 Metabolism; Lipase Type II Porcine Pancreas

The diagrams (Diagram 27 to Diagram 29) show the measured area under the curve of SNAP-7941 after incubating with the protease in different points in time. All of them show a straight line between the the different points in sample taking, so it can be assumed that there was no decrease of SNAP-7941, also no peak of the SNAP-acid could be seen in the chromatogram.

3. Discussion

Pretests to verify the functionality of selected Enzymes

One aim set for this thesis, was to improve assays to test the activity of proteases, esterases and lipases. Another aim was to test the selected enzymes if they are active and optimize their concentration for the enzymatic conversion of SNAP-7941. Both, the concentration level and the optimum working concentration of used enzymes could be established in the General Hospital of Vienna (AKH).

Proteases

Some adaptions were made to the method of Sigma's non-specific protease activity assay (37). For instance, the samples were centrifuged instead of filtrated, to handle samples simultaneously. This was necessary to enable a quicker process of work.

The results show that the concentration, in which the proteases worked best cleaving casein to L-trypsin was 1mg/ml. A longer incubation time led to a higher L-trypsin concentration. For this reason, it can be said that for further experiments a concentration of 1mg/ml and an incubation time above 20 minutes would be an optimal set-up.

Lipases

Some modifications were made to the method of Hermansyah et al (41). Briefly, the lipase was dissolved in 250µl 20mM phosphate buffer, pH 7.4 instead of 50mM phosphate buffer which was described in this paper. With this adaption a better comparability to the other experiments with SNAP-7941 was assured. Also, the mobile phase has been modified. Due to better agreement with the used HPLC, 2-propanol-hexane was left out in order to guarantee the transparency of

the oleic acid peak. One test run showed that the 2-propanol-hexane is overlapping withe the oleic acid peak.
The results show that the concentration of oleic acid using selected lipases were increasing with longer incubation time, which lead to the conclusion that all tested lipases were working. The different concentrations of oleic acid could be correlated with the different triolein concentrations. In this assay the educt concentration was adjusted to the enzyme's concentration.

Esterases

The assay to verify the functionality of the esterase was improved by Zarko Solajic for the AKH Wien (40).

The results show that the p-NPA concentration was increasing and the p-NP concentration was decreasing. This demonstrates that the esterase was working. In the sample without esterase the p-NP and p-NPA concentration stayed the same except for a small raise of p-NPA and reduction of p-NP which could be lead to a decomposition of p-NP to p-NPA at 20°C and atmospheric conditions.

Enzymatic conversion of SNAP-7941 via selected Enzymes

The second aim of this thesis mentioned before was to ascertain to convert SNAP-7941 to SNAP-acid via selected enzymes, because SNAP-acid is needed as a precursor compound for the synthesis of [^{11}C]SNAP-7941. It has been proven that the used enzymes are not able to metabolise SNAP-7941 to SNAP-acid.

It seems that from a chemically point of view, the conversion of SNAP-7941 to SNAP-acid using the selected enzymes did not work because of stereospecific characteristics of SNAP-7941. Lipases are mainly designed to metabolize fatty acid esters which are too differently structured to SNAP-7941. Also the benzene ring with the two fluorine atoms could be a stereospecific obstacle for SNAP-7941 not fitting into the enzymes active site.

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Zusammenfassung

Das Melanin-Concentrating-Hormon (MCH)-System wirkt auf Ingestion, Energiehomöostase und Schlafverhalten, es wird deswegen mit Erkrankungen wie Adipositas und Depressionen in Verbindung gebracht. Zur Visualisierung und Quantifizierung des Melanin-Concentrating-Hormon Rezeptors 1 (MCH-R1) und zur Berechnung der Dosisanpassung des MCH-R1 Antagonisten wurde der PET-Tracer [^{11}C]SNAP-7941 entwickelt. Für die Synthese des Tracers wird die synthetisch aufwändig herzustellende Vorläufersubstanz (Präcursor) SNAP-Säure benötigt. Da die Synthese von SNAP-7941 einfach und in einer guten Ausbeute zu bewerkstelligen ist, war primäres Ziel dieser Arbeit, mit Hilfe von Enzymen den Präcursor SNAP-Säure aus SNAP-7941 herzustellen.

Bei der Arbeit mit Enzymen ist es essentiell, diese auf ihre Aktivität zu überprüfen. Deshalb war die Etablierung von Protease- und Lipaseaktivitätsassays am AKH Wien ein weiteres Ziel dieser Arbeit.

Die Metabolisierung von SNAP-7941 zur SNAP-Säure konnte mit Hilfe der verwendeten Enzyme nicht bewerkstelligt werden.

Jedoch konnten sowohl der Protease- als auch der Lipaseaktivitätsassay am AKH Wien erfolgreich etabliert werden.

Abstract

The melanin concentrating hormone (MCH)-System affects ingestion energy homeostasis and sleeping behaviour. Therefore, it is associated with obesity and depression. For visualisation and quantification of melanin concentrating hormone-receptor 1 (MCH-R1) and for scaling the dose of MCH-R1 antagonists the PET tracer [^{11}C]SNAP-7941 was developed. For the synthesis of [^{11}C]SNAP-7941 the precursor SNAP-acid is needed. In difference to SNAP-acid, SNAP-7941 is easily synthesised in a high yield. For that reason, the main aim of this thesis was to produce SNAP-acid through enzymatic cleaving of SNAP-7941.

While working with enzymes it is essential to verify their activity. Therefore, an other aim of this work was to establish assays to verify the activity of proteases and lipases.
The metabolisation of SNAP-7941 to SNAP-acid was not achieved with the chosen enzymes.

However, it was possible to establish the assays to verify the activity of proteases and lipases in the AKH Wien.

SNAP-7941/SNAP-acid

SNAP-7941 Regression line; Sample 1					
	AUC (mAU*s)				Standard deviation (AUC)
Dilution µg/mL	Injection 1	Injection 2	Injection 3	Mean value	
10	164.0846	168.5212	167.3567	166.6542	2.3002
7.5	124.6492	122.8475	123.3199	123.6055	0.9342
5	87.42344	83.72584	80.4713	83.8735	3.4784
2.5	44.44952	40.04266	39.96651	41.4862	2.5666
1	15.31052	15.29114	15.75685	15.4528	0.2635
0.5	7.889188	7.272491	7.69431	7.6187	0.3152
0.1	2.280473	2.404434	2.360774	2.3486	0.0629

Table 17: SNAP-7941 Regression line; Sample 1

SNAP-7941 Regression line; Sample 2					
	AUC (mAU*s)				Standard deviation (AUC)
Dilution µg/mL	Injection 1	Injection 2	Injection 3	Mean value	
10	161.1711	162.7844	161.811	161.9222	0.8124
7.5	127.76	120.3474	125.0246	124.3773	3.7484
5	84.17841	81.52295	82.49707	82.7328	1.3433
2.5	39.76436	40.38435	40.68611	40.2783	0.4699
1	15.05339	14.69346	14.67239	14.8064	0.2141
0.5	8.298835	7.96173	7.82207	8.0275	0.2451
0.1	1.759003	1.438132	1.466018	1.5544	0.1778

Table 18: SNAP-7941 Regression line; Sample 2

SNAP-acid Regression line; Sample 1					
	AUC (mAU*s)				Standard deviation (AUC)
Dilution µg/mL	Injection 1	Injection 2	Injection 3	Mean value	
10	28.80515	28.8938	29.39095	29.0300	0.3157
7.5	21.77135	21.8462	21.16305	21.5935	0.3747
5	14.27339	14.28531	14.53661	14.3651	0.1486
2.5	6.102494	6.017662	5.718859	5.9463	0.2015
1	2.355014	2.419077	2.347938	2.3740	0.0392
0.5	0.959837	1.000187	0.929529	0.9632	0.0354
0.1	0.41698	0.367423	0.296493	0.3603	0.0606

Table 19: SNAP-acid Regression line; Sample 1

SNAP-acid Regression line; Sample 2					
	AUC (mAU*s)				Standard deviation (AUC)
Dilution µg/mL	Injection 1	Injection 2	Injection 3	Mean value	
10	28.6203	29.73584	29.49833	29.2848	0.5876
7.5	20.80468	20.19896	20.66737	20.5570	0.3176
5	13.36345	13.33256	13.23719	13.3111	0.0658
2.5	5.708766	5.841568	5.759912	5.7701	0.0670
1	2.048035	2.177464	2.253244	2.1596	0.1038
0.5	1.063459	0.99123	0.940251	0.9983	0.0619
0.1	0.451238	0.49273	0.590511	0.5115	0.0715

Table 20: SNAP-acid Regression line; Sample 2

Proteases

Pretests to verify the functionality of selected Proteases

Acid Protease II Amano				
Measurement number	Concentration of protease (mg/6mL) Minutes	0.0001	0.01	1
		Absorption of the sample (λ_{max} at 660nm)		
1	0	0	0.001	0.006
2		0.002	0.001	0.006
Mean value 1&2		**0.001**	**0**	**0.006**
Standard deviation		0.001	0.001	0
3	10	0.002	0.002	0.021
4		0.002	0.003	0.017
Mean value 3&4		**0.002**	**0.003**	**0.019**
Standard deviation		0	0.001	0.003
5	20	0.003	0.005	0.034
6		0.005	0.008	0.031
Mean value 5&6		**0.004**	**0.007**	**0.033**
Standard deviation		0.001	0.002	0.002

Table 21: Pretests to verify the functionality of Acid Protease II Amano

Chirazym P-Z				
Measurement number	Concentration of protease (mg/6mL) Minutes	0.0001	0.01	1
		Absorption of the sample (λ_{max} at 660nm)		
1	0	0.007	0.015	0.112
2		0.003	0.012	0.107
Mean value 1&2		**0.005**	**0.014**	**0.110**
Standard deviation		0.003	0.002	0.003
3	10	0.075	0.098	0.127
4		0.073	0.093	0.123
Mean value 3&4		**0.074**	**0.096**	**0.125**
Standard deviation		0.001	0.004	0.003
5	20	0.099	0.109	0.13
6		0.094	0.104	0.126
Mean value 5&6		**0.097**	**0.107**	**0.128**
Standard deviation		0.004	0.004	0.003

Table 22: Pretests to verify the functionality of Chirazym P-Z

Papain of Carica Papaya				
Measurement number	Concentration of protease (mg/6mL) Minutes	0.0001	0.01	1
		Absorption of the sample (λ_{max} at 660nm)		
1	0	0.002	0.001	0.023
2		0	0	0.018
Mean value 1&2		**0.001**	**0.001**	**0.021**
Standard deviation		0.001	0.001	0.004
3	10	0	0.002	0.127
4		0	0.003	0.124
Mean value 3&4		**0**	**0.003**	**0.126**
Standard deviation		0	0.001	0.0021
5	20	0	0.002	0.134
6		0	0.003	0.12
Mean value 5&6		**0**	**0.003**	**0.127**
Standard deviation		0	0.001	0.010

Table 23: Pretests to verify the functionality of Papain of Carica Papaya

Proleather Amano				
Measurement number	Concentration of protease (mg/6mL) Minutes	0.0001	0.01	1
		Absorption of the sample (λ_{max} at 660nm)		
1	0	0.003	0.001	0.011
2		0	0.003	0.012
Mean value 1&2		**0.002**	**0.002**	**0.012**
Standard deviation		0.002	0.001	0.001
3	10	0	0.002	0.121
4		0.001	0.002	0.122
Mean value 3&4		**0**	**0.002**	**0.122**
Standard deviation		0.001	0	0.001
5	20	0	0.003	0.130
6		0	0.004	0.118
Mean value 5&6		**0**	**0.004**	**0.124**
Standard deviation		0	0.001	0.009

Table 24: Pretests to verify the functionality of Proleather Amano

Protease A Amano				
Measurement number	Concentration of protease (mg/6mL) Minutes	0.0001	0.01	1
		Absorption of the sample (λ_{max} at 660nm)		
1	0	0	0	0.036
2		0	0	0.035
Mean value 1&2		**0**	**0**	**0.0355**
Standard deviation		0	0	0.001
3	10	0.002	0.008	0.124
4		0.001	0.009	0.124
Mean value 3&4		**0.002**	**0.009**	**0.124**
Standard deviation		0.001	0.001	0
5	20	0	0.013	0.127
6		0	0.013	0.131
Mean value 5&6		**0**	**0.013**	**0.129**
Standard deviation		0	0	0

Table 25: Pretests to verify the functionality of Protease A Amano

Protease M Amano				
Measurement number	Concentration of protease (mg/6mL) Minutes	0.0001	0.01	1
		Absorption of the sample (λ_{max} at 660nm)		
1	0	0	0	0.017
2		0	0.003	0.017
Mean value 1&2		**0**	**0.002**	**0.017**
Standard deviation		0	0.002	0
3	10	0	0.001	0.086
4		0	-0.001	0.084
Mean value 3&4		**0**	**0**	**0.085**
Standard deviation		0	0.001	0.001
5	20	0.001	0.001	0.115
6		0	0.001	0.115
Mean value 5&6		**0,001**	**0.001**	**0.115**
Standard deviation		0.001	0	0

Table 26: Pretests to verify the functionality of Protease M Amano

Protease N Amano				
Measurement number	Concentration of protease (mg/6mL) Minutes	0.0001	0.01	1
		Absorption of the sample (λ_{max} at 660nm)		
1	0	0	0.004	0.005
2		0	0	0
Mean value 1&2		**0**	**0.002**	**0.003**
Standard deviation		0	0.005	0.004
3	10	0	0.017	0.022
4		0	0.018	0.02
Mean value 3&4		**0**	**0.018**	**0.021**
Standard deviation		0	0.001	0.001
5	20	0.012	0.02	0.063
6		0.012	0.02	0.063
Mean value 5&6		**0.012**	**0.02**	**0.063**
Standard deviation		0	0	0

Table 27: Pretests to verify the functionality of Protease N Amano

Protease S Amano				
Measurement number	Concentration of protease (mg/6mL) Minutes	0.0001	0.01	1
		Absorption of the sample (λ_{max} at 660nm)		
1	0	0	0	0.041
2		0	0	0.04
Mean value 1&2		**0**	**0**	**0.041**
Standard deviation		0	0	0.001
3	10	0.002	0.003	0.111
4		0.005	0.001	0.113
Mean value 3&4		**0.004**	**0.002**	**0.112**
Standard deviation		0.002	0.001	0.001
5	20	0.002	0.006	0.123
6		0.003	0.004	0.121
Mean value 5&6		**0.003**	**0.005**	**0.122**
Standard deviation		0.001	0.001	0.001

Table 28: Pretests to verify the functionality of Protease S Amano

Prozyme 6 Amano				
Measurement number	Concentration of protease (mg/6mL) Minutes	0.0001	0.01	1
		Absorption of the sample (λ_{max} at 660nm)		
1	0	0	0.002	0.074
2		0	0.002	0.07
Mean value 1&2		**0**	**0.002**	**0.072**
Standard deviation		0	0	0.003
3	10	0	0.035	0.14
4		0	0.035	0.137
Mean value 3&4		**0**	**0.035**	**0.139**
Standard deviation		0	0	0.002
5	20	0	0.063	0.145
6		0.002	0.059	0.137
Mean value 5&6		**0.001**	**0.061**	**0.141**
Standard deviation		0.001	0.003	0.006

Table 29: Pretests to verify the functionality of Prozyme 6 Amano

Acid Protease II Amano					
	AUC (mAU*s)				
Hours	1	2	3	Mean Value	Standard deviation
Sample 1					
0	-	1026.511	1049.042	**1037.777**	15.932
1	1048.212	1060.759	1046.835	**1051.935**	7.673
2	1051.844	1067.83	1075.752	**1065.142**	12.179
3	1080.73	1060.979	1078.572	**1073.427**	10.834
24	1088.243	1085.357	1078.402	**1084.001**	5.059
Sample 2					
0	1075.937	1091.061	1084.580	**1083.859**	7.588
1	1059.326	1063.721	1071.212	**1064.753**	6.010
2	1072.892	1071.463	1073.120	**1072.492**	0.898
3	1021.873	1042.133	1043.626	**1035.877**	12.151
24	1035.121	1116.608	1084.723	**1078.817**	41.063
Sample 3					
0	1068.548	1084.998	1076.606	**1076.717**	8.226
1	-	1091.717	1096.275	**1093.996**	3.223
2	1041.826	1065.142	1063.436	**1056.801**	12.997
3	1049.647	1046.401	1077.087	**1057.712**	16.858
24	1073.530	1061.690	1099.259	**1078.160**	19.207

Table 30: SNAP-7941 Metabolism; Acid Protease II Amano

Chirazym P-Z					
	AUC (mAU*s)				
Hours	1	2	3	Mean Value	Standard deviation
Sample 1					
0	1013.234	1058.634	1056.235	**1042.701**	25.547
1	1037.392	1044.832	1043.656	**1041.960**	3.999
2	1034.169	1039.836	1038.053	**1037.353**	2.898
3	1030.643	1048.285	1049.322	**1042.750**	10.498
24	1039.897	1040.326	1033.447	**1037.890**	3.854
Sample 2					
0	1022.925	1024.584	1024.065	**1023.858**	0.849
1	1002.892	1003.137	985.968	**997.332**	9.843

2	1024.019	1030.628	1028.356	**1027.668**	3.358
3	1035.168	1004.013	1046.678	**1028.620**	22.073
24	1010.394	1013.723	1022.455	**1015.524**	6.229
Sample 3					
0	1037.419	1041.250	1034.287	**1037.652**	3.487
1	1018.985	1036.855	1041.530	**1032.457**	11.899
2	1023.418	1011.575	1027.629	**1020.874**	8.324
3	1031.241	1030.421	1028.343	**1030.002**	1.494
24	1053.241	1045.720	-	**1049.481**	5.318

Table 31: SNAP-7941 Metabolism; Chirazym P-Z

Papain of Carica Papaya					
	AUC (mAU*s)				
Hours	1	2	3	Mean Value	Standard deviation
Sample 1					
0	1091.167	1129.768	1121.943	**1114.293**	20.406
1	1139.240	1136.624	1118.350	**1131.405**	11.381
2	1184.717	1173.580	1172.901	**1177.066**	6.635
3	1129.949	1126.972	1113.436	**1123.452**	8.801
24	1162.089	1162.355	1170.228	**1164.891**	4.624
Sample 2					
0	1130.120	1117.228	1054.611	**1100.653**	40.391
1	1131.155	1135.871	1157.610	**1141.545**	14.111
2	1313.385	1327.755	1320.658	**1320.599**	7.185
3	1160.770	1158.933	1125.775	**1148.493**	19.696
24	1139.869	1143.377	1147.100	**1143.449**	3.616
Sample 3					
0	1294.824	1335.046	1323.242	**1317.704**	20.675
1	1347.029	1347.132	1347.132	**1347.098**	0.059
2	1162.363	1160.567	1141.603	**1154.844**	11.502
3	1358.140	1370.258	1358.282	**1362.227**	6.956
24	1335.246	1357.416	1371.239	**1354.634**	18.157

Table 32: SNAP-7941 Metabolism; Papain of Carica Papaya

Proleater Amano					
	AUC (mAU*s)				
Hours	1	2	3	Mean Value	Standard deviation

Sample 1					
0	1099.939	1124.134	1134.049	**1119.374**	17.546
1	1094.879	1114.829	1115.086	**1108.265**	11.593
2	1096.349	1100.939	1088.350	**1095.213**	6.371
3	1099.982	1112.082	1138.797	**1116.954**	19.861
24	1047.519	1102.827	1116.452	**1088.933**	36.507
Sample 2					
0	1078.483	1065.894	1073.941	**1072.773**	6.375
1	1085.400	1086.706	1096.456	**1089.521**	6.042
2	1054.002	1057.153	1051.828	**1054.328**	2.677
3	1061.788	1085.195	1066.156	**1071.046**	12.446
24	1068.325	1067.225	1071.866	**1069.139**	2.425
Sample 3					
0	1043.986	1030.192	1037.339	**1037.172**	6.899
1	1075.464	1106.683	1075.654	**1085.934**	17.970
2	1018.066	1028.013	1021.532	**1022.537**	5.049
3	1014.334	988.4124	1009.377	**1004.041**	13.760
24	1049.902	1039.293	1053.798	**1047.664**	7.507

Table 33: SNAP-7941 Metabolism; Proleater Amano

Protease A Amano					
	AUC (mAU*s)				
Hours	1	2	3	Mean Value	Standard deviation
Sample 1					
0	931.4180	929.3160	906.2900	**922.3413**	13.9405
1	765.3789	802.9730	767.5880	**778.6466**	21.0962
2	686.4130	664.8910	693.7850	**681.6963**	15.0134
3	738.0950	773.5649	765.9090	**759.1896**	18.6652
24	770.6530	873.8890	-	**822.2710**	72.9989
Sample 2					
0	832.5340	849.8680	-	**841.2010**	12.2570
1	907.5400	957.9130	957.9450	**941.1327**	29.0921
2	602.9980	626.0770	630.3004	**619.7918**	14.6964
3	428.2600	446.2700	448.6048	**441.0449**	11.1335
24	509.3910	597.8560	594.4240	**567.2237**	50.1139
Sample 3					

0	915.3590	772.0300	-	**843.6945**	101.3489
1	977.745	1130.581	1108.453	**1072.2597**	82.5964
2	727.1516	735.9690	722.2900	**728.4702**	6.9342
3	719.6530	846.2990	649.2069	**738.3863**	99.8726
24	759.3740	660.9165	659.1500	**693.1468**	57.3612

Table 34: SNAP-7941 Metabolism; Protease A Amano

Protease M Amano					
	AUC (mAU*s)				
Hours	1	2	3	Mean Value	Standard deviation
Sample 1					
0	1623.060	1352.542	1404.682	**1460.095**	143.520
1	1334.996	1418.615	1432.301	**1395.304**	52.675
2	1352.639	1338.964	1401.020	**1364.208**	32.605
3	1344.790	1406.108	1411.933	**1387.610**	37.198
24	1315.221	1513.316	1462.231	**1430.256**	102.846
Sample 2					
0	1145.941	1137.387	1124.970	**1136.099**	10.545
1	1210.020	1221.123	1203.703	**1211.615**	8.819
2	1197.086	1220.863	1217.658	**1211.869**	12.902
3	1220.500	1201.622	1202.070	**1208.064**	10.772
24	1370.005	1346.258	1348.329	**1354.864**	13.153
Sample 3					
0	1047.595	1054.215	1047.910	**1049.907**	3.734
1	1286.512	1282.471	1284.790	**1284.591**	2.028
2	1172.851	1291.708	1332.866	**1265.808**	83.092
3	1266.645	1256.590	-	**1261.618**	7.110
24	1319.052	1332.024	1336.376	**1329.151**	9.012

Table 35: SNAP-7941 Metabolism; Protease M Amano

Protease N Amano					
	AUC (mAU*s)				
Hours	1	2	3	Mean Value	Standard deviation
Sample 1					
0	1197.086	1187.378	1186.019	**1190.161**	6.036
1	-	1163.982	1187.973	**1175.978**	16.964
2	1060.065	1156.657	1153.152	**1123.291**	54.784
3	1170.191	1170.413	1153.580	**1164.728**	9.655

24	1102.513	1116.464	1153.297	**1124.091**	26.237
Sample 2					
0	1018.777	1054.230	1024.809	**1032.605**	18.969
1	1098.223	1046.882	1091.158	**1078.754**	27.827
2	950.711	1053.714	1087.283	**1030.569**	71.167
3	1107.621	1094.872	1094.534	**1099.009**	7.460
24	1089.484	1100.564	1084.533	**1091.527**	8.208
Sample 3					
0	1022.055	1024.809	1012.300	**1019.721**	6.573
1	1023.546	1026.835	1024.375	**1024.919**	1.711
2	1027.420	1019.691	1028.960	**1025.357**	4.967
3	1057.430	1040.444	1041.588	**1046.487**	9.494
24	1048.914	1055.061	1050.282	**1051.419**	3.227

Table 36: SNAP-7941 Metabolism; Protease N Amano

Protease S Amano					
	AUC (mAU*s)				
Hours	1	2	3	Mean Value	Standard deviation
Sample 1					
0	892.0619	903.6610	907.7728	**901.165**	8.147
1	1025.211	1027.113	1021.652	**1024.659**	2.772
2	988.8505	996.4630	1000.3320	**995.215**	5.842
3	1003.070	1019.651	1031.142	**1017.954**	14.113
24	999.4397	1033.524	1028.241	**1020.402**	18.345
Sample 2					
0	891.8362	821.8232	897.9669	**870.542**	42.303
1	920.9775	890.3829	881.1753	**897.512**	20.837
2	866.9311	881.9963	871.9561	**873.628**	7.671
3	865.3500	860.5817	863.6359	**863.189**	2.415
24	888.2634	881.8873	874.1282	**881.426**	7.079
Sample 3					
0	976.1197	998.8422	989.6410	**988.201**	11.430
1	967.8751	1017.924	1004.190	**996.663**	25.860
2	973.1234	967.0322	994.6960	**978.284**	14.536
3	985.0188	971.9900	973.5989	**976.869**	7.103
24	1043.701	1064.846	1076.787	**1061.778**	16.755

Table 37: SNAP-7941 Metabolism; Protease S Amano

Prozyme 6 Amano					
	AUC (mAU*s)				
Hours	1	2	3	Mean Value	Standard deviation
Sample 1					
0	934.6184	1041.688	970.2414	**982.1826**	54.524
1	871.8458	848.4631	949.2116	**889.8402**	52.7296
2	877.6619	968.9690	869.9585	**905.5298**	55.0748
3	901.7600	895.5075	895.7346	**897.6674**	3.5461
24	900.1169	875.8594	869.5398	**881.8387**	16.1417
Sample 2					
0	1210.285	1043.397	1185.505	**1146.396**	90.056
1	937.1529	912.1425	916.6332	**921.976**	13.334
2	924.7700	926.6710	1024.5180	**958.653**	57.0487
3	933.0920	947.8629	935.8191	**938.925**	7.860
24	903.0820	918.6640	901.0892	**907.612**	9.623
Sample 3					
0	1092.818	1081.695	1092.006	**1088.840**	6.201
1	1009.112	999.290	1009.407	**1005.936**	5.758
2	1024.518	1021.116	1031.191	**1025.608**	5.125
3	1009.643	1016.979	1024.702	**1017.108**	7.530
24	975.9283	991.0021	996.2778	**987.736**	10.561

Table 38: SNAP-7941 Metabolism; Prozyme 6 Amano

Esterases

Pretests to verify the functionality of selected Esterase

Esterase Activity Samples					
pNPA	AUC (mAU*s)				
Minutes	Sample 1	Sample 2	Sample 3	Mean Value	Standard deviation
0	31.4031	30.8631	32.0646	**31.4436**	0.6018
0.5	34.3491	26.8150	25.3446	**28.8362**	4.8306
1	29.1848	20.4363	17.2841	**22.3017**	6.1658
2.5	8.4278	6.5818	6.9363	**7.3153**	0.9796
5	0.5833	1.1179	0.9620	**0.8877**	0.2749
p-NP					
0	114.0881	96.3818	80.31546	**96.9285**	16.8930
0.5	185.1082	197.1007	139.1692	**173.7927**	30.5785
1	264.8411	271.6537	277.4310	**271.3086**	6.3020
2.5	346.0297	433.2873	412.9320	**397.4163**	45.6511
5	357.8490	518.7391	482.6892	**453.0924**	84.4297

Table 39: Esterase Activity Samples

Esterase Activity Blank				
pNPA	AUC (mAU*s)			
Minutes	Sample 1	Sample 2	Mean Value	Standard deviation
0	1.3141	0.7604	**1.0373**	0.3915
0.5	0.5540	0.5334	**0.5437**	0.0146
1	0.5990	0.7085	**0.6538**	0.0774
2.5	1.8731	1.8554	**1.8643**	0.0125
5	4.4888	4.4476	**4.4682**	0.0291
p-NP				
0	36.7338	38.2060	**37.4699**	1.0410
0.5	38.5800	36.3317	**37.4559**	1.5898
1	37.5214	38.6981	**38.1098**	0.8321
2.5	38.2343	38.5246	**38.3795**	0.2053
5	37.6856	37.7779	**37.7318**	0.0653

Table 40: Esterase Activity Blank

Butyrylcholinesterase					
	AUC (mAU*s)				
Hours	1	2	3	Mean Value	Standard deviation
Sample 1					
0	1053.586	1074.526	1105.603	**1077.905**	26.173
1	1116.486	1080.211	1086.753	**1094.483**	19.334
2	1026.516	1069.197	1027.106	**1040.940**	24.473
3	1145.375	1116.853	1133.661	**1131.963**	14.337
24	932.905	916.360	885.235	**911.500**	24.204
Sample 2					
0	1046.971	1058.206	1063.640	**1056.272**	8.501
1	1071.941	1046.024	1083.944	**1067.303**	19.381
2	1057.960	1028.654	1046.964	**1044.526**	14.804
3	1103.123	1097.847	1103.659	**1101.543**	3.212
24	1031.323	1050.182	1039.048	**1040.184**	9.481
Sample 3					
0	996.064	1002.081	993.272	**997.139**	4.502
1	1031.918	1015.128	-	**1023.523**	11.872
2	1032.838	1028.212	1036.518	**1032.523**	4.162
3	1036.542	1022.421	1021.111	**1026.691**	8.556
24	1123.135	1118.163	1130.698	**1123.999**	6.312

Table 41: SNAP-7941 Metabolism; Butyrylcholinesterase

Lipases

Pretests to verify the functionality of selected Lipases

Amano Lipase PS Burkholderia cepacia		
	AUC (mAU*s)	
Hours	Sample 1	Sample 2
0	0	0
1	15963.46	7318.909
3	26466.99	14239.42

Table 42: Pretest; Amano Lipase PS Burkholderia cepacia

Lipase Candida cylindracea		
	AUC (mAU*s)	
Hours	Sample 1	Sample 2
0	0	0
1	5930.116	11588.55
3	7959.11	17971.52

Table 43: Pretest; Lipase Candida cylindracea

Lipase Type II Porcine Pancreas		
	AUC (mAU*s)	
Hours	Sample 1	Sample 2
0	0	0
1	4526.692	3341.138
3	7309.813	9623.668

Table 44: Pretest; Lipase Type II Porcine Pancreas

Amano Lipase PS Burkholderia cepacia					
	AUC (mAU*s)				
Hours	1	2	3	Mean Value	Standard deviation
Sample 1					
0	1845.561	1867.833	1901.501	**1871.632**	28.163
1	1901.513	1904.004	1907.563	**1904.360**	3.041
2	1822.391	1867.443	1888.436	**1859.423**	33.745
3	1812.129	1843.47	1858.006	**1837.868**	23.446
24	1905.682	1903.651	1910.652	**1906.662**	3.602
Sample 2					
0	799.2616	814.4438	794.0848	**802.5967**	10.5813
1	810.907	819.808	815.8226	**815.5125**	4.4586
2	811.9404	823.5324	836.0009	**823.8246**	12.0329
3	802.4577	828.8097	827.6636	**819.6437**	14.8945
24	791.4675	817.6098	813.0922	**807.3898**	13.9729
Sample 3					
0	2190.635	2286.841	2296.701	**2258.059**	58.599
1	2215.638	2221.325	2270.324	**2235.762**	30.066
2	2194.205	2239.448	2265.584	**2233.079**	36.113
3	2201.468	2242.989	2244.086	**2229.514**	24.295
24	2634.494	2733.784	2671.815	**2680.031**	50.152

Table 45: SNAP-7941 Metabolism; Amano Lipase PS Burkholderia cepacia

Lipase Candida cylindracea					
	AUC (mAU*s)				
Hours	1	2	3	Mean Value	Standard deviation
Sample 1					
0	440.5006	441.3039	-	**440.9023**	0.5680
1	427.6783	431.4404	431.298	**430.1389**	2.1321
2	425.0673	428.3639	433.0374	**428.8229**	4.0048
3	427.2472	436.7067	433.7143	**432.5561**	4.8349
24	424.8983	432.1097	430.4397	**429.1492**	3.7749
Sample 2					
0	1725.885	1760.206	1795.716	**1760.6023**	34.9172
1	1777.591	1767.327	1799.279	**1781.3990**	16.3128
2	1743.825	1782.348	1806.631	**1777.6013**	31.6709

3	1739.439	1759.121	1811.606	**1770.0553**	37.3053
24	1763.73	1770.41	1804.148	**1779.4293**	21.6660
Sample 3					
0	580.7505	584.0578	584.0803	**582.9629**	1.9160
1	587.1418	590.9882	586.1303	**588.0868**	2.5631
2	589.225	589.3448	593.8626	**590.8108**	2.6436
3	593.3894	590.1354	588.803	**590.7759**	2.3593
24	602.8653	577.3023	583.1947	**587.7874**	13.3861

Table 46: SNAP-7941 Metabolism; Lipase Candida cylindracea

Lipase Type II Porcine Pancreas					
	AUC (mAU*s)				
Hours	1	2	3	Mean Value	Standard deviation
Sample 1					
0	1771.174	1716.983	1724.479	**1737.545**	29.363
1	1738.036	1746.99	1759.763	**1748.263**	10.919
2	1752.56	1779.016	1763.855	**1765.144**	13.275
3	1734.666	1770.413	1781.429	**1762.169**	24.447
24	1784.202	1804.191	1825.23	**1804.541**	20.516
Sample 2					
0	1094.596	1097.986	1108.591	**1100.391**	7.301
1	1085.352	1111.566	1107.604	**1101.507**	14.130
2	1104.35	1099.367	1108.323	**1104.013**	4.487
3	1080.165	1092.057	1112.766	**1094.996**	16.498
24	1111.947	1126.041	1120.652	**1119.547**	7.112
Sample 3					
0	699.6543	678.9885	705.7416	**694.7948**	14.0230
1	701.3988	707.3441	701.3573	**703.3667**	3.4446
2	710.0883	707.4544	716.1515	**711.2314**	4.4598
3	708.6046	694.7894	718.9376	**707.4439**	12.1159
24	700.6925	706.8743	-	**703.7834**	4.3712

Table 47: SNAP-7941 Metabolism; Lipase Type II Porcine Pancreas