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„Identification and quantification of Peptides

via HPLC and MS“

verfasst von / submitted by

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Bhagavadgita XI Sloka 38

Tvam Adidevah Purusha Puranas

Tvam Asya Visvasya Param Nidhanam

Vetta´si Vedyam Ca Param Ca Dhama

Tvaya Tatam Visvam Anantarupa

You are the First among the Gods, the Primordial Purusha, the supreme refuge.
You are the knower and the knowing and the highest goal.
The entire universe is permeated by You, oh Lord of infinite form.

I would like to thank Ao. Univ.-Prof. Dr. Mag. pharm. Ernst Urban for his supervision and for giving me the possibility to work in such a great team and writing my diploma thesis in the pharmaceutical chemistry.

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A further thank you goes to my friends who supported me in every situation in this study, especially Jelena, Cathrin and Sebastian. You are not only friends to me, but above all you belong to my family.

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Abstract

Proteomics is a massive scientific field using tools, including High performance liquid chromatography and mass spectrometry. This large scale study of proteins is one of the most popular disciplines for characterizing gene function and for giving insight into the mechanism of biological processes. **[Zhu *et al.,* 2003]** In this study, the sample P02769 derives from a cortical region, dentate gyrus that is composed of three layers, namely the molecular layer, the granule cell layer and the polymorphic cell layer **[Amaral, Scharfman *et al.,* 2007]**. Based on some researches, hippocampal neurogenesis is reported to play an important role in both psychiatric and neurological disorders including addiction, schizophrenia, epilepsy and depression. **[Eisch, Cameron *et al*., 2008]**. In this study we demonstrate that amino acids can be separated under various pH conditions, performing it as preliminary studies for bovine serum albumin. In the practical part, amino acids were separated, fractionated as well as used as external and internal standards. These studies were important for the final separation and pre-fractionation of the sample P02769.

Further experiments were performed with BSA and amino acids in combination. In this case we could reveal the amount of Tryptophan in BSA. Based on HPLC fractionation and the standard addition method, we compare the results to determine which method minimizes the loss of the sample.

In case of BSA the limit of detection (lower LOD = 0.002 µg/µL, upper LOD = 2.727 µg/µL) was measured. Furthermore, the limit of quantification (LOQ =0.559 µg/µL) was calculated. BSA was used in a preliminary study for the sample P02769. Since we were able to perform HPLC runs in a pH of 10 on BSA, we found that the method is suitable to measure brain samples.

The protein mixture, P02769, was finally pre-fractionated by HPLC at a pH of 10 and measured with LC-MS at a pH of 3. Pre-fractionation is a very important tool as the protein complexity can be reduced and thus more protein groups can be identified. **[Smidak *et al*., 2016]**

Kurzfassung

Die Proteomik ist ein riesiges wissenschaftliches Gebiet, das unter anderem die Hochleistungs-Flüssigkeitschromatographie und die Massenspektrometrie nutzt. Diese groß angelegte Untersuchung von Proteinen ist eine der beliebtesten Disziplinen zur Charakterisierung der Genfunktion und zur Aufklärung des Mechanismus biologischer Prozesse. **[Zhu *et al.*, 2003]** In dieser Studie stammt die Probe P02769 aus einer kortikalen Region, Dentate Gyrus, die aus drei Schichten besteht, nämlich der molekularen Schicht, der Granulatzellschicht und der polymorphen Zellschicht **[Amaral, Scharfman *et al.,* 2007].** Basierend auf einigen Untersuchungen wird berichtet, dass die Hippocampus-Neurogenese sowohl bei psychiatrischen als auch bei neurologischen Erkrankungen wie Sucht, Schizophrenie, Epilepsie und Depression eine wichtige Rolle spielt. **[Eisch, Cameron *et al.,* 2008].** In dieser Studie zeigen wir, dass Aminosäuren unter verschiedenen pH-Bedingungen aufgetrennt werden können und führen sie als Vorstudie für Rinderserumalbumin durch. Im praktischen Teil wurden Aminosäuren getrennt, fraktioniert sowie als externe und interne Standards verwendet. Diese Studien waren wichtig für die endgültige Trennung und Vorfraktionierung der Probe P02769.

Weitere Experimente wurden mit BSA und Aminosäuren in Kombination durchgeführt. In diesem Fall konnten wir die Menge an Tryptophan in BSA nachweisen. Basierend auf der HPLC-Fraktionierung und der Standardadditionsmethode vergleichen wir die Ergebnisse, um festzustellen, welche Methode den Verlust der Probe minimiert.

Bei BSA wurde die Nachweisgrenze (untere LOD = 0,002 µg/µL, obere LOD = 2,727 µg/µL) gemessen. Weiterhin wurde die Grenze der Quantifizierung (LOQ = 0,559 µg/µL) berechnet. BSA wurde in einer Vorstudie für die Probe P02769 verwendet. Da wir in der Lage waren, HPLC-Läufe bei einem pH-Wert von 10 bei BSA durchzuführen, fanden wir heraus, dass die Methode geeignet ist, Gehirnproben zu messen.

Die Proteinmischung P02769 wurde schließlich durch HPLC bei einem pH-Wert von 10 vorfraktioniert und mit LC-MS bei einem pH-Wert von 3 gemessen. Die Vorfraktionierung ist ein sehr wichtiges Instrument, da die Komplexität der Proteine

reduziert werden kann und somit mehr Proteingruppen identifiziert werden können. **[Smidak *et al*., 2016]**

1. Introduction

1.1 Protein

1.1.1 What is a protein?

Proteins **(see figure 1)** are macromolecules containing more than 100 amino acids. They are polymeric compounds that include, apart from amino acids, also other structures, like fatty acids, mono-, oligo- or polysaccharides among others. **[Teuscher *et al*, 2012]**

One of their main tasks is to identify and bind other molecules. Proteins are highly selective. Each protein has a unique surface profile. The surface profile is characterized by the side chain of the amino acid. **[Müller- Esterl, 2011]**

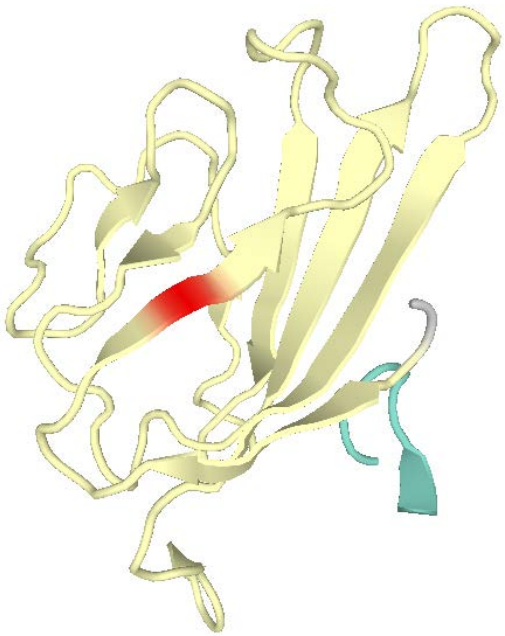


Figure 1 Bovine serum albumin.
This figure shows the protein structure of BSA. (Image taken from https://swissmodel.expasy.org/interactive/x2q8Ry/models/).(22.07.2016)

In biochemistry and molecular biology total protein determination is used very often. Lots of assays were developed. The protein assays can be divided in two categories: 1) UV absorbance and 2) colorimetric or fluorimetric dye binding assays. Three different aromatic amino acids are part of the proteins that contain benzene, phenol and indole rings and thus it is possible that these functional groups are stimulated to fluorescence by UV light. **[Wisńiewski *et al.*, 2015]**

1.1.2 Protein structure

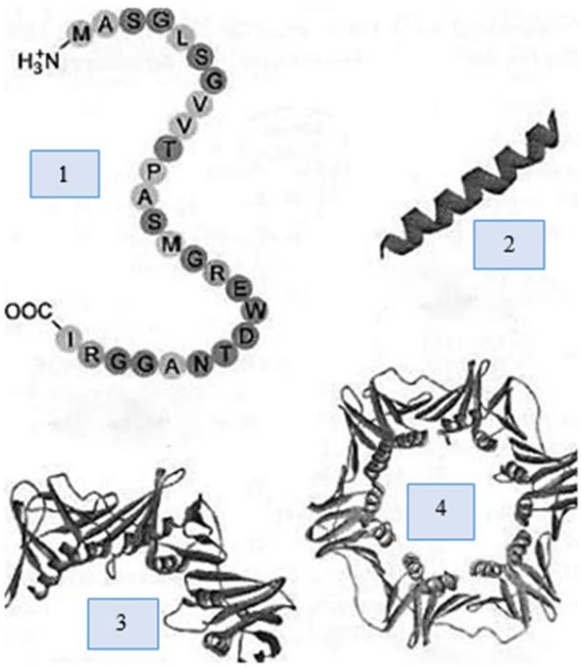


Figure 2 Protein structure formations.

This picture shows the primary, secondary, tertiary and quaternary structure of proteins.
(Image was taken from Müller- Esterl, 2011).

1) The primary structure is described as the linear sequence of amino acids. They are bound through a covalent linkage.

2) The secondary structure defines the spatial organisation of the adjacent amino acids in the linear sequence.

3) The tertiary structure is a 3D arrangement of the whole protein.

4) At the end a finished folded polypeptide chain can self-assemble with chains of other polypeptides forming a quaternary structure. **[Müller- Esterl , 2011]**

1.1.3 Tryptophan

Tryptophan is an amino acid which has the 3- letter code Trp and 1- letter code W. In this study tryptophan was used as the internal standard to determine the content of Trp in BSA **(see experimental studies).** In biochemistry, the fluorescence of tryptophan was mainly used for the analysis of protein structures and their functions. Although several studies have demonstrated that the tryptophan content in proteins could be determined by fluorescence measurement, this method was rarely used for total protein quantification. **[Wisńiewski *et al.*, 2015]**

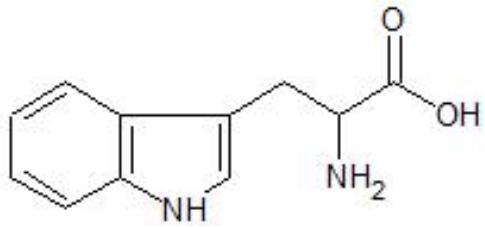
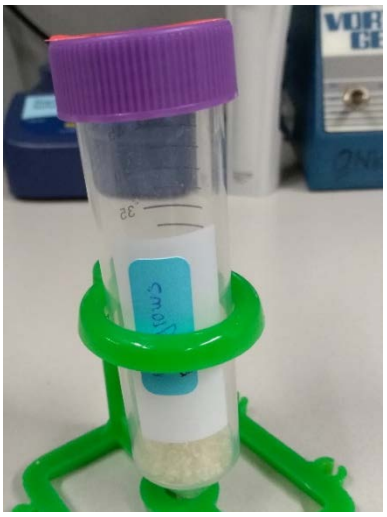


Figure 3 Tryptophan.

This image shows the structural formula of TRP. (drawn with the program chem sketch).

1.1.4 Bovine Serum Albumin



BSA is a protein with a molecular mass of 66.4 kDa. It contains 583 amino acid residues and 17 pairs of disulfide bridges. BSA is similar to Human serum albumin. However, BSA is more available and has lower costs. Thus, BSA is chosen more often as a model protein. **[Zhang *et al.*, 2016]**

Figure 4 Bovine Serum albumin.

This figure shows BSA lyophilized.

1.2 Analytical methods

1.2.1 Chromatographic methods

For characterization, identification and quality control of medical substances, the separation of substance mixtures and quantification of single substances, is required. Generally, chromatographic methods apply stationary and mobile phases for separation of substances.. The separation is based on different retention periods of the analytes in the stationary and mobile phase. **[Dominik *et al.*, 2013]**

Table 1 Various forms of chromatography. [Dominik *et al*, 2013]

mobile phase	stationary phase	process
gaseous	solid	gas chromatography
gaseous	liquid	gas chromatography
liquid	solid	column chromatography
		thin layer chromatography

Chromatographic processes can be divided according to their execution technology into:

Column chromatography: the stationary phase is packed in a glass, synthetic material or metal column, the mobile phase flows through column.

Gas chromatography (GC): This is also a column chromatography, however, a gas is used as the mobile phase. The stationary phase is liquid or a polymer inside a column. Therefore, some special apparatuses, e.g. gas separator are needed.

Thin layer chromatography: The stationary phase is applied as thin layer to a carrier, for example a glass plate or an aluminium film. The mobile phase is a liquid and has to be different from the stationary phase.

1.2.2. HPLC- High performance liquid chromatography

HPLC is an important analytical tool of drug discovery in the pharmaceutical industry. New potential drugs have to be tested during drug development. Therefore, the metabolism of the potential drugs has to be determined followed by preclinical and clinical trials. **[Kazakevich, Lobrutto, 2017]**

History of the discovery and the early development

Michail Semjonowitsch Zwet discovered liquid solid chromatography (LCS) in the early 1900. **[Tswett, M. S, 1905].**

Further improvements of this technology resulted in additional chromatographic methods such as gas chromatography, thin layer and liquid- liquid chromatography. Liquid-liquid chromatography as it exists in its current form can be attributed mostly to the work of Prof.C.Horvath at Yale University. He constructed an instrument that allowed the continuous flow of liquid through a column containing a different liquid. As a result, the contemporary high performance liquid chromatography arose. **[Kazakevich, Lobrutto, 2017]**

Principal of high performance liquid chromatography

High performance liquid chromatography (HPLC) is a liquid- solid- chromatography. HPLC is a special form of column chromatography, where the sample is pushed through the column by pressure generated by a pump. **[Rücker *et al.*, 2013]** However, in column chromatography the mobile phase flows through gravitation through a separation column, which is filled with the stationary phase. **[Dominik *et al.*, 2013]**

1.2.2.1 Reversed phase HPLC

Reversed phase (RP)

In reversed phase chromatography, materials, which are chemically modified, are used. The polar surface of a carrier (for example silanol groups of silica gel) in the production of RP particles will be made hydrophobic through the implementation of alkylchlorsilane. This way the surface gets a cover with lipophilic alkyl groups. Here the stationary phase is more apolar than the mobile phase. This can be achieved by the esterification of the polar silanol groups from the silica gel with organosilicon compounds. **[Rücker *et al.*, 2013]**

Table 2 chromatographic specifications.

This table shows typical chromatographic specifications of analytical HPLC applications. **[Dominik *et al.*, 2013]**

Specifications	Examples
separation principles	adsorptions chromatography and partition chromatography (Normal Phase and Reversed Phase), ion-pair chromatography, ion exchange chromatography, exclusion chromatography
column	particle size: 3-10 µm column length : 10 – 50 cm column diameter : 4 – 10 mm
flow improvers	water, acetonitrile, methanol, dichlormethane, propanol
flow improvers additions	Trifluoroacetic acid, Acetic acid, salts, puffer
detectors	UV/VIS detector, fluorescence detector, electrochemical detector etc...

1.2.2.2 Isocratic elution and gradient elution

One refers to an *isocratic elution* when the composition of the flow medium is constant during a chromatographic run.

Isocratic elution can be applied, if the substances have similar retention times. **[Dominik *et al.*, 2013]**

Substances with different chromatographic characteristics and therefore varying retention times, require the modification of the flow medium mixture during the chromatographic separation to elute the substance by their polarity. This is known as gradient elution. **[Dominik *et al.*, 2013]**

1.2.2.3 Requirement to the mobile phase

The mobile phase used in the HPLC has to be highly pure and should be degassed before utilization. By performing sonication (in a vacuum), by using an inert-rinsing with helium or by filtration through filter membranes with limiting pores, degassing can be accomplished. The function of the pumps can be disrupted by gas bubbles. They arise, if the flow medium is not degassed correctly. This causes disturbing signals during detection. **[Dominik *et al.*, 2013]**

Flow rate

The separation performance of a chromatographic system depends on the elution speed. **[Dominik *et al.*, 2013]**

1.2.2.4 Set-up of an HPLC device

Figure 5 Construction of a HPLC.

This image shows the schematic building of a HPLC and its various regions.

https://www.shimadzu.com/an/hplc/prominence/lc20.html (08.05.2018)

HPLC Pumps

For analytical applications, flow rates of 0.1 -10 mL/min and pressures up to 400 bar are used. The pumps insure that the flow is constant, which is very important for the mobile phase. During detection, the pumps should work with low pulsation. This ensures that signal fluctuations will be avoided. **[Dominik *et al.*, 2013]**

Auto sampler

An auto sampler is useful if there are several samples and enables an automatic sample application. In this way series of trials can be programmed and performed without constant manual injection. **[Dominik *et al*., 2013]**

Separation Column

Depending on their requirements, analytical columns have a length of 5 to 30 cm and an internal diameter of 2 to 8 mm. The column packing is determined by the particle size, material, pore size and texture of the surface. **[Rücker *et al.*, 2013]**

UV/VIS Detector

The most commonly used detector in HPLC is the UV/VIS detector **(see schematic presentation of UV/VIS in figure 6)**. The light absorption of the eluate will be measured. The structure corresponds to a photometer, which has a flow through cuvette. If a substance gets in the measurement cell of the detector, the light absorption increases and the substance will be registered as a peak in the chromatogram.

There are UV/VIS detectors, which can measure in one or more specified wavelengths and others that have a variable wavelength (measured wavelengths can be chosen). Photo diode array detectors register numerous wavelengths of the light absorption from the eluate simultaneously. This gives an UV spectrum of the eluate at each time point of the chromatogram. In this ways peaks can be identified also by their UV spectrum and not only by their retention times. **[Dominik *et. al*, 2013]**

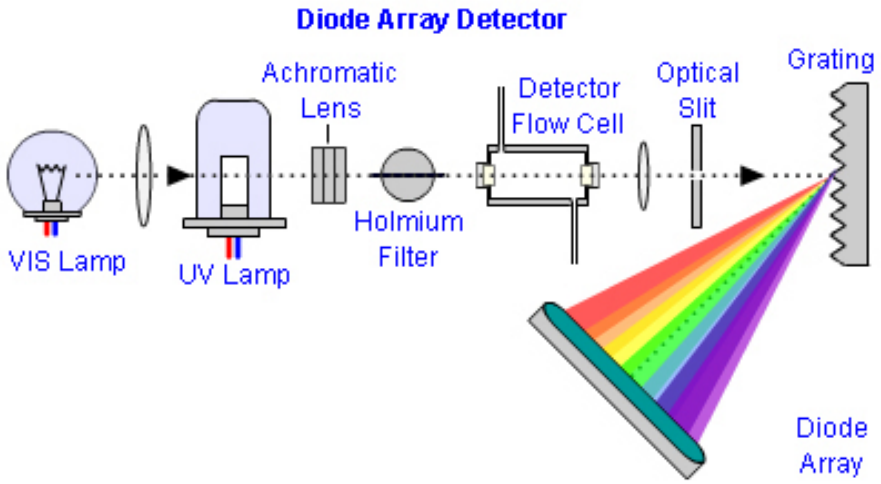


Figure 6 Diode array detector.

This image shows the schematic construction of a diode array detector and how the different wavelengths result. (http://www.crawfordscientific.com/Chromatography-Technical-Tips-Diode-Array-Detector-Settings.html) (20.12.2017)

1.2.3 Peptide and Proteins Separation with HPLC

Reversed phase HPLC is a very important tool in the separation and analysis of proteins and peptides. It is of utmost importance for the separation of peptides from digested proteomes before protein quantification by mass spectrometry is performed. HPLC is also able to separate similar proteins. Proteins are very often broken down into pieces. This is done using protease enzymes. The resulting peptide fragments are then analysed by reversed-phase HPLC. The most common enzyme applied in the cleavage of proteins is trypsin **(see figure 7).** There are five steps in the digestion of proteins by trypsin: denaturation, reduction of disulphide bonds, carboxymethylation of free cysteines, removal of salt, trypsin digestion.
Proteins as well peptides can be separated with HPLC. Very similiar proteins that only differ in three amino acids can be detected. And peptides only have to differ in one amino acid. **[David Carr]**

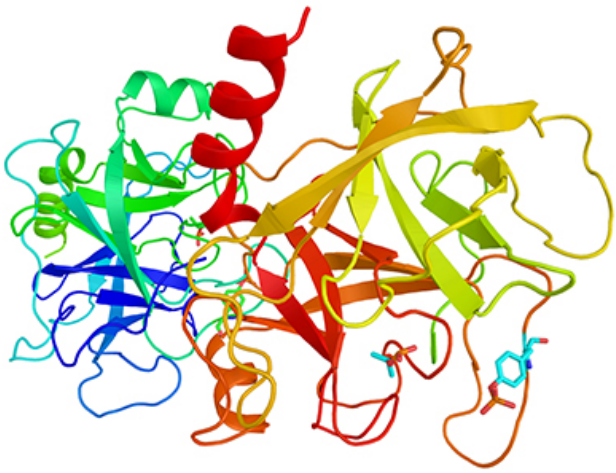


Figure 7 Trypsin.
This figure depicts the structure of Trypsin, which is a very important enzyme in protein digestion.

https://www.merckmillipore.com/AT/de/20170616_152646?ReferrerURL=https%3A%2F%2Fwww.google.at%2F&bd=1 (19.05.2018)

1.2.4 Proteomics

The protein species composition of an organism, or only a part of it, is called proteome. **[Jungblut, Schlüter]** The goals of proteome research are comprehensive, quantitative descriptions of protein expression and possible changes that can be caused by biological disorders due to drug treatment or illness. **[Anderson N.L, Anderson N.G, 1998].**

In proteomics **(see figure 8)**, three areas are important, including the fractionation of proteins and peptide mixtures, as well the MS that is necessary for the collection of data, because this enables the identification of proteins, and finally bioinformatics. Bioinformatics analyses and compiles MS data. **[Yu, Stewart *et al.,* 2010]**

Mass spectrometry is the method of choice for both proteome analysis and protein identification. Furthermore, liquid chromatographic separations have also proven their worth. When low resolution devices are used, experimental methods such as tandem MS (MS/MS) are preferred. This ensures better identification. **[Hixon, Lopez-Ferrer *et al.*, 2010]**

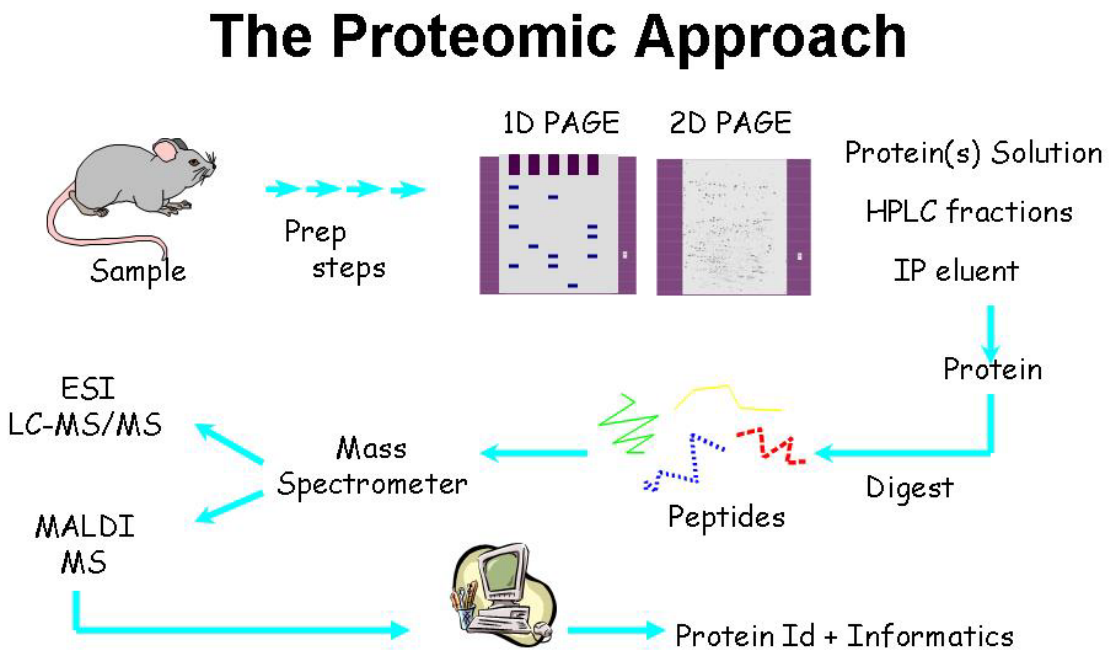


Figure 8 Proteomics

This figure demonstrates the way from sample to analysed data.

http://proteomics.arizona.edu/ (19.05.2018)

1.2.5 Mass spectrometry (MS)

Areas in which Mass Spectrometry is used

This technique is used for routine and research purposes in industrial and academic settings.

The following list clarifies the major mass spectrometric applications:

• *Pharmaceutical:* drug discovery, combinatorial chemistry, pharmacokinetics, drug metabolism,

• *Clinical:* neonatal screening, haemoglobin analysis, drug testing,

• *Environmental:* water quality, food contamination,

• *Geological:* oil composition,

• *Biotechnology:* the analysis of proteins, peptides. **[Hassan, 2012]**

Commitment of mass spectrometry in pharmaceutical analytic

In connection with HPLC, mass spectrometry allows, as a fast, reproducible and high sensitive analytical process, the detection of impurities and degradation products. **[Sonsmann, 2001]**

Table 3 Applications of mass spectrometry.

This table shows the different fields where mass spectrometry can be used. https://www.thermofisher.com/at/en/home/life-science/protein-biology/protein-biology-learning-center/protein-biology-resource-library/pierce-protein-methods/overview-mass-spectrometry.html

Field of Study	Applications
Proteomics	Determine protein structure, function, folding and interactions
	Identify a protein from the mass of its peptide fragments
	Detect specific post-translational modifications throughout complex biological mixtures
	Quantitate (relative or absolute) proteins in a given sample
	Monitor enzyme reactions, chemical modifications and protein digestion
Drug Discovery	Determine structures of drugs and metabolites
	Screen for metabolites in biological systems
Clinical Testing	Perform forensic analyses such as confirmation of drug abuse
	Detect disease biomarkers (e.g., newborns screened for metabolic diseases)

1.2.5.1 Q-Exactive Plus mass spectrometer

Since we worked with Q-Exactive Plus mass spectrometer, a short overview about some important functions

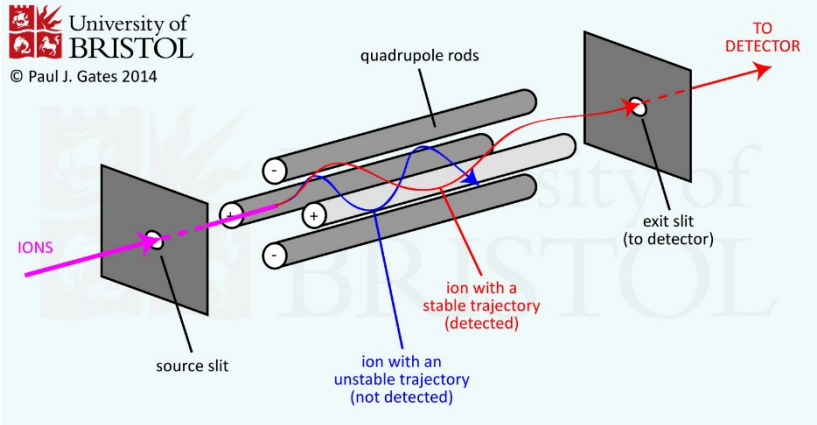
Orbitrap mass analyzers developed into the most frequently used mass analyzers in proteomics. They have an excellent combination of sequencing speed and high resolution, sensitivity, mass accuracy and dynamic range. **[Kelstrup *et al*., 2014]**

Orbitrap Functional principle: Ions are captured via a central spindle electrode. An outer electrode is coaxial to the inner electrode. The mass-/ charge values are measured from the frequency of the ion oscillations along an axis which lies in an electric field. **[Hu *et al*., 2005]**

Quadrupol function:

The ion beam of the substance to be analyzed is guided in the longitudinal direction between four parallel metal rods. With the opposite pair of rods, DC voltage is applied which interferes with an AC voltage, creating an electric field in the interior between the rods. This electric field is only for ions which have a certain m/z ratio and only these reach the rods via a stable oscillating trajectory in longitudinal direction. All other ions with different m/z ratios collide with the rods as they fly on unstable orbits. **[Rücker *et al*., 2007]**

Figure 9 Quadrupol mass analysis.
The figure schematically represents quadrupole.
http://www.chm.bris.ac.uk/ms/quadrupole.xhtml (03.06.2018)



1.2.5.2 Peptide preparation to enter in the MS

The first step after the purification of proteins is to break them down into peptides by using a protease, which is sequence specific. A mass spectrometer is able to measure the mass of intact proteins, but there are a lot of reasons why peptides and not proteins are analysed. Not all proteins might be soluble under same conditions, and are thus difficult to handle. Additionally, the mass spectrometer is much more sensitive when analyzing peptides rather than proteins. Furthermore, if the aim is to identify a protein, the sequence information is needed. The mass spectrometer is better at receiving sequence information of peptides than of whole proteins. After protein digestion, the peptides are first separated by HPLC, which is connected to the mass spectrometer. The peptides are eluted using a solvent gradient, in order to elute the peptides depending on their hydrophobicity. Hydrophilic peptides will be eluted first and hydrophobic peptides later on. The mass spectrometer is able to differentiate the peptides by its masses, so there is no reason to separate them into non-overlapping chromatographic peaks. Chromatographic columns are produced as small as packing allows and plugging can be avoided. These columns have an inner diameter between 50 and 150 µm. **[Steen, Mann, 2004]**

1.3 Validation of analytical methods

1.3.1 The limit of detection (LOD)

For any analytical procedure, the limit of detection (LOD), is the point where analysis is feasible. **[Armbruster *et al.*, 1994]**
LOD gives a signal-to-noise-ratio of about 3:1. **[Kazakevich, Lobrutto, 2017]**

1.3.2 Limit of Quantitation (LOQ)

LOQ is the lowest concentration at which the analyte can be detected.
[**Armbruster, Pry, 2008]**

Every separation method has a minimal concentration at which the sample can be detected and quantitated. LOD gives a signal-to-noise-ratio of about 3:1. A peak in limit of quantification gives a signal to noise ratio of about 10:1.
From the injected sample, a peak will be selected when the height is about 3 to 10 times larger than the noise. [**Kazakevich, Lobrutto**, **2017**]

1.3.3 Calibration

Calibration is a very important step during analysis. The concentration of samples cannot be measured directly in spectrometric methods and therefore it is necessary to use the physical measurement of the absorbance of a solution. A theoretical relationship can be demonstrated between the quantity and the concentration of the analyte. The most auspicious calibration function is linear. In practice, deviations appear due to possible impurities of the sample from the ideal calibration line. The calibration equation is $Y = a + bX$. **[Kapil, 2011]**

1.3.4 Linearity

A linear correlation should be calculated over the whole range of the analytical spectrum. It can be superimposed directly on the drug substance (by dilution of a standard stock solution). By visual inspection the linearity should be analyzed of a plot of signals as a function of analyte concentration or content. For the determination of linearity, a minimum of 5 concentrations is advised. **[ICH Expert Working Group, 1994, 2005]**

1.3.5 Standardaddition-method

More than 50 years ago, Chow and Thompson first described the standard addition method. **[Kelly, MacDonald *et al*., 2008]** The standard addition method is excellent for correcting matrix effects. **[Cimetiere, Soutrel *et al*., 2013]** Furthermore, this method is mainly used for chemical determinations. In the standard addition method, different amounts of analytes (x0 < x1 < x2...) are added to unknown solutions of constant volumes. Solutions with the same concentration are produced, but the concentration of the analyte increases. **[Kelly, MacDonald et al., 2008]** Standard addition method is combined with an empty addition and is usually added at the same distance. **[Steliopoulos, 2015], [Kelly, MacDonald *et al*.,2008]**

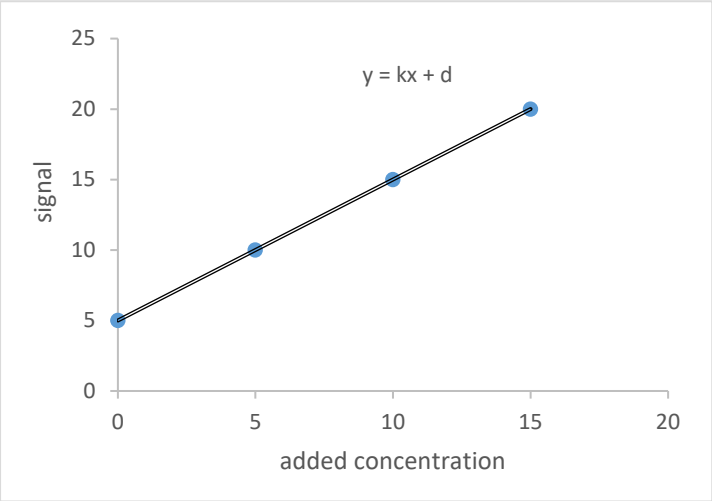


Figure 10 Standardaddition.
In this figure the standard addition method is described. By adding an analyte, you create a linear line. The formula is y=kx+d -> d denotes the section on the y-axis, k is the slope of the linear line.

1.4 Hippocampus

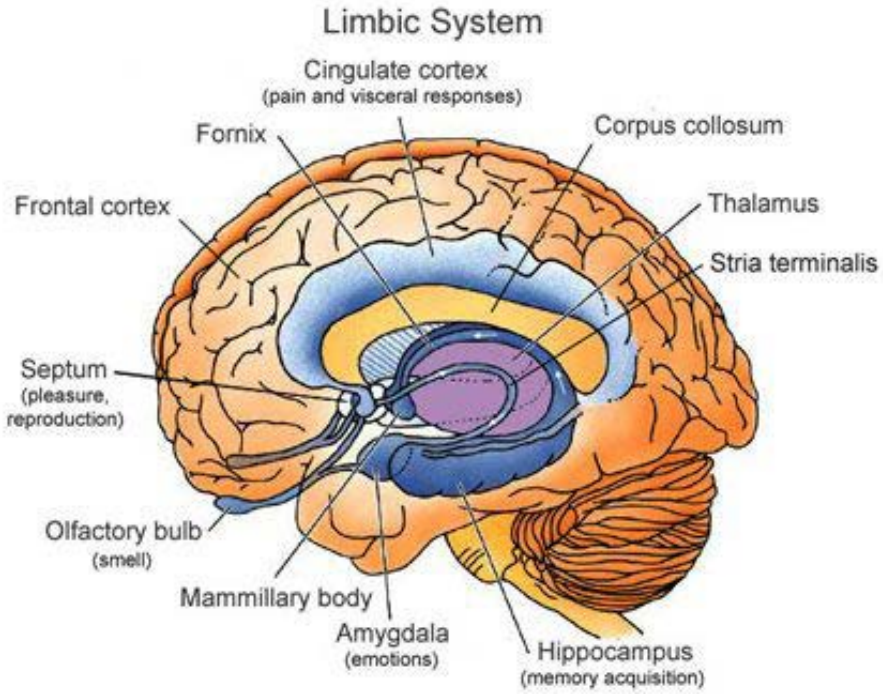
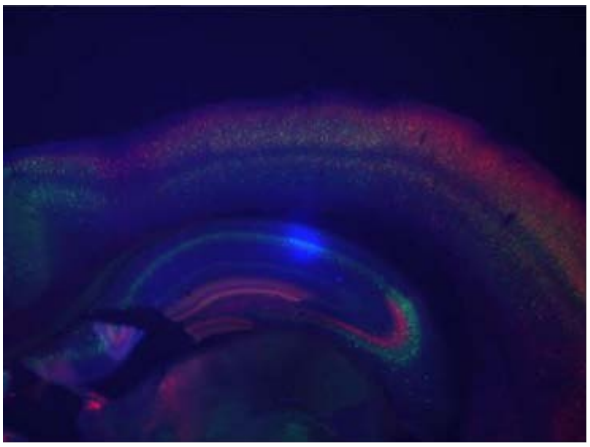


Figure 11 Limbic System.

This figure represents the various regions of the limbic system. In our study we focused on the hippocampus and especially on the dentate gyrus.

http://heritance.me/anatomy-of-brain-psychology/anatomy-of-brain-psychology-neuroscience-touch-and-the (20.05.2018)

The limbic system lies on the brainstem hood shaped. It is a phylogenetical old brain part (archicortex with periarchicortex). This includes:



- ➔ hippocampus
- ➔ gyrus parahippocampalis
- ➔ gyrus cinguli
- ➔ bulbus olfactorius

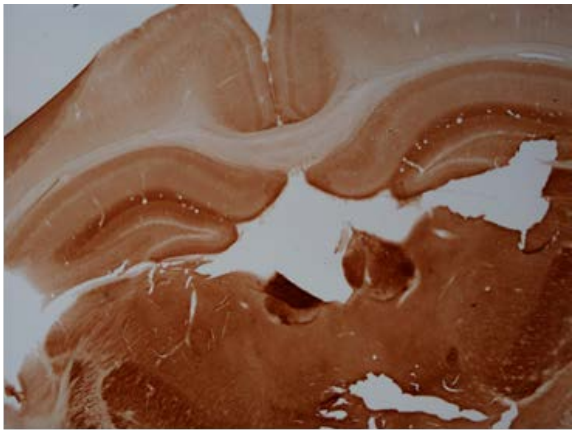
Figure 12 hippocampus cross section.

This image demonstrates the hippocampus of a mongolian gerbil (*Meriones unguiculatus).*
(Photo was taken by Dr. Sonja Brosel)

From the hippocampus a fiber web extends, the fornix, which is arched to the hypothalamus, more specifically to the corpus mamillare. **[Thews, Mutschler, 1999]** To the limbic system also belongs the subcortical nucleus, particularly the nucleus amygdalae and associated brain structures. This system plays a central role during experience and memory formation. Also motor control will be influenced. **[Bannerman *et al.,* 2014]**

The hippocampus is of great importance for long-term memory**. [Bird, Burgess, 2008]** One of the few brain regions showing adult neurogenesis is the formation of the hippocampus and, above all, the dentate gyrus. **[Abellan, Desfilis *et al.*, 2014]** The dentate gyrus is a cortical region and is composed of three layers, which are the molecular layer, the granule cell layer and the polymorphic cell layer. **[Amaral, Scharfman *et al.*, 2007]**

According to some evidence, hippocampal neurogenesis is reported to play an important role in both psychiatric and neurological disorders, such as addiction, schizophrenia, epilepsy and depression. **[Eisch, Cameron *et al.*, 2008]** Very important for survival is the ability to learn and remember spatial location. The hippocampus is affiliated with the spatial navigation system and spatial memory in rodents as well as in humans. **[Bannerman *et al.*, 2014]**



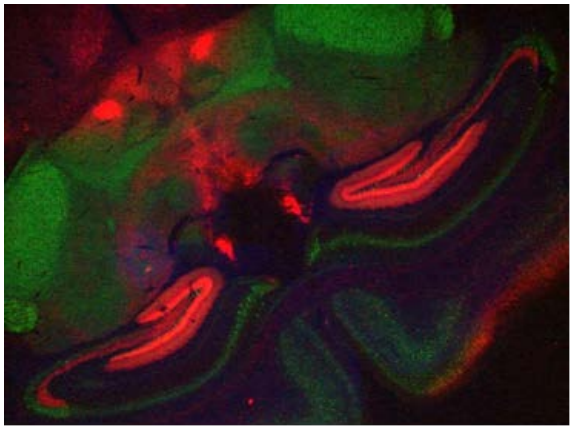


Figure 13 and 14 cross section of mongolian gerbil. (Image was taken by Dr. Sonja Brosel)

2. Material and Methods

BSA – Digestion

Urea, 99.5%, for molecular biology, DNAse, RNAse, and Protease free BioRad 161-0745-MSDS

Iodoacetamide, Sigma Aldrich I1149-25G

TEAB - Triethylammonium bicarbonate buffer, Sigma Aldrich 17902

1,4-Dithiothreit DTT, Carl Roth 6908.2

Incubator: Heidolph Unimax 1010

Trypsin, Sequencing Grade, Promega V5111

Trifluoroacetic acid: Merk Millipore1082620100

BSA: Bovine Serum Albumin – Standard (Batch 18889912); Product # Roche: 238031/1g; Manufacturer: Originally Boehringer Mannheim, from 2002 Roche

Solid phase extraction

Acetonitrile - ROTISOLV; LC-MS Grade Carl Roth AE70.1

Vacuum Centrifuge: Centrifuge 5417R, Eppendorf

Column: Macro Spin Column, Silica C18; 50-450 µL loading, 30-300 µg capacity (The Nest Group)

Evaporation: Concentrator plus, Eppendorf

Peptides of Rat Brain and BSA

HPLC instrument: HPLC Shimadzu Prominence with FRC

Detector: UV/VIS Photodiode array detector, “prominence diode array detector” SPD-M20A, (CAT No: 228-45005-38; Serial No: L20154370038 US F, 220-230 V, 50/60Hz, 150 VA)

Auto Sampler: Sil-20AC prominence Auto Sampler

Column Oven: prominence Column oven CTO-20AC

Fraction Collector: FRC-10A Shimadzu Fraction collector

Column: C-18 column: Kinetex® 2.6µm EVO C18 100 A; Size: LC Column 150 x 2.1 mm; S/No.: H15-213924
Phenomenex

Reagents:

Mobile phase A: (5% ACN, 20 mM NH_4FA, pH 10)
Ingredients: Acetonitrile, water: and ammonium formate

a) **Water:** HPLC gradient grade water, Rotisolv © HPLC Gradient Grade, Art.-Nr. A511.2, Carl Roth GmbH + Co. KG, Charge 1088411
b) **ACN:** HPLC gradient grade acetonitrile, Rotisolv © HPLC Gradient Grade, Art.-Nr. 8825.2, Carl Roth GmbH + Co. KG, Charge 1088961
c) **Ammonium formate** (*Stock solution*): ammonium hydroxide solution, formic acid and water, pH 10

Formic acid: Formic acid Rotipuran ® ≥ 98%, p.a., ACS, Art.-Nr. 4724.3, Carl Roth GmbH + Co.KG, Charge 475236476

Ammonia solution: Ammonia solution 25%, Ph.Eur., Art.-Nr. 2610.1, Carl Roth GmbH + Co.KG, Charge 495232536

Mobile phase B: (90% ACN, 20 mM NH_4FA, pH 10)

Ultrasonic bath: Branson 5200

pH meter: inoLab WTW series pH720

Tecan: infinite M200 TECAN

Well plates: Cellstar- greiner bio one 96 well Cell cultur plate, sterile F bottom with lid

LC-MS: Thermo Q-Exactive Plus mass spectrometer (ThermoFisher Scientific, Austria) with Dionex Ultimate 3000 nano-LC system

MS: maXis HD Bruker

Amino Acids – Preliminary:

HPLC instrument: LC-2010 A HT (Cat. No. 228-45102-38; Serial No. C21244404642, 230 V, 50-60 Hz 700 VA), Liquid Chromatograph from Shimadzu Corporation

Column: C-18 column: EC 250/4.6 Nucleosil 100 -5 C18, Cat No.:720014.46, Ser. No.: 3115855, Batch 3119.

Mobile phase:A: HPLC gradient grade water, Rotisolv © HPLC Gradient Grade, Art.-Nr. A511.2, Carl Roth GmbH + Co. KG, Charge 1088411 (**)

Mobile phase B: HPLC gradient grade acetonitrile, Rotisolv © HPLC Gradient Grade, Art.-Nr. 8825.2, Carl Roth GmbH + Co. KG, Charge 1088961 (*)

HPLC instrument: LC-20AD XR prominence liquid Chromatography UFLC XR Shimadzu

Autosampler: SIL-20AC HT prominence autosampler Shimadzu

Fluorescence detector: RF- 20ª XS prominence fluorescence detector Shimadzu

Diode array detector: SPD-M20A prominence diode array detector Shimadzu

Column oven: CTO-20AC prominence column oven Shimadzu

Fraction collector: FRC-10A fraction collector Shimadzu

Content of Amino Acids - Preparation:

Protein powder

Membrane filter: CHROMAFIL®Xtra PES-45/25, 0.45µm

Absorbance

Instrument: UV-visible Recording Spectrophotometer UV- 160A, Shimadzu

Cuvettes: HELLMA 111-QS

IR Spectrometry

IR Spectrometer: PERKIN ELMER FT-IR Spectrometer, SPECTRUM 1000

KBr: Sigma Aldrich, Potassium bromide, 221864 – 100G Lot# SZBG0080V

Compactor: PERKIN ELMER, Hydraulic press

General equipment

Pipets 100-1000 µL Eppendorf Research
2- 20 µL Eppendorf Reference
50-200 µL Eppendorf Reference

Vortex: Vortex Genie 2

Computer Softwares

Microsoft Word
Microsoft Excel
Lab Solutions
Chem sketch

Used Databanks

Pubmed

Methods

Method A – HPLC measurement of amino acids

Time	Module	Command	Valve
0.01	**Autosampler**	**Rinse**	
0.02	Pump	Solvent C conc.	0
35.00	Pump	Solvent C conc.	50
35.01	Pump	Solvent C conc.	90
37.00	Pump	Solvent D conc.	100
40.00	**Controller**	**Stop**	

Method B - fraction collecting of amino acids

Time	Module	Command	Value
0.01	**Autosampler**	**Rinse**	
2.02	Pumps	Solvent D conc.	0
10.00	Pumps	Solvent D conc.	50
10.01	Pumps	Solvent D conc.	100
11.00	Pumps	Solvent D conc.	0
13.00	**Controller**	**Stop**	

Method C - fraction collecting of BSA and biological sample P02769

Time	Module	Command	Value
0.01	**Autosampler**	**Rinse**	
1.00	Pumps	Solvent B conc.	0
6.00	Pumps	Solvent B conc.	0
47.00	Pumps	Solvent B conc.	35
51.00	Pumps	Solvent B conc.	70
53.00	Pumps	Solvent B conc.	100
58.00	Pumps	Solvent B conc.	100
58.01	Pumps	Solvent B conc.	0
78.00	**Controller**	**Stop**	

3. AIMS

The aim of this diploma thesis was the identification of amino acids by UV /VIS, HPLC and high resolution MS, the separation of an amino acid mixture, containing His, Phe, Trp and Tyr and the quantification of several amino acids in one HPLC run. Further separation of amino acids were with phenomenex/ Kinetex® at pH10, which in turn was a preliminary study to BSA.

Due to the previous experimental part of the amino acids, we started using BSA following the final step with the biological sample P02769.
Thus, the limit of detection and the limit of quantification were determined by injecting different concentrations of BSA (20 µL per sample) into HPLC at pH10 with formate buffer, starting with a concentration of 1 mg/mL as the highest.
The development of a new HPLC method using an external standard led to further investigation included the recovery of Trp in BSA, by comparing the result to those of the standard addition.

Based on our previous data, the experiment with the sample P02769 were performed under the same pH conditions as BSA. This sample was pre- fractionated with HPLC and the recovered fractions were measured with the LC- MS (Thermo Q-Exactive Plus mass spectrometer (Thermo Fisher Scientific, Austria) with Dionex Ultimate 3000 nano-LC system).

Finally, generating diagrams of the identified peptides show that the fractionated sample P02769 afforded greater amount of peptides comparing to unfractionated.

4. EXPERIMENTAL PRELIMINARY STUDY

4.1 Experiments with amino acids

For the amino acid analysis proline, alanine and tryptophan were chosen. Each amino acid was prepared at a concentration of 1 mg/mL in water containing 0.1% TFA (Trifluoroacetic acid). In order to entirely dissolve the amino acid proline, the solution was treated in an ultrasonic bath for 10 minutes. Subsequently, absorbance of the amino acid was monitored (200-230nm) with the UV/VIS (Ultraviolet-visible spectroscopy), (**see table 4 and figure 16**). For that purpose, first one pipetted 1200 µL of the sample and then 1200 µL of the solvent TFA in H_2O in a cuvette (volume capacity 2500 µL) resulting in a 1:1 dilution. For His 20% ACN was selected because His dissolved better in this solvent. The maxima of Pro is at 224 nm and His at 226 nm. The peak max. of absorbance of Ala and Trp were not measured.

Then an HPLC measurement was performed. For that purpose, a 1:10 dilution of each amino acid was prepared directly in a vial pipetting 100 µL of the sample and 900 µL of the 0.1% TFA+H_2O.
For each run 20 µL of the samples were injected. For separation, a solvent gradient **(see method A, 0%-50%-90%, solvent C [ACN], Solvent D [H_2O with 0.1% TFA], see method A)** was applied, and 0.1%TFA+H_2O and ACN were used.

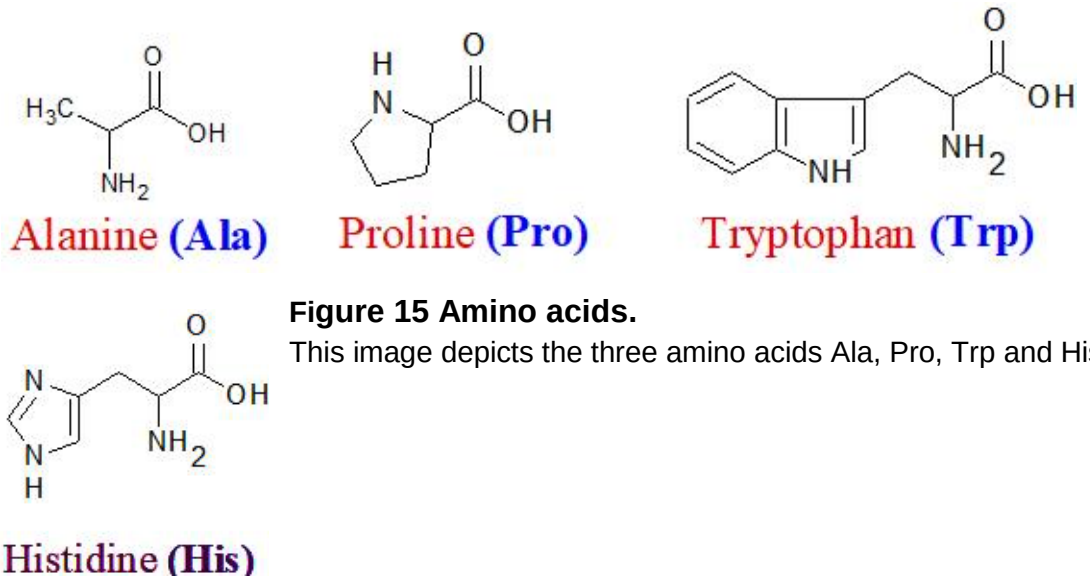


Figure 15 Amino acids.
This image depicts the three amino acids Ala, Pro, Trp and His.

Table 4 Absorbance measurement of various amino acids.
This table shows an absorbance scan for the AS Pro, Ala, and Trp from the wavelengths 200 to 230 nm. The maxima of Pro is at 224 nm and of His at 226 nm. The peak max of absorbance of Ala and Trp were not measured.

wavelenght[nm]	Pro	Ala	Trp	His
200	0.26	0.22	0.91	1.09
202	0.30	0.27	1.24	1.41
204	0.29	0.28	1,41	1.56
206	0.26	0.26	1.55	1.66
208	0.23	0.24	1.68	1.74
210	0.20	0.21	1.79	1.83
212	0.18	0.19	1.90	1.90
214	0.15	0.17	2.00	1.96
216	0.14	0.15	2.10	2.01
218	0.12	0.13	2.18	2.05
220	0.11	0.12	2.25	2.07
222	0.10	0.11	2.30	2.09
224	0.88	0.09	2.35	2.11
226	0.08	0.08	2.41	2.11
228	0.07	0.07	2.46	2.00
230	0.06	0.06	2.48	1.55

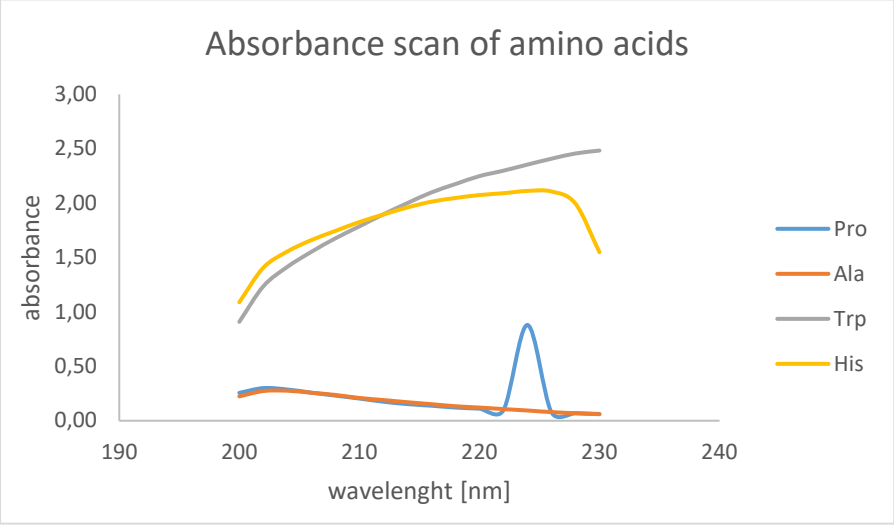


Figure 16 Uv/vis absorbance .

This graph demonstrates the absorbance of Ala, Pro, His and Trp.

After all single scans **(see appendix page 84- 86)** of the amino acids were performed, all amino acids were pooled together that had been easily detected at a wavelength of 215 nm, taking into account their retention times, followed by a separation chromatogram. (**see figure 17**).

The amino acids we have chosen were:

L-Histidin

L-Phenyalanine

L-Tryptophan

L-Tyrosine

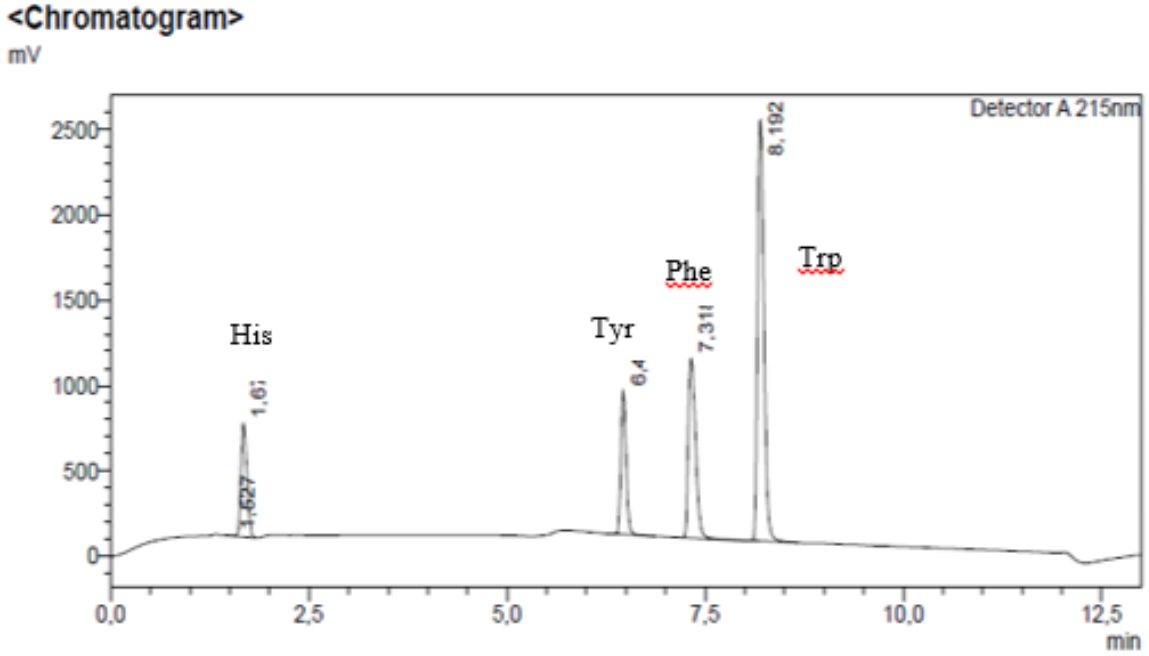


Figure 17 Separation chromatogram.
This depiction shows a separation of the chosen amino acids.

Amino acid	**Retention time**
His	1.7
Tyr	6.5
Phe	7.3
Trp	8.2

For further studies, His and Phe (**see figure 17**) were chosen due to their greater difference in retention time.

10.00 mg of His and 10.00 mg of Phe were weighted into a vial and dissolved in 1 mL of 20% ACN. Subsequently, HPLC measurement was performed by injecting 20 µL of the His and Phe mixture. The Measurement was taken at a wavelength of 215 nm. In the next step a total of 10 fractions were collected to separate these two amino acids. First, a method was developed to ensure that the fractions could be properly collected. A time program was created for this. The vial volume was 1 mL, measurements were taken at a wavelength of 190-747 nm.

Before the collection of fractions was started, a simulation spectrum was taken. For this purpose, a spectrum of previous measurements was utilized. This allowed us to show which amino acid arrived in which fraction tube. 1 mL was collected per tube (containing solvent and the amino acids) and the correct spectrum was recorded. According to calculations, 20 µL sample injection contained 0.2 mg of substance. Since 1 g was needed, to perform the IR, five passes had to be made to collect enough substance. Using previous measurements with amino acids, **see Figure 17**, the approximate time at which amino acid would result in a peak was possible to determine. For this reason, two amino acids with different retention times were chosen. His appeared in previous experiments at a retention time of 1.7 min and Phe reached at 7.3 min.

In the spectrum shown in **figure 18 (see appendix page 87)**, His only appeared at a retention time of 3.2 min and Phe, only at 11.9 min. Now the question arises why the retention times are different or why the amino acids only resulted in a peak so late. This is due to a different method to the previous experiment, see method A in comparison to method B, as well as possible disturbance factors such as impurities. In **figure 18** it is demonstrated that His showed a sharp peak, but a second peak was attached to it. This could be the aforementioned contamination. His was collected in fraction tubes 1 and 2. Phe also showed minimal changes in the peak. An impurity can also be assumed here. Phe was collected in fraction tubes 8 and 9. The liquids of the amino acids separated into fractions were pooled together in a flask, then lyophilized, which means that the solvent is removed and the solid remains. Finally an IR spectrum in order to determine the purity of the amino acids was performed. To record an IR spectrum, 1.3 mg His and 2.0 mg Phe were weighed in and mixed with potassium bromate. This mixture was placed in a pressing tool

and then a tablet was produced. This was inserted into the IR and a spectrum was detected. (**see figure 19 & 20, [appendix page 97,98], Method B, solvent gradient from 0%-50%-100%-0%, for Solvent D [ACN]**). On the basis of these control spectra we were able to assign our amino acids 1 and 2.

For the fractionation a general formula to calculate the time for fraction collecting, was created. This formula allowed to determine when all the fractions would be collected, which made it possible to optimize our fraction collection method.

Table 5 Time method.

This time method was developed for collecting fractions of the amino acids.

Fraction	Vial	Start Time[min]	End Time[min]
1	0	1.200	2.400
2	1	2.410	3.610
3	2	3.630	4.830
4	3	4.840	6.040
5	4	6.050	7.250
6	5	7.260	8.470
7	6	8.480	9.680
8	7	9.690	10.890
9	8	10.910	12.110
10	9	12.120	13.010

$$\frac{(Fr)_n \times V_{(vial)}}{F_L(pump)} = t_0$$

$$t_0 + t_1 = t_2$$

$$t_0 + t_2 = t_3$$

$(F_R)_n$ = fraction tube
$V_{(vial)}$= Volume which is collected
t= time to collect fractions
$F_{L(pump)}$ = flow

t_0… calculated time from the formula

t_1… Time out of the program

t_2… Half of the time where fractions were taken

t_3… Time when all fractions were collected

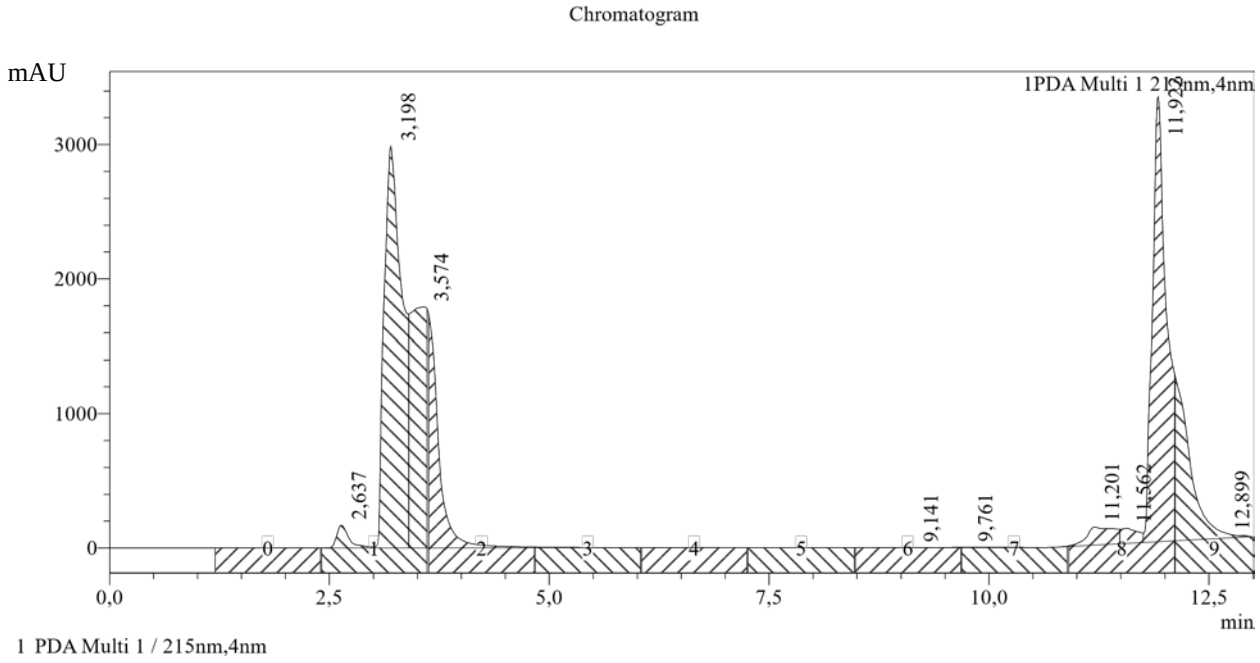


Figure 18 Separation chromatogram.
This figure depicts the HPLC measurement of the two amino acids and at which time they were collected.

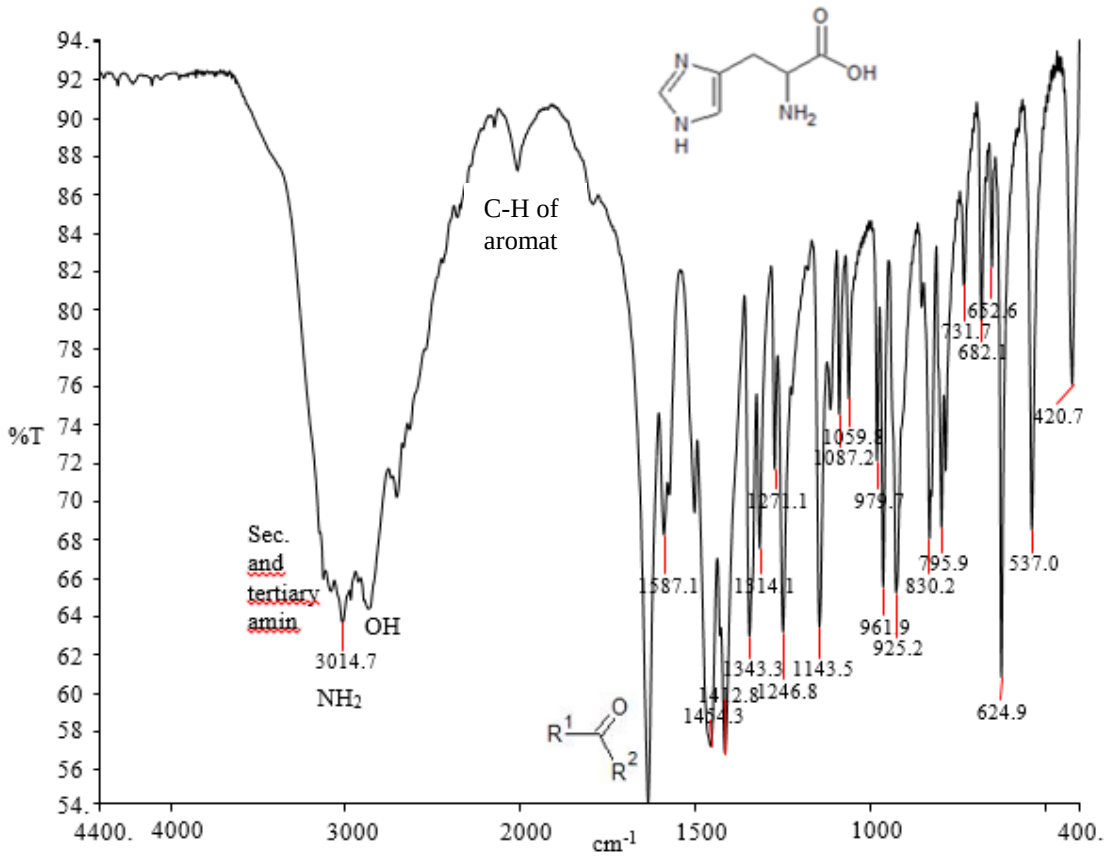


Figure 19 IR spectra of His.
This graphic shows distinctly three bands, which are NH_2, OH, a keton and a small low fourth band, of a sec and tertiary amin.

Table 6 assignment of bands and their wave numbers of His.

structure	range [cm^{-1}]
NH_2	3014.7
OH	~2800
keton	~1680
Sec. & tertiary amin	3100
C-H of aromatic ring	~2000

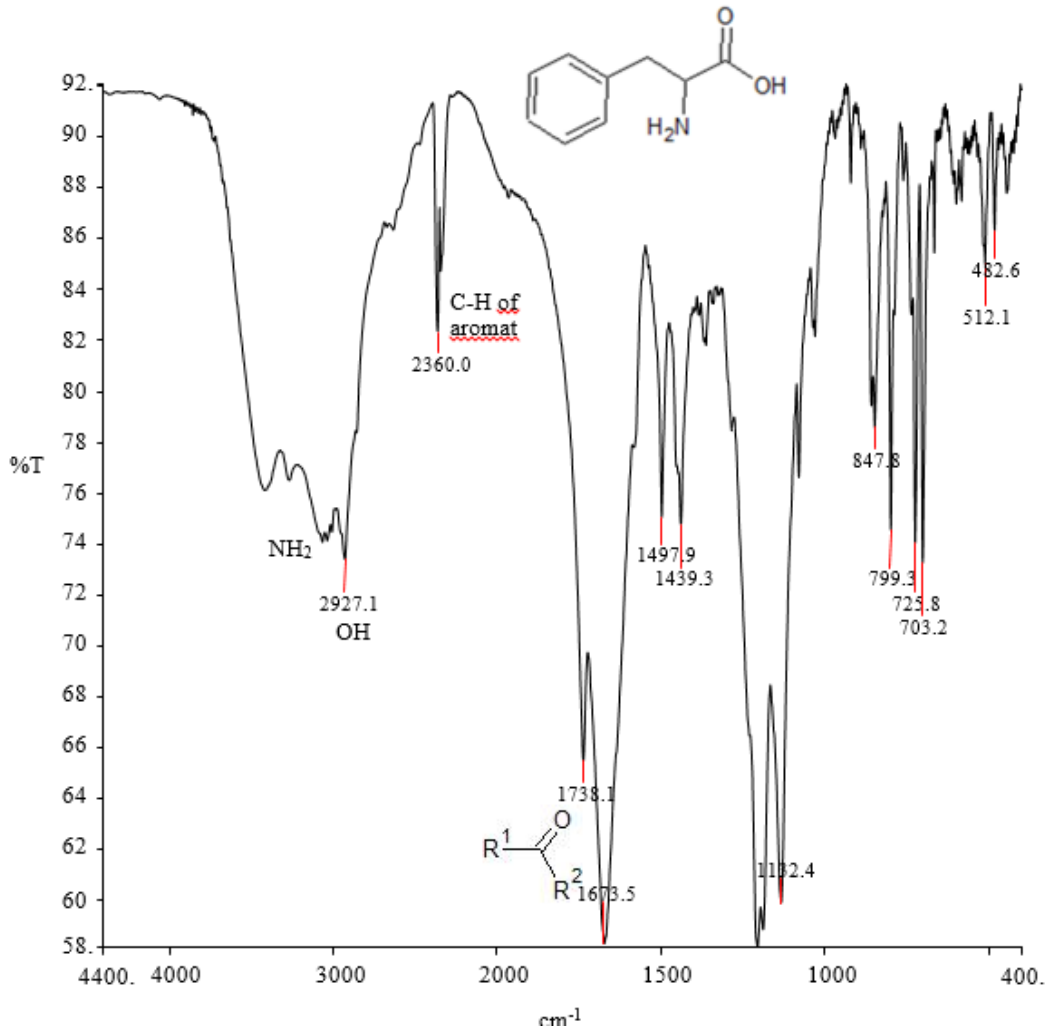


Figure 20 IR spectra of Phe.

This figure presents the IR spectra of Phe and shows the various Bands and the chemical assignement to it. Thus, you can see clearly the Bands of NH_2, O-H, C-H of an aromatic ring and a keton.

Table 7 assignment of bands and their wave numbers of Phe.

structure	range [cm^{-1}]
OH	2927.1
keton	1738.19
NH_2	~3100
C-H of aromatic ring	2360

The spectrum of His **(see figure 19 and table 6)** demonstrates very distinct intensive bands, clearly predominant here were the bands in the range around 3000 cm^{-1}, the O-H band lies around 2800 cm^{-1}, furthermore a band of 3014 cm^{-1}, a somewhat weaker band, originating from the secondary and tertiary amine was around approx. 3100 cm^{-1}. Further bands that could be assigned to the His were a band about 2000 cm^{-1} and another very pronounced one at 1680 cm^{-1}. The band at about 2000 cm^{-1} indicates a C-H valence vibration and the band of 1680 cm^{-1} indicates a ketone.

The spectrum of Phe **(see figure 20 and table 7)** showed much intensified bands in the range of 1700 cm^{-1} to 3000 cm^{-1}, the band of the O-H vibration is 2927.14 and the NH_2 band was 3100 $cm^{-1.}$ The C-H valence vibration of the aromatic ring was 2360.08 cm^{-1} and the ketone had a wave number of 1738.19 cm^{-1}. Based on these assignments, we determined that the respective fractions contained the correct amino acids.

4.1.1 Calibration curve of the amino acids

Before starting to generate a calibration curve of BSA, a calibration curve of Phe and Trp was performed. (**see Method B, solvent gradient from 0%-50%-100%-0%, for Solvent D [ACN]**) For the calibration curve of Phe, a concentration of 10 mg/mL was used. In the next step dilution series were created**. (see table 8 and figure 21)** Based on the created equation y = 3.8927x + 0.0091 the LOD and LOQ was calculated mathematically, shown in **table 9.** Thus, it was very important to know the LOD and LOQ, because in the next chapter **(see chapter 4.1.2)** the detection and quantification via the external standard of Phe and Trp in a protein powder mixture was performed.

Table 8 Dilution serie of Phe.
This table conveys the different concentration, in which 0.02 µg/µL represents the LOD.

concentration [µg/µL]	peak area of Phe [10^{-6}]
10.00	24.75
5.00	10.75
2.50	7.67
1.25	4.90
0.63	2.40
0.31	1.21
0.16	0.61
0.08	0.30
0.04	0.17
0.02	0.12

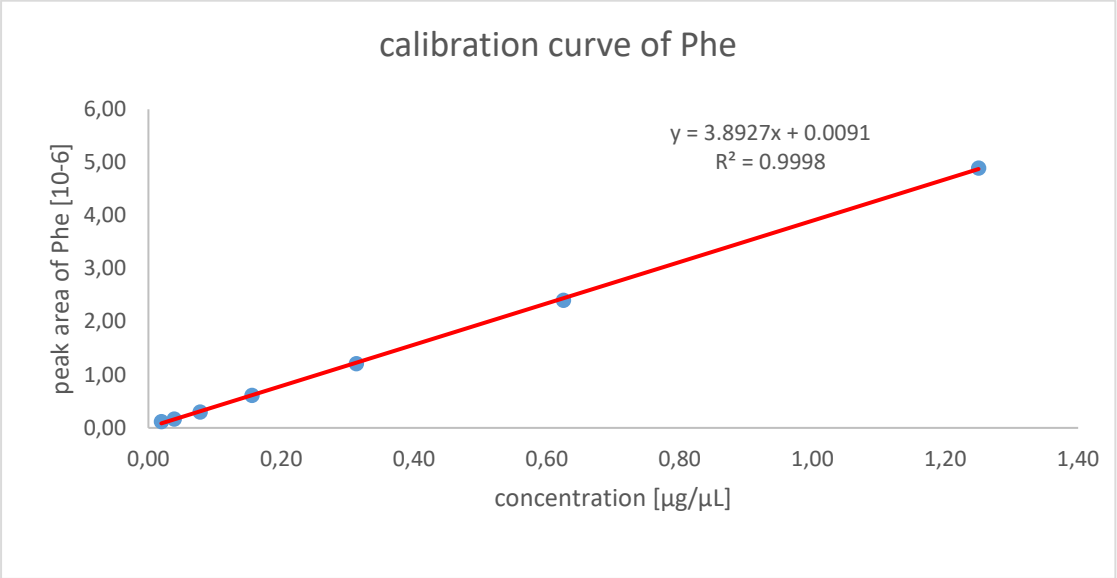


Figure 21 Calibration curve of Phe.
This image is a graphical representation of the data in table 6. The resulting calibration curve is linear. From the calibration curves the content of the amino acid in the sample was calculated. (see section 4.1.2)

SD of intercept = SE of intercept *√n **(see chapter 1.3)**

LOD= 3.3*(SD of intercept / slope)

LOQ= 10 * (SD of intercept / slope)

Table 9 LOD, LOQ.
This table depicts the mathematical calculation of LOD and LOQ.

SE of intercept	0.0130
SD of intercept	0.0909
LOD [µg/µL]	0.0771
LOQ [µg/µL]	0.2335

For the calibration curve of Trp a stock solution with a concentration of 1 mg/mL was chosen. This solution was applied for a dilution series, which was (**see table 10 and figure 22**) measured at 215 nm.

Table 10 Dilution serie of Trp.

This table is intended to demonstrate Trp's dilution series

concentration [µg/µL]	**area of Trp [10^{-6}]**
1,000	38.375
0,500	22.099
0,250	11.728
0,125	6.682
0,063	4.050
0,031	1.648
0,016	0.808
0,008	0.395
0,004	0.203
0,002	0.102
0,001	0.067

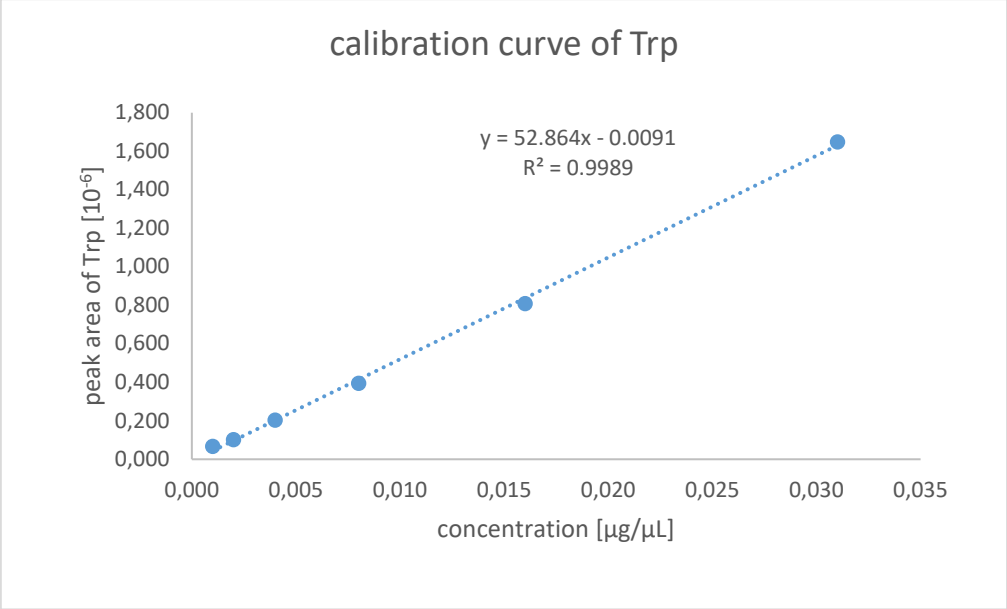


Figure 22 Calibration curve Trp.

This image shows the linearity of Trp. Based on the coefficient of determination [R^2], we can describe the degree of linearity. At a perfect linear correlation R^2 would be 1. In this case R^2=0.9989.

Table 11 LOD, LOQ of Trp.
This table depicts the mathematical calculation of LOD and LOQ

SE of intercept	0.0131
SD of intercept	0.0787
LOD [µg/µL]	0.0049
LOQ [µg/µL]	0.0149

As can be seen in **figures 21 and 22**, both Phe and Trp showed a linear progression. R^2 is the correlation coefficient and describes the linearity. The optimum would be R^2 =1, in our case R^2 [Phe] = 0.9998 and R^2 [Trp] = 0.9989. This was very important, because Phe and Trp were used as external standard in later experiments. Furthermore, the detection limit of Phe and Trp, was experimentally determined, in which dilution was performed until no signal was detected in the HPLC measurements. The LOD of Phe is 0.020 µg/µL and of Trp is 0.001µg/µL. Since we also wanted to know the LOQ **(see table 9 and 11)** of the two amino acids, we proved this mathematically. A more detailed description of the LOQ and LOD can be read in chapter **see chapter 4.1.2** on **page 25**.

4.1.2 Quantification *via* external standards for the protein mix

In order to verify the analytical methods, a protein powder which comprises several amino acids was analyzed. **(see table 12 and Method B, solvent gradient from 0%-50%-100%-0%, for Solvent D [ACN])**

Table 12 protein powder.
This table shows the composition of the protein powder, which was used for content measurement.

amino acids	g/100g protein mix
L-alanine	3.91
L-arginine	2.89
L-cysteine	1.37
L-glutamin acid	18.40
L-glycin	1.80
L-histidine	2.34
L-isoleucin	5.93
L-leucin	9.54
L-lysin	8.13
L-methionin	2.44
L-phenylalanine	3.91
L-proline	7.71
L-serin	5.50
L-threonin	5.53
L-tryptophane	1.48
L-tyrosin	4.11
L-valin	6.22
L-asparagin acid	8.78

Calculation with external standards

The purpose of this experiment was to detect the amount of Phe in 0.391 mg/mL of the protein powder. Thus, a fraction collecting of the protein mix (wavelength 215 nm) was performed.
With the equation (**see page 48**) of the calibration curve the amount of the Phe could be calculated. **(see figure 21)**

sample concentration : 0.391 mg/mL
area of phenylalanine [10^{-6}] : 0.217774

Measured content

$$y = 3.8927x + 0.0091$$

$$0.217774 = 3.8927\text{x} + 0.0091$$

$$\text{x} = 0.054 \text{ mg/mL}$$

Theoretical content

$$\left(\frac{3.91}{100}\right) * 100 = 3.91\% \text{ (Phe/protein mix)}$$

$$\left(\frac{3.91}{100\ \%}\right)$$

$$\left(\frac{x}{3.91\%}\right)$$

$$\frac{(3.91*3.91)}{100} = 0.152\ mg \text{ Phe/10 g flask}$$

$$\left(\frac{0.1529 - 0.054}{0.1529}\right) * 100 = 65\%$$

The same was performed with Trp. A sample weight of 0.19 mg/mL was taken. Repeating the same process using the formula of the calibration curve **(see figure 22)**.

sample weight : 0.19 mg/mL protein mix
area of TRP [10^{-6}]: 0.090604

Measured content

$$\boldsymbol{y = 52.864x - 0.0091}$$

$$0.090604 = 52.864\text{x} - 0.0091$$
$$\text{x} = 0.0019\ \text{mg/mL}$$

Theoretical content

$$\left(\frac{1.48}{100}\right) * 100 = 1.48\%\ \text{(Trp/protein mix)}$$
$$\left(\frac{1.48}{100\ \%}\right)$$
$$\left(\frac{x}{1.48\%}\right)$$

$$\frac{(1.90*1.48)}{100} = 0.028\ mg\ \text{Trp/10 g flask}$$

$$\left(\frac{0.0028 - 0.0019}{0.0028}\right) * 100 = 32.14\%$$

The reason why in both cases the measured content was lower than the theoretical content, could be due to a loss during reconditioning. These losses are caused by

the repeated utilization of a filter, which was reused to get a clear solution. In percentage terms, the measured content of both experiments is 65% and 32% lower than the theoretical content, respectively.

4.1.3 Calibration of Trp at pH= 10

For the calibration curve of BSA we first generated a calibration curve of Trp in order to find out if Trp can be used as internal standard for BSA. Since we are working with BSA in milieu of pH=10 we also worked with Trp in the same milieu. **(see method C, 0%-0%-35%-37%-100%-100%-0%, solvent A [5%ACN + 20mM Ammonium formate], solvent B [90%ACN+20mM Ammonium formate], appendix page 88 and 89).**Therefore, we used the same buffer solutions (solvent A and B), applied for the BSA. The concentration of Trp was 1 mg/mL. Next step was to make a dilution series. Absorbance scans using a photometer and single runs with the HPLC of each sample were recorded. For the absorption scan of Trp glass tubes were used which allowed a suitable filling quantity. Since the UV-160A UV-visible Recording Spectrophotometer was used and quartz cuvettes with a filling volume of approx. 2.5 mL were utilised, enough solution had to be prepared from each individual dilution to allow the light to pass through from the UV device. The absorption scan was performed to find out, if Trp showed a linearity at 215 nm. The correlation coefficient is $R^2 = 0.999$, which represents a good linearity. **(see table 13 and figure 23)** However, as we found that BSA is better detectable with HPLC measurements at a wavelength of 230 nm, we also set the HPLC measurements of Trp at 230 nm. **(see figure 25)** The dilution solutions previously prepared for the absorbance measurement, were used for this purpose. From each dilution 20 µL were injected and single HPLC spectra were determined. The result of these measurements was that Trp has a linearity $R^2 = 0.9989$ at a wavelength of 230 nm and a ph= 10. **(see table 14 and figure 24)** As a result, Trp could be used as an internal standard in experimental studies with BSA.

Table 13 Absorbance measurement with the Photometer (215nm).

This table shows a dilutions serie of Trp.

concentration Trp µg/µL	absorbance [215nm]
1.000	0.125
0.500	2.224
0.250	1.813
0.125	0.645
0.063	1.213
0.031	0.720
0.016	0.524
0.008	0.256
0.002	0.031

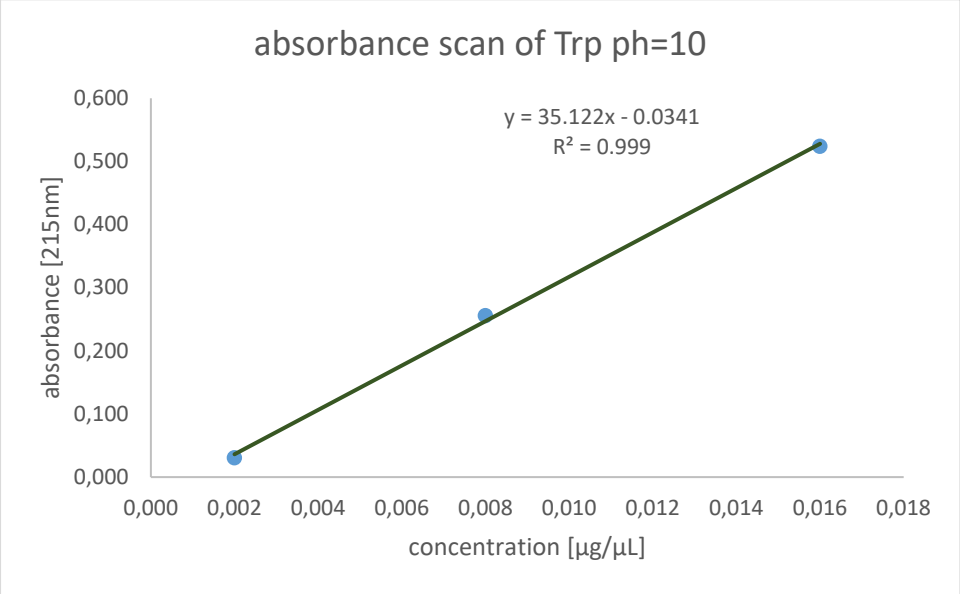


Figure 23 calibration curve of Trp in pH=10.

This figure depicts the graphical presentation of the table 13, and represents a calibrations curve.

Table 14 HPLC measurement at 230nm.

This table shows the dilutions serie of Trp, but measured with HPLC.

concentration µg/µL TRP	area [10^{-6}]Trp
0.500	125.025
0.250	89.949
0.125	56.669
0.063	39.537
0.031	22.035
0.016	12.470
0.008	7.711
0.004	3.251
0.002	1.259
0.001	0.485

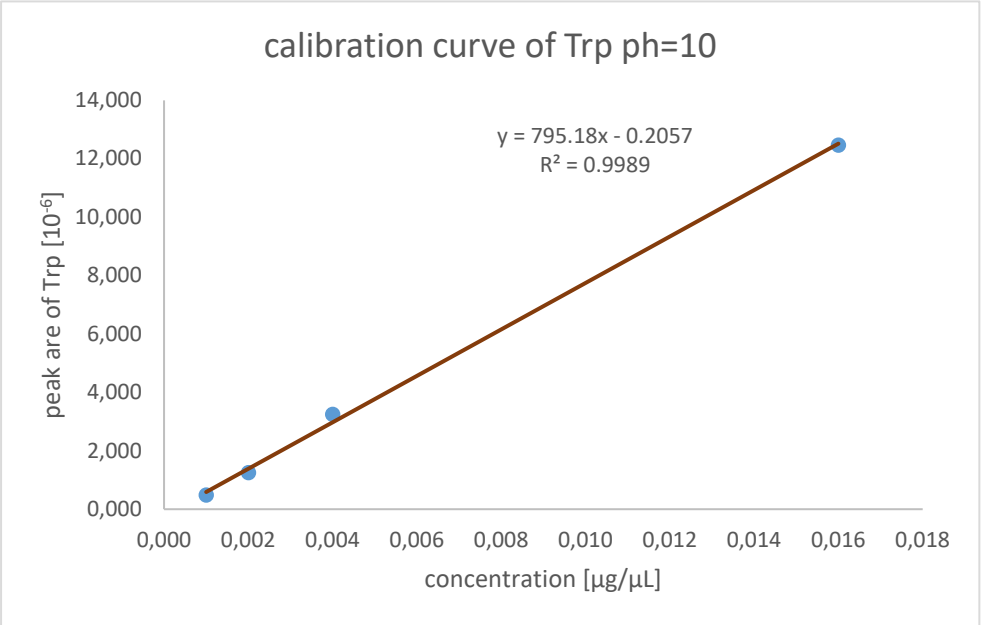


Figure 24 Graphical representation of calibration curve.

This figure shows that the calibration curve has a linearity and therefore Trp can be used as an internal standard for further experiments.

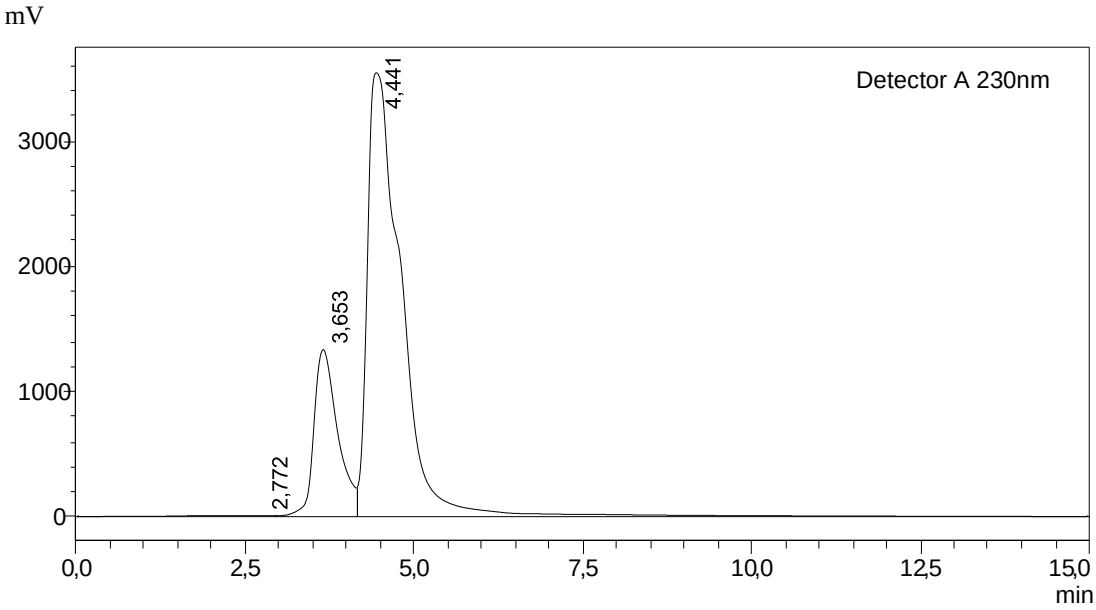


Figure 25 HPLC chromatogram of Trp in pH=10.
This figure depicts the measurement of Trp under high pH (=10) conditions.

4.2 Experiments with BSA

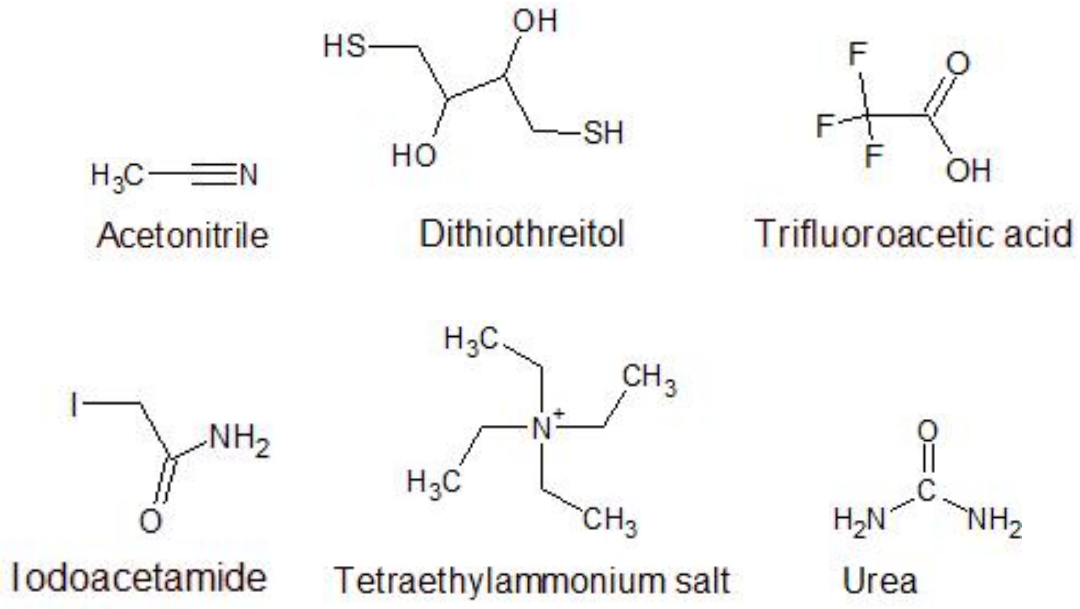
For experiments with BSA, prior digestion is required.

Part 1

The first step was to take an 8 µL aliquot of BSA stock solution and add 96 µL of 100 mM TEAB. In the second step BSA has to be denatured by adding 100 µL of 6M urea. Urea prevents hydrogen bonding and the protein will be unfolded. The next step is to reduce BSA by adding 20 mL of 100 mM DTT followed by incubation for 1h at 56°C. After that the solution was cooled down to room temperature. Futhermore, BSA was alkylated by adding 20 µL of 0.5 M iodoacetamide and incubation in the dark for 30 min at room temperature. The incubation in the dark was important, because iodoacetamide is photoinstabil. Then 500 µL of 100 mM TEAB buffer was added to dilute the urea. The pH was checked and if necessary adjusted to be 8.

Further step was to add 40 µL trypsin solution and incubate at 37°C overnight. Then the trypsin had to be quenched by adding 20 µL of 30%TFA. The final peptide concentration was 800 µg/ 800µL (1µg/µL) solution.

Figure 26 Structures of used reagents.



Part 2

For the second part, a solid phase extraction (SPE) was performed. The first step was to check the pH, which should be acidic for SPE. Since the capacity is a max. of 300 µg, we had to use 3 columns, followed by activating the columns with 100% ACN and then centrifuge. The flow through was mostly discarded. Then equilibration with a stage tip buffer (STP) was performed and again centrifuged. In the next step we had to load the sample and centrifuge. Next, we had to wash the sample, which was loaded onto a SPE column with 0.1% TFA followed centrifugation. Then we had to elute with SPE elution buffer (pH 8). This flow through of the three columns was collected and pooled together into a brown vial.

Part 3

In part three the solvent had to be partially evaporated in the vacuum centrifuge at 45°C until 150 µL remained. These 150 µL were transferred to a clean vial with insert. The brown vial had to be washed with ACN to be sure that no BSA is lost and then the 100 µL had to be added to the clear vial.

TEAB was added until any bubbles were could be seen. Liquid was evaporated and 160 µL of H_2O was added. At the end, 8 aliquots had been made, which means 20 µL of the BSA solution was in the vial. The concentration amounts to 100 µg/20 µL in every vial.

4.2.1 BSA- LOD and LOQ

In this experiment different concentrations of BSA were injected to the HPLC. We worked in a milieu at pH 10. **(see method C, 0%-0%-35%-37%-100%-100%-0%,solvent A [5%ACN + 20mM Ammonium formate], solvent B [90% ACN+20mM Ammonium formate]),** Solvent A was used to dilute the BSA stock solution.

The following dilution 2.727 µg/µL, then 1.818 µg/µL, 0.909 µg/µL, 0.818 µg/µL, 0.727 µg/µL, 0.182 µg/µL, 0.091 µg/µL, 0.045 µg/µL, 0.005 µg/µL, 0.002 µg/µL were prepared. **(see appendix page 90 - 95)** The latter indicates the LOD. Below this concentration detection was no longer possible. At a concentration of 2.727 µg/µg saturation of the column was demonstrated, and therefore we described it as upper LOD. The selected concentrations, which are represented in **table 15**, depict a linear range. This was graphed in **figure 27**. This has a very well visible linearity and a very good coefficient of determination [R^2], shown in **figure 28.** The LOQ was calculated mathematically and results for BSA 0.559 µg/µL.

Table 15 dilutions serie of BSA.

concentration [µg/µL]	peak area of BSA [10^{-6}]
2.727	53.108
0.091	1.153
0.045	0.403
0.005	0.279
0.002	0.154

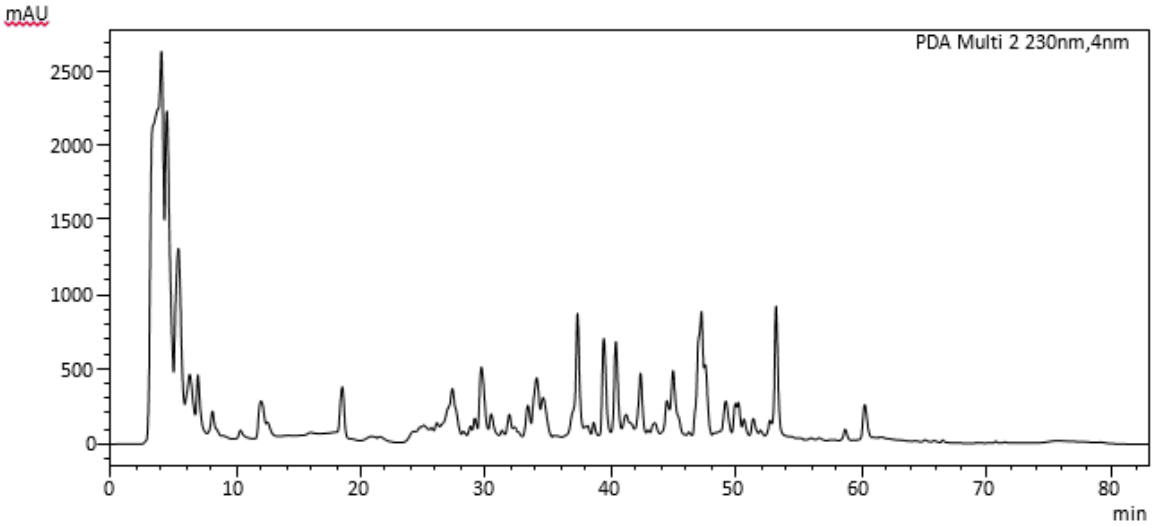


Figure 27 HPLC measurement of BSA.

This figure demonstrates a HPLC measurement of BSA at a concentration of 2.7 µg/µL. And at the same time the upper limit of detection.

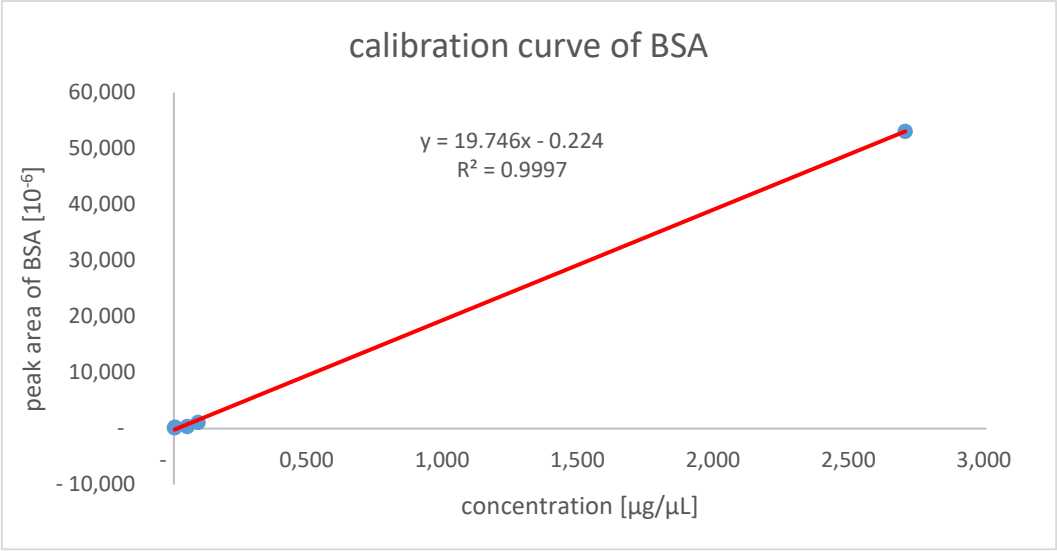


Figure 28 calibration curve of BSA.

This figure depicts the calibrations curve of BSA. The upper limit of detection 2.727 µg/µL and the lower limit of detection 0.002 µg/µL were determined.

4.2.2 Recovery and content measuring of Trp in BSA.

10	20	30	40	50
MK**W**VTFISLL	LLFSSAYSRG	VFRRDTHKSE	IAHRFKDLGE	EHFKGLVLIA
60	70	80	90	100
FSQYLQQCPF	DEHVKLVNEL	TEFAKTCVAD	ESHAGCEKSL	HTLFGDELCK
110	120	130	140	150
VASLRETYGD	MADCCEKQEP	ERNECFLSHK	DDSPDLPKLK	PDPNTLCDEF
160	170	180	190	200
KADEKKF**W**GK	YLYEIARRHP	YFYAPELLYY	ANKYNGVFQE	CCQAEDKGAC
210	220	230	240	250
LLPKIETMRE	KVLASSARQR	LRCASIQKFG	ERALKA**W**SVA	RLSQKFPKAE
260	270	280	290	300
FVEVTKLVTD	LTKVHKECCH	GDLLECADDR	ADLAKYICDN	QDTISSKLKE
310	320	330	340	350
CCDKPLLEKS	HCIAEVEKDA	IPENLPPLTA	DFAEDKDVCK	NYQEAKDAFL
360	370	380	390	400
GSFLYEYSRR	HPEYAVSVLL	RLAKEYEATL	EECCAKDDPH	ACYSTVFDKL
410	420	430	440	450
KHLVDEPQNL	IKQNCDQFEK	LGEYGFQNAL	IVRYTRKVPQ	VSTPTLVEVS
460	470	480	490	500
RSLGKVGTRC	CTKPESERMP	CTEDYLSLIL	NRLCVLHEKT	PVSEKVTKCC
510	520	530	540	550
TESLVNRRPC	FSALTPDETY	VPKAFDEKLF	TFHADICTLP	DTEKQIKKQT
560	570	580	590	600
ALVELLKHKP	KATEEQLKTV	MENFVAFVDK	CCAADDKEAC	FAVEGPKLVV
STQTALA				

Figure 29 MW of tryptophan / MW of BSA.

According to the depiction, a section of the sequence of BSA shown above, we can find the amino acid Trp thrice.

For this experiment digested BSA was utilized. The existing section of the sequence of BSA **(see figure 29)** contains the amino acid Trp thrice. For the recovery of the Trp content 1.8 µg/µL of BSA was used. During the HPLC measurement 10 (FR 0 – Fr 9) fractions were collected (**see figure 30**, **method C, 0%-0%-35%-37%-100%-100%-0%, solvent A [5% ACN + 20mM Ammonium formate], solvent B [90% ACN+20mM Ammonium formate]).** The fractions were applied to a 96 well plate and fluorescence was measured with the instrument TECAN (**see figure 31**). According to the **figure 31**, fraction 6 and 7 contain a high amount of Trp,

Figure 32 shows especially fraction 6 and 7. These two containing a high amount of Trp 78 mAU at 37 min and 30mAU at 43 min, respectively. Because of the fact that the fluorescence intensity is very high at 295nm, the separation was performed at this wavelength.

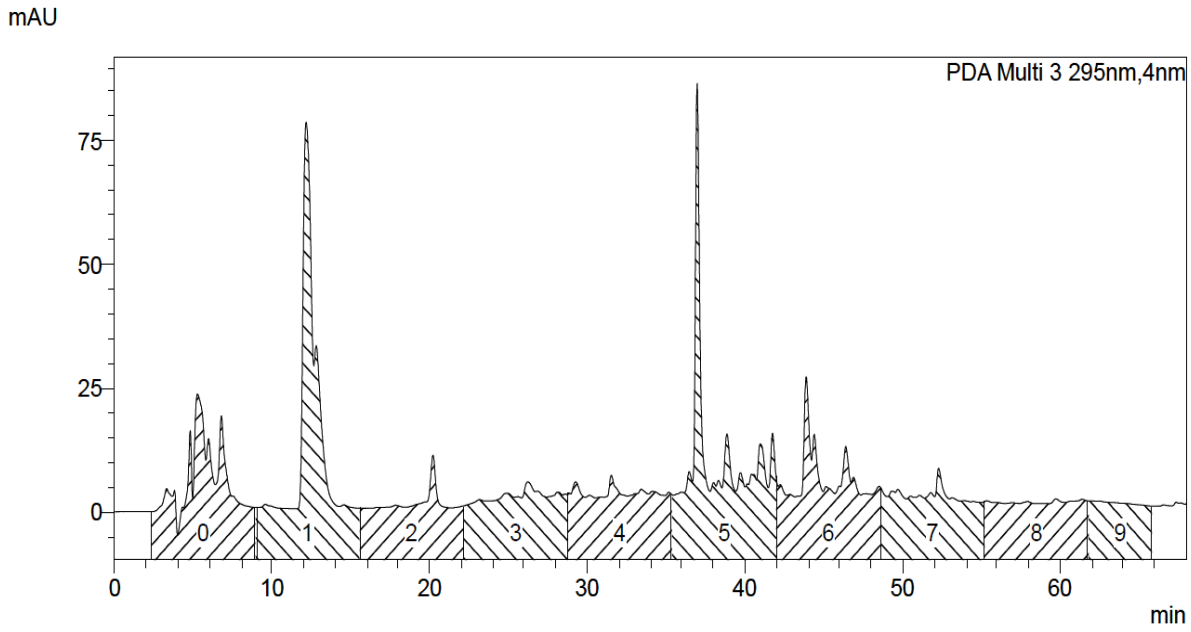


Figure 30 HPLC measurement.

This figure demonstrates the amount of collections and also the intensity of Trp in the individual fractions of BSA, due to this measurement FR 5 and 6 deserves more detailed consideration.

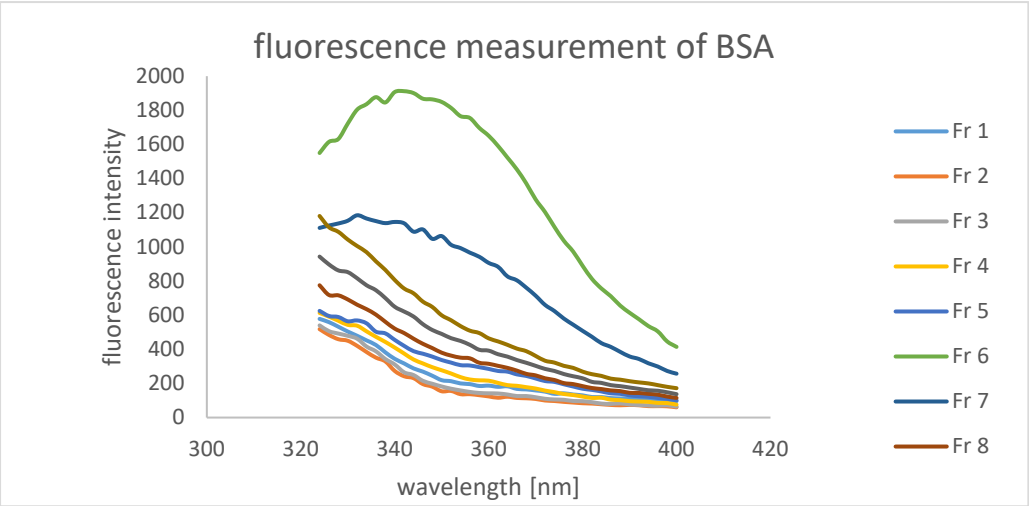


Figure 31 graphical presentation of the fluorescence.

This image depicts the measurement of the fluorescence by TECAN. Here we can see that two fractions show a very good fluorescence intensity. FR 5 and FR 6.

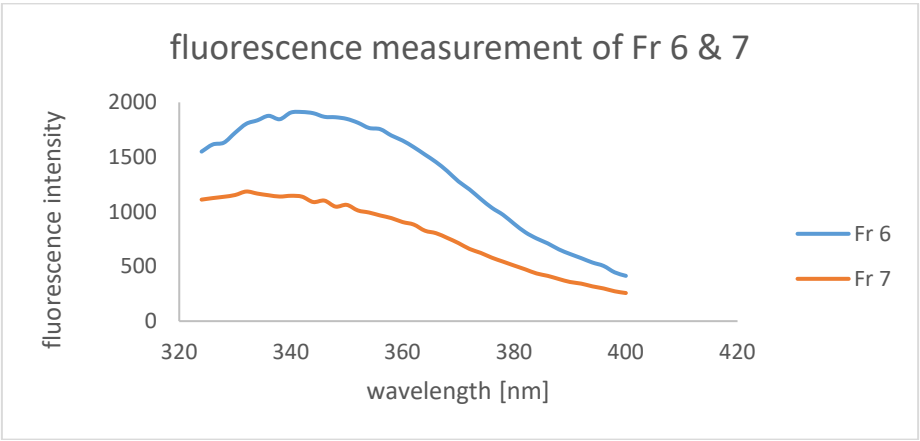


Figure 32 Graphic of fluorescence measurement of Fr 6 and 7.

This figure is specifically intended to represent the fluorescence of fraction 5 and 6, as they show the highest content of Trp

The next step was to inject fraction 6 **(see appendix page 96)** into the HPLC and determine which peak belongs to the Trp. Then a calibration curve of Trp was generated. Thus, a stock solution of 1 mg/mL of TRP was made. Then Trp was diluted so that the final concentration of the solution was 0.01 µg/µL. Then a dilution series was performed and furthermore the calibration curve was established. **(see table 16 and figure 33).** The calibration curve was made on the one hand to determine the concentration of Trp in BSA via an external standard and on the other hand to obtain a fluorescence intensity of Trp, which also functions as an internal standard, which is close to the intensity of BSA and thus the concentration of Trp in

BSA could be detected **(see figure 34 and table 17).** A 96 well plate was used to measure the fluorescence of Trp.

Table 16 Dilution serie of Trp and measured fluorescence intensity.

Trp concentration µg/µL	flurescence
0.0100	4829
0.0050	2512
0.0025	1350
0.0013	768
0.0006	460
0.0003	309
0.0002	239
0	155

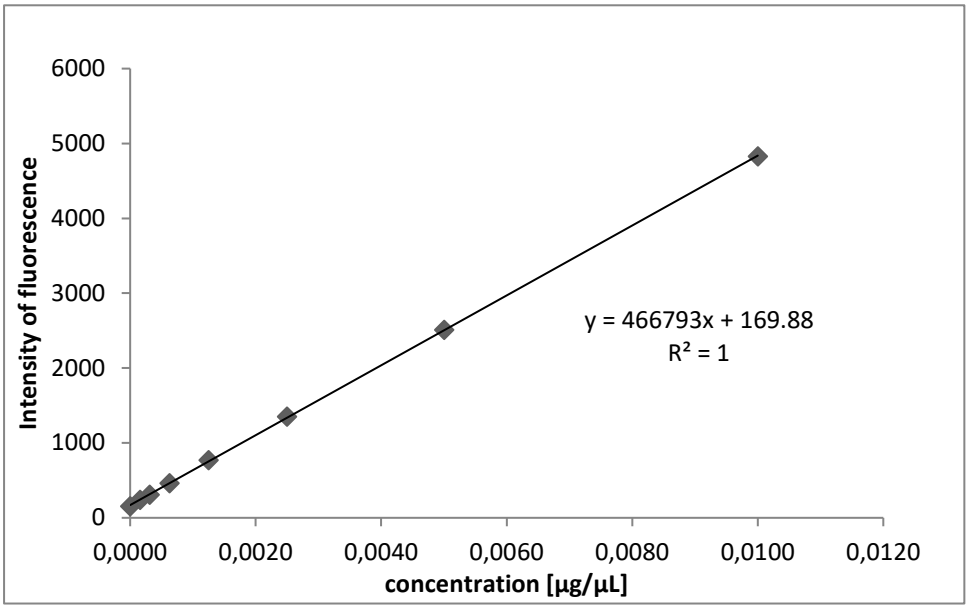


Figure 33 Calibration curve of Trp.
This figure depicts the calibration curve of Trp and the measured fluorescence intensity. Since [R^2] showed an excellent linearity, Trp provided to be the internal standard for further experiments with BSA.

Taking the calibration curve into account, the peptides which contain TRP were identified. The concentration of the individual fractions were determined by using the equation.

$$y = 466793x + 169.88$$

$$R^2 = 1$$

Table 17 Quantification of Trp in BSA via measurement of fluorescence intensity.

Fraction	Fluorescence intensitiy	concentration [µg/L]
1	219	0.11
2	154	-0.03
3	182	0.03
4	277	0.23
5	337	0.36
6	**1849**	**3.60**
7	**1063**	**1.91**
8	380	0.45
9	490	0.69
10	599	0.92

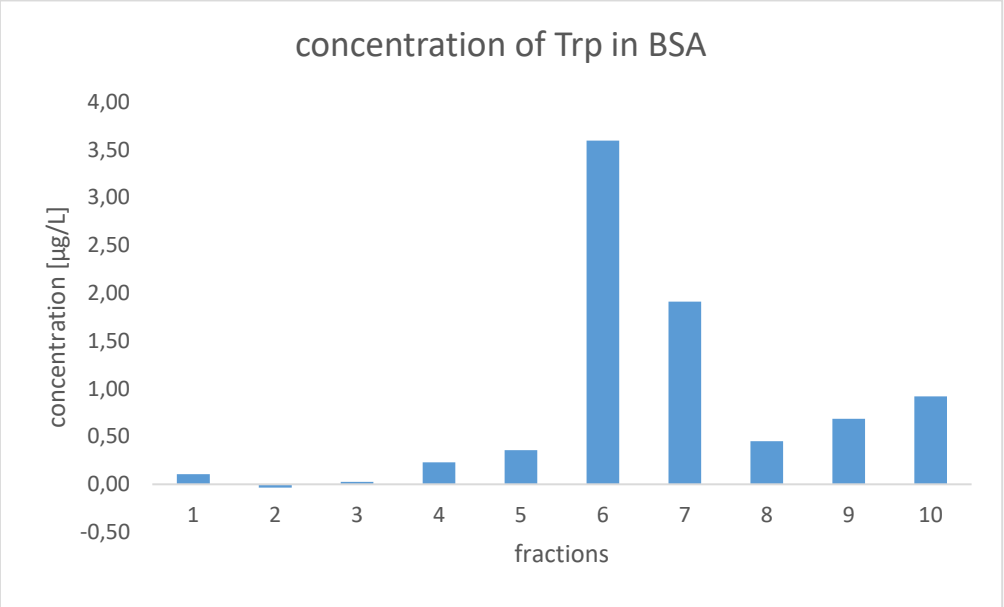


Figure 34 Graphical presentation of Trp in BSA.
This figure depicts the concentration of Trp in different fractions of BSA.

4.2.3 Recovery and quantification of Trp in BSA by standard addition

Since the previous experiment worked well, the whole experiment was repeated with a concentration of 0.82 µg/µL of BSA. Then again fractions were taken by HPLC **(see method C, 0%-0%-35%-37%-100%-100%-0%,solvent A [5% ACN + 20mM Ammonium formate], solvent B [90% ACN+20 mM Ammonium formate]),** and the fractions were pipetted into a 96 well plate, following by the measurement of the fluorescence. Then the previously determined equation was used to calculate the concentrations, shown in table **18 and figure 35**.

Table 18 Content measurement of Trp in 0.82 µg/µL BSA.

Fraction	Fluorescence intensity	concentration [µg/L]
1	224	0.13
2	189	0.06
3	254	0.19
4	189	0.06
5	288	0.27
6	**1028**	**1.82**
7	**1117**	**2.00**
8	310	0.31
9	328	0.35
10	**892**	**1.53**
11	529	0.77
12	526	0.76

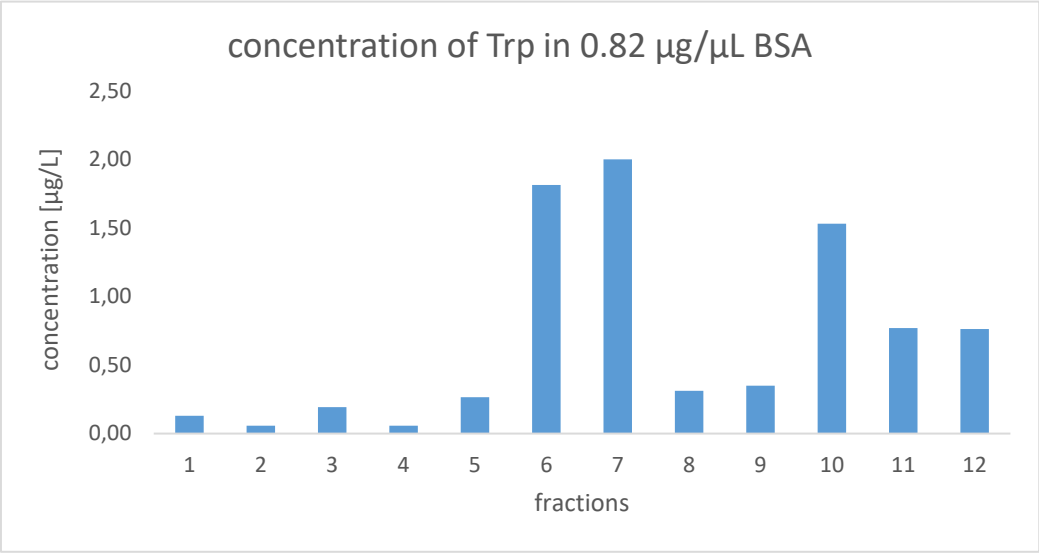


Figure 35 graphical depiction of content measurement of Trp in 0.82 µg/µL BSA.

Since we mainly worked with HPLC, we wanted to verify whether our method, the external standard, could detect a loss of our sample BSA, or whether we would lose anything at all. For this purpose, we prepared a comparison and used the standard addition method as a comparison method. These observations were important to us, since in later experiments the samples we worked with, were the peptides originating from the brain of rats. To ensure a precise method, we first carried out these experiments with BSA.

Table 19 Investigation of concentration for standard addition.

addition of 5µL	concentration [µg/L]
205	0.006
210	0.012
215	0.017
220	0.023
225	0.028

Table 20 Application of the standard addition method to fraction 6.
FR 6

concentration [µg/L]	fluorescence intensity
0	1028
0.006	1041
0.012	1058
0.017	1099
0.023	1128
0.028	1141

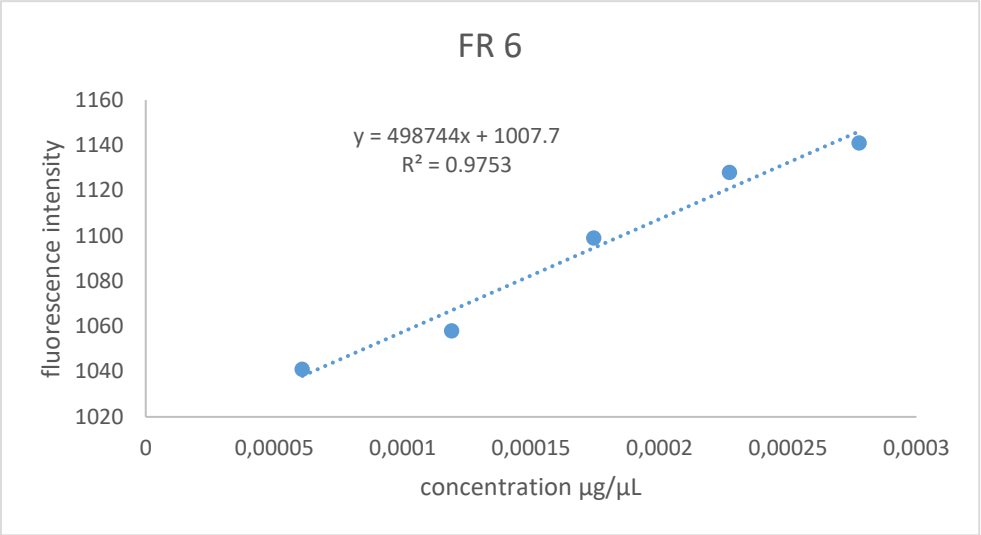


Figure 36 graphical presentation of fraction 6 using standard addition method and generating a calibration curve.

This figure shows a calibration curve, performed by using standard addition method.

Table 21 Application of the standard addition method to fraction 7.

FR 7

concentration [µg/L]	fluorescence intensity
0	1117
0.006	1120
0.012	1138
0.017	1069
0.023	1084
0.028	1101

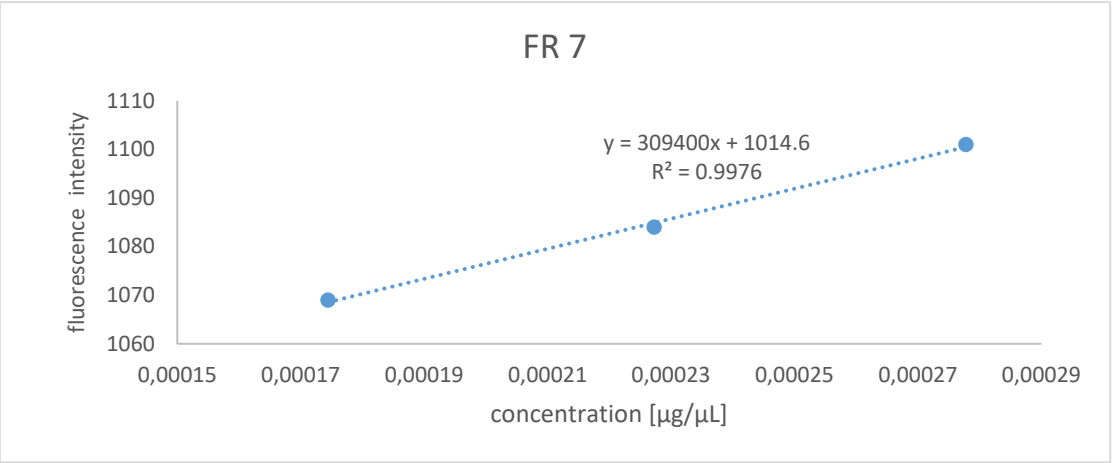


Figure 37 graphical presentation of fraction 7 using standard addition method and generating a calibration curve.
This figure depicts a calibration curve, performed by using standard addition method.

Table 22 Application of the standard addition method to fraction 10.

FR 10

concentration [µg/L]	fluorescence intensity
0	892
0.006	976
0.012	1026
0.017	1035
0.023	1079
0.028	1099

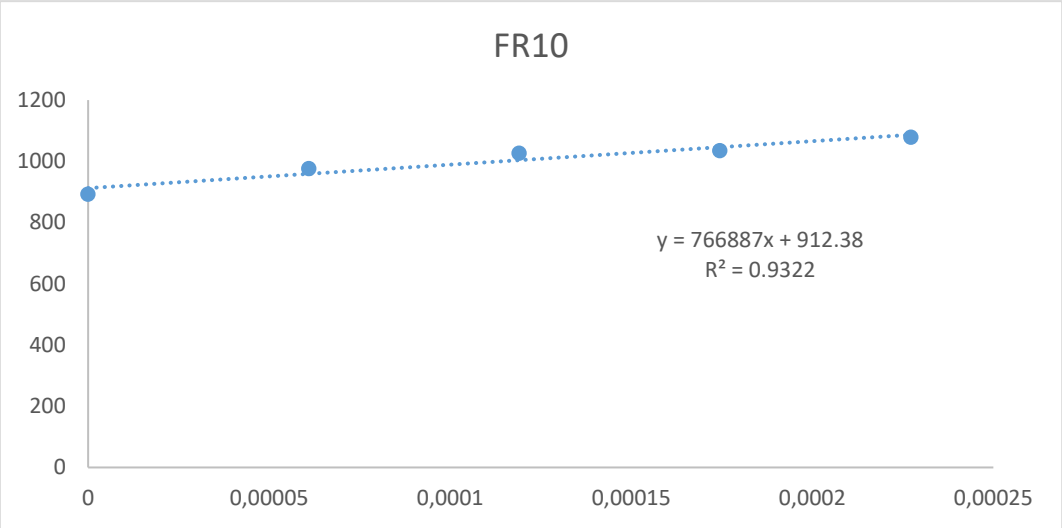


Figure 38 Presentation of fraction 10 using standard addition method and generating a calibration curve.
This figure shows a calibration curve, performed by using standard addition method.

FR 6

$$y = 0$$
$$0 = 498744x + 1007.7$$
$$x = |-\mathbf{0.002}|\ \mu\boldsymbol{g}/\mu\boldsymbol{L}$$

FR 7

$$y = 0$$
$$0 = 309400x + 1014.6$$
$$x = |-\mathbf{0.003}|\ \mu\boldsymbol{g}/\mu\boldsymbol{L}$$

FR10

$$y = 0$$
$$0 = 766887x + 912.38$$
$$x = |-\mathbf{0.001}|\ \mu\boldsymbol{g}/\mu\boldsymbol{L}$$

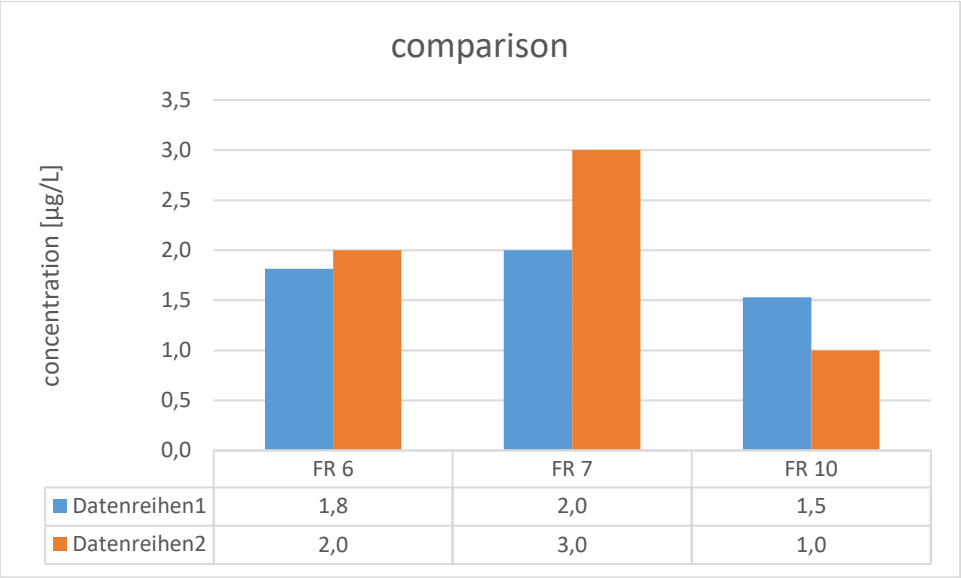


Figure 39 Comparison of external standard to standard addition.
This figure depicts the comparison of the previously discussed fractions 6,7 and 10. The orange colour represents the determined concentrations with the standard addition method and the blue one represents the data calculated with external standard.

For the standard addition a concentration with an intensity close to the intensity of the fractions was applied. According to the previous dilution series, shown in **table 16 and figure 34**, the fluorescence intensity of Trp corresponds approximately to the intensity to BSA. Thus, a concentration of 0.0013 µg/µL was selected and periodically added to the fractions 6, 7 and 10. **(see table 19)**

As depicted in **table 20, 21 and 22** intensities were detected by using standard addition method, and thereby calibration curve were generated. **(see figures 36,37 and 38)** Based on the equation of this calibration curve the final concentrations were determined. **(see page 68)** Finally a comparison of external standard to standard addition method was performed, by using our determined concentrations, depicted in **figure 39**. Based on this graph we could see, that due to standard additions method the losses are reduced.

Based on the **figure 39**, comparing these two methods described above, external standard had in fractions 6 and 7 a higher loss than standard addition method, a loss of 10% and 66%, respectively. By contrast, the fraction 10 showed a higher percentage of the loss using the external standard namely a loss of 150%, comparing to the standard addition.

4.3 Sample P02769

In this study proteins were extracted from the dentate gyrus, a subregion of the hippocampus with the ongoing neurogenesis throughout adulthood. **(for detailed information see chapter 1.4, page 26-27)**.

In shotgun proteomics, the proteins are first digested by protease trypsin to produce peptides that are separated and analyzed by liquid chromatography coupled to mass spectrometry (LC-MS). In online reversed-phase (RP) LC, peptides are retained on the analytical column through hydrophobic interactions with a C18 stationary phase in the presence of an acidic modifier, such as formic acid. The peptides are subsequently separated and eluted by applying acetonitrile gradient. In case of more complex protein samples the peptides can be separated by two-dimensional (2D) procedure to lower the sample complexity, increase the resolution power of the analysis and the number of identified proteins. Offline high-pH reversed-phase (RP) fractionation is wildly used as a first dimension peptide separation technique in which the peptide mixture is pre-fractioned using C18 stationary phase at high pH (~10). The individual fractions are further subjected to LC-MS analysis with online C18-based LC system representing the second dimension of peptide separation at low pH (~2.5).

In our experiment, a total amount of 300µg of proteins was injected into the HPLC and 50 fractions were collected. In the fraction tubes, 5% formic acid was present in each case. HPLC solvent A contained 20 mM NH4OH pH 10 in 5% acetonitrile and solvent B contained of 20 mM NH4OH pH 10 in 90% acetonitrile. Peptides were separated at 35 °C with a flow rate of 100 µL/min and eluted from the column with a 41 min gradient from 0 to 35% solvent B, then a 4 min gradient from 35 to 70% solvent B and, finally, a 2 min gradient from 70 to 100% solvent B. The fractions were pooled together to get a higher amount to identify the peptides via LC MS.

In the **figure 40** below the identified protein groups and peptides are shown. Figures 1 and 2 showed an increase in the number of the identified protein groups and peptides in the combined fraction in compare to the individual fractions or the unfractionated sample.

Figure 3 and 4 present the comparison of the numbers of identified peptide spectral matches (PSMs) and total MS/MS spectra.

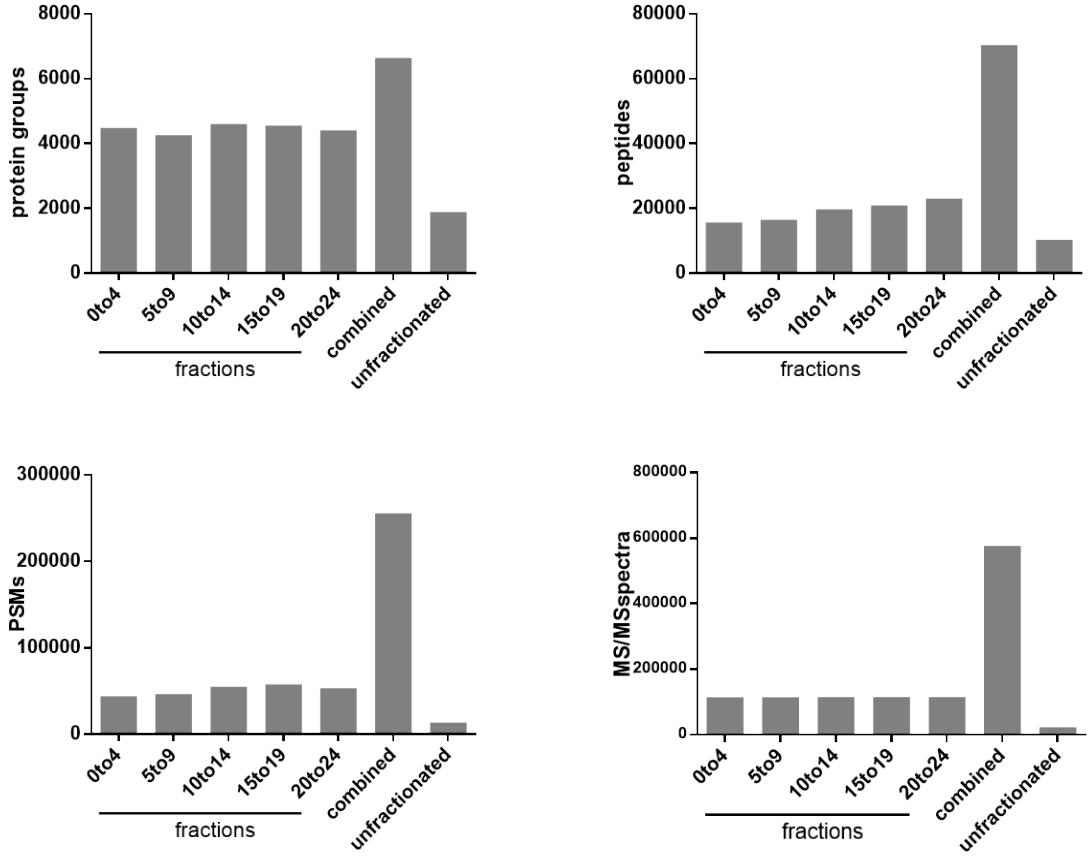


Figure 40 Comparison of identified peptides, fractionated *versus* unfractionated.

This figure presents the peptides, identified from the dentate gyrus.

5. Conclusion

Based on the experiments with amino acids (Trp, His, Phe,) we could demonstrate the easy separation of them, using the methods HLPC and standard addition.

The aim of the work was among others to demonstrate the separation of the amino acids under various pH conditions, performing it as preliminary studies for BSA. In the practical part, amino acids were separated, fractionated as well as used as external and internal standards.

It was possible to create an amino acid mixture and fractionate in order to regain the required amino acids again with less impurities. This could be supported by HPLC and IR.
A substantial point in our study was the recovery of Trp in BSA by performing a comparison of the content measurement of Trp with HPLC and using of standard addition. Besides, we could determine the amount of the loss and based on the data, in our case we have a negligible loss by fractionating with HPLC.
Further experiments with BSA concerning LOD (upper LOD 2.7 µg/µL and lower LOD 0.002 µg/µL) and LOQ (0.56 µg/µL) revealed, that a low amount of BSA can be used for the detection and quantification.

Experiments with the sample **P02769** show that fractionation is a very important tool. The combined fractions contained more protein groups or peptides in contrast to the unfractionated sample. Moreover, a great amount of MS spectra could be performed (ca. 60.000) and thus, peptides and its sequences can be identified.

Zusammenfassung

Basierend auf den Experimenten mit Aminosäuren (Trp, His, Phe,) konnten wir die einfache Trennung mit den Methoden HLPC und Standardaddition demonstrieren.

Ziel der Arbeit war es unter anderem, die Trennung der Aminosäuren unter verschiedenen pH-Bedingungen zu demonstrieren und als Vorstudie für BSA durchzuführen. Im praktischen Teil wurden Aminosäuren getrennt, fraktioniert sowie als externe und interne Standards verwendet.

Es war möglich, ein Aminosäurengemisch herzustellen und zu fraktionieren, um die benötigten Aminosäuren mit weniger Verunreinigungen wiederzugewinnen. Dies könnte durch HPLC und IR unterstützt werden.
Ein wesentlicher Punkt in unserer Studie war die Rückgewinnung von Trp in BSA durch einen Vergleich der Inhaltsmessung von Trp mit der HPLC und der Verwendung von Standardaddition. Außerdem konnten wir die Höhe des Verlustes bestimmen und haben in unserem Fall einen vernachlässigbaren Verlust durch Fraktionierung mit HPLC.
Weitere Experimente mit BSA bezüglich LOD (oberer LOD 2,7 µg/µL und unterer LOD 0,002 µg/µL) und LOQ (0,56 µg/µL) ergaben, dass eine geringe Menge BSA für den Nachweis und die Quantifizierung verwendet werden kann.

Versuche mit der Probe P02769 zeigen, dass die Fraktionierung ein sehr wichtiges Werkzeug ist. Die kombinierten Fraktionen enthielten mehr Proteingruppen oder Peptide als die unfraktionierte Probe. Außerdem konnte eine große Anzahl von MS-Spektren durchgeführt werden (ca. 60.000) und so können Peptide und ihre Sequenzen identifiziert werden.

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Images

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7. Appendix

7.1 List of Tables

7.2 List of Figures

7.3 List of Abbreviations

AC	alternating current
ACN	acetonitrile
Ala	alanine
AS	amino acid
BSA	bovine serum albumin
cm	centimetre
3D	three-dimensional
DC	direct current
DNA	deoxyribonucleic acid
DTT	1,4- Dithiothreit
Fr	fraction
g	gramm
GC	gas chromatography
His	histidine
HPLC	High performance liquid chromatography
IR	Infrared spectroscopy
kDA	kilo Dalton
LCS	liquid solid chromatography
LOD	limit of detection
LOQ	limit of quantification
µg	microgram
µL	microliter
µm	micro metre
min	minute
mL	mililiter
mm	milimetre
nm	nanometre
MS	Mass spectrometry
MW	molecular weight
m/z	mass-to-charge-ratio
Phe	phenylalanine
Pro	proline

RP	reversed phase
RNA	ribonucleic acid
SD	standard deviation
SE	standard error
SPE	Solid phase extraction
STP	stage tip buffer
TEAB	triethylammonium bicarbonate buffer
TFA	trifluoroacid
Trp	tryptophan
UV/ VIS	ultraviolet/ visible

HPLC CHROMATOGRAMS

SHIMADZU LabSolutions Analysis Report

<Sample Information> **Amino acid chromatogram**

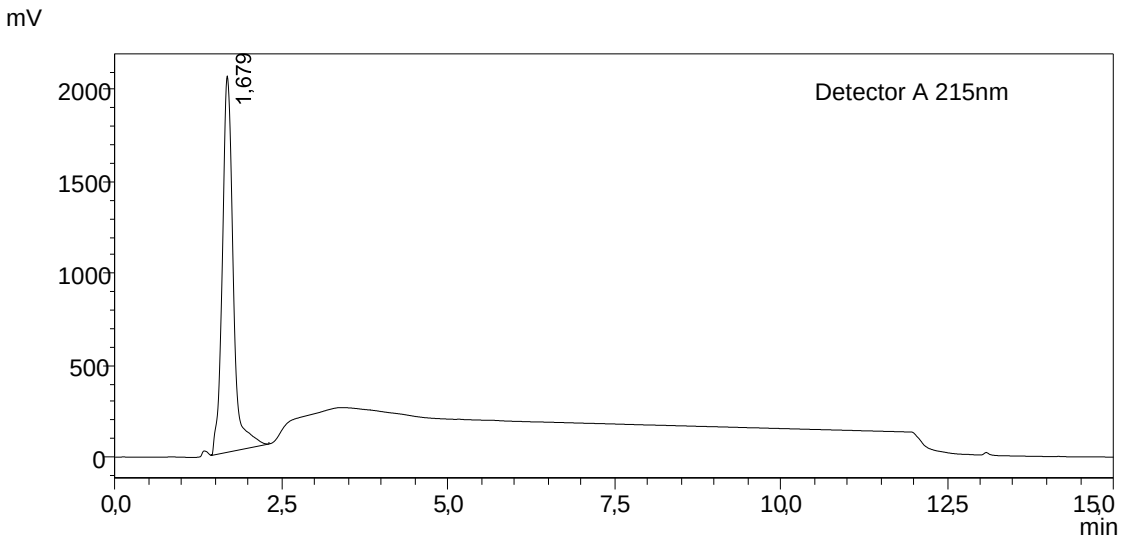
Sample Name
2016_02_03_His

Sample ID
0.1 mg/mL
0.2
Data Filename
2016_02_03_Histidin.lcd

Method Filename
2016_02_03_AS MIX.lcm

Batch Filename
2016_02_03_AS MIX_001_.lcb

Vial #	1-40
Injection Volume	20 µL



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SHIMADZU
LabSolutions Analysis Report

<Sample Information> **Amino acid chromatogram**

Sample Name
2016_02_03_Phe

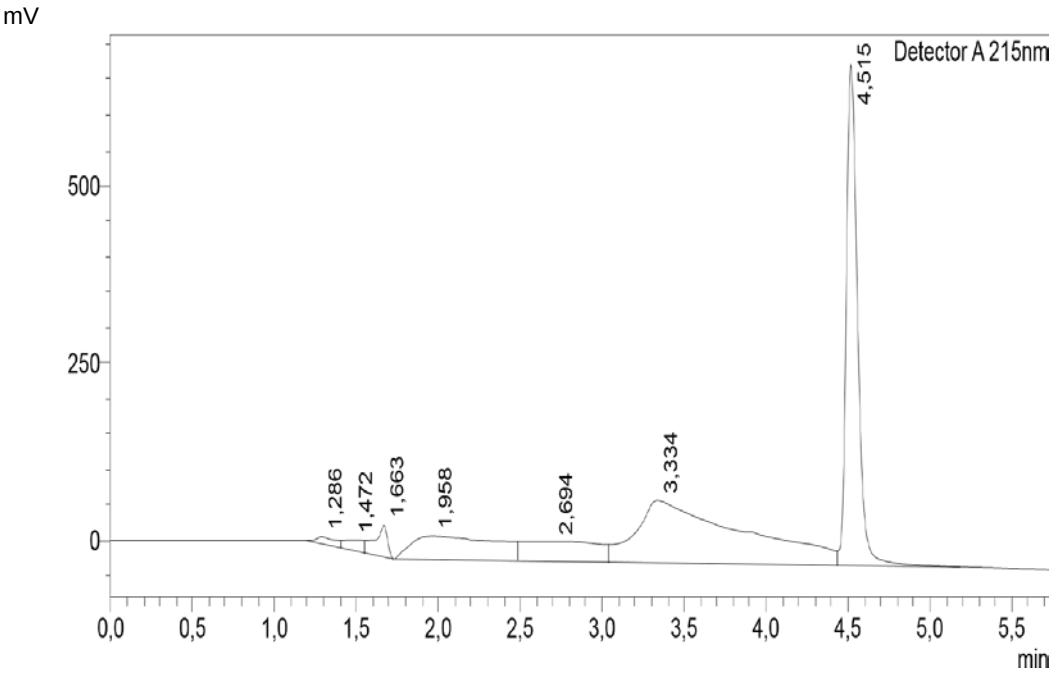
Sample ID
0.1 mg/mL

Data Filename
2016_02_03_PHE_001.lcd

Method Filename
2016_02_02 ACN 0.1 TFA_001_.lcm

Batch Filename
2016_02_03_AS MIX_001_.lcb

Vial #	1-39
Injection Volume	20 µL



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SHIMADZU
LabSolutions Analysis Report

<Sample Information> **Amino acid chromatogram**

Sample Name
2016_02_03_Trp

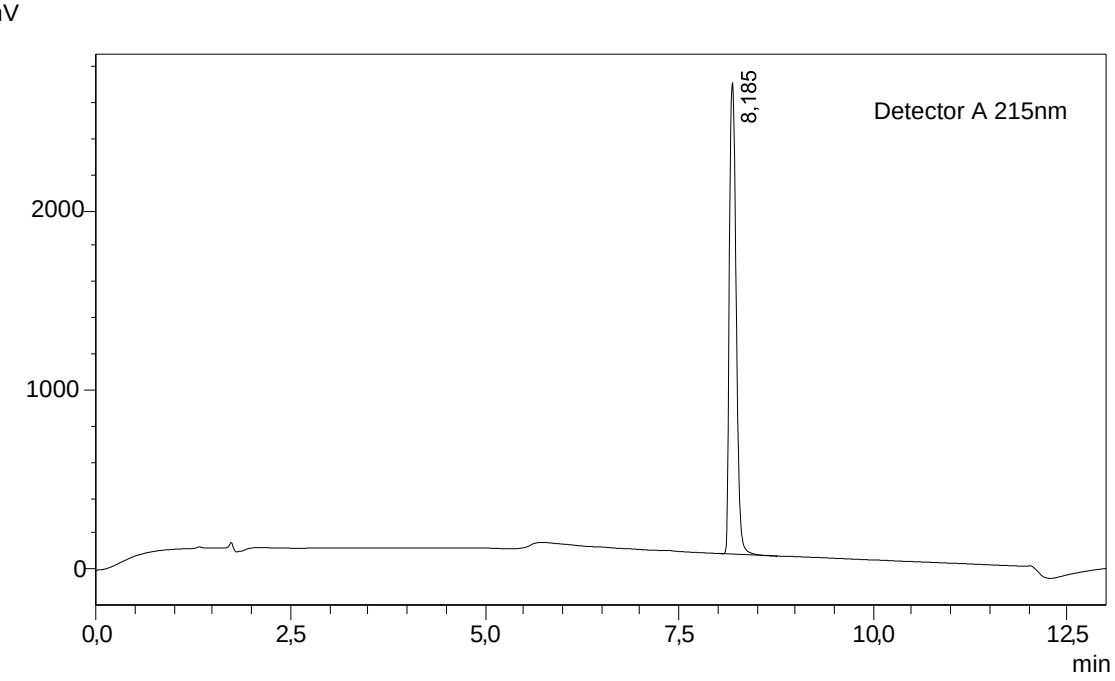
Sample ID
0.1 mg/mL

Data Filename
2016_02_03_Tryptophan_004.lcd

Method Filename
2016_02_03_AS MIX_002.lcm

Batch Filename
2016_02_03_AS MIX_002_.lcb

Vial # : 1-40
Injection Volume : 20 µL



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==== Shimadzu LabSolutions Analysis Report ===

Sample Information: **Amino acid separation chromatogram of His and Phe**

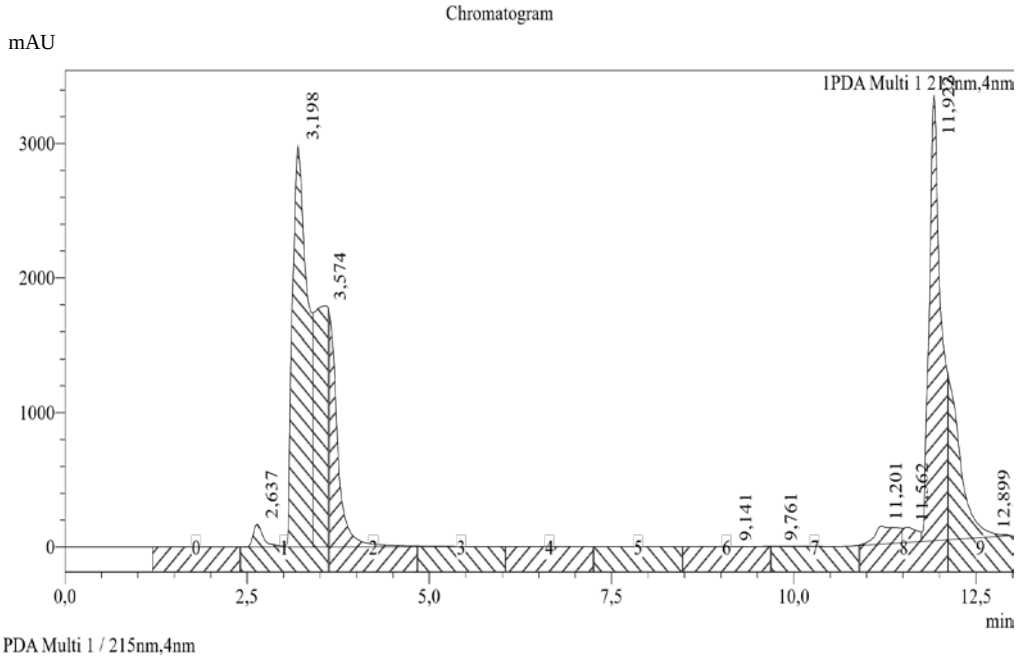
Sample Name
2016_02_11_HIS_PHE_FR_20%ACN

Sample ID :
2016_02_11_HIS_PHE_FR_20%ACN

Data Filename :
2016_02_11_HIS_PHE_20%ACN 1. FR1.lcd

Method Filename: 2016_02_10_ HIS_PHE _20 % ACN.lcm

Vial # : 1-42
Injection Volume : 20 uL



SHIMADZU
LabSolutions Analysis Report

<Sample Information> **Dilution serie of Trp, Trp measured in ph=10**

Sample Name TRP1

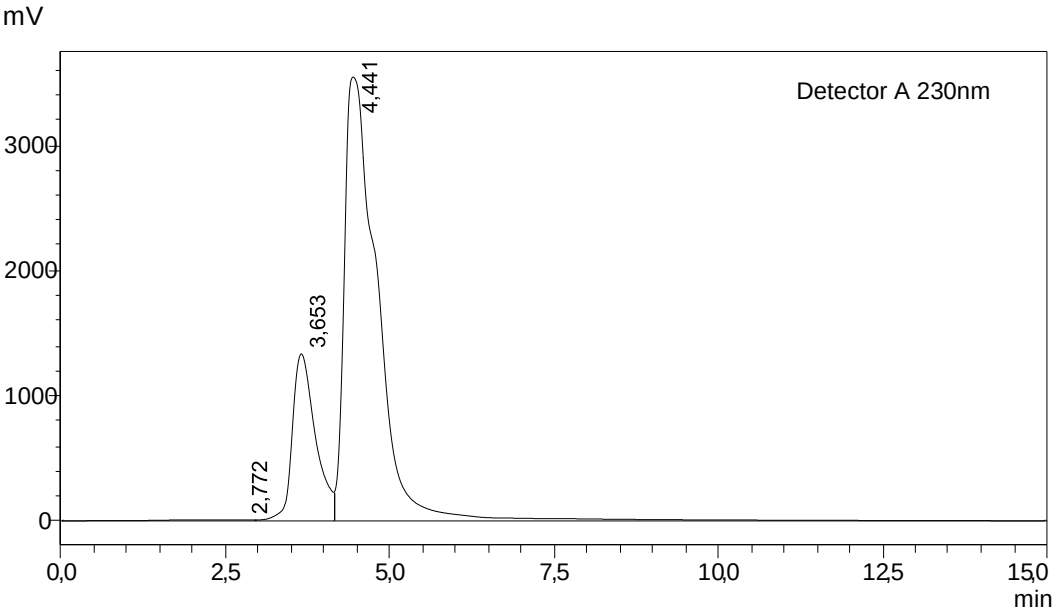
Sample ID
TRP 1 µg/µL

Data Filename
2016_04_15_TRP1.lcd

Method Filename
2016_04_15_calibration of BSA_pH10.lcm

Batch Filename
2016_04_18_calibration of TRP_pH10.lcb

Vial #	1-70
Injection Volume	20 uL



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SHIMADZU
LabSolutions Analysis Report

<Sample Information> Dilution serie of Trp, Trp measured in ph=10

Sample
Name TRP3

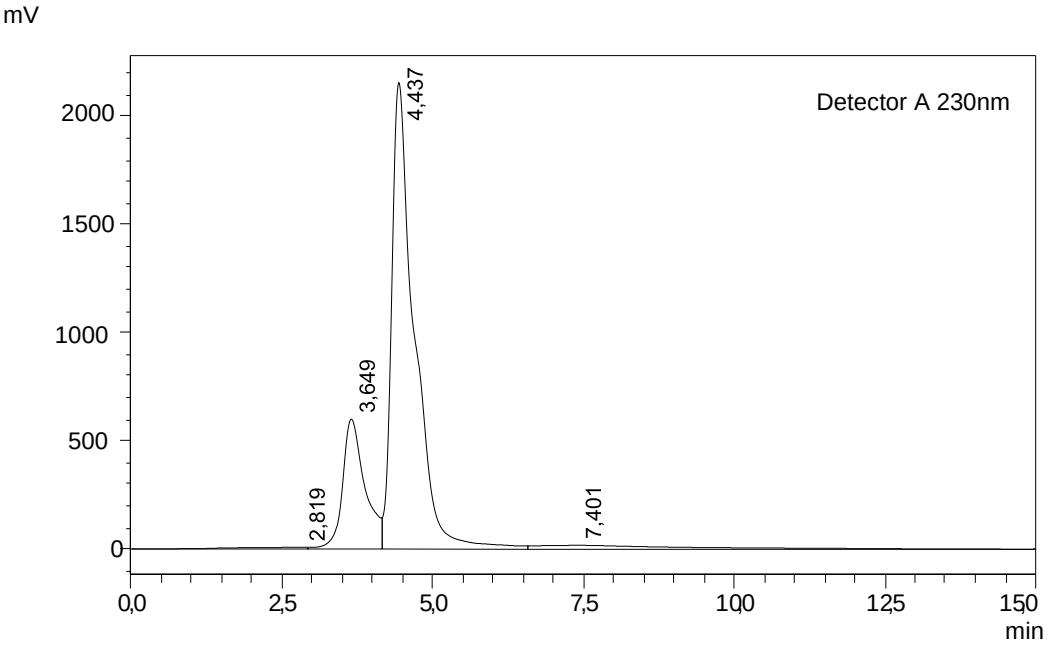
Sample ID
TRP 0.25
µg/µL

Data Filename
2016_04_15_TRP3.lcd

Method Filename
2016_04_15_calibration of BSA_pH10.lcm

Batch Filename
2016_04_18_calibration of TRP_pH10.lcb

Vial # : 1-68
Injection Volume : 20 uL



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SHIMADZU
LabSolutions Analysis Report

<Sample Information> Measurement of 2.73 µg /µL BSA

Sample Name
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Sample ID
2016_09_05_BSA_calibration_2.73 µg/µL

Data Filename
2016_09_05_BSA_calibration_2.73 µg/µL.lcd

Method Filename
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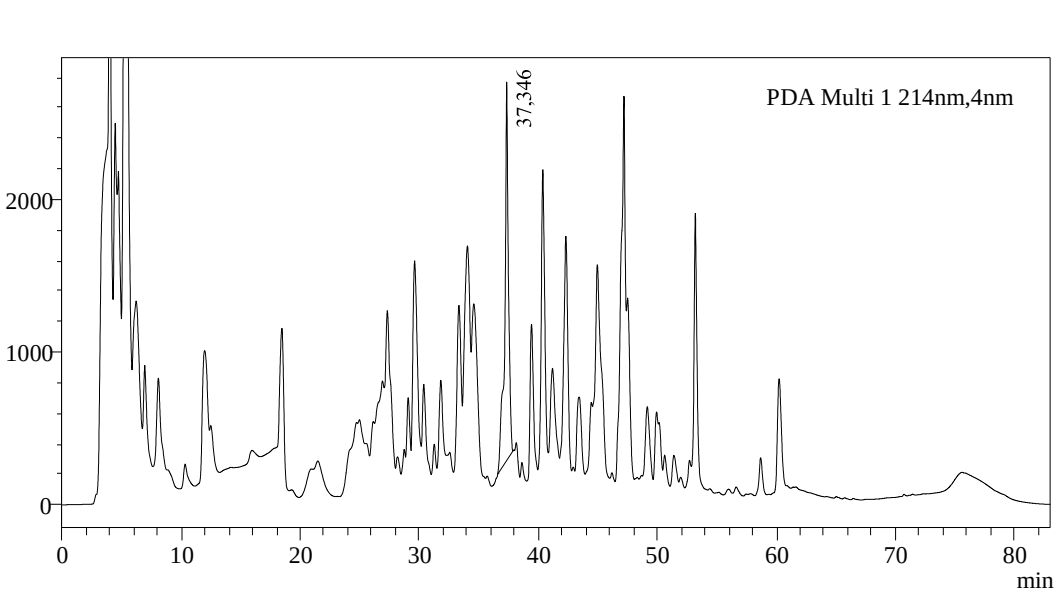
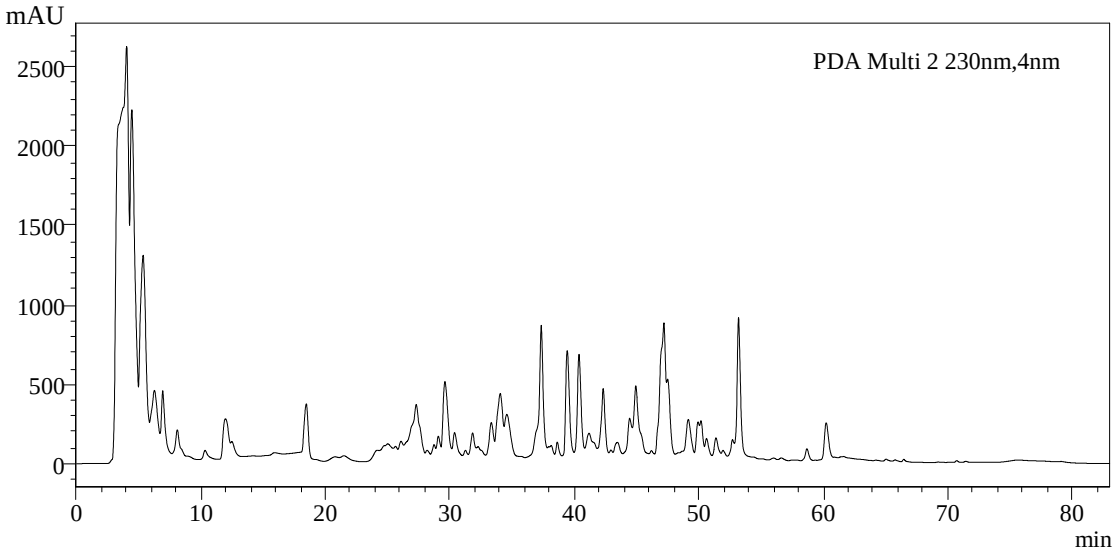
Vial # : 1-1
Injection Volume : 100 uL

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2500
2000
1500
1000
500
0
PDA Multi 2 230nm,4nm
0
10
20
30
40
50
60
70
80
min

SHIMADZU
LabSolutions Analysis Report

<Sample Information> **Measurement of 1.73 µg /µL BSA and collecting fractions**

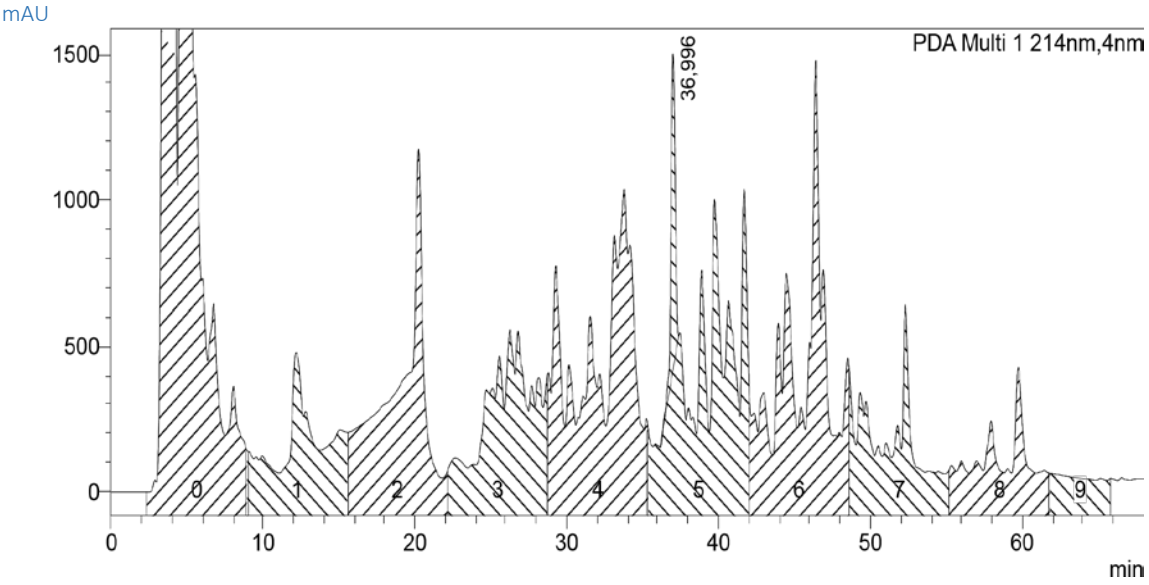
Sample Name
2016_07_08_BSA 1.82 µg/µL

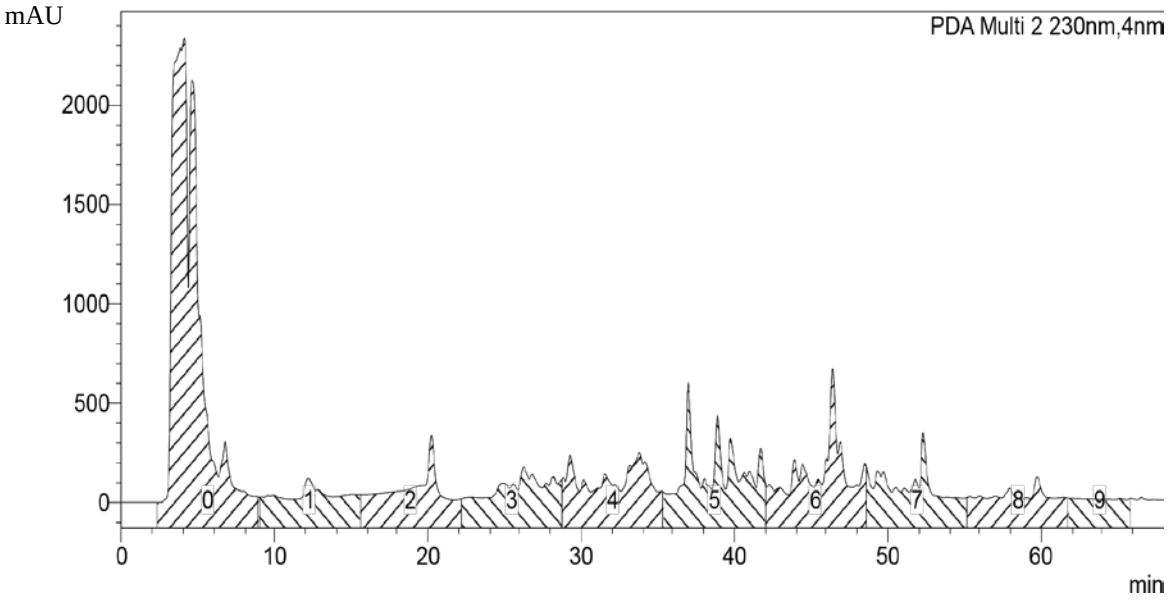
Sample ID
2016_07_08_BSA 1.82µg/µL

Data Filename
2016_07_08_BSA 200µg in110µL.lcd

Method Filename
2016_07_08_BSA_10Fractions.lcm

Vial # : 1-1
Injection Volume : 100 uL

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1500
1000
500
0
0
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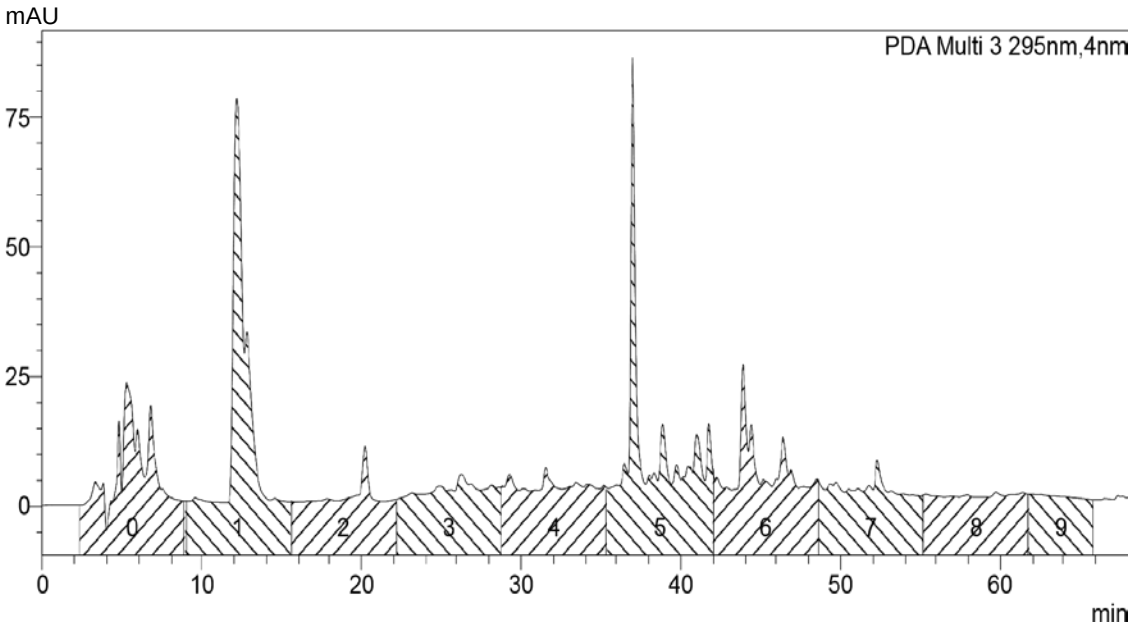
2
3
4
5
6
7
8
9
0
10
20
30
40
50
60
min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PDA Multi 3 295nm,4nm
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50
25
0
0
1
2
3
4
5
6
7
8
9
0
10
20
30
40
50
60
min

SHIMADZU
LabSolutions Analysis Report

<Sample Information> **Measurement of 0.05 µg /µL BSA**

Sample Name
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Sample ID
2016_07_27_BSA fractions_10_0.05 µg/µL

Data Filename
2016_07_27_BSA fractions_10_0.05 µg/µL.lcd

Method Filename
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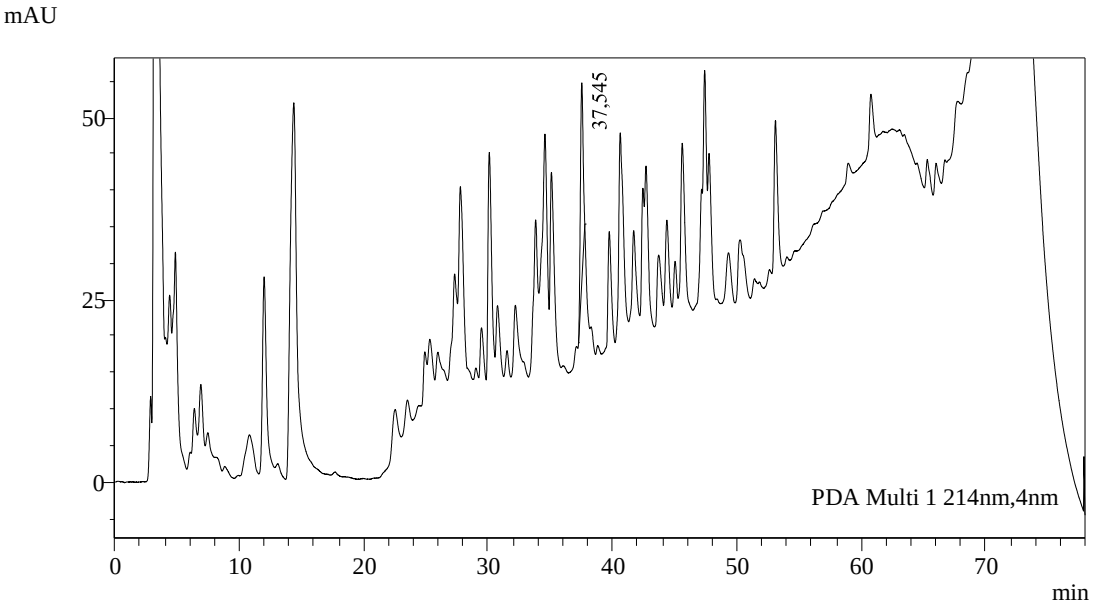
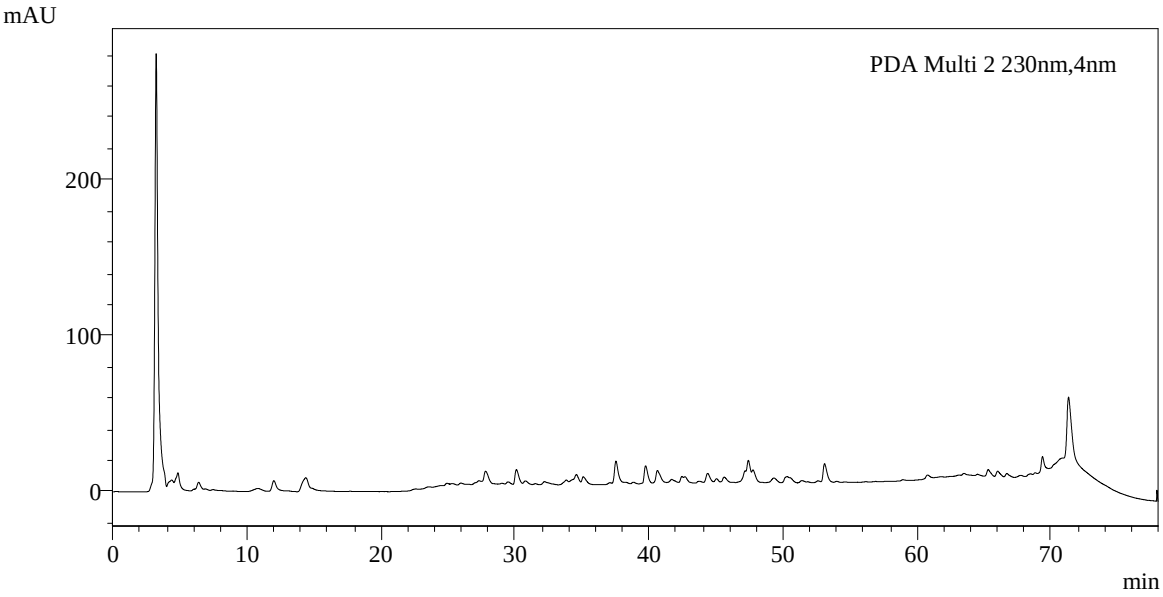
Vial # : 1-1
Injection Volume : 5 uL




mAU
PDA Multi 2 230nm,4nm
200
100
0
0
10
20
30
40
50
60
70
min

SHIMADZU
LabSolutions Analysis Report

<Sample Information> **Measurement of Fr 6 of BSA containing Trp**

Sample Name
Fraction F6

Sample ID
Fraction F6

Data Filename
Fraction F6 BSA Trp FLUORESCENCE.lcd

Method Filename
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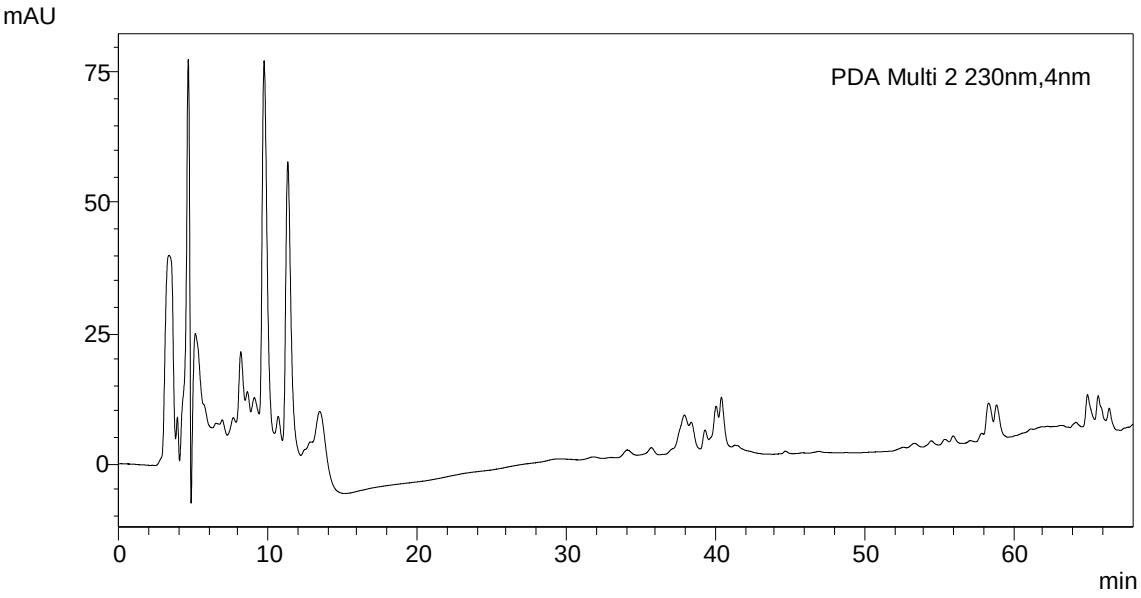
Vial # : 1-1
Injection Volume : 100 uL



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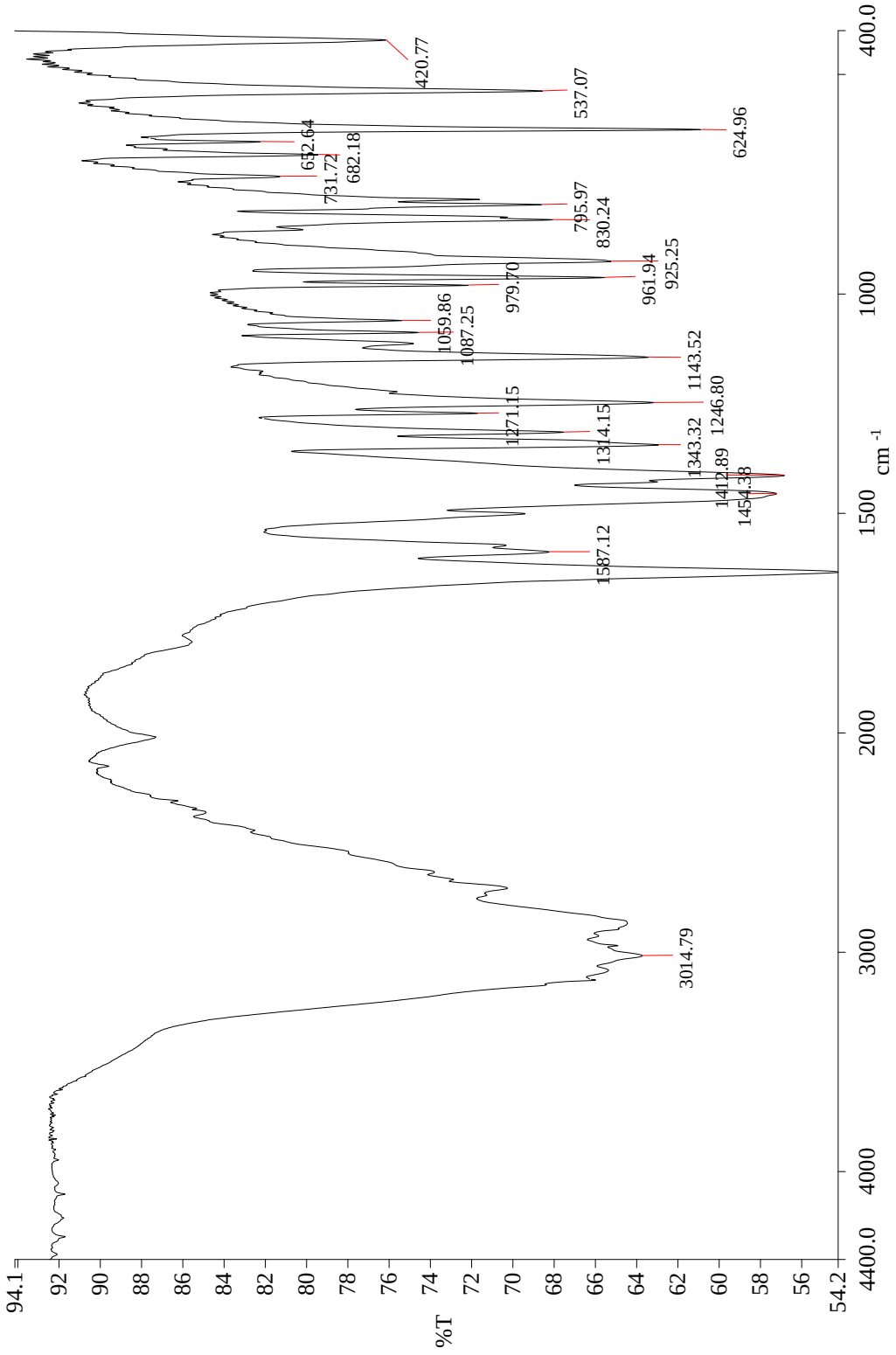

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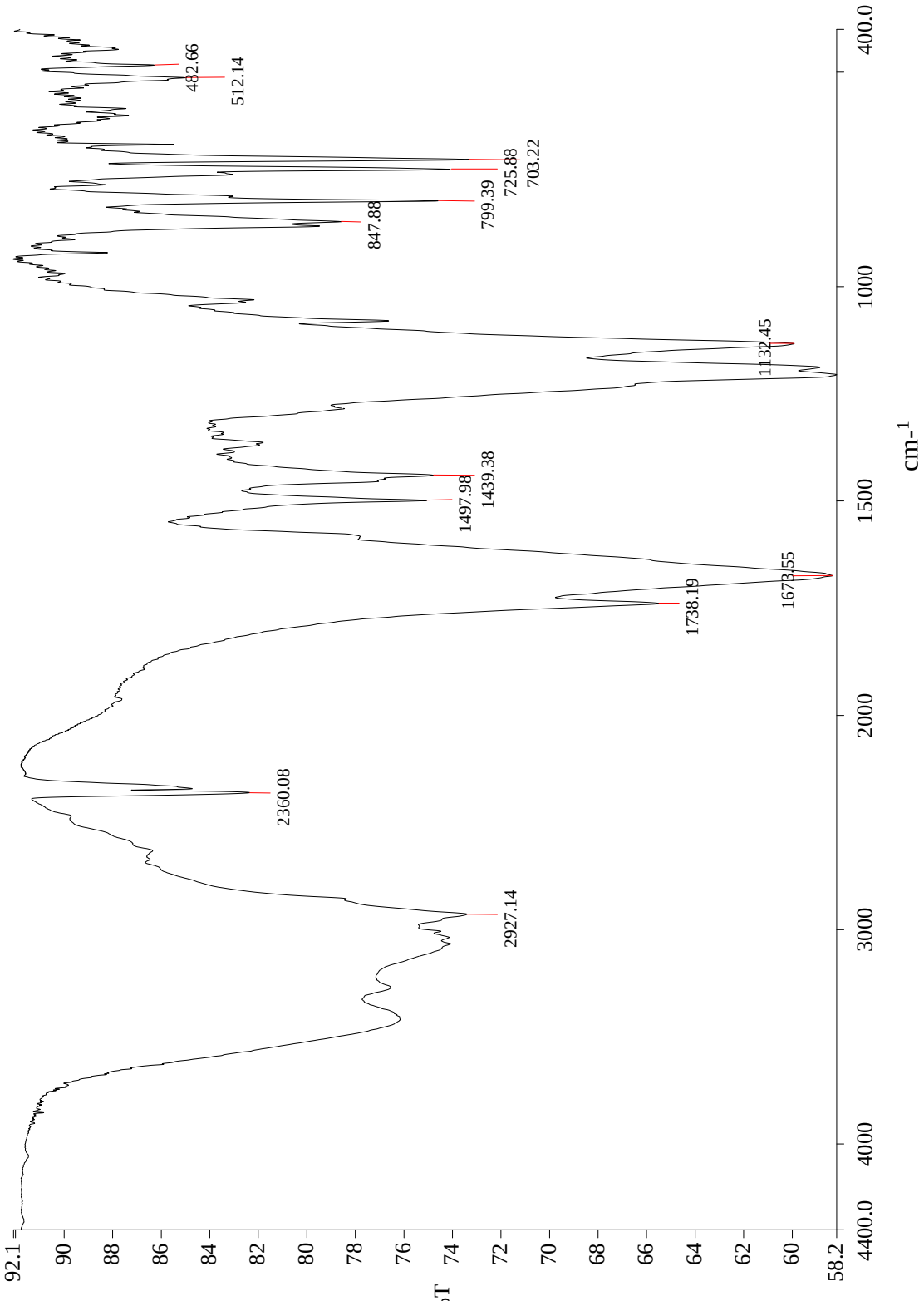

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