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„Identification of Lipids and quantification of drugs in Plasma samples by LC-MS“

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1. Abstract

In general, the subject of this thesis is to analyse rat plasma with LC-MS. On the one hand lipidomics of rat plasma are performed comparing the lipid profile of old rats with younger ones to see if this profile is linked to any diseases like hypercholesterolemia or cardiovascular diseases. Furthermore, the plasma lipid profile is compared with the lipid profile in the brain. The results demonstrate that the plasma includes especially fatty acids and glycerophospholipids and thus contains mainly low MWs molecules. Contrary, in the brain several lipids with much higher MWs are located. On the other hand, CE-123, a derivative of Modafinil, is analysed in course of pharmacokinetic trials. CE-123, with Modafinil as IS, is not successfully quantified with HPLC with absorbance detection due to baseline separation problems and limited sensitivity. However, we could show that quantification with LC-MS enables sensitive and selective detection of these drugs in plasma. For that purpose a change of column will be necessary in order to optimize baseline separation while shorten the retention time allowing for fast detection.

2. Introduction

High-performance liquid chromatography (HPLC), the major analytical tool for separating complex mixture components [1], combined with mass spectrometry (MS) which provides beside speed, high sensitivity and isotopic specificity [1] advances in ionization, fragmentation, high mass accuracy analysis [2] of 1ppm and even lower [3] and a resolution over 20 000 [3] – also known as liquid chromatography mass spectrometry (LC-MS) is an indispensable tool in the drug discovery and development in pharmaceutical industries and scientific research [1] due to the synergistic employment that leads to a rapid characterization and quantification of complex mixtures. [1]

Metabolomics has as well a major place in the drug discovery. Beginning the analysis in one single cell, going to complex clinical studies and continuing with defining the biochemical state of an organism for discovering biomarkers is enabled by MS as the ultimate detection tool [4], particularly combined with separation techniques such as HPLC greatly expanded the power of lipidomics-based examinations and therefore facilitate the identification of biomarkers, even the disease mechanisms, and also the efficacy of the therapy. [2] Lipids play an essential role in life, they are present in the human body e.g., in membranes, cells and serum. They are associated with many human diseases like diabetes, atherosclerosis, cancer, psychiatric disorders and neurodegenerative diseases. For this reason, the research on lipids has become a unique discipline called “Lipidomics“. [6] The field of Lipidomics deals with identifying the structures of cellular lipids, quantifying them and examine the interactions with other lipids, metabolites or proteins. Based on new research in this field lipid-associates diseases may be prevented in the future. [6] Lipidomics can help in drug discovery and development by determining changes in lipid states and looking for pathogenic mechanism that leads to lipid associated diseases. [6]

The process of bringing a new drug to the market is divided into two areas: drug discovery and drug development. [14] Pharmacokinetic trials, accomplished through these two areas, attain enhancement in either the variety as well as the number of newly synthesized drug candidates. LC-MS is one of the most used tool in determining drug concentrations in biological matrices due to the unique combination of sensitivity and selectivity. [1]

Modafinil is a wake promoting drug with a potential for cognitive enhancement [7] [8] [9] [10]. However, it has a limited specificity for DAT, as it blocks serotonin and noradrenaline transporters as well, which leads to adverse side effects [8] [11], including increased impulsivity [8] [16]. It was proven that a series of compounds targeting DAT, enhance the memory and cognitive performance most probably by reuptake inhibition. [12] [13]

In this thesis on the one hand the lipidomic profile of rat plasma is analysed and these lipids are compared with those in the brain according to the publication of Smidak *et al.* 2017 [15], on the other hand CE-123, a derivative of Modafinil is detected in plasma by LC-MS.

2.1. Liquid Chromatography – Mass Spectrometry (LC-MS)

Bruker maXis HD is a high-resolution electrospray- quadrupole time-of-flight mass spectrometer (ES-QTOF-MS) coupled with an ultra-high performance liquid chromatography (UHPLC) separation system. Thus, it enables measurements of exact masses and their isotope pattern with ultra-high-resolution even for complex samples. [17]

LC-MS has become a unique tool to determine drug concentrations in plasma or other biological matrices. Due to its high sensitivity and selectivity it is surely used in pharmacokinetic trials in the course of drug development to support *in vivo* studies. [1]

The UHPLC system is operating as a reversed-phase UHPLC, gradient elution is applied and a deuterium lamp with a diode array detector is used. [1] [18] ESI technique is used as it has the ability to connect with chromatographic substances [3]. ESI is commonly used for drug metabolism applications because the ions have little energy, hence barely a fragmentation and the mass of the stable non-fragmented molecular ion is provided. [14] As mass analyzer Q-TOF is applied, where first the ions which stay stable in the Q are transported through the TOF tube due to their masses, to the detector. [1] Q-TOF provides high sensitivity and exact mass measurements with high resolution. This indispensable tool has become one of the first choices in the drug metabolism area. [14]

2.1.1. **High Performance Liquid Chromatography (HPLC)**

High-performance liquid chromatography (HPLC) is the major analytical tool, utilized in drug discovery, development and production, in the course of preclinical and clinical trials to completely screen the drugs. [1]

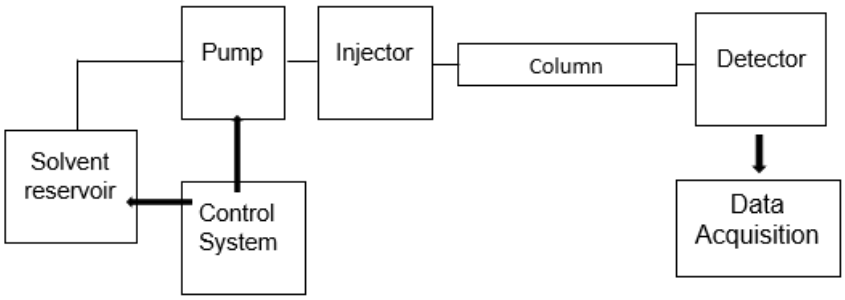

Fig.1: HPLC schemata.

HPLC system components are *Solvent Reservoirs, Pump, Injector, Column, Detector, Data Acquisition and Control System* as described in *Fig.1*. HPLC solvents are stored to keep the system in continuous operation (solvent reservoirs). With the aid of the pump, a continuous flow of the mobile phase is provided. The injector or autosampler is the inlet of the samples into the mobile phase. [1]

The column is an essential part and the heart of HPLC separation processes [18] in which the separation takes place. [1] The separation is based on the transport of the mobile phase in which the analytes are dissolved through the stationary phase where interactions due to the polarity of the analytes lead to different migration times. [1] Different affinities of molecules to the adsorbent/stationary phase surface lead to different retention times, the weaker the interaction, the less the analyte is retained. [1] The most commonly applied column packings are silica particle (SiO_2) with a bonded organic surface layer such as C18 or C8, displayed in *Fig.2* and *Fig.3*. [18] [1] The smaller the particle size, the faster the separation. General particle sizes in analytical separations are 5 µm, for ultra performance liquid chromatography (UPLC) particle diameters are below this value. [18]

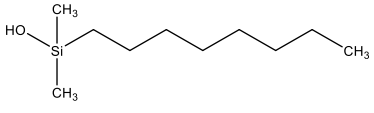

Fig.2: C8 silica.

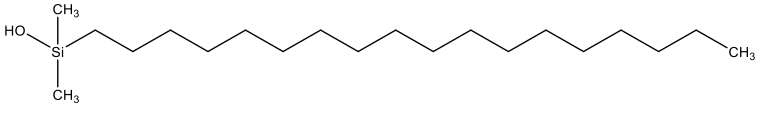

Fig.3: C18 silica.

In Normal-phase HPLC (NP HPLC) the stationary phase is polar due to the OH groups covering the surface. NP HPLC is operating with nonpolar solvents; this chromatographic system is suitable for hydrophobic compounds which are insoluble in polar solvents. [1] Reversed-phase HPLC (RP HPLC) is exactly the opposite: hydrophobic or van der Waals interactions predominate, the stationary phase is hydrophobic and the mobile phase is polar. For that purpose, water-based solutions are used. [1] RP-HPLC signifies that the column is less polar than the mobile phase. This means non-polar samples remain longer in the column due to sample-stationary phase-interactions and the polar compounds eluate first. [18]

Isocratic separation signify that the same mobile phase composition is used during the entire separation e.g., 50% ACN in water. In contrast, by using gradient elution the composition of the mobile phase changes during the run e.g., from 5 to 95% ACN in water. By operating with RP, ACN is the stronger solvent and its concentration is therefore increasing during the run. In order to start the next run with the same solvent composition as the run before, it is necessary to wait until the column is equilibrated. Thus, in total gradient elution takes more time than isocratic separations. [18]

The light source is a deuterium lamp, which provides light with wavelength from 190 to 400 nm. [18] Ultraviolet (UV) detection is the most used one, either with a variable-wavelength (VWD) or a diode array detector (DAD). [18] A certain UV absorbance is continuously registered and monitored. When the detector detects the analyte and its absorbance is stronger than the one of the mobile phase, the absorbance changes and a positive signal is obtained. [1] With VWD detectors it is possible to select the wavelength used for detection (e.g., 254 nm). This wavelength selection is realised with a diffraction grating. [18] With DADs chromatograms at all wavelengths between 190 - 400 nm are collected during a single run. DADs therefore offer more information than a single wavelength run. [18]

Finally, data acquisition and control systems, usually computer based systems, - control all parameters of the HPLC instrument and provide data from the detector. [1]

2.1.2. **Mass Spectrometry (MS)**

Owing to the rapid development, MS becomes a more and more important tool for structural characterization of small molecules, especially in the drug discovery area. Combining it with chromatographic separation techniques, in particular HPLC, its role expands in the pharmaceutical research and development. [1] MS offers speed, has a high sensitivity and isotopic specificity is provided. Mixtures of ions are separated based on mass-to-charge (m/z) ratio. Thus, this technique supplies the MWs of the compounds. LC-MS – applied for quantification and identification of either known as well as unknown organic substances - [1] allows measuring of relative masses with a resolution of over 20 000 and most of them provide accuracy of 1 ppm or even lower. [3]

The components of a MS are the *sample inlet* to introduce the sample, *ion source* where the ionization takes place, *mass analyzer* for sorting the ions by their m/z ratios, and *detector* which delivers the output e.g., mass spectra, as shown in *Fig.4*. [1] [14] [3]

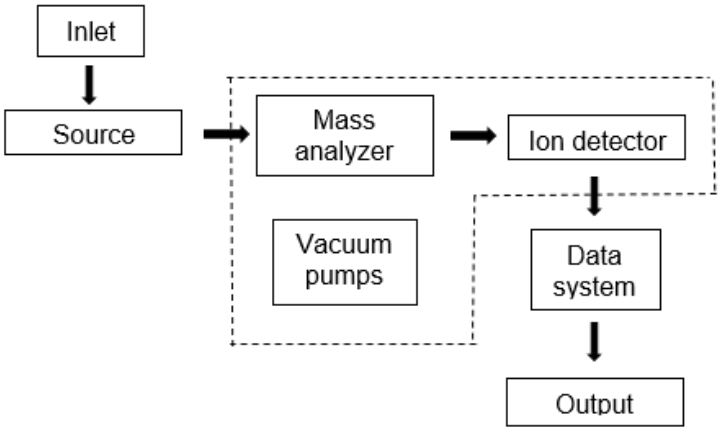

Fig.4: MS schemata.

Inlet

There are two different principles of intake systems: direct inlet and direct infusion. By using the direct infusion, it is possible to couple MS with chromatographic methods. Direct infusion is suitable for samples, which are easily evaporated. The sample is dosed from the reservoir *via* a capillary continually into the mass spectrometer. Operating with solid or liquid samples requires the use of direct inlet. [3]

Ion Source

To be able to detect the molecules by their m/z, it is necessary to ionize them to convert the neutral molecules to detectable ions. [3] [1] The ionization [3] and thus the generation of ions [1] take place in the ion source and can be performed with two different ionization methods: positive mode leading to protonation (cations) and negative mode (anions). [3]

Several ionization methods such as *chemical ionization (CI), electron impact (EI), desorption ionization (DI), matrix-assisted laser desorption ionization (MALDI)* and *electrospray ionization (ESI)* are evolved. [1] [3] Ionization techniques are divided in 3 categories: Evaporation method, which is used for volatile substances, for instance EI or CI. Desorption method, used for substances, which are hard to evaporate, and atomization method for substances in solutions. [3]

One of the most common ionization technique is ESI due to the connecting ability with chromatographic methods like HPLC. It also possesses high sensitivity and is suitable for polar and non-polar substances. [3] In ESI charged droplets are created under a high electrical field, then evaporated with thermal desolvation or N_2 (drying gas) and finally due to droplet fragmentation the analyte ions are created. [1] In case of large molecules, e.g., peptides and proteins, multiply charged ions are formed enabling their detection as the m/z is measured. [1] [14] A mass accuracy of 0.01% or even better can be realised for masses up to 100 kDa. [1] Furthermore, ESI is a soft ionization, meaning the analytes expire little fragmentation. [1] [14] The ability of introducing the sample at atmospheric pressure grant the coupling with HPLC. [1] Plenty ion types, such as $[M + H]^+$, $[M + Na]^+$ or $[M + NH_4]^+$ in positive-ion mode or $[M - H]^-$ in negative-ion mode can be created. [14] ESI is commonly used for drug metabolism applications because the ions have little energy, hence barely a fragmentation and the mass of the stable non-fragmented molecular ion is provided. [14]

CI and EI are only used to ionize volatile compounds. The ionization in EI is performed owing to collisions of the molecules with energetic electrons, which are produced from a heated filament and provide the molecule with high quantities of residual energy. [3] [1] Extensive fragmentation and resulting absence of molecular ions are the consequence. [1] In contrary, CI – a soft ionization – where ions are generated by ion-molecule-reactions – little fragmentation is the result. [3] [1] When the sample is non-volatile, polar or has a high MW, DI is used. Here energetic particles or photons impact the samples. MALDI, a DI method, has a UV or IR absorbing matrix. This matrix absorbs photon energy from the laser irradiation and interacts in this way with the sample. [1]

Mass analyzer

The ions are transported to the mass analyzer by magnetic or electric fields e.g., *time-of-flight* (TOF) analyzer or *quadrupole* mass analyzer. [3]

Applying TOF analyzers, the ions are accelerated and fly through a flight tube to the detector. The time, which the ion takes to reach the detector, is direct proportional to its m/z. Heavy ions with higher m/z need more time to reach the detector as the ones with lower m/z. [1] [14] Hence, the ions are separated due to the time they take to reach the detector depending on their m/z. [3] [14]

Another frequently used mass analyzer is quadrupole (Q). [3] The quadrupole mass analyzer consists of four parallel rods with electrical potentials, where the ions move through and only the desired ones pass to the detector. Only the ones which stay stable (mass-selective stability) reach the detector. Other ions with lower or higher masses are eliminated. [1] Q-TOF analyzers provide high sensitivity and exact mass measurements with high resolution. This indispensable tool has become the first choice in the drug metabolism area. [14]

2.1.3. **Quantification**

Bioanalytical methods like HPLC or MS have to be validated to reveal the performance of the method, as well as its reliability and to demonstrate its adequacy for the intended purpose. [19] [1] This is important for preclinical and clinical research of the drug development and an essential part of pharmacokinetic trails, especially registration of new discovered drugs. [19]

The validation of pharmaceuticals starts during the preclinical and clinical trials, going through the marketing of the drug and should also be monitored while its application to control the performance of the drug by measuring parameters such as accuracy, precision, reproducibility, limit of detection (LOD), limit of quantitation (LOQ), quantification range, recovery, standard curve, stability and robustness. [19] [1]

Accuracy

Accuracy describes the closeness between the obtained results and the nominal value, [19] [1] or rather the deviation of the observed concentrations to the nominal/true value. [19] The result is multiplied with 100 to get the relative error (% RE). [19] A minimum of 3 concentrations, which are replicated at least 3 times, i.e. 9 determinations should be assessed. [1] The recovery percent and relative standard deviation (RSD) of the measured concentrations are calculated to get the accuracy. [20] The mean value should be inside the range of 15% of the nominal value, except lowest limit of quantification (LLOQ), which are not allowed to deviate by more than 20%. To determine the accuracy it is necessary to analyse control samples spiked with the analyte or compare the method with a reference method. [19]

Reproducibility

Reproducibility expresses the precision between laboratories. The method has to be able to yield similar concentrations for one sample on different occasions. Reproducibility shows to the performance of the method from lab-to-lab, from day-to-day, from analyst-to-analyst, and from instrument-to-instrument, in either qualitative as well as quantitative terms. [19]

Precision

Precision – the random error – is the closeness of various measurements to each other. [19] The acceptable tolerance is between ±15% and only for LLOQ sample it is ±20%. [21] The recovery percent and RSD of the measured concentrations are calculated to get the precision. [20] There are three stages of precision. First stage is the injection precision which refers to exactness of repeated injections, where a single preparation is multiplied injected on the same day. The next stage is the analysis repeatability, where the same chemist analyses multiple preparations and arranges multiple injections of one sample on the same day. The last one is the intermediate precision, used for determining the variability of the analytical test, on a different system, performed by different analysts, on a different day, but on the same sample. For instance, %RSD is used to evaluate the adequacy of the precision has been achieved. [1]

LOD/LOQ

LOD is the lowest detectable (but not quantified) concentration of the analyte [19] [1] and is defined for a peak with a signal-to-noise ratio of about 3:1. [1] LOQ is the lowest concentration in the sample that can be measured with an acceptable level of accuracy and precision. [1] LOQ has a signal-to-noise ratio of 10:1. The prepared solutions are injected five or six times and then the %RSD of the peak area is calculated. The values should have a %RSD of ≤ 20% for LOD and %RSD of ≤ 10% for LOQ. [1] The LOD/LOQ range for non-peptide/protein-related products is between 0.01% and 0.2% and for protein/peptides between 0.1% and 0.5%. [1]

Quantification Range

Quantification Range includes all concentrations between and including LLOQ and ULLOQ that can be quantified with a high reliability and reproducibility. [19]

Recovery

Recovery describes the extraction efficiency of an analytical process (given in %). The results of extracted samples measured at three concentrations (low, medium and high) are compared with un-extracted standards which have 100% recovery. [19]

Standard Curve

The calibration curve is the relation between instrument response and known concentrations of the substance. When measuring the analyte in plasma, also the calibration curve concentrations have to be spiked with plasma and measured at the same way. Blank sample (matrix sample without IS), zero sample (matrix sample with IS) and 6-8 other concentrations including LLOQ should be taken in the curve. When the precision of 20% and an accuracy of 80 - 120% are available and the analyte response is at least five times higher than the blank, the lowest standard in the calibration curve can be accepted as the limit of quantification. [19]

Stability

Stability tests are used to detect any analyte degradation during preparation of the sample, its analysing and storing until the analyte is not used anymore. At least two concentrations should be evaluated and the results should be within 15% of nominal concentrations. [19]

Short - term stability: To evaluate the stability of the analyte in the matrix at ambient temperature, three aliquots of low and high concentrations are kept for at least 24 hours and then analysed. [19]

Long - term stability: This term refers to the stability of analyte, where the biological matrix has to have a stable condition between the time of the first sample collection and the last sample analysis. [19]

Freeze and Thaw Stability: At least three freeze-thaw cycles have to be evaluated to see whether the stability of the analyte stays equal or not. [19]

Robustness

Robustness is the capacity of an analytical procedure of remaining unaffected by small variations and demonstrates the reliability during its usage [19] (ICH guidelines – *The **I**nternational **C**ouncil for **H**armonisation of Technical Requirements for Pharmaceuticals for Human* [26]). The reproducibility of the method in different laboratories or under other conditions can also be shown. [19]

2.2. Lipidomics

2.2.1. **Metabolomics**

Lipidomics is a part of Metabolomics - *the quantitative and qualitative analysis of endogenous small molecule metabolites*. [4] A metabolome involves small molecules which are modified by medication, diet, environmental exposure or coexisting organisms. The biochemical state of an organism is reflected by the metabolome and the measurement of the metabolome changes due to external stressors, e.g., genetic modification, drug treatment, environmental changes can lead to determine the relation between a perturbation and affected biochemical pathways and eventually to the discovery of biomarkers. In drug discovery the comparative mode is applied where two or more groups are compared with each other, such as normal state/healthy individuals and perturbed state/drug administration. Thus, qualitative and quantitative changes are determined that can be related to the perturbation. [4]

2.2.2. **Lipids and Lipidomics**

Lipids play an essential role in life, they are present in the human body e.g., in membranes, cells and serum and therefore associated with many human diseases like diabetes, atherosclerosis, cancer, psychiatric disorders, neurodegenerative diseases and infectious diseases. For this reason, the research on lipids has become a unique discipline called “Lipidomics“. [6]

The field of Lipidomics deals with identifying the structures of cellular lipids, quantifying them and examine the interactions with other lipids, metabolites or proteins. Because of this scientific region, lipids can be more explained which could lead to the prevention of lipid-associates diseases in the future. [6]

Lipidomics is the identification and quantification of lipids in biological systems, which plays an important role in cellular function. The advances of coupling LC with MS and the resulting high mass accuracy, higher resolutions etc. facilitate the identification of biomarkers of diseases, their mechanisms and the efficacy of the therapy. Lipids have pleiotropic roles in cellular functions. The lipid assembly of cellular membranes serves as regulator of transmembrane protein activities, like ion channels and receptors. Furthermore, thousands of lipids serve several signalling functions that provide suitable cellular responses to external perturbations during health, however even accelerate pathologic changes in the metabolic network, which initialise disease processes. [2]

Lipidomics is divided in three main approaches, involving several sample preparation/separation and following mass spectrometric analysis. [2] Shotgun lipidomics provides a possibility of analysing cellular lipids rapid with a high reproducibility. More than 20 lipid classes and hence thousands of individual lipid molecular species can be analysed directly from lipid extracts, based on multidimensional mass spectrometric array analyses after sample preparation and separation. [5] With this rapidly expanding technology [5] lipid extracts are directly injected into the MS and analysed using a variety of fragmentation strategies. [2] Specialized derivatization methods are developed for targeted identification and sensitive quantification of lipids. Moreover, lipids have pleiotropic roles in disease processes and this field will continue to grow more and more. [2]

Table1: Lipid classes and some representatives [6] [22] [23].

Fatty acyls (FA) (fatty acids and conjugates)	Octadecanoids Eicosanoids Docosanoids Dodecanoic acid Fatty alcohols Fatty aldehydes Fatty esters Arachidonic acid (20:4) Prostaglandin PGH2

Glycerolipids (GL)	Monoacylglycerols (MG) Diacylglycerols (DG) Triacylglycerols (TG/TAG)
Glycerophospholipids (GP/GPL)	Phosphatidic acids (GPA) Phosphatidylcholines (GPCho/PC) Phosphatidylserines (GPSer) Phosphatidylglycerols (GPGro) Phosphatidylethanolamines (GPEtn)
Sterol lipids (ST)	Sterols Steroids Secosteroids Bile acids and derivatives Linoleic acid (18:2) Cholesterol
Sphingolipids (SP)	Sphingoid bases Ceramides (Cer) Phosphosphingolipids Phosphonosphingolipids Neutral glycosphingolipids Acidic glycosphingolipids

Table 1 shows the lipid classes and some representatives. Other lipid maps even involve prenol lipids, saccharolipids and polyketides as lipid categories. [23]

Fatty acids have two components, the chain which is lipophilic and the head which is hydrophilic, this characteristic is called amphiphilic. There are many other lipid classes which are dominantly hydrophobic and only have small parts like carbonyl or hydroxyl groups that are hydrophilic. In general, they are nonpolar molecules that do not dissolve in water. [6]

2.2.3. **Diseases**

Lipidomics can help in drug discovery and development by determining changes in lipid states and looking for pathogenic mechanism that leads to lipid associated diseases. [6] All the abbreviations of the lipids mentioned in this chapter can be found in *Table 1*.

Metabolic syndrome

Caloric food and high-fat diet lead to obesity, diabetes, atherosclerosis, hypertension, myocardial infarction and finally to congestive heart failure and stroke. Chronic increases of serum lipids cause changes in cellular lipid metabolism and eventually leads to intracellular lipid accumulation. [5] Diabetes mellitus is a metabolic disease, which is characterized by high blood glucose, insulin resistance and relative insulin deficiency. The causes for this disease and other metabolic disorders like hyperlipidaemia are not only because of genetic factors, but also due to a false nutrition attitude, such as eating an unhealthy amount of food with a high content of fat. [24]

Cardiovascular diseases

Hypertension is a high risk for cardiovascular diseases. Therefore, people with high blood pressure were tested for their lipid levels. In this manner, lipidomics is applied for valuing the efficacy of antihypertensive drug therapy. [6] According to the publication of Hu *et al.* [27] the levels of PC and TAG species were significantly increased in patients with hypertension and after treatment with beta-blockers (BBs) or a combination of 2-4 antihypertensive drugs such as diuretics, BBs, Angiotensin-converting enzyme inhibitor (ACEIs), angiotensin receptor blockers (ARBs) and calcium channel blockers (CCBs), e.g., Bisoprolol or Amlodipine/Bisoprolol or Felodipine/Bisoprolol/Valsartan (CCs/BBs/ARBs) even the total cholesteryl esters were significantly decreased. [27].

Non-alcoholic fatty liver disease (NAFLD)

NAFLD is a common type of chronic liver disease, which is associated with a high accumulation of lipids in the liver - nonalcoholic steatohepatitis (NASH) is the most extreme form of NAFLD. Numerous lipids like free cholesterol, cholesteryl ester, Cer,

and GPL accumulate, therefore Lipidomics is the best tool to determine the changes in these lipid levels and to find the mechanism leading to the accumulation. [6]

Alzheimer's disease

Alzheimer's disease (AD) is a progressive neurodegenerative disorder. It is characterized clinically by progressive cognitive disturbance and neuropathologically by the appearance of diffusible amyloid-beta and neuritic plaques. Even though there are not any drugs available to prevent AD, it is important to discover the disease as early as possible to delay the onset by, e.g., changing the lifestyle. Many studies were carried out and showed that late stages lead to neurodegeneration and neuronal loss caused by lipid level changes and membrane disruptions. Moreover, nutritional conditions are changed in later stages of AD altogether leading to changes in brain lipid components and signalling. [6]

Psychosis

Changes in lipid concentrations are associated with psychosis e.g., changed brain membrane lipid composition is related with the development of schizophrenia. A cohort study revealed that schizophrenia is associated with increased serum levels of specific TAG species (few double bonds & shorter chains of FAs). It was also discovered, that treatment with antipsychotic drugs manage significant changes in serum lipid profiles within 2 weeks. [6]

Cancer

Lysophospholipids activate the proliferation in breast cancer cells and thus lead to breast cancer progression and growth. Levels of lysophosphatic acid (LPA), a bioactive lysophospholipid are increased in patients with ovarian cancer, so it was suggested that LPA can be used as biomarker for cancer. [5] Eicosanoids – created from the arachidonic acid cascade - play beside inflammation an essential role in pathogenesis of cancer. Cyclooxygenase (COX) and lipoxygenase (LOX) which metabolize arachidonic acid to eicosanoids, are involved in cancer progression. [5]

2.3. Modafinil and its derivatives

2.3.1. **Modafinil**

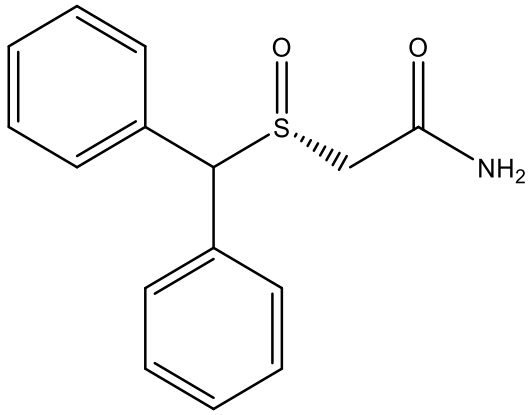

Fig. 5: The structure of Modafinil with a MW of 273.35 g/mol and a sum formula of $C_{15}H_{15}NO_2S$.

2-[(Diphenylmethyl)sulfinyl]acetamide - shown in *Fig. 5* - better known as Modafinil, is a psychostimulant and belongs to the group of indirect alpha-sympathomimetica. [25] Modafinil - a wake promoting drug - has the potential for cognitive enhancement [7] [9] [10] [8] and is used for the treatment of excessive daytime sleepiness in narcolepsy and other sleeping disorders. [9] [10] As a psychostimulant, Modafinil increases wakefulness, improves attention and even enhances mood among shift workers. [10] Beside the cognitive enhancement it is even able to improve working memory [28] [13], fear memory [29], spatial memory [29], avoidance learning [13] [30], impulsive behavior [31], attention [31], and even speed of response and accuracy. [31] [9] The underlying mechanisms are still not understood in detail. [9]

Modafinil is a dopamine transporter (DAT) targeting drug leading to reuptake inhibition and increase of the dopamine concentration in the synaptic cleft. [10] [9] Elevated extracellular dopamine concentrations are associated with cognitive improvements. [12] As this drug blocks serotonin and noradrenaline transporters as well, it has a limited specificity for DAT which leads to adverse side effects [8] [11], including increased impulsivity. [8] [16] The dopaminergic system has an important role in improvement of learning and memory. [13]

Modafinil - a non-cocaine-non-methamphetamine-like DAT reuptake inhibitor - is well studied in rodents and humans. [13]

In *in vitro* binding assays the IC_{50} [9] (half maximal inhibitory concentration – the efficacy of a substance in inhibiting a target or a specific biological/biochemical process by half) [38] value for Modafinil is determined and is 11.11 µM for DAT. The both, serotonin transporter (SERT) and noradrenaline transporter (NET) are blocked with even lower affinities (IC_{50} values of 1547 µM and 182.3 µM), as shown in *Table 2*. [9]

R-Modafinil binds with an about three times higher affinity to DAT than the S-enantiomer. The R-enantiomer has higher plasma concentrations than its S-enantiomer and they show comparable halftimes. [9]

Medicines containing Modafinil were first marketed in Europe in 1992. They are available in many countries of Europe with different names such as Modasomil, Modiodal, Provigil and Vigil, and as generic medicines. [32] This drug was associated with serious psychiatric disorders such as suicidal thoughts, depression, mania and symptoms of psychosis (delusion). Furthermore, skin reactions (especially children are concerned), and cardiovascular adverse reactions, such as hypertension and irregular heart beat were observed in patients treated with this drug. Therefore, the Pharmacovigilance Working Party (PhVWP) – a part of the European Medicines Agency (EMA) - reviewed the safety of Modafinil-containing medicines. The effectiveness of Modafinil in narcoleptic patients has been shown. Due to these serious side effects Modafinil is only registered for narcolepsy and not for obstructive sleep apnoea, shift-work sleep disorder and idiopathic hypersomnia given by the negative benefit-risk balance. [32]

Cognitive abilities are improved by drugs which have dopamine transporters (DAT) as target. Modafinil for example is such a drug which also blocks noradrenaline and serotonin transporters, leading to unwanted side effects. Therefore, CE-123 and other Modafinil derivatives were tested. [8]

2.3.2. **The derivatives**

It was proven that a series of compounds targeting DAT, enhance the memory and cognitive performance most probably by reuptake inhibition. [12] [13] More and more compounds with a lower toxicity are researched. [13] Several Modafinil analogs were synthesized since they may improve cognitive functions and working memory, moreover may show a high specificity and therefore decreased neurotoxicity. [13]

By substituting the amide component with five- and six-membered aromatic heterocycles, some components show similar or higher activities on DAT. Moreover, they are more selective toward DAT *versus* NET/SERT than Modafinil and even indicate an absence of neurotoxicity. [10] *Table 2* shows the IC_{50} values of chosen Modafinil derivatives synthetized and tested. CE-111, which specifically targets DAT and even inhibits dopamine reuptake, improves spatial memory performance in male Sprague-Dawley rats in the radial arm maze (RAM). [12] Similar to Modafinil, CE-111 permeates the blood brain barrier according to the publication of Saroja *et al.* [12] CE-111 specifically blocks dopamine uptake, while weakly blocks NET and has insignificant effects on SERT. [12] CE-125 displayed much higher potency for inhibiting uptake DAT as compared to NET and SERT, as shown in *Table 2*. [13]

Table 2: IC_{50} values of Modafinil, CE-111, CE-125 and CE-123 [12] [13] [9] [8].

	DAT	NET	SERT
Modafinil	11.11 µM	1547 µM	182.3 µM
CE-111	IC_{50} = 3.25 ± 0.23 µM	IC_{50} = 174 ± 15.5 µM	IC_{50} = 272291 ± 3453 µM
CE-125	IC_{50}=4.1 ± 0.8 µM; n=3	IC_{50}=687.2 ± 152.8 µM; n=3	IC_{50}=436.1 ± 129.0 µM; n=3
S- CE-123	IC_{50}=2.7 ± 0.3 µM; n=4	IC_{50}=137.7 ± 11.89 µM; n=3	not specific
R- CE-123	IC_{50}=18.6 ± 2.8 µM; n=4	IC_{50}=84.1 ± 9.86 µM; n=3	not specific

2.3.3. **CE-123**

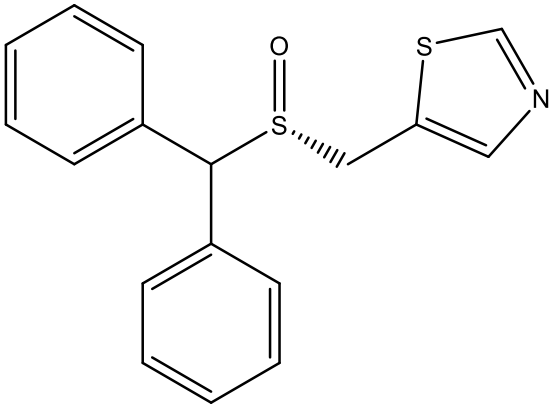

Fig. 6: The structure of CE-123 with a MW of 314.07 g/mol and a sum formula of $C_{17}H_{16}NOS_2$.

CE-123 - 2-[(Diphenylmethyl)sulfinyl]methylthiazole - (*Fig. 6*) shows higher specificity for DAT. It was tested in male rats in an attentional set-shifting task (ASST). ASST is a task, which verify cognitive flexibility and a 5-choice serial-reaction time task (5-CSRTT) measuring visuospatial attention and impulsivity. [8] Rats treated with R-Modafinil showed increased premature responses that indicates an increased impulsivity, compared to vehicle-treated rats. Only this visuospatial attention differs between R-Modafinil and CE-123. Hence, CE-123 enhanced cognitive flexibility with reduced impulsivity. [8]

2.4. Diazepam

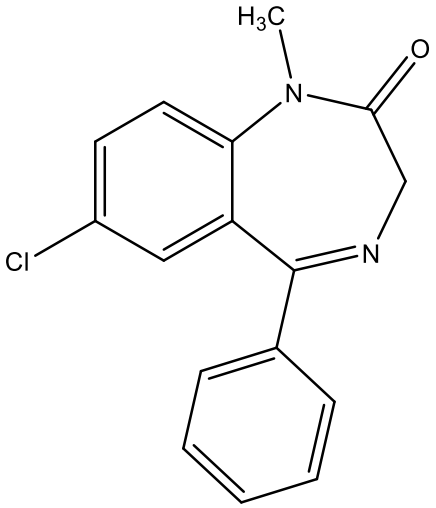

Fig. 7: The structure of Diazepam.

In order to quantify Modafinil and CE-123 in plasma samples, an internal standard (IS) is applied. For that purpose, Diazepam can be applied.

Diazepam (*Fig. 7*), a benzodiazepine with a long half-life of about 30 hours, is a psychotropic drug with a sedative, anxiolytic, hypnotic, muscle-relaxant and anticonvulsant/antiepileptic effect. These effects result from targeting the $GABA_A$ receptor. Benzodiazepines are indicated for the prophylaxis and treatment of convulsive disorders, epilepsy, anxiety, preeclampsia and eclampsia. Owing to their large therapeutic index, they are even prescribed to neonates/children and during pregnancy with the risk of causing adverse effects in infants due to their ability of crossing the placental barrier and accumulating in the breast milk. [37]

3. Material and methods

3.1. Technical equipment

- High Performance Liquid Chromatography (HPLC)
  - Shimadzu
  - LC-20AD liquid chromatography
  - SIL-20AC Autosampler
  - SPD-M20A prominence diode array detector
  - CTO-20AC prominence column oven
  - CBM-20A prominence communications bus module

- Ultra High Performance Liquid Chromatography (UHPLC)
  - Shimadzu CORP. Serial NO: L203054, 62310; Nexera XR
  - LC-20AD XR liquid chromatography
  - SIL-20AD XR Autosampler
  - SPD-M20A prominence diode array detector
  - CTO-20AC prominence column oven
  - CBM-20A prominence communications bus module

- Liquid Chromatography Mass Spectrometry (LC-MS) [33]
  - UltiMate 3000 RSLC-series system
    (Dionex; Thermo Fisher Scientific, Inc., Germering, Germany)
  - maXis HD ESI-Qq-TOF mass spectrometer
    (Bruker Corporation, Bremen, Germany)
  - DIONEX UltiMate 3000 RS Column Compartment
  - DIONEX UltiMate 3000 RS Diode Array Detector

- ACQUITY UPLC HSS T3 Column, 1.8 µm, 2.1 mm X 30 mm [34]
  - Column Length: 30mm
  - Particle Size: 1.8 µm; Pore Diameter: 100Å
  - store in 100% acetonitrile (ACN)
  - pH Range is 2 to 8
  - Pressure: up to 1241 bar
  - Temperatures between 20–45 °C

- Compass DataAnalysis 4.2 (Bruker Corporation)

- Eppendorf Centrifuge 5804 R

- Eppendorf Thermomixer comfort

3.2. Chemicals

- LiChrosolv Reag. Ph Eur **Acetonitrile** gradient grade for liquid chromatography (Merck KGaA, CAS-No: 75-05-8)

- LiChrosolv Reag. Ph Eur **Methanol** gradient grade for liquid chromatography (Merck KGaA, CAS-No: 67-56-1)

- **2-Propanol** (J.T.Baker, CAS-No: 67-63-0, Lot: 1609815009)

- **Formic acid** (Carl Roth GmbH + Co. KG, Art.-Nr. 4724.3)

- **Chloroform** stabilized with ethanol for UV, IR, HPLC (PanReac AppliChem, Lot: 0000923914)

- **Water** ROTISOLV HPLC Gradient Grade (Carl Roth GmbH + Co. KG, Art.-Nr. A511.3)

- **Butylated hydroxytoluene** (SIGMA-ALDRICH, Lot# MKBZ3580V)

- **Tert-Butyl methyl ether** ROTISOLV HPLC (Carl Roth GmbH + Co. KG, Art.-Nr. T175.1, UN 2398)

3.3. Lipidomics

In order to prepare the plasma samples for HPLC and MS analysis, different methods were applied according to the following publications: Patterson *et al*. 2015 [35] and Christinat *et al.* 2016 [36].

3.3.1. **Sample preparation**

Folch lipid extraction (FLE) [35]: 160 µL of methanol containing 1 mM butylhydroxytoluole (BHT) as a stabilizer and 320 µL of chloroform (ratio 1:2) are added to 40 µL of plasma. Folch sample is vortexed, incubated on ice for 20 minutes and vortexed again. After addition of 150 µL of water to induce phase separation, the sample is incubated on ice for 10 minutes and centrifuged for 5 minutes at 4500 rpm at room temperature (RT). The bottom layer (organic phase) is transferred to a clean microcentrifuge tube. The top layer (aqueous) is reextracted with 250 µL of chloroform:methanol (2:1). This extract is vortexed and centrifuged for 5 minutes. Furthermore, the organic layers are combined and dried with argon gas. Finally, they are reconstituted in 200 µL isopropanol (IPA) and transferred to HPLC vials.

Matyash Lipid Extraction (MLE) [35]: 40 µL of plasma are added to 300 µL of methanol (+1mM BHT) and 1 mL of Methyl-tert-butylether (MTBE). The sample is placed on shaker at 700 rpm at room temperature for 1 hour. 250 µL of water are added, after 10 minutes of incubation at RT with vortexing, the sample is centrifuged for 10 minutes at RT and 4500 rpm. The upper (organic) phase is removed. The bottom layer is reextracted with 2 mL of MTBE:methanol:water (10:3:2.5). The tubes are vortexed and centrifuged for 10 minutes. The organic layers are combined, dried under argon gas and reconstituted in 200 µL IPA.

Isopropanol Lipid Extraction (ILE): In order to precipitate proteins, 50 µL of plasma are added to 450 µL of IPA. The sample is shaken for 30 min at 700 rpm in a Thermomixer at 7 °C, then centrifuged for 10 min at 1500 rpm at 4 °C. 150 µL of the supernatant is placed into a new microcentrifuge tube. Solvent evaporation is performed with argon gas. The extract was reconstituted in 75 µL of ACN−water (1:1) and shaken for 5 min at 1000 rpm in the Thermomixer maintained at 4 °C.

3.3.2. **HPLC conditions**

ACQUITY UPLC HSS T3 Column (100Å, 1.8 µm, 2.1 mm X 30 mm) is used for HPLC and LC-MS measurements. The mass spectrometer is operated in both, positive and negative mode. Measurements take place at a constant flow rate of 0.1 mL/min and the temperature is set to 40 °C. As mobile phase A 60:40 ACN:water with 10 mM ammonium formate (with 0.1% formic acid) and phase B 90:10 IPA:ACN with 10 mM ammonium formate (0.1% formic acid) are used. 10 µL of the sample is injected. First, a gradient from 0% to 95% ACN (see *Table 3*) is carried out to detect the distribution of the analytes in the chromatogram. The selected chromatography gradient starts, like demonstrated in *Table 3*, at 0% of solvent B and increases to 95% solvent B during 40 minutes, is then maintained constant for 10 minutes in order to flush the column and then set to 0% solvent A again for equilibration reasons. This HPLC condition is applied to all sample preparing methods to be able to compare them with each other.

Table3: HPLC and LC-MS gradient.

time	**module**	**command**	**value**
0.01	Pumps	Pump B Conc.	0
40.00	Pumps	Pump B Conc.	95
50.00	Pumps	Pump B Conc.	95
50.01	Pumps	Pump B Conc.	0
60.00	Controller	Stop	

3.3.3. **LC-MS conditions**

The ESI ion source is operated as follows: capillary voltage: 3500/4500 V, nebulizer: 0.8 bar (N_2), dry gas flow: 7 L/min (N_2), and dry temperature: 200 °C. Mass spectra are recorded in the range of m/z 50–2800 in the positive-ion mode and m/z 50-2250 in the negative-ion mode. The sum formulas are determined by using Bruker Compass DataAnalysis 4.2 based on the mass accuracy (Δ m/z ≤ 5 ppm). It is operated with the same gradient as displayed in *Table 3* and the same HPLC conditions are used.

3.4. CE-123

3.4.1. **Sample preparation**

In order to measure the samples with HPLC and LC-MS, CE-123 and *R*-Modafinil (IS) are dissolved in ACN. First, the samples dissolved in ACN are measured (CE-123, Modafinil and a mix of both, each in a concentration of 50 µg/mL – 0.001 g/ 20mL weighed exactly). Then these solutions are mixed 1 + 1 with plasma (20 µL + 20 µL) and a blank with ACN is carried out. ACN has the advantage of precipitating proteins. The four samples are vortexed for few seconds and then centrifuged with a speed of 4500 rpm for 2 minutes. 20 µL of the supernatant is then carefully pipetted and transferred into the HPLC vial.

3.4.2. **HPLC conditions**

ACQUITY UPLC HSS T3 Column (100Å, 1.8 µm, 2.1 mm X 30 mm) is used for HPLC and LC-MS measurements. The mass spectrometer is operated in positive mode. Measurements take place at a constant flow rate of 0.2 mL/min and the temperature is set to 24 °C. 10 µL of the sample is injected. As mobile phase A water (with 0.1% formic acid) and phase B ACN are used. The chromatography gradient starts at 5% of solvent B and increases to 95% solvent B in 30 minutes, is maintained constant for 10 minutes in order to flush the column and then set to 5% for equilibration reasons, like shown in *Table 4*.

Table 4: HPLC and LC-MS gradient.

time	**module**	**command**	**value**
0.01	Pumps	Pump B Conc.	5
30.00	Pumps	Pump B Conc.	95
30.01	Controller	Stop	

3.4.3. **LC-MS conditions**

The ESI ion source is operated as follows: capillary voltage: 3500/4500 V, nebulizer: 0.8 bar (N_2), dry gas flow: 7 L/min (N_2), and dry temperature: 200 °C. Mass spectra are recorded in the range of m/z 50–2800 in the positive-ion mode.

4. Results and discussion

4.1. Lipidomics

The spectra are analysed with Compass DataAnaylsis 4.2 (Bruker Corporation). The formula, m/z and the intensity are determined. Intensity is given in %, this means that the highest peak is 100% and the others are shown in relation to this peak.

4.1.1. **Comparison of the plasma lipids with those in the brain**

The aim of this thesis is a non-targeted lipidomic analysis to determine the lipids present in rat plasma. According to the recent publication of Smidak *et al.* 2017 [15] the lipids of young and old rats show differences in the profile of brain lipids. Thus, the differences of the lipid profile of young and old rat plasma will be identified with the developed methods. Finally, these lipids will be compared to the ones present in the brain in order to evaluate if there is a connection. The samples were measured with LC-MS in both, positive and negative ion mode. Beside fatty acids, LC-MS data was examined for glycerophosphocholines and -serines, triacylglycerols, cholesterol esters and ceramids, which are present in the brain in positive mode, without result. Other glycerophosphocholines are found in plasma sample prepared by ILE in the positive ion mode, but they have lower molecular masses than the lipids in the brain (see *Table 5)*.

4.1.2. **HPLC measurements at 254 nm**

After preparing the plasma samples with the methods described in chapter 3.3.1, they are measured with the HPLC with the gradient, displayed in *Table 3*, to obtain the chromatograms at the UV wavelength of 254 nm. Due to a poor absorbance of the lipids at this wavelength (*Fig. 8*), LC-MS measurements are carried out to detect the m/z.

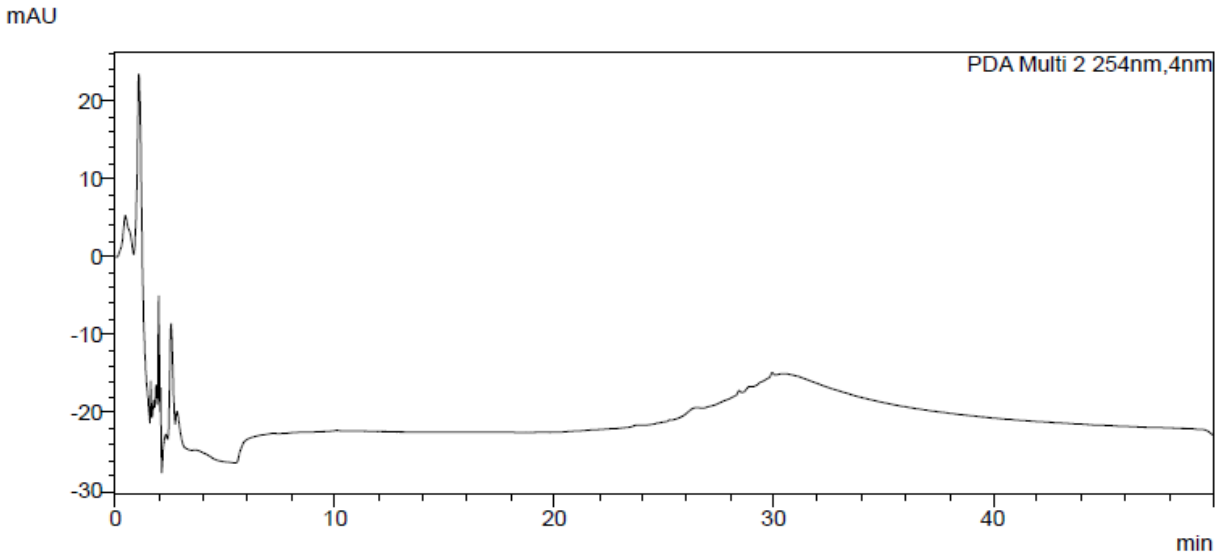

Fig. 8: Exemplary UV chromatogram (254nm) of plasma samples.

4.1.3. **Positive ion mode**

In this mode, protons are added *via* ESI to the molecule to make them charged positively. The ability to bind this proton depends on the functional groups of the molecule. In our case, lipids are of interest. Plasma samples mainly contain fatty acids preferring to donate a proton rather than accept one due to their carboxylic acid group. Hence, in positive mode fatty acids could hardly be detected. However, lipids such as glycerophospholipids, which have nitrogen groups are able to accept a proton and are therefore efficiently detected in positive mode, as shown in *Table 5*.

LC-MS measurements are performed for each sample preparation method: First, samples prepared by ILE according to the publication of *Christinat et al.* 2016 [36] are measured: with stabilizer (BHT), without stabilizer (IPA), an old sample (stored in the freezer at -80°C and defrosted for 3 times) to investigate what happens with the sample while storage (OLD) as well as a blank (MeOH).

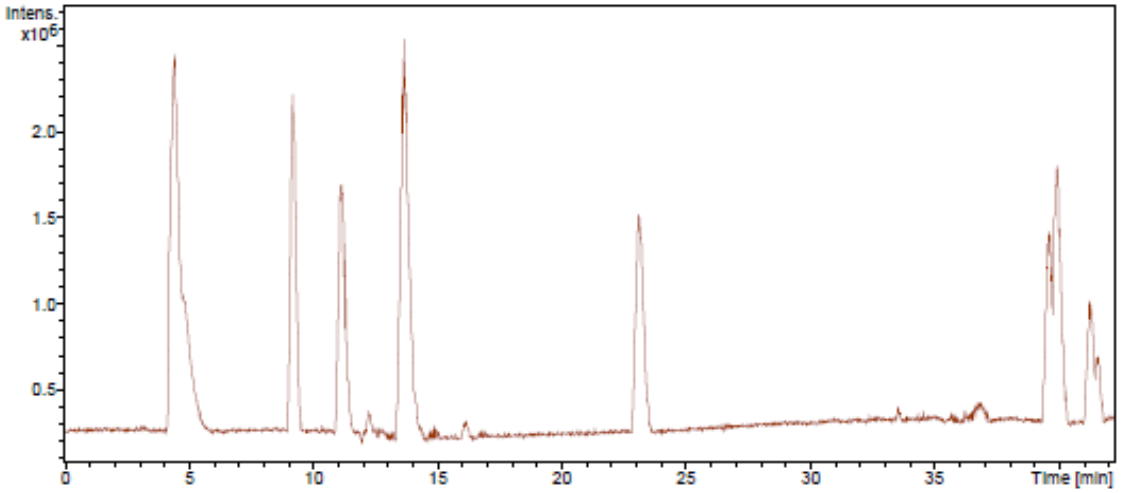

Fig. 9: LC-MS chromatogram of plasma sample "BHT" prepared by ILE.

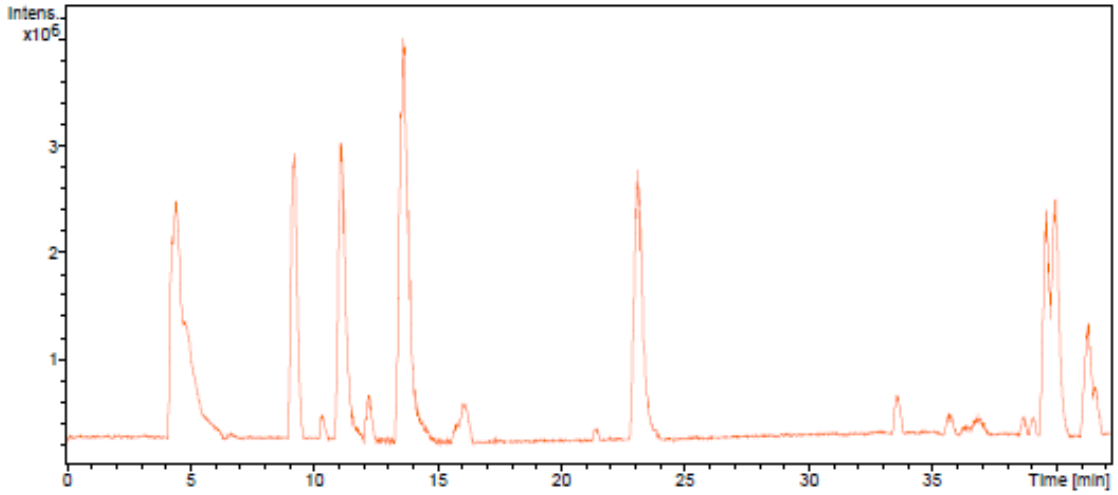

Fig. 10: LC-MS chromatogram of plasma sample "IPA" prepared by ILE.

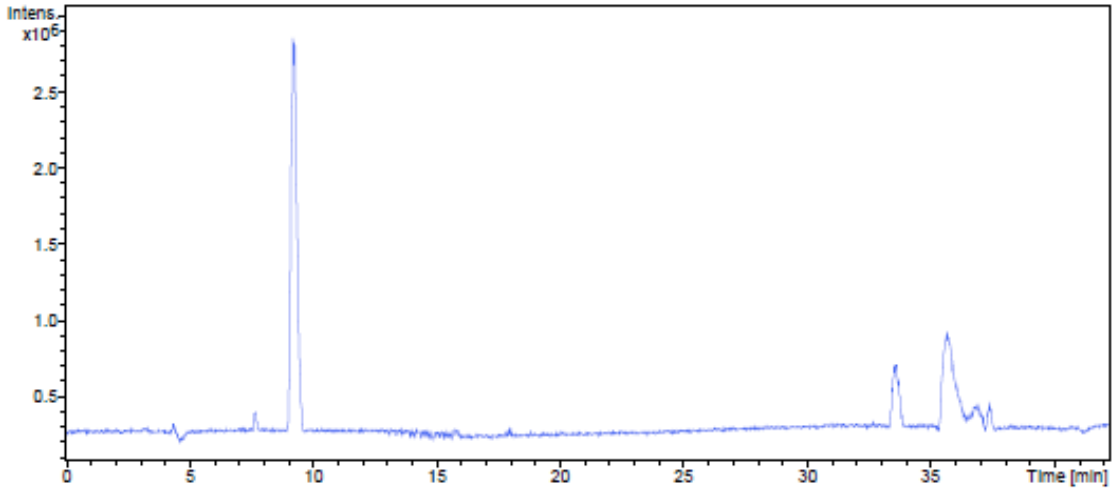

Fig. 11: LC-MS chromatogram of plasma sample "OLD" prepared by ILE.

Fig. 9-13 show the LC-MS chromatograms of three different samples prepared *via* ILE sample preparation, the methanol blank and the ACN/H_2O (1:1) blank, which is exactly prepared as the plasma samples although without plasma. *Fig. 11* depicts the main components of the "old" ILE sample, which demonstrates that repeated freezing of the sample results in fewer signals than in the freshly prepared sample. Thus, the samples have to be prepared directly before measurements and are not suitable for refreezing. The signals, which appear at minute 11 and in the last 10 minutes are present in all sample preparations and the ACN/H_2O blank. These signals do not correspondent to the plasma sample itself. Lipids are mainly detected between minute 10 and 30. Thus, all presented data is related to this chromatogram region.

Table 5: A list of phospholipids detected in the plasma sample prepared by ILE.

m/z	formula	Intensity (I)	Lipid
520.4304	**$C_{26}H_{51}NO_7P$**	854,369	Linoleoylglycerophosphocholine
496.3405	**$C_{24}H_{51}NO_7P$**	1,218,297	Palmitoyllysophosphatidylcholine
522.3561	**$C_{26}H_{53}NO_7P$**	305,937	Oleoylglycerophosphocholine
524.3722	**$C_{26}H_{55}NO_7P$**	778,084	Stearoylglycerophosphocholine

In the publication of Smidak *et al.* 2017 [15] glycerophosphocholines (PCs; 32-48 C-atoms), - ethanolamines (PE; 38-44 C-atoms), -serines (PS; 46 C-atoms), triacylglycerols (TG; 46-60 C-atoms), cholesterol esters (CE; 20 C-atoms), ceramides (Cer; 20-36 C-atoms) and sphingomyelins (SM; 22-24 C-atoms) are detected in the brain. These lipids have high MWs and are therefore not found in plasma. Indeed, other phospholipids are found, which are listed in *Table 5.* They have lower MWs and are therefore accessible to the plasma. The trivial names are obtained from PubChem.

Table 6: Comparison of the intensities of the main compounds between fresh prepared IPA with stabilizer (BHT), without stabilizer (IPA) and the old IPA (old) obtained from LC-MS.

m/z	formula	Intensity (BHT)	Intensity (IPA)	Intensity (old)
118.0869	$C_5H_{12}NO_2$	1,060,933	1,634,106	-
102.1279	$C_6H_{16}N$	251,748	242,866	260,267
393.2871	$C_{27}H_{37}O_2$	841,438	1,454,744	1,352,528
768.5377	$C_{46}H_{74}NO_8$	80,388	156,339	167,510
102.1278	$C_6H_{16}N$	233,452	248,724	-
496.3405	$C_{31}H_{46}NO_4$	1,218,297	445,663	-
524.3722	$C_{33}H_{50}NO_4$	778,084	1,229,394	-
758.5711	$C_{49}H_{76}NO_5$	1,190,487	1,385,810	-
419.3163	$C_{26}H_{43}O_4$	122,539	115,230	170,801

Table 6 shows components, which are detected in the presented sample preparations to show the difference in the intensities of the fresh prepared sample, the stabilized sample and the old one. The old sample contains fewer components due to decomposition of multiple substances because of repeated refreezing. For instance, $C_{49}H_{76}NO_5$ shows comparable intensities in the fresh prepared samples, with an intensity of 1,190,487 in BHT and 1,385,810 in IPA, but is not detected in the frequently refrozen sample, which indicates a deviation of 10^6.

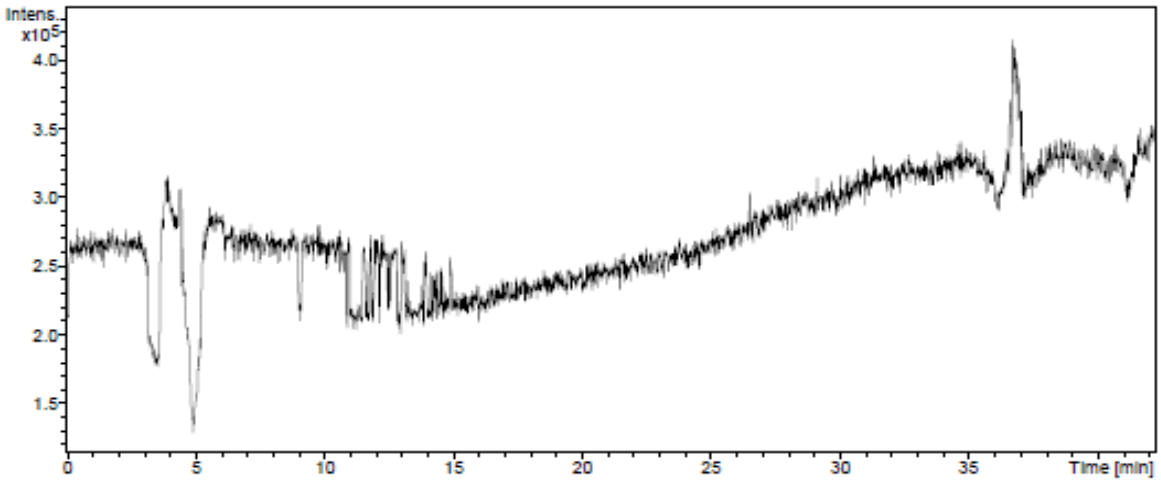

Fig. 12: LC-MS chromatogram of the MeOH blank.

The methanol blank has to be recorded in order to determine whether the column is contaminated with substances from the samples. As shown in *Fig. 12*, the LC-MS chromatogram of the MeOH blank barely shows peaks compared the ones from the samples. In conclusion, the MeOH blank mainly possesses noise.

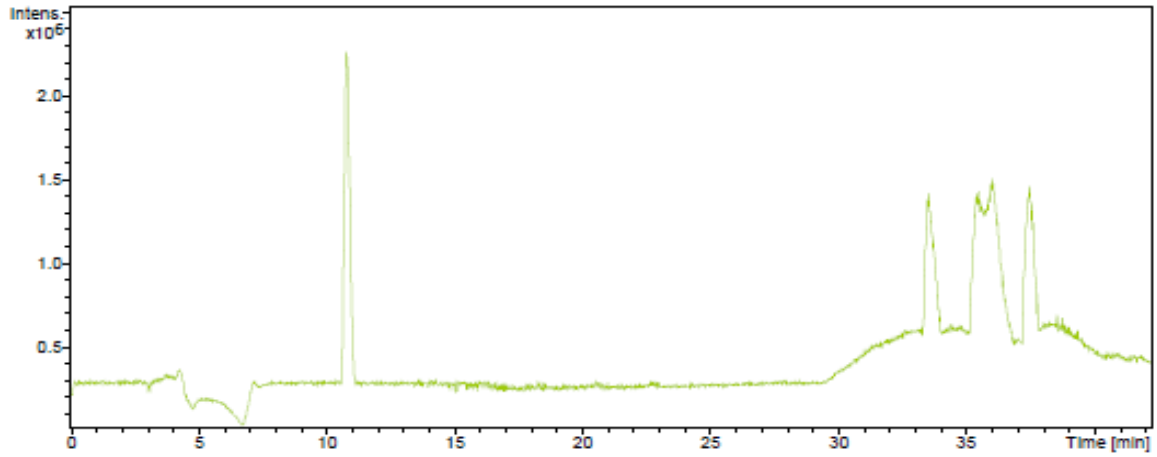

Fig. 13: LC-MS chromatogram of ACN/H_2O without plasma prepared by ILE.

Fig. 13 contains the chromatogram of ACN/H_2O and serves as a blank since ILE uses ACN/H_2O as solvent. The difference between the MeOH blank (*Fig. 12*) and ACN/H_2O (*Fig. 13*) is that the blank only consists of methanol which is directly injected in the LC-MS without preparing it. In contrast, ACN/H_2O is exactly prepared as the plasma samples although without plasma.

4.1.4. **Negative ion mode**

The three sample preparation methods presented in chapter 3.3.1. are compared to each other: FLE, ILE and MLE. Following the recorded chromatograms depicted in *Fig. 14-17* and their fatty acids in *Tables 7 – 9*. Folch - the most applied lipid extraction - delivers the best results (*Fig. 14*, *Table 7*), showing more fatty acids than the other extraction methods. With $CHCl_3$ and MeOH as solvents (FLE) the same lipids as in the other methods are detected and even more. *Table 9* shows the detected lipids with IPA as solvent. Apparently only these two fatty acids are found: $C_{20}H_{31}O_2$ and $C_{18}H_{31}O_2$ and thus with this method the fewest lipids are determined in negative mode.

Applying the sample preparations FLE, ILE and MLE, fatty acids like linoleic acid ($C_{18}H_{32}O_2$), palmitic acid ($C_{16}H_{32}O_2$), oleic acid ($C_{18}H_{34}O_2$), vaccenic acid ($C_{18}H_{34}O_2$), stearic acid ($C_{18}H_{36}O_2$), dihomo-γ-linolenic acid ($C_{20}H_{34}O_2$), docosatetraenoic acid ($C_{22}H_{36}O_2$), docosanoic acid ($C_{22}H_{44}O_2$) and arachidonic acid ($C_{20}H_{32}O_2$) are detected. As this data is recorded in negative mode, the fatty acids donate a proton and show a lower MW. For example, arachidonic acid has $C_{20}H_{32}O_2$ as a structural formula and a molar mass of 304.47 g/mol. It is detected as $C_{20}H_{31}O_2$ with a molar mass of 303.23 m/z. In negative mode, more fatty acids are found compared to positive mode since they have one main functional group – the carboxylic acid – and this group is likely to donate a proton and obtains a negative charge.

UV-chromatograms are not available since the lipids have a poor absorbance in the UV range as shown at the wavelength of 254nm, depicted in chapter 4.1.2. (*Fig. 8*).

LC-MS measurements are performed for each sample preparation method: The three sample preparation methods FLE (“$CHCl_3$”), ILE (“IPA”) and MLE (“MTBE”) are compared to each other.

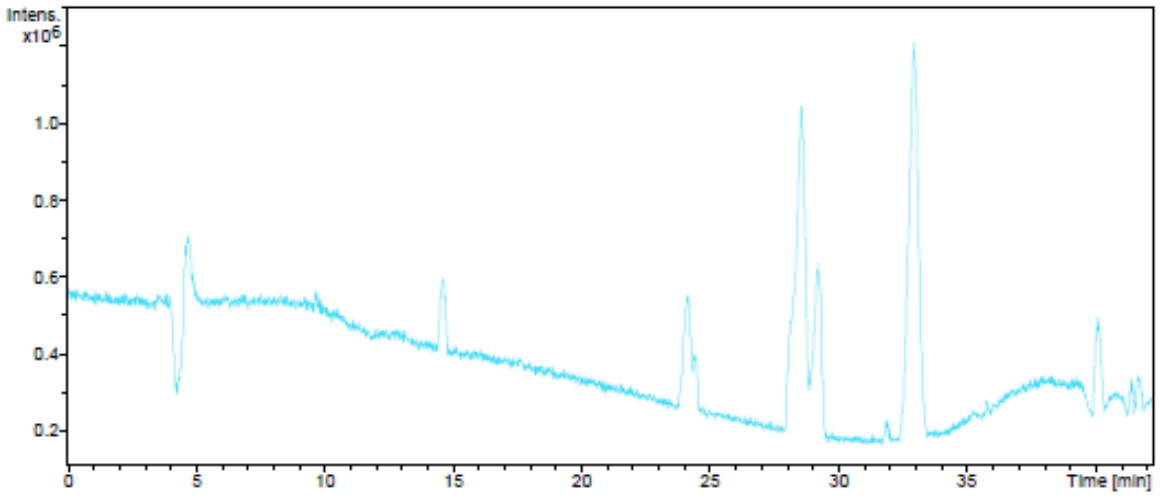

Fig. 14: LC-MS chromatogram of plasma sample "$CHCl_3$" prepared by FLE.

Folch - the most applied lipid extraction - delivers the best results (*Fig. 14*, *Table 7*), showing more fatty acids than the other extraction methods.

Table 7: Fatty acids of the plasma sample preparation "$CHCl_3$" (with stabilizer) by FLE.

m/z	formula	Intensity (I)	I%
283.2614	$C_{18}H_{35}O_2$	4,593	0.9
297.2309	$C_{18}H_{31}O_2$	201,956	47.2
303.2305	$C_{20}H_{31}O_2$	153,024	25.8
255.2308	$C_{16}H_{31}O_2$	164,231	94.4
281.2459	$C_{18}H_{33}O_2$	10,029	5.8
305.2456	$C_{20}H_{33}O_2$	3,994	2.3
331.2610	$C_{22}H_{35}O_2$	18,049	10.4
307.2612	$C_{20}H_{35}O_2$	4,765	0.9
339.3235	$C_{22}H_{43}O_2$	5,648	2.5
303.2304	$C_{20}H_{31}O_2$	153,963	36.0
279.2308	$C_{18}H_{31}O_2$	513,606	100
255.2308	$C_{16}H_{31}O_2$	199,970	100
281.2461	$C_{18}H_{33}O_2$	161,496	24.0

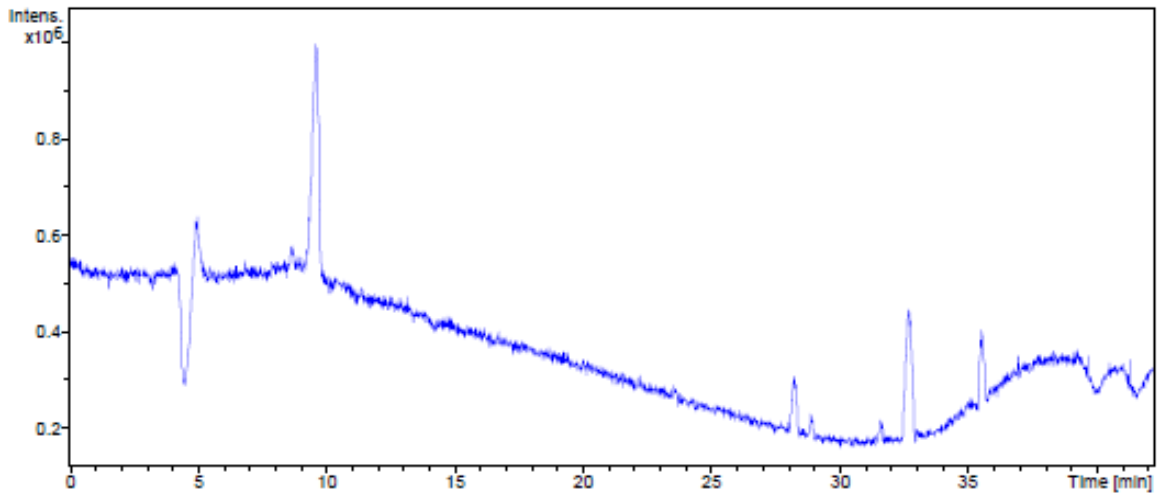

Fig. 15: LC-MS chromatogram of plasma sample "MTBE" prepared by MLE.

Table 8: Fatty acids of the plasma sample preparation "MTBE" (with stabilizer) by MLE.

m/z	formula	Intensity (I)	I%
279.2307	$C_{18}H_{31}O_2$	26,302	13.7
283.2618	$C_{18}H_{35}O_2$	4,869	2.5
303.2305	$C_{20}H_{31}O_2$	62,248	32.3
255.2312	$C_{16}H_{31}O_2$	135,701	78.7
281.2463	$C_{18}H_{33}O_2$	17,023	9.9
269.2465	$C_{17}H_{33}O_2$	2,750	1.0
303.2310	$C_{20}H_{31}O_2$	112,088	43.5
281.2467	$C_{18}H_{33}O_2$	43,879	12.4
283.2626	$C_{18}H_{35}O_2$	301,860	100

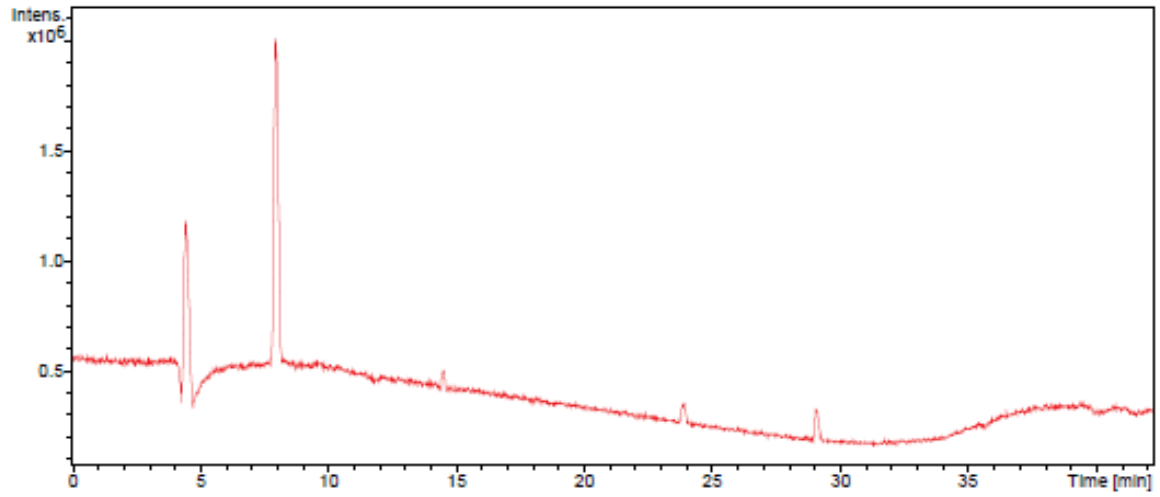

Fig. 16: LC-MS chromatogram of plasma sample "IPA" prepared by ILE.

Apparently, only these two fatty acids are found: $C_{20}H_{31}O_2$ and $C_{18}H_{31}O_2$ and thus with this method the fewest lipids are determined in negative mode (*Table 9*).

Table 9: Fatty acids of the plasma sample preparation "IPA" by ILE.

m/z	formula	Intensity (I)	I%
303.2300	$C_{20}H_{31}O_2$	17,838	9.6
279.2305	$C_{18}H_{31}O_2$	242,818	100

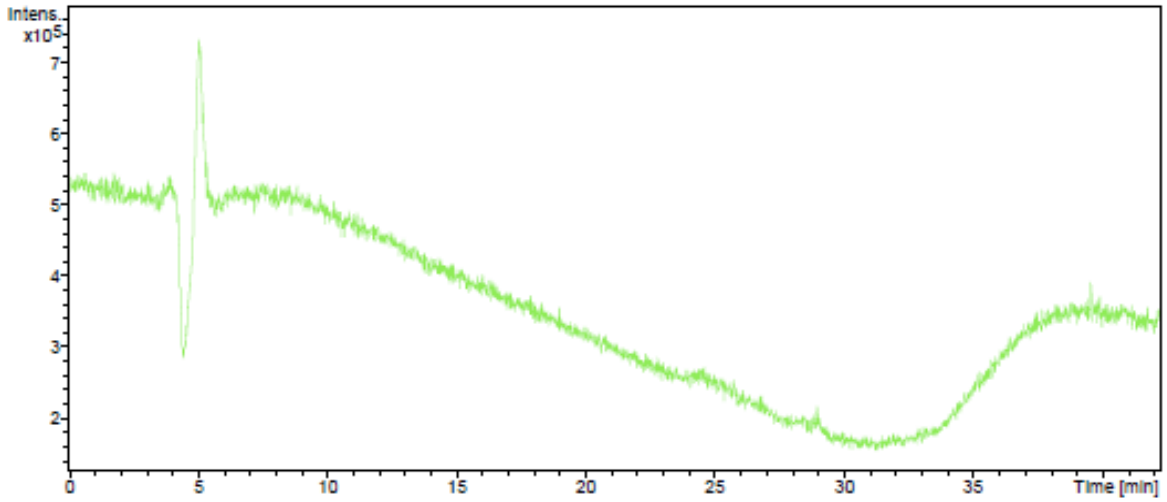

Fig. 17: LC-MS chromatogram of the MeOH blank.

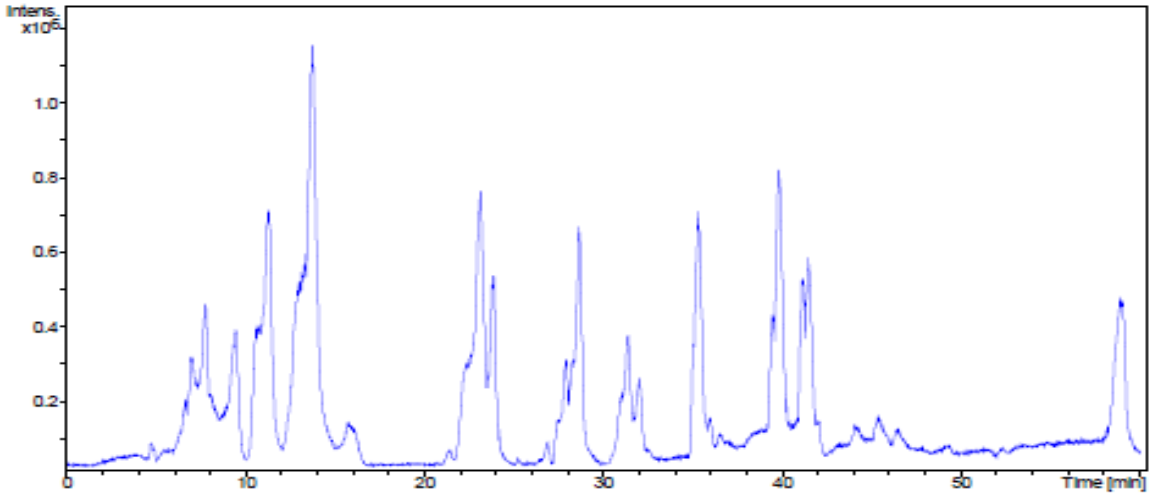

Fig. 18: LC-MS chromatogram of plasma sample "$CHCl_3$" prepared by FLE.

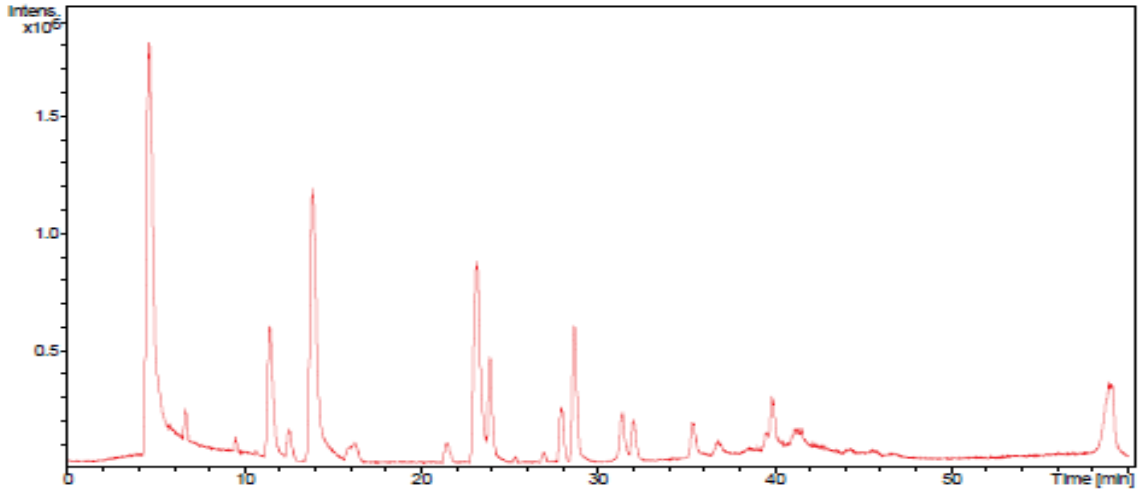

Fig. 19: LC-MS chromatogram of plasma sample "BHT" prepared by ILE.

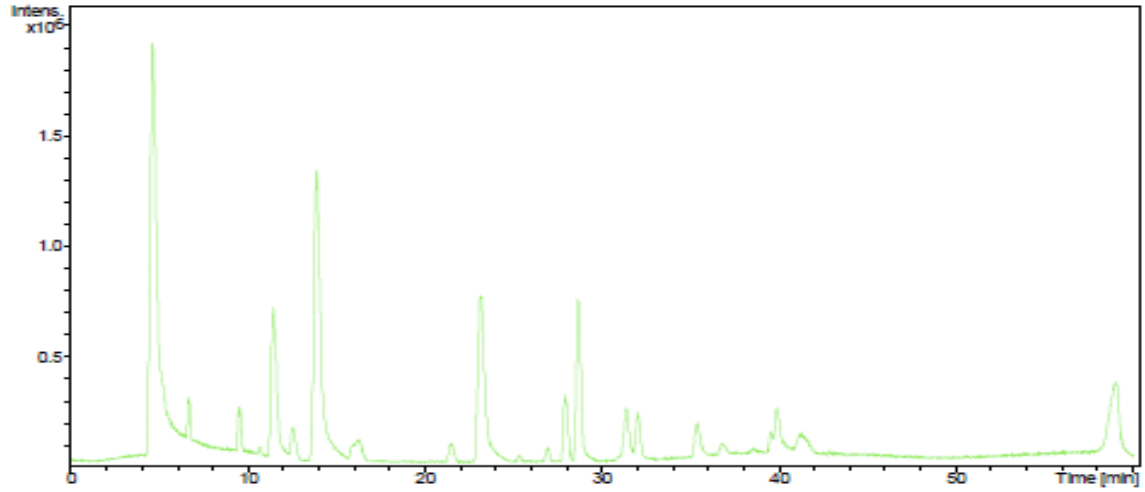

Fig. 20: LC-MS chromatogram of plasma sample "IPA" prepared by ILE.

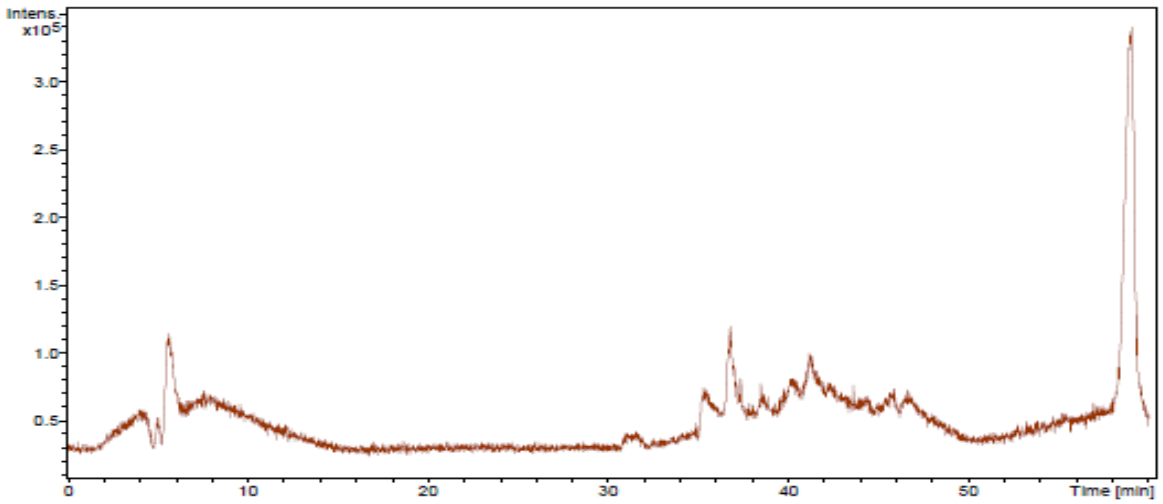

Fig. 21: LC-MS chromatogram of the MeOH blank.

Fig. 14-21 show that by repeating the measurements of the three sample preparations, different and more signals appear, hence this method possesses a low reproducibility. In *Fig. 18-20* more signals are detected, e.g., by comparing *Fig. 14* with *Fig. 18* there is a large peak appearing in minute 20, which is not in the other chromatogram although it is the same sample preparation, which indicates that the column might be polluted. Overall, this method is not suitable for our purpose due to the limited reproducibility. The chromatograms in *Fig. 18-20* possess the same lipids as the chromatograms in *Fig. 14-17*, though they contain more other substances visible through the number of signals. *Fig. 21* shows the MeOH blank, which has more signals than the MeOH blank displayed in Fig.17. This is as well a sign of limited reproducibility.

4.1.5. **Discussion**

Glycerophosphocholines with various chains are detected in positive ion mode, since a high concentration of these phospholipids can cause several diseases such as hyperlipidemia, it is necessary to compare the lipids from young rats with those of the old ones to detect the differences and prevent the arise of such diseases at early stages.

Several fatty acids are detected in the negative ion mode, like in the publication of Christinat *et al.* 2016 [36] and other publications. This indicates that rat plasma includes fatty acids like linoleic acid ($C_{18}H_{32}O_2$), palmitic acid ($C_{16}H_{32}O_2$), oleic acid ($C_{18}H_{34}O_2$), vaccenic acid ($C_{18}H_{34}O_2$), stearic acid ($C_{18}H_{36}O_2$), dihomo-γ-linolenic acid ($C_{20}H_{34}O_2$), docosatetraenoic acid ($C_{22}H_{36}O_2$), docosanoic acid ($C_{22}H_{44}O_2$) and arachidonic acid ($C_{20}H_{32}O_2$) which can as well influence the health of the rats, especially when they are present in plasma at high levels. Saturated fatty acids - as part of triglycerides - can lead in high concentrations to hyperlipidemia, followed by arteriosclerosis and cardiovascular diseases.

Due to poor reproducibility of the developed method, it was not possible to compare the plasma from young and old rats with each other. However, the comparison with the brain lipids indicates the plasma includes other lipids, especially fatty acids and glycerophospholipids with lower MWs. Contrary, in the brain glycerophosphocholines, -ethanolamines, -serines, triacylglycerols, cholesterol esters, ceramides and sphingomyelins with much higher MWs are located.

4.2. CE-123

The second part of this thesis deals with the quantification of CE-123 by using Modafinil as internal standard (IS) in plasma by LC-MS. First, these drugs are dissolved in ACN and measured with HPLC to reveal their separation. After detecting these two separated peaks, LC-MS is performed to analyse the MW of the peaks. Furthermore, a calibration curve with its LOD is established, as described in chapter 4.2.4. The following step is to spike the plasma with Modafinil and CE-123 to determine if the detection of this drug in plasma with LC-MS is possible. The last step is to measure the plasma of the rat, which was treated with CE-123 and to quantify the concentration of the drug.

4.2.1. **Solutions of Modafinil and CE-123**

Both, CE-123 and Modafinil are dissolved to 50 µg/mL in ACN. 10 µL of each solution are injected at a flow rate of 0.2 µL/min into the HPLC and measured according to the gradient shown in *Table 4*. With the available high resolution MS all the measurements occur at a high mass accuracy analysis.

Modafinil

High-resolution electrospray ionization mass spectrometry (HRESIMS) spectra are calculated by using the Modafinil precursor ion HRESIMS m/z 274.0896 [M+H$^+$] (calculated for $C_{15}H_{16}NO_2S$, Δ = -3.9 ppm deviation and a score of 92.35). Applying UPLC-MS, the peak of Modafinil appears at minute 16 with an intensity of $13 \cdot 10^5$ and a sum formula of $C_{15}H_{15}NO_2S$, verified by data analysis with the Smart Formula Manually, as shown in *Fig. 21*. *Fig. 22* represents the HPLC chromatogram at 254 nm of Modafinil, where the IS appears at minute 16 as well.

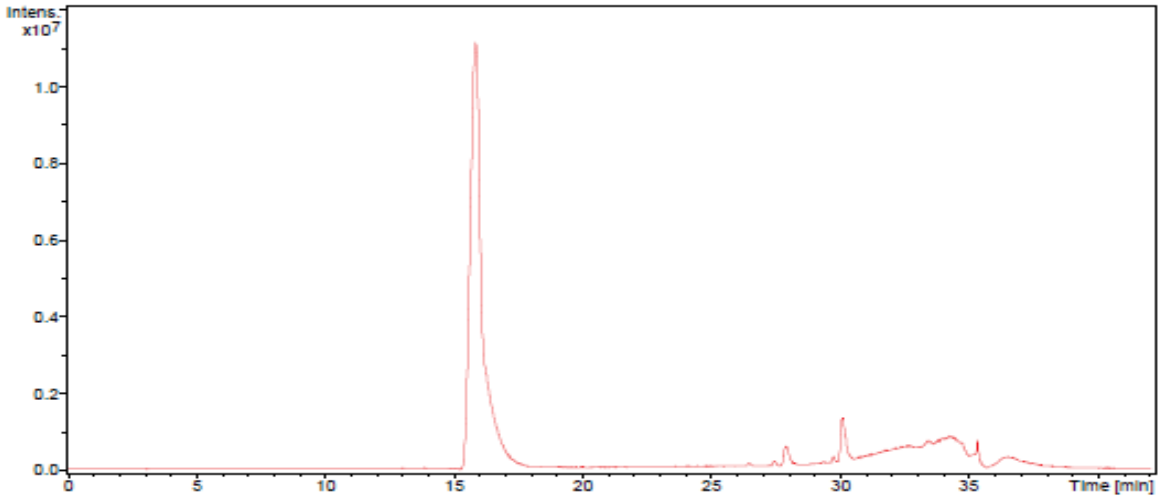

Fig. 21: LC-MS chromatogram of Modafinil at a concentration of 50 µg/mL.

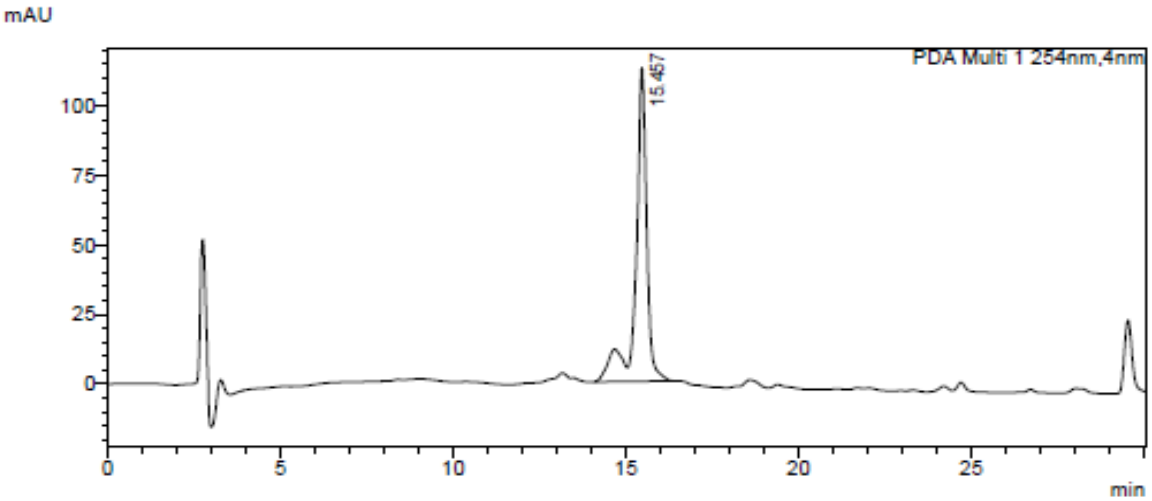

Fig. 22: HPLC chromatogram at 254 nm of Modafinil at a concentration of 50 µg/mL.

CE-123

According to the LC-MS chromatogram, the peak of CE-123 appears at minute 18 with an intensity of $52 \cdot 10^5$ and a sum formula of $C_{17}H_{16}NOS_2$, verified by data analysis with the Smart Formula Manually, as shown in *Fig. 23*. CE-123 is determined from the precursor ion HRESIMS m/z 314.0668 [M+H$^+$] (calculated for $C_{17}H_{16}NOS_2$, Δ = -4.3 ppm deviation and a score of 92.35). *Fig. 24* represents the HPLC chromatogram at 254 nm of CE-123, the derivative appears at minute 18 as well.

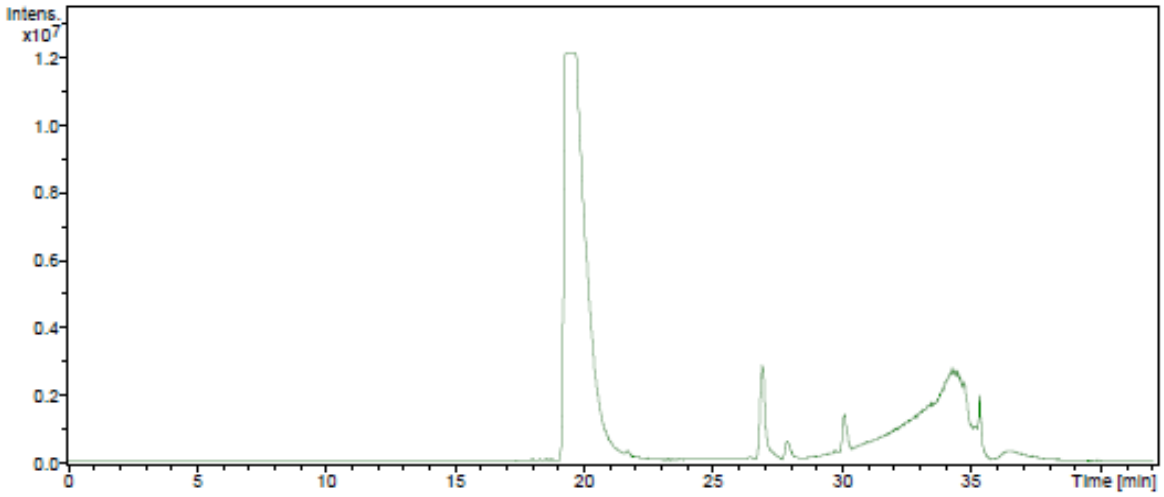

Fig. 23: LC-MS chromatogram of CE-123 at a concentration of 50 µg/mL.

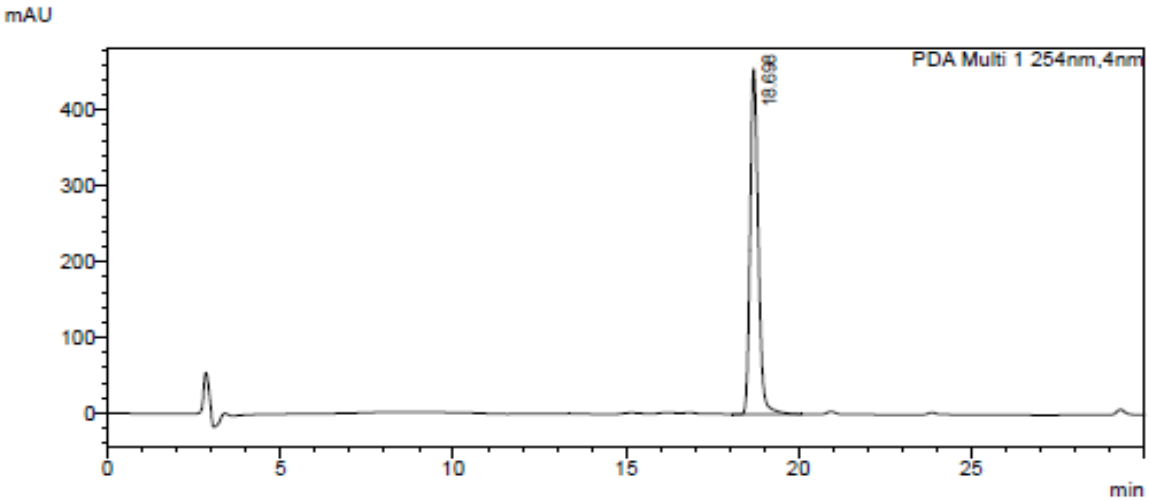

Fig. 24: HPLC chromatogram at 254 nm of CE-123 at a concentration of 50 µg/mL.

Mix

Fig. 25 and *26* show the mix of both, CE-123 and Modafinil dissolved in ACN. The drugs are detected at a wavelength of 254 nm. Modafinil is eluated first, owing to its lower hydrophobicity. The arrows indicate which peak is from CE-123 and which belongs to the IS Modafinil. The small signals at the end of the measurement come from the blank. The elution times of CE-123 and Modafinil occur at minutes 15 and 18, as mentioned by *Fig. 21* and *Fig. 23*. There is barely a difference between the LC-MS and HPLC chromatogram (*Fig. 25* and *Fig. 26*) in the elution times of both drugs. However, the HPLC peaks possess lower intensities than those of the LC-MS. Hence, the limited sensitivity of the HPLC towards LC-MS is not visible due to utilized high concentrations of the drugs.

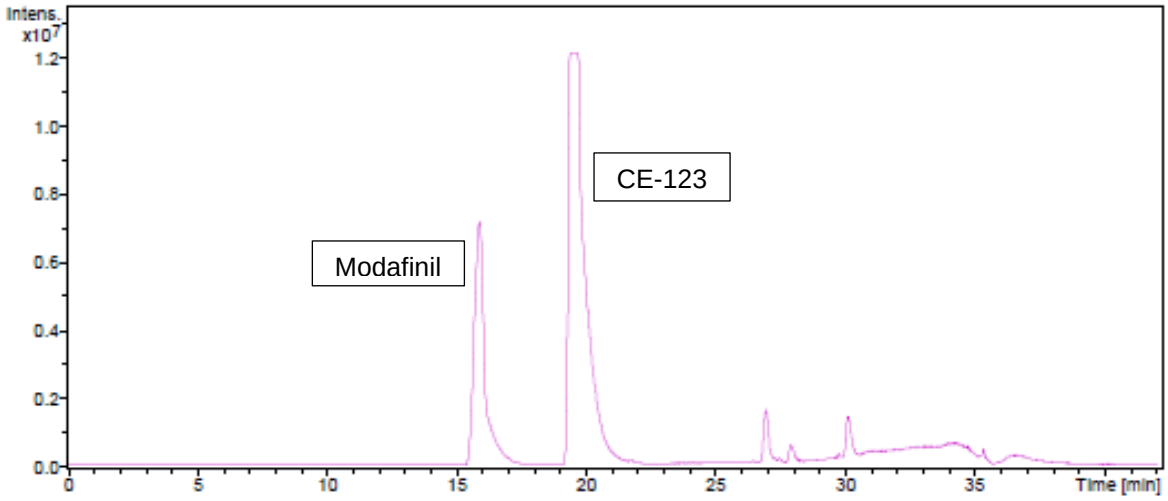

Fig. 25: LC-MS chromatogram of CE-123 with IS, each at a concentration of 50 µg/mL.

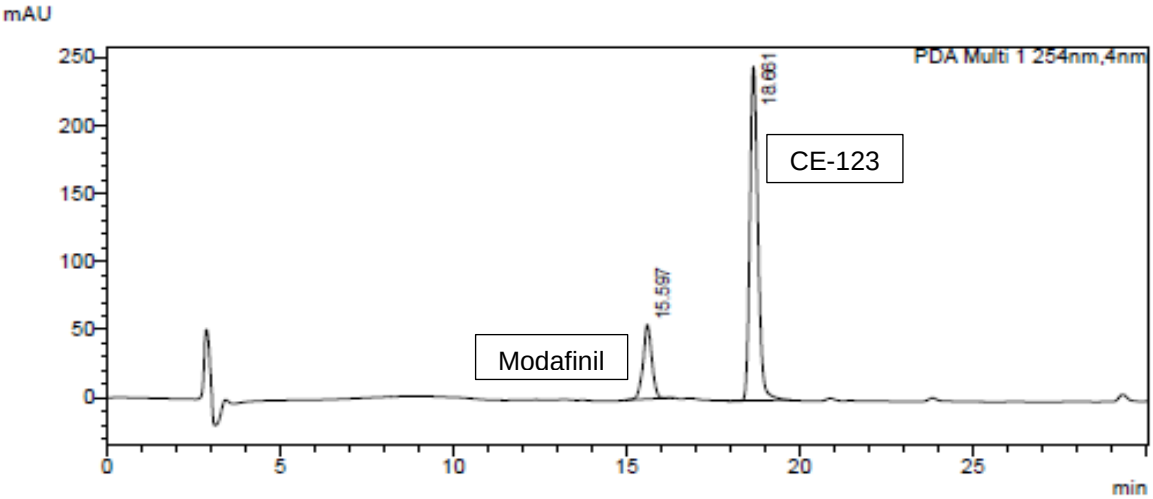

Fig. 26: HPLC chromatogram at 254 of CE-123 with IS, each at a concentration of 50 µg/mL.

Blank

Fig. 27 and *Fig. 28* show ACN, the blank. The LC-MS chromatogram of the blank contains the signals, which are in all LC-MS chromatograms of CE-123 and Modafinil (minute 27 to minute 38), as ACN is the solvent used in the preparation. The intensity of these signals is so low, so that the peaks are hardly recognizable in the HPLC chromatogram (*Fig. 28*) due to lower sensibility of the HPLC towards the LC-MS.

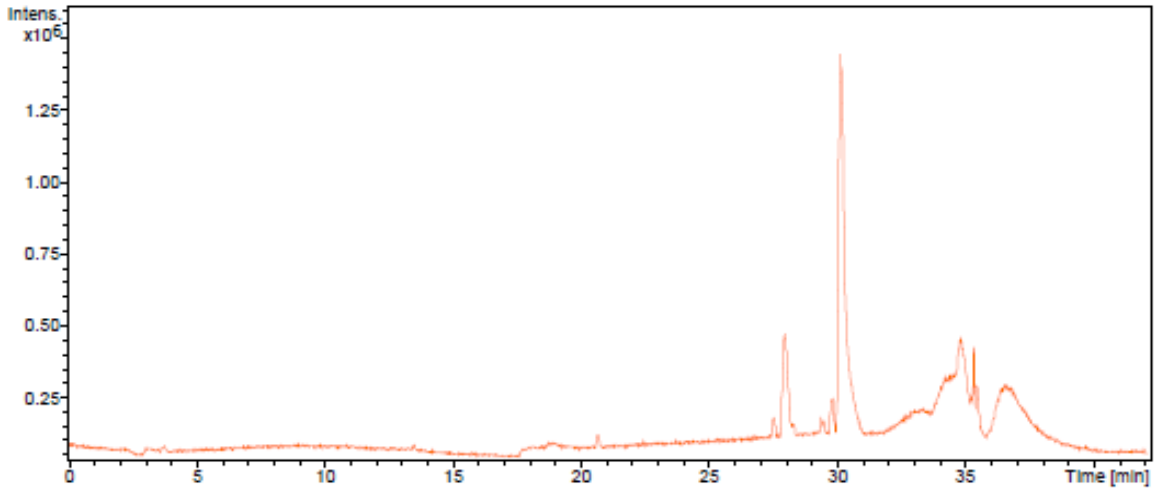

Fig. 27: LC-MS chromatogram of ACN.

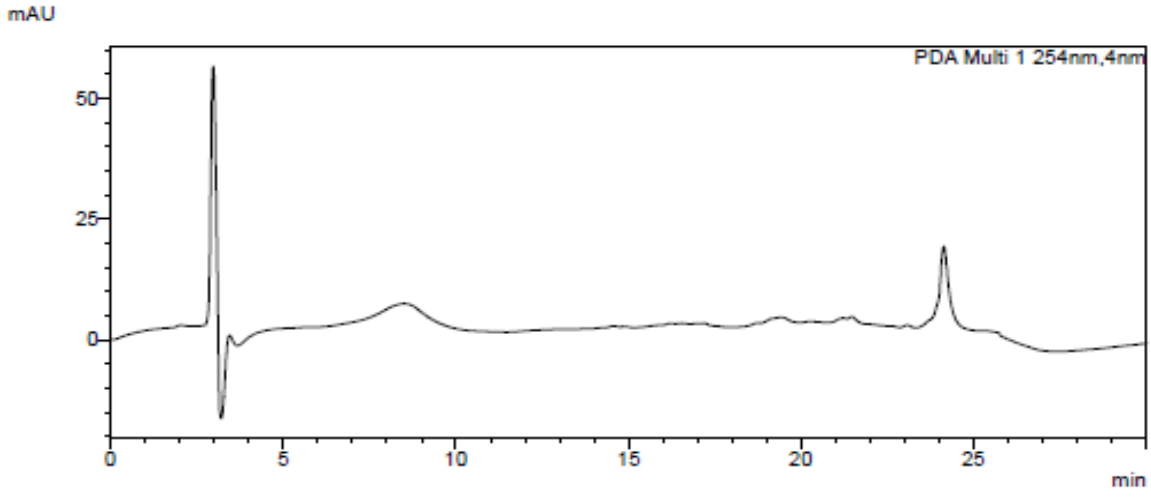

Fig. 28: HPLC chromatogram at 254 nm of ACN (blank).

4.2.2. **Spiked plasma**

Plasma is spiked with CE-123 to reveal the difference in CE-123 concentration between spiked plasma and plasma of the rat after being treated with CE-123. This serves information about the pharmacokinetic of the rat and how its metabolism is working. ACN is used as solvent due to its precipitating properties. Molecules with high masses, e.g., proteins which are in plasma, have to be precipitated and removed out of it since they disturb the measurement and their peaks could overlap those of CE-123 or Modafinil. Both, CE-123 and/or Modafinil are dissolved to 50 µg/mL (0.001 g / 20 mL) in ACN. The plasma is spiked by mixing it with ACN (0.001 g CE-123/Modafinil in 20 mL ACN) in a ratio of 1:1 (20 µL + 20 µL). The samples are vortexed and centrifuged for 2 minutes. 20 µL of the supernatant is then carefully pipetted and transferred to the vial. 10 µL of the solution is injected at a flow rate of 0.2 µL/min into the HPLC and measured according to the gradient shown in *Table 4*.

Modafinil in Plasma

The peak of Modafinil has a lower intensity in plasma than dissolved in ACN: $14 \cdot 10^4$, like shown in *Fig. 29. Fig. 30* demonstrates the HPLC chromatogram of Modafinil in plasma, Modafinil appears at minute 16. As the spiked plasma is measured, the chromatogram shows the signals of the plasma components at the beginning (before minute 5) and at the end of the chromatogram (minute 20 to minute 35) in the LC-MS chromatogram (*Fig. 29*), but even near to the peak of Modafinil in the HPLC chromatogram (*Fig. 30*). Several peaks, especially those at minutes 25-31 have higher concentrations than the peak of Modafinil, which is obviously noticeable in the LC-MS chromatogram (*Fig. 29*). A not existing baseline separation at the HPLC chromatogram (*Fig. 30*) leads to quantification problems. The HPLC is not as sensible as the LC-MS and this difference is clearly observable in *Fig. 29* and *Fig. 30*. Hence, the LC-MS is the suitable tool for quantification.

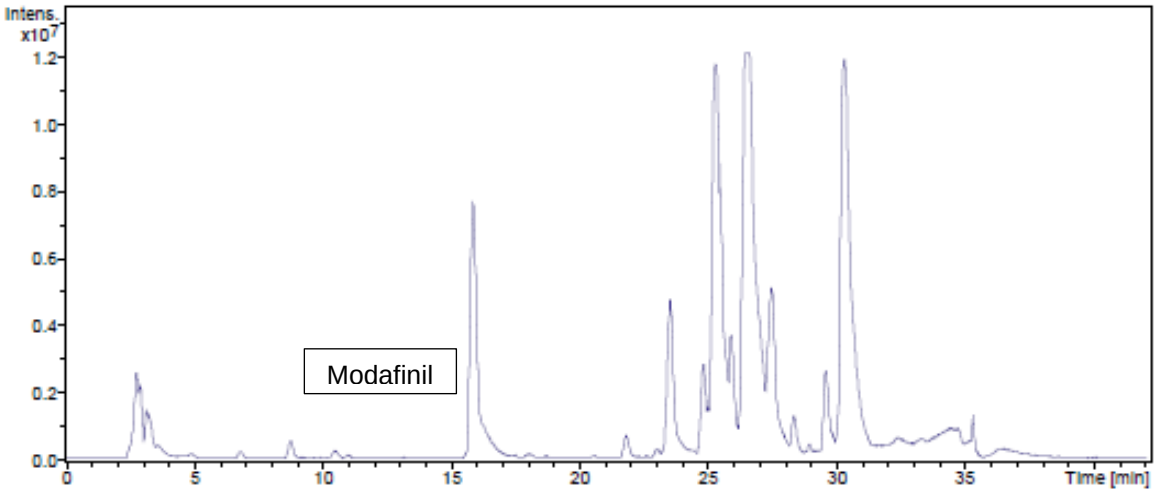

Fig. 29: LC-MS chromatogram of Modafinil (50 µg/mL) in plasma.

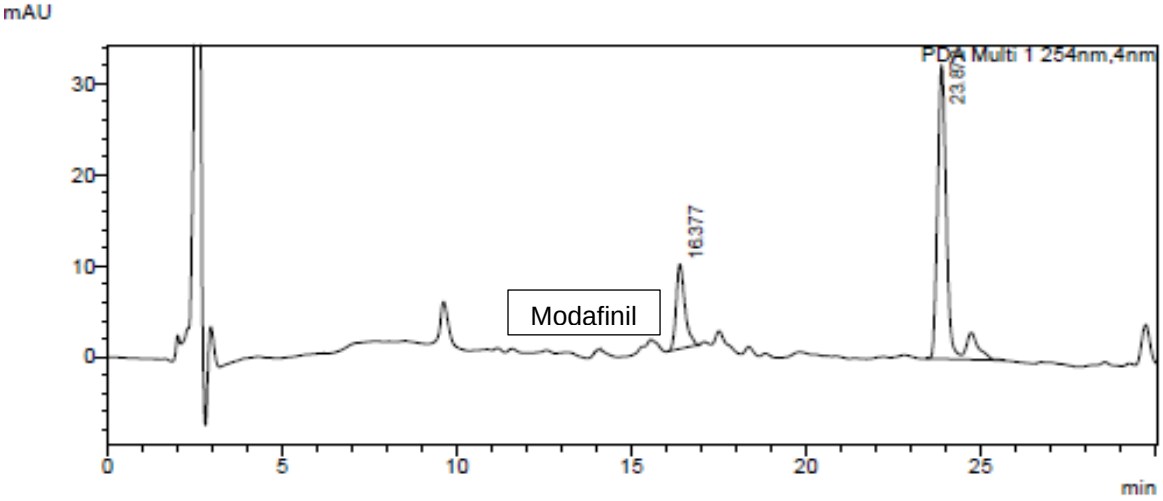

Fig. 30: HPLC chromatogram at 254 nm of Modafinil (50 µg/mL) in plasma.

CE-123 in Plasma

As shown in *Fig. 31*, CE-123 in spiked plasma has a lower intensity than in ACN: $36 \cdot 10^5$ and $52 \cdot 10^5$ respectively. The HPLC chromatogram of CE-123 reveals the derivative at minute 20, as shown in *Fig. 32*. CE-123 in a concentration of 50 µg/mL possess a high intensity and is therefore either in the LC-MS (*Fig.31*) as well as in the HPLC (*Fig. 32*) chromatogram, without problems as Modafinil, visible. The peak does not overlap with the plasma peaks and therefore a quantification of CE-123 with this gradient (*Table 4*) is possible. Owing to the higher sensibility of LC-MS towards HPLC more signals are detected and better identifiable than the signal in the HPLC chromatogram.

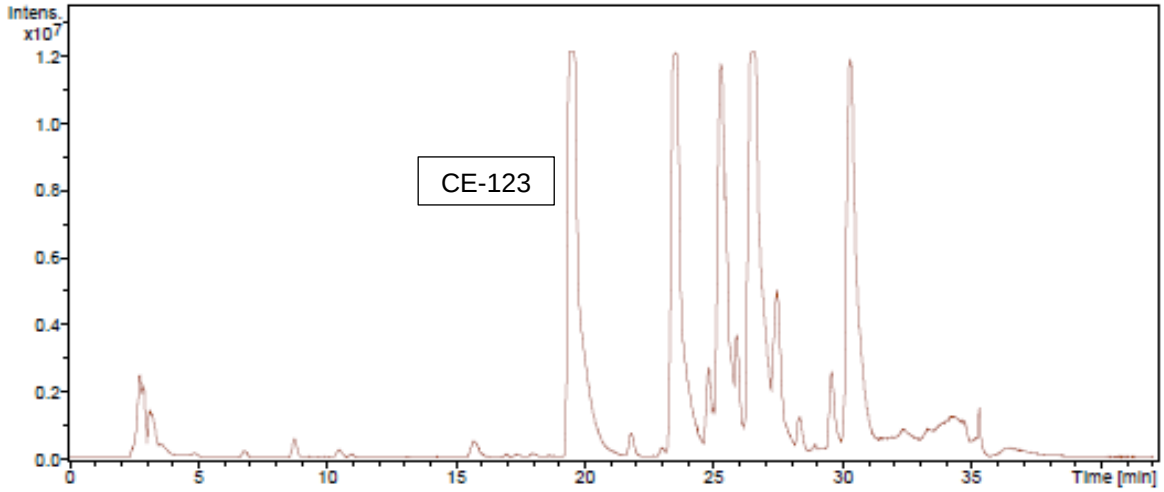

Fig. 31: LC-MS chromatogram of CE-123 (50 µg/mL) in plasma.

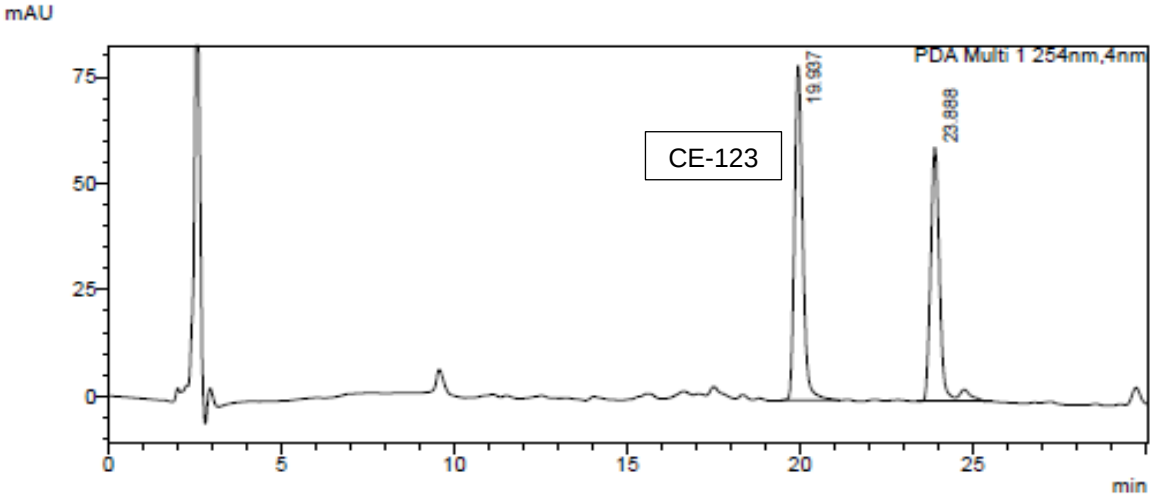

Fig. 32: HPLC chromatogram at 254 nm of CE-123 (50 µg/mL) in plasma.

Mix in Plasma

Fig. 33 and *Fig. 34* show the mix of both, CE-123 and Modafinil in plasma. Although Modafinil and CE-123 are prepared the same way, with the same concentration of 50 µg/mL and of both samples 10 µL is injected in the HPLC, Modafinil has a lower intensity than its derivative and is barely visible in the HPLC chromatogram measured at a wavelength of 254 nm (*Fig. 34*). Hence, it is better to utilize another IS to be able to quantify CE-123. For that purpose, Diazepam can be applied (see chapter 4.2.5.).

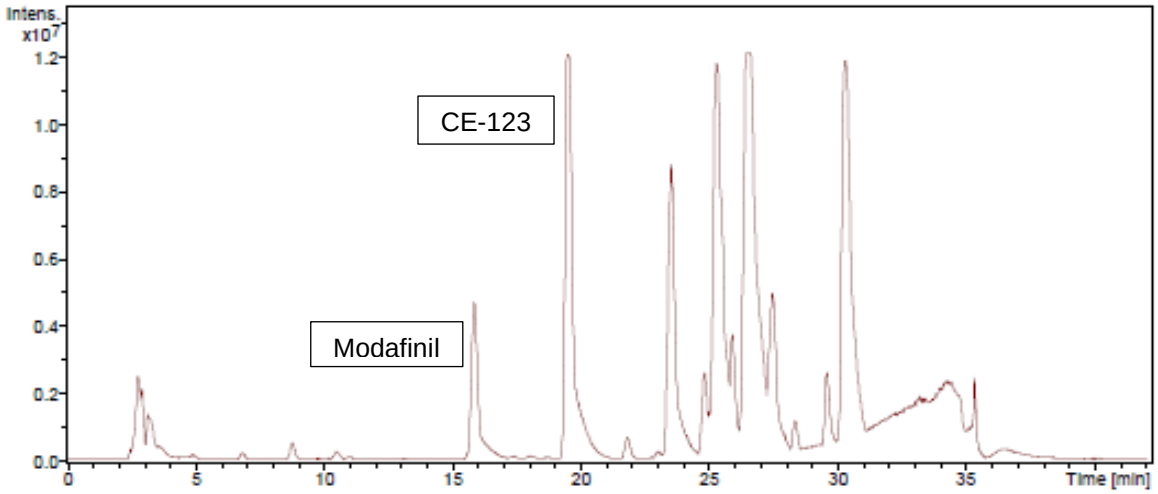

Fig. 33: LC-MS chromatogram of CE-123 and IS in plasma, each at a concentration of 50 µg/mL.

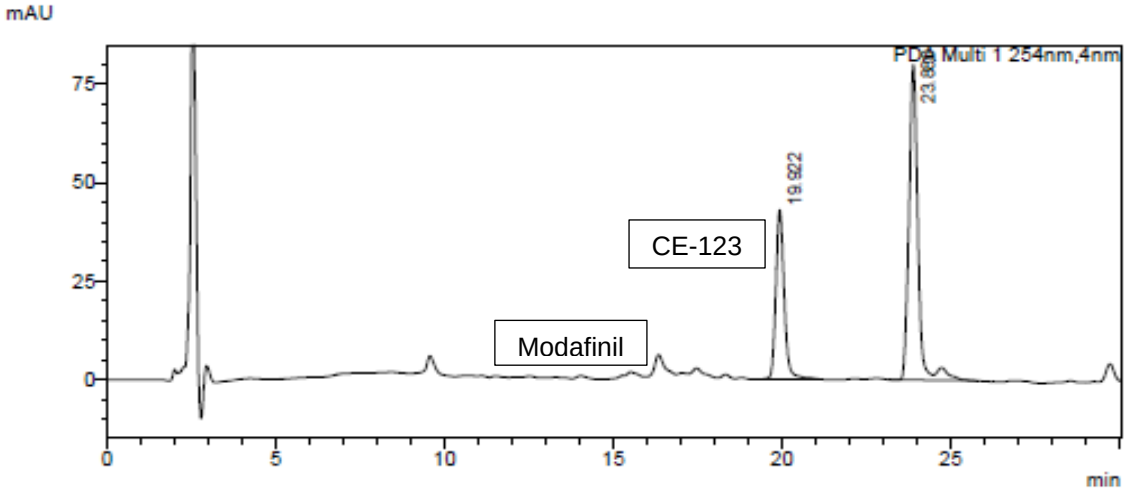

Fig. 34: HPLC chromatogram at 254 nm of CE-123 and Modafinil (each at 50 µg/mL) in plasma.

ACN (blank) in Plasma

Fig. 35 and *36* show the blank (ACN) to determine the plasma peaks and the difference between this chromatogram and the ones with CE-123/IS are the peaks from the drugs. The ACN blank is prepared exactly like the CE-123 and Modafinil plasma sample preparations, with the difference that only ACN is used without any analyte concentration. 20 µL of the supernatant is pipetted and 10 µL is injected. Due to higher sensibility of the LC-MS more signals are detected with the instrument.

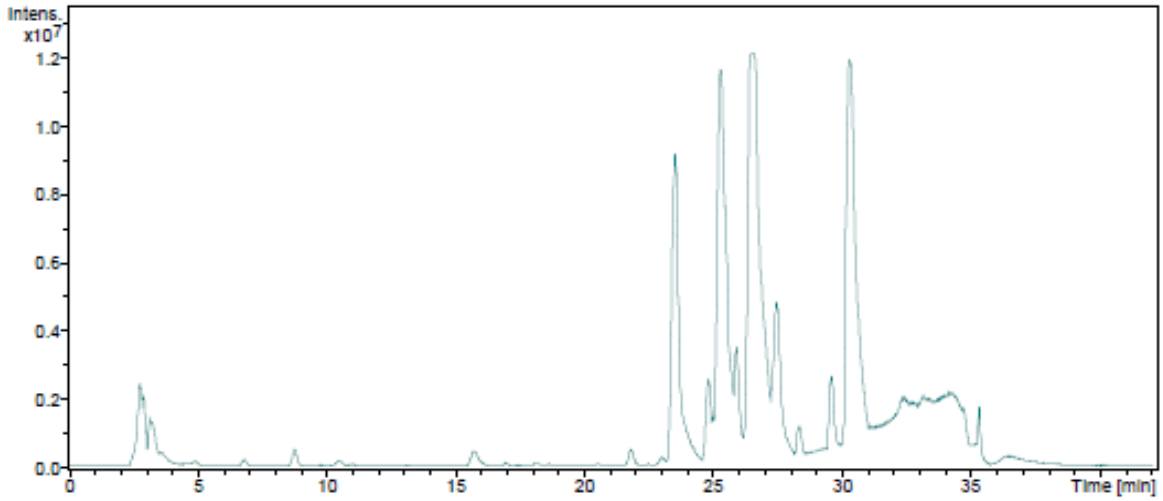

Fig. 35: LC-MS chromatogram of ACN blank.

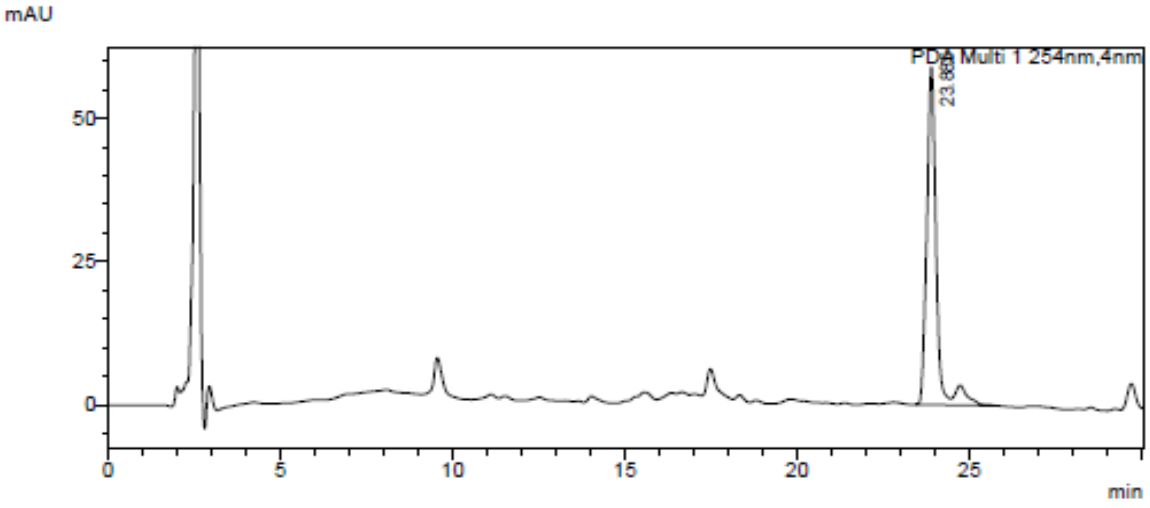

Fig. 36: HPLC chromatogram at 254 nm of ACN in plasma.

4.2.3. **Extracted Ion Chromatogram (EIC)**

The signals from the LC-MS chromatogram of CE-123 and Modafinil are further analysed with Compass DataAnalysis 4.2 (Bruker Corporation) with the aid of "Extracted Ion Chromatogram". The peaks of CE-123 and Modafinil appearing as $[M + H]^+$ and $[M + Na]^+$ are extracted according to their masses with a deviation of +/- 0.01 g/mol as shown in *Table 10***.** *Table 11* demonstrates the respective areas and intensities revealing the differences in intensities between the solutions of CE-123 and Modafinil in ACN (0.001 g / 20mL) and the spiked plasma samples. In pure solvent, CE-123 and Modafinil possess higher intensities as the signals are not influenced by the matrix of the plasma, which lower their intensities. For the integration of the peaks the m/z of 314.065 for CE-123 and 296.069 for Modafinil are chosen since they possess a high resolution/intensity. The EIC chromatogram of $[CE\text{-}123 + Na]^+$ and $[Modafinil + H]^+$ cannot be correctly integrated due to decreased intensities and noise issues, compared to the cations of $[CE\text{-}123 + H]^+$ and $[Modafinil + Na]^+$.The extracted ion chromatograms are depicted in *Fig. 37-48*.

Table 10: m/z values of ionised CE-123 and Modafinil ($+H^+$ or $+Na^+$).

	$[M + H]^+$	$[M + Na]^+$
CE-123	**314.065**	336.045
Modafinil	274.089	**296.069**

In theory CE-123 has a MW of 314.07 g/mol, Modafinil 273.35 g/mol, H 1.008 g/mol and Na 22.9898 g/mol. By subtracting the m/z of the drugs in *Table 10* with H or Na, another MW is the result. Thus, these is a difference between the theory and the detected m/z. The deviation is lower than 0.3 g/mol in Modafinil and approximately 1.01 g/mol with CE-123.

Table 11: Depicition of the areas and intensities of CE-123 and Modafinil in ACN and spiked plasma samples: Mix of CE-123 (C) and Modafinil (M), CE-123 and Modafinil in ACN/plasma.

	Area	Intensity		Area	Intensity
Mix (C)	589,646,464	$52 \cdot 10^5$	**Mix_plasma**	290,981,888	$36 \cdot 10^5$
(M)	10,873,127	$13 \cdot 10^5$		7,558,570	$14 \cdot 10^4$
CE-123	740,523,712	$52 \cdot 10^5$	**CE-123_pl**	429,172,800	$36 \cdot 10^5$
Modafinil	17,245,528	$13 \cdot 10^5$	**Modafinil_pl**	12,095,240	$14 \cdot 10^4$

Mix

The EIC of CE-123 [$+H^+$] with a MW of 314.065 (*Fig. 37*) and CE-123 [$+Na^+$] with a MW of 336.04 (*Fig. 40*) indicate that the derivative appears at minute 18, as described in the chapters before. Extracting this signal and analysing it by itself supplies better results in regard of integration. The EIC of Modafinil [$+Na^+$] with a MW of 296.069 (*Fig. 38*) and Modafinil [$+H^+$] with a MW of 247.08 (*Fig. 39*) prove that Modafinil appears at minute 15. The EIC chromatogram of $[CE\text{-}123 + Na]^+$ and $[Modafinil + H]^+$ cannot be correctly integrated due to decreased intensities and noise issues, compared to the cations of $[CE\text{-}123 + H]^+$ and $[Modafinil + Na]^+$. Thus, for the integration of the peaks the m/z of 314.065 for CE-123 and 296.069 for Modafinil are chosen since they possess a high resolution/intensity.

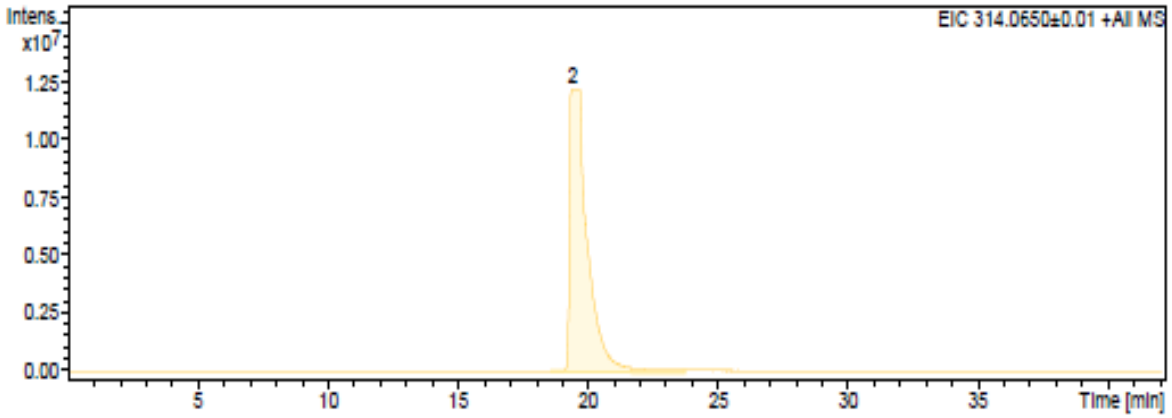

Fig. 37: Extracted Ion Chromatogram of CE-123 [$+H^+$] with a MW of 314.065.

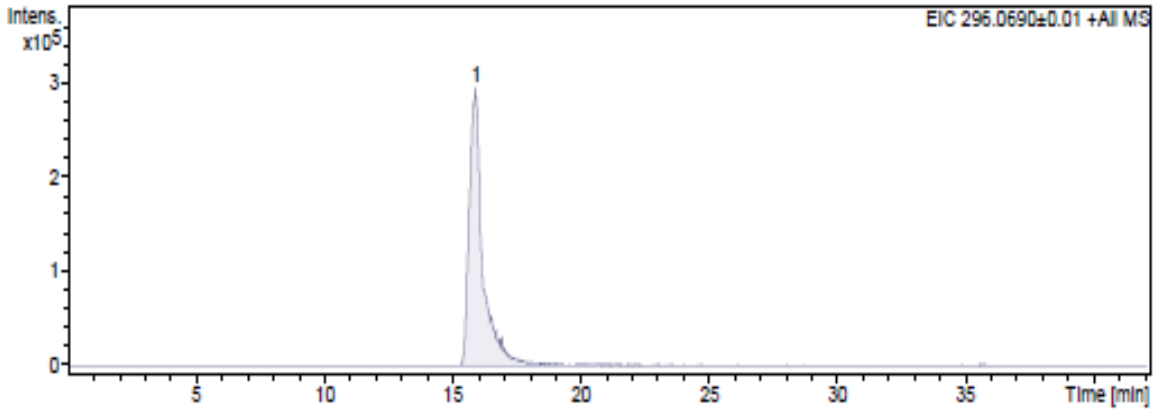

Fig. 38: Extracted Ion Chromatogram of Modafinil [+Na⁺] with a MW of 296.069.

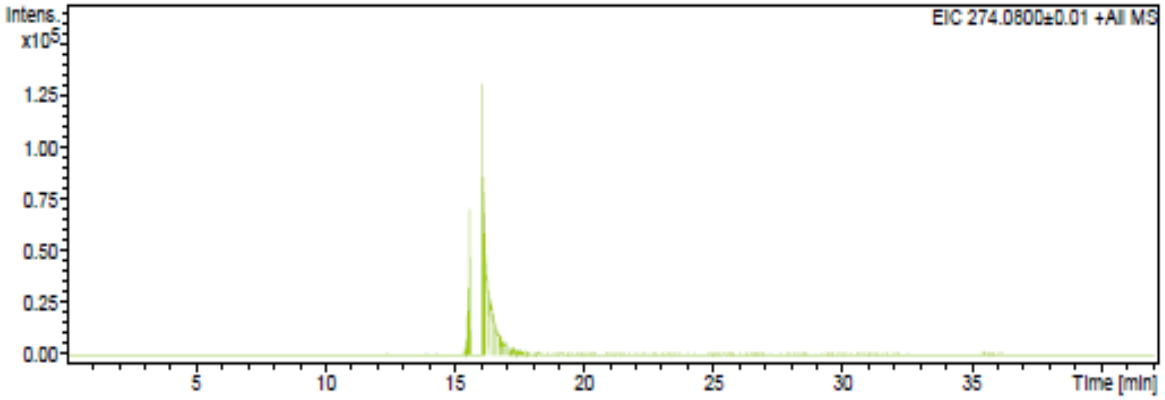

Fig. 39: Extracted Ion Chromatogram of Modafinil [+H⁺] with a MW of 247.08.

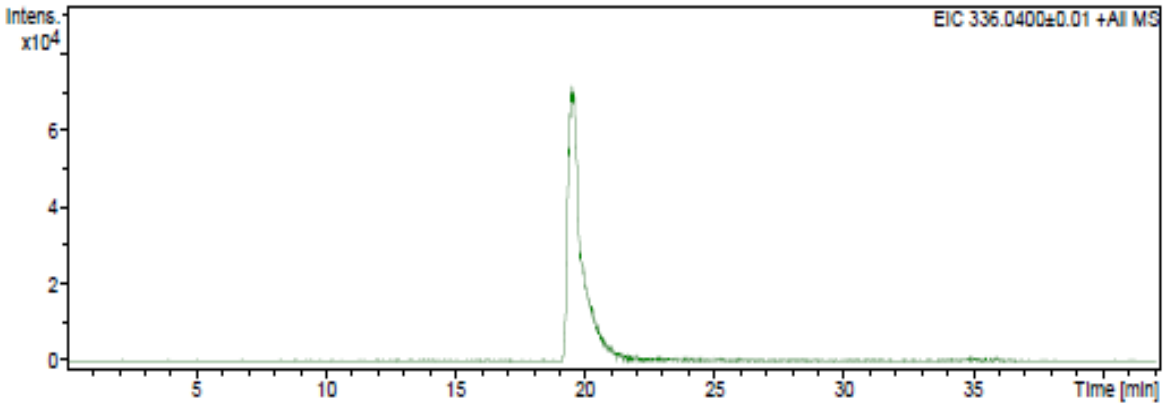

Fig. 40: Extracted Ion Chromatogram of CE-123 [+Na⁺] with a MW of 336.04.

CE-123

The EIC of CE-123 [+H^+] with a MW of 314.065 (*Fig. 41*) shows CE-123 at minute 18. CE-123 with a MW of 314.065 is chosen for the integration and has an area of 740,523,712, a bit higher than its area in the mix (*Fig. 37, Table 11*). A reason can be that the presence of Modafinil lowers the intensity.

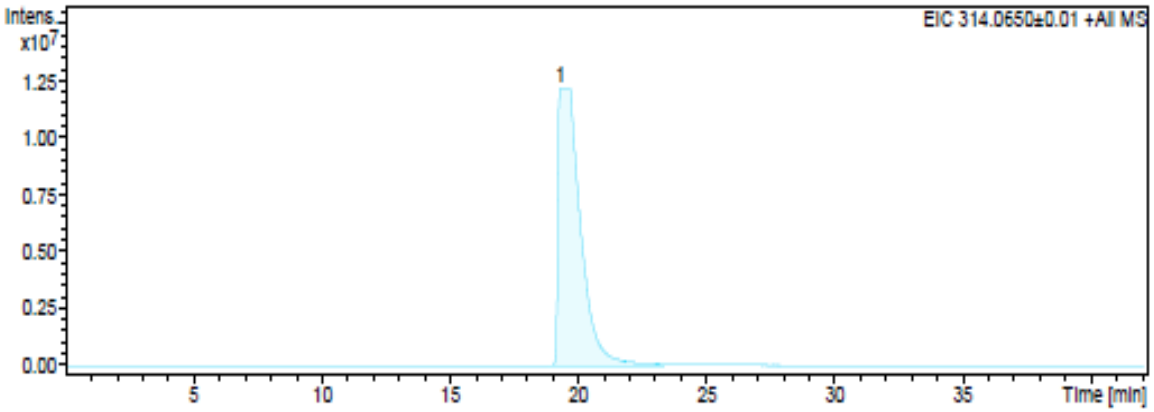

Fig. 41: Extracted Ion Chromatogram of CE-123 [+H^+] with a MW of 314.065.

Modafinil

The EIC of Modafinil [+Na^+] with a MW of 296.069 is shown in *Fig. 42*. This peak is chosen for the integration and an area of 17,245,528 is the result.

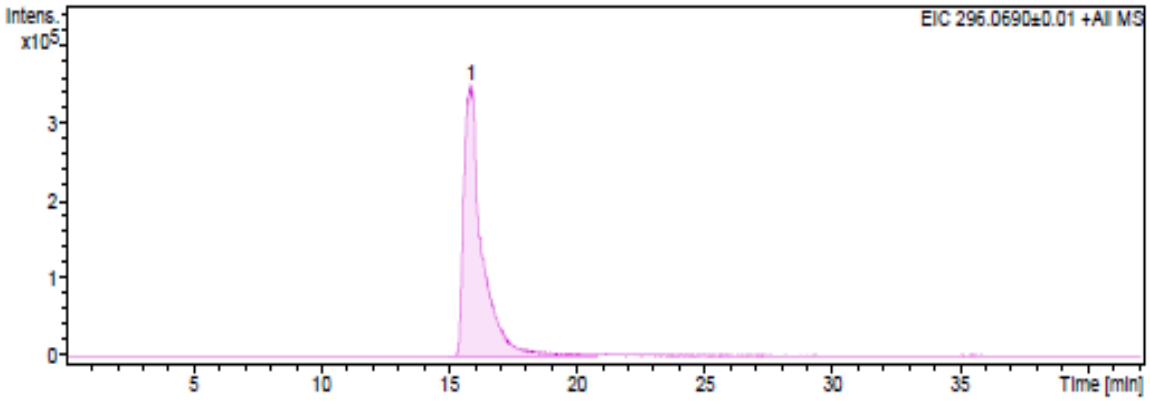

Fig. 42: Extracted Ion Chromatogram of Modafinil [+Na^+] with a MW of 296.069.

Plasma mix

The EICs of the plasma sample preparations (*Fig. 43 - 48*) indicate much lower intensities, as the drugs are spiked with plasma and the plasma composition lowers the drug intensities. The peak areas of CE-123 (290,981,888), Modafinil (7,558,570) in mix, CE-123 (429,172,800) and Modafinil (12,095,240), in plasma are much lower than the intensities in ACN (*Table 11*) due to plasma substances that reduce the areas and intensities of the drugs.

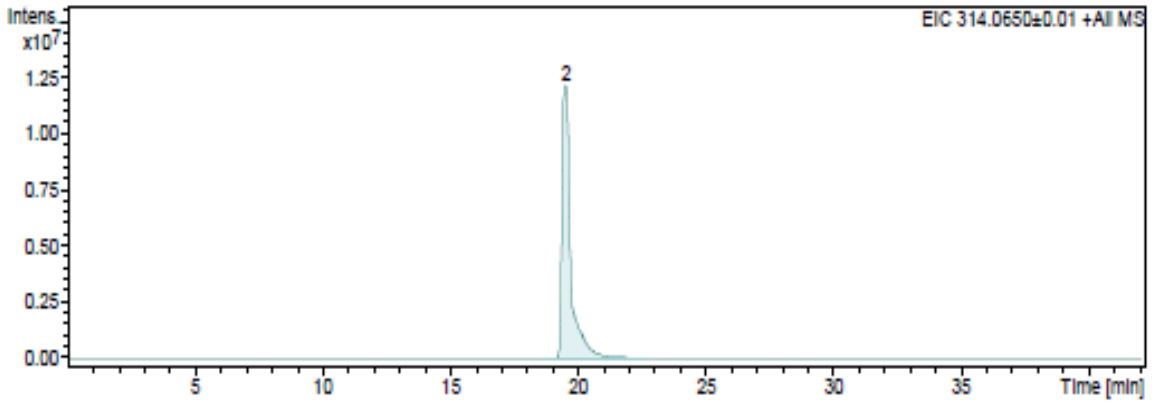

Fig. 43: Extracted Ion Chromatogram of CE-123 [+H⁺] with a MW of 314.065.

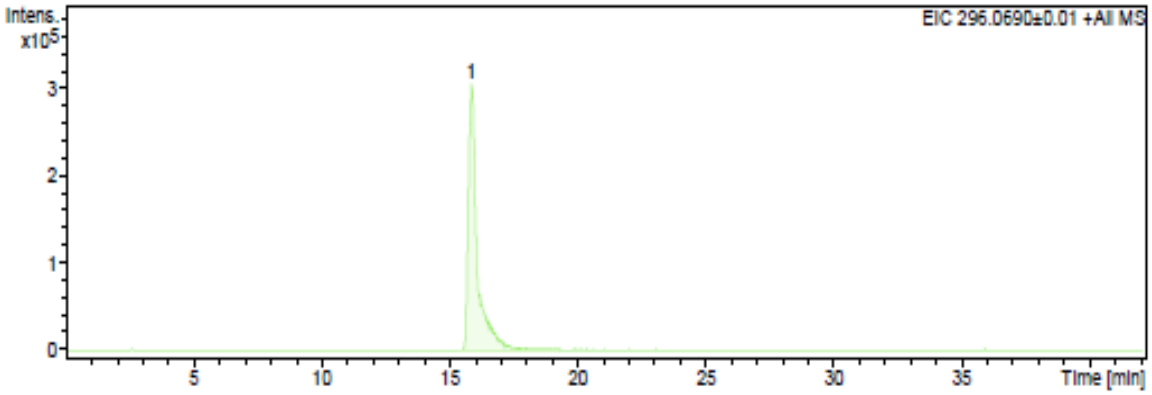

Fig. 44: Extracted Ion Chromatogram of Modafinil [+Na⁺] with a MW of 296.069.

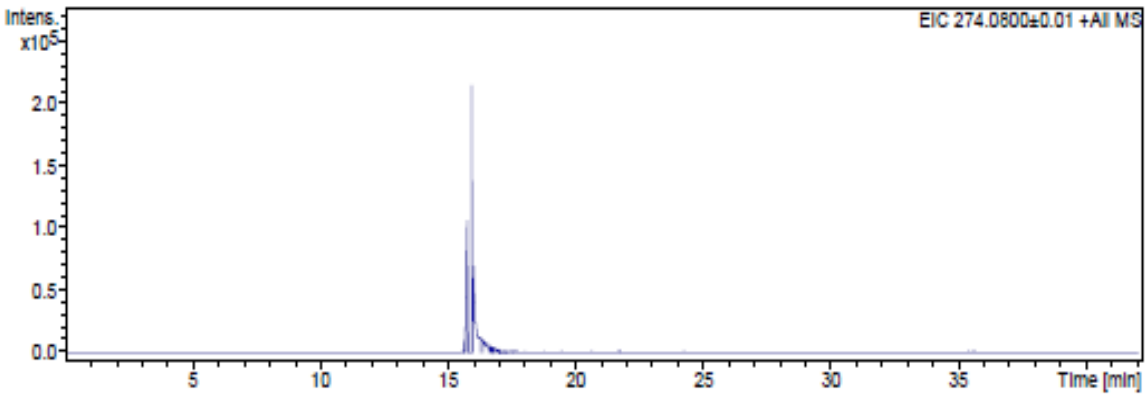

Fig. 45: Extracted Ion Chromatogram of Modafinil [+H⁺] with a MW of 274.08.

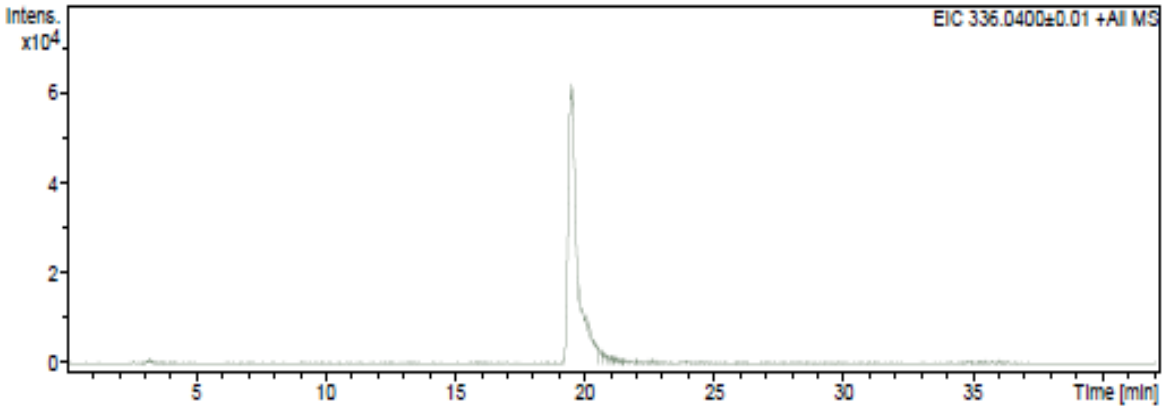

Fig. 46: Extracted Ion Chromatogram of CE-123 [+Na⁺] with a MW of 336.04.

Plasma CE-123

This peak (*Fig. 47*) is chosen for the integration and has an area of 429,172,800.

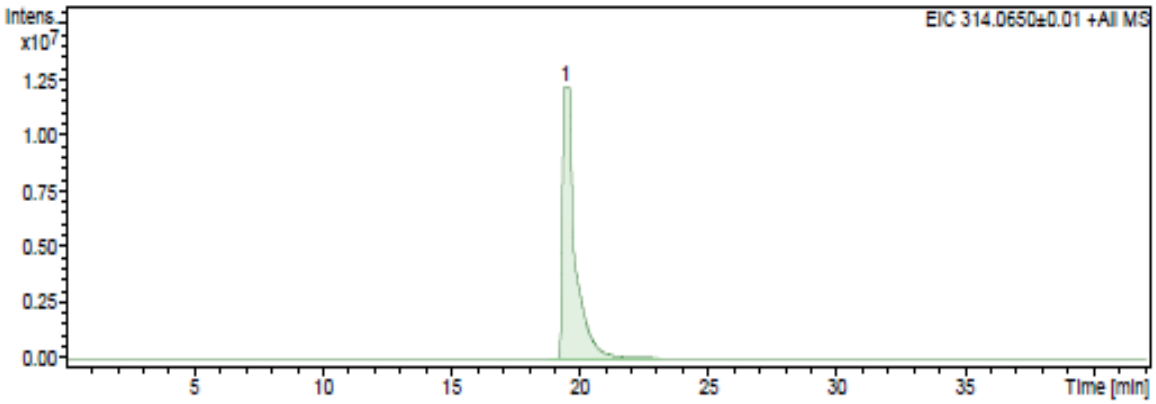

Fig. 47: Extracted Ion Chromatogram of CE-123 [+H⁺] with a MW of 314.065.

Plasma Modafinil

This peak (*Fig. 48*) is chosen for the integration and has an area of 12,095,240.

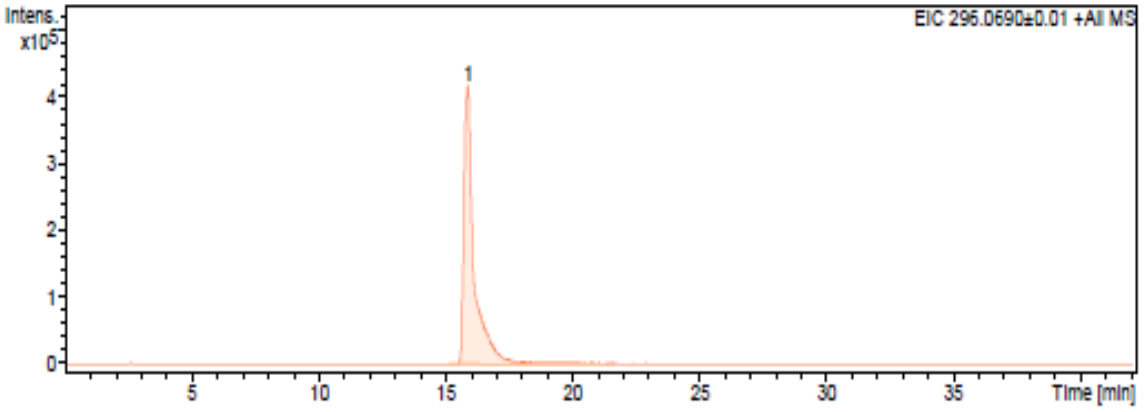

Fig. 48: Extracted Ion Chromatogram of Modafinil [+Na⁺] with a MW of 296.069.

4.2.4. Quantification - Calibration curves

CE-123 is diluted in ACN to get the lowest concentration detected and a calibration curve is established, as shown in *Fig. 49* and *Table 12*. 0.001 g of CE-123 is dissolved in 20 mL of ACN. This stock solution is diluted as described in *Table 12*. First, 1:10, then 1:20, 1:100, 1:1000, 1:2000 and finally 1:20,000. 10 µL of these dilutions are injected in the HPLC and measured with the conditions described in chapter 3.4.2., the measurements are repeated once again, so two peak areas are obtained and a mean can be calculated. However the concentration of 0.0025 µg/mL is below the LOD. Therefore, we can predict that the LOD is between 0.025 µg/mL and 0.0025 µg/mL.

Table 12: Serial dilution of CE-123, the peak areas and the mean value.

Stock solution:	50.00 µg/mL	Peak area 1	Peak area 2	Mean
1:10	5.00 µg/mL	493,502	499,289	496,395.5
1:20	2.50 µg/mL	247,280	246,004	246,642
1:100	0.50 µg/mL	61,228	60,326	60,777
1:1000	0.05 µg/mL	4,601	3,107	3,854
1:2000	0.025 µg/mL	2,331		2,331
1:20,000	0.0025 µg/mL			

Fig. 49 shows the calibration curve of the values presented in *Table 12*. A coefficient of determination R^2 of 0.9994 (*Fig. 49*) indicates that the dilution is correctly implemented. The peak areas are similar, there is not a massive difference between them, e.g., 247,280 and 246,004 (*Table 12*), hence the dilution is conformable.

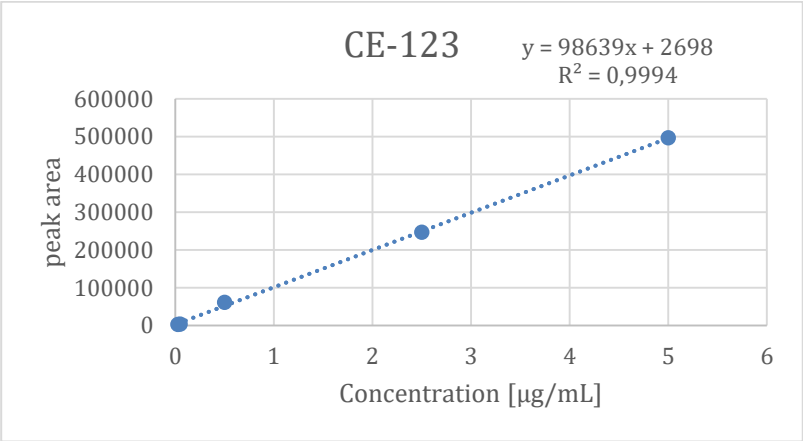

Fig. 49: calibration curve of CE-123 in ACN without IS.

A serial dilution of CE-123 is carried out, with Modafinil as IS in a constant concentration of 0.50 µg/mL, 100 µL of the IS is added. 0.50 µg/mL of Modafinil are used because this concentration is in the middle of the chosen concentrations of CE-123. A stock solution of 1 mg/mL of CE-123 in ACN (0.01 g CE-123 / 10 mL ACN exactly weighted) is established. This stock solution is diluted to a concentration of 0.0125 µg/mL, the calibration curve obtained is displayed in *Fig. 50*. The stock solution is diluted 1:100 twice, then 1:10 and then 1:5 with following resulting concentrations: 10.00 µg/mL, 0.10 µg/mL, 0.01 µg/mL and 0.002 µg/mL. The same stock solution is taken and another serial dilution is established with these concentrations: 5.00 µg/mL, 0.05 µg/mL, 0.025 µg/mL, 0.0125 µg/mL, 0.005 µg/mL and 0.0025 µg/mL. 1 mL of CE-123 with these concentrations was measured with 100 µL of the IS. Hence, 1100 µL are added in the vail and thus the measured concentrations of CE-123 are divided through 1.1 and the values depicted in *Table 13* are received.

Table 13: Serial dilution of CE-123.

[µg/mL]	peak area
909.09	35,443,774
9.09	473,437
4.55	259,170
0.09	26,734
0.05	27,756
0.02	25,875
0.011	25,140
0.009	26,916
0.005	23,927
0.0023	26,348
0.0018	27,149

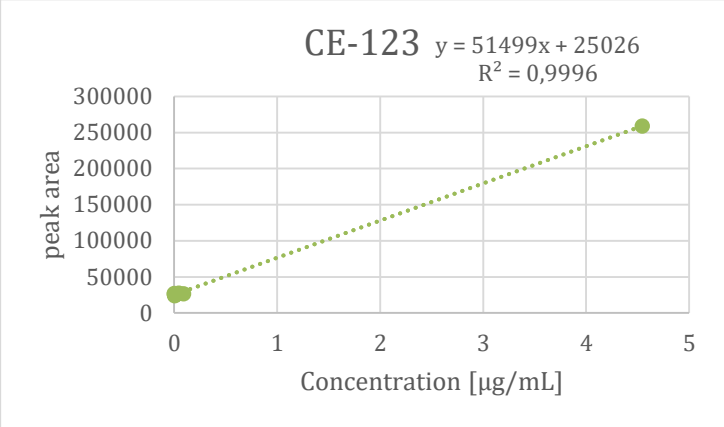

Fig. 50: calibration curve of CE-123 in ACN with IS.

The coefficient of determination R^2 of 0.9996 is obtained (*Fig. 50*), this indicates a high accuracy, though the measurements are not sensitive enough.

Table 14: Serial dilution of CE-123.

[ng/mL]	Peak area
90.91	26,734
45.45	27,756
22.73	25,875
11.36	25,140
9.09	26,916
4.55	23,927
2.27	26,348
1.82	27,149

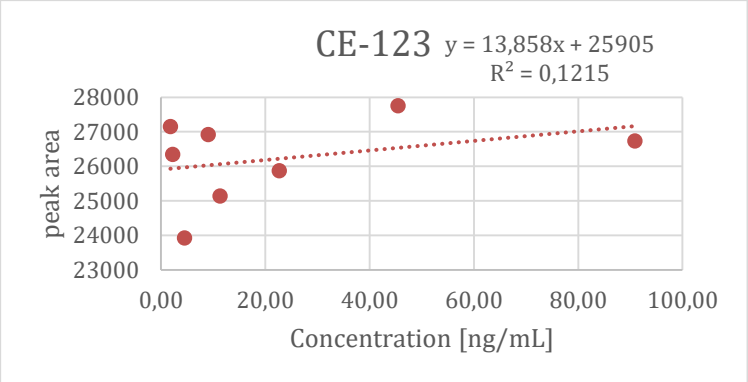

Fig. 51: Calibration curve of CE-123 in ACN.

Thus, by removing the high values R^2 of 0.9996 decreases to 0.1215, as shown in *Fig. 50* and *Fig. 51*.

4.2.5. **Optimization – Diazepam**

The intensity of Modafinil in the desired concentration range of 50 µg/mL is in the lower limit of detection and the peak is overlapped from the plasma peaks, hence a quantification is not possible. Thus, performing HPLC measurements, is better to utilize Diazepam as an IS owing to its higher intensity and not overlapping with the peaks of the plasma. In order to increase sensitivity, LC-MS measurements are performed.

The peak of Diazepam, which appears at minute 20 with a concentration of 19.832 in ACN (*Fig. 52*) and 19.832 in plasma (*Fig. 54*), shows a higher intensity than the other IS though the same concentration as Modafinil is used. The diazepam peak even is not overlapped with the peaks of the plasma. *Fig. 52-55* indicate this statement.

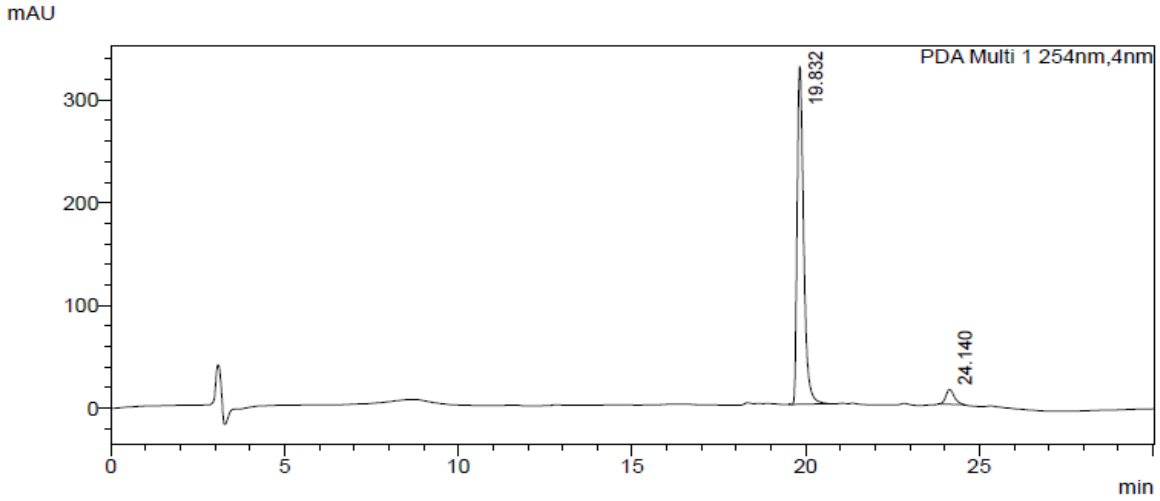

Fig. 52: HPLC chromatogram at 254 nm of Diazepam (50 µg/mL) in ACN.

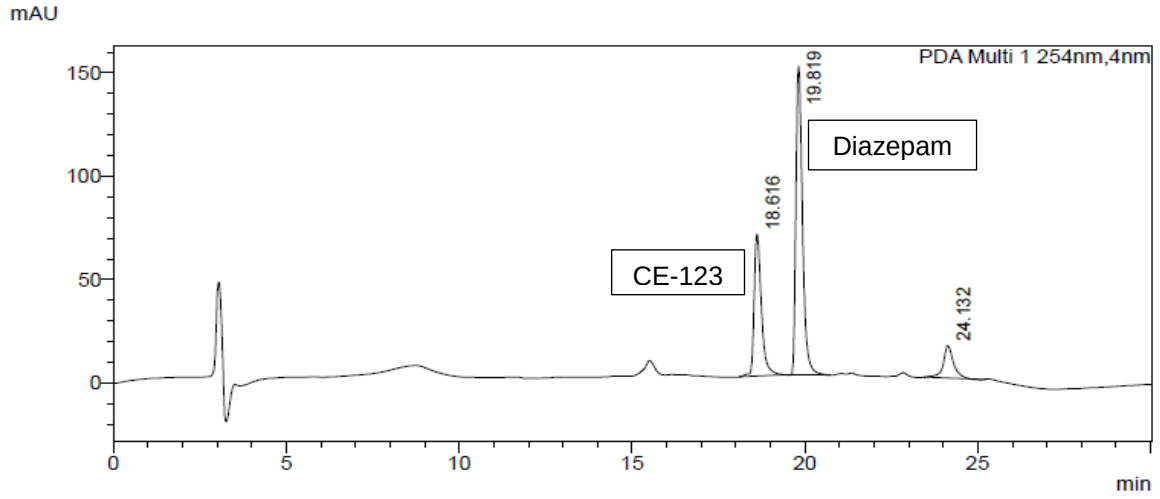

Fig. 53: HPLC chromatogram at 254 nm of CE-123 (50 µg/mL) and Diazepam (50 µg/mL) in ACN.

In plasma other substances which are hydrophilic such as ion compounds are present -shown in *Fig. 54* and *Fig. 55* - and due to the chromatographic conditions they appear at the begin of the run. Thus, it is advantageous that diazepam and CE-123 are lipophilic and not nearby these substances (to prevent overlapping).

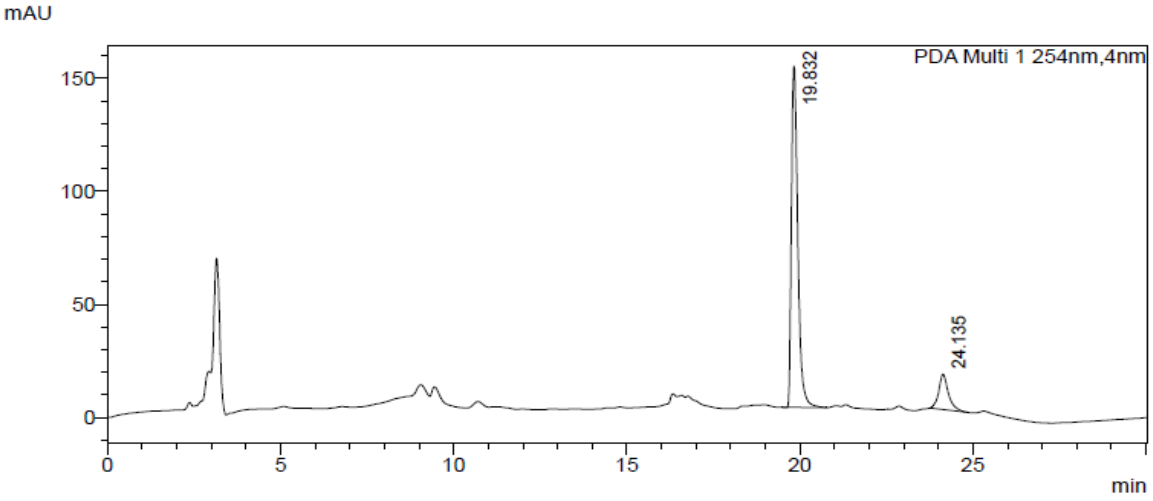

Fig. 54: HPLC chromatogram at 254 nm of Diazepam (50 µg/mL) in plasma.

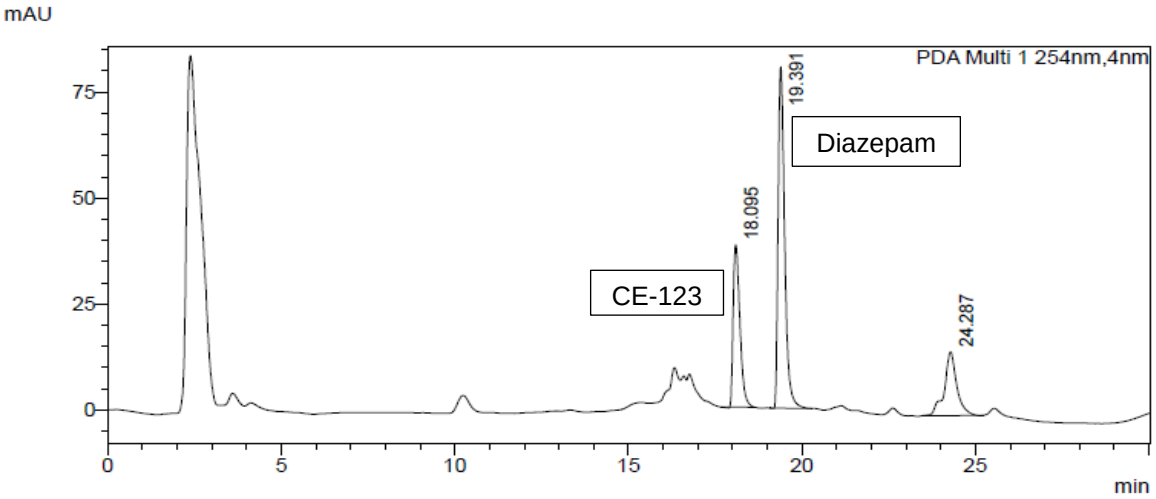

Fig. 55: HPLC chromatogram at 254 nm of CE-123 and Diazepam (each 50 µg/mL) in plasma.

4.2.6. **Discussion**

Comparing the peaks of CE-123 and Modafinil, dissolved in ACN with those spiked with plasma, both drugs show lower intensities in plasma, owing to other components that are in the plasma and therefore decrease the intensities.

The peak of Modafinil has a too low intensity, maybe due to the peak with the mass of 296, which interfere with Modafinil and therefore reduce its intensity (*Fig. 56*)

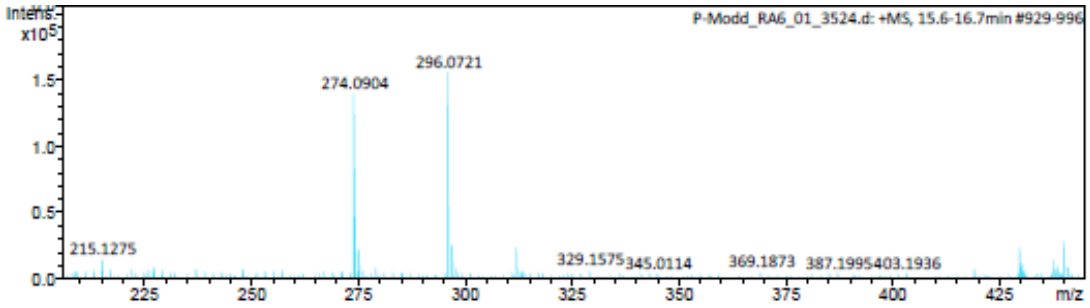

Fig. 56: MS signals of Modafinil in plasma.

The calibration of CE-123 as carried out is not successful owing to irregularity of the values. Hence, it is better to use another column to receive better results. Due to lower sensibility of the HPLC in contrast to the LC-MS, the calibration of CE-123 shows an irregularity of the values. Thus, performing these measurements with LC-MS will deliver better results, proven by the chromatograms of *Fig. 33* and *34*, which indicate the difference between HPLC and LC-MS measurements especially regarding the peak of Modafinil.

The intensity of Modafinil in the desired concentration range of 50 µg/mL is in the lower limit of detection and the peak is overlapped from the plasma peaks, hence a quantification is not possible. Thus, performing HPLC measurements, is better to utilize Diazepam as an IS owing to its higher intensity and not overlapping with the peaks of the plasma. In order to increase sensitivity, LC-MS measurements are performed.

5. Conclusion

According to Smidak *et al.* 2017, [15] glycerophosphocholines (PCs; 32-48 C-atoms), - ethanolamines (PE; 38-44 C-atoms), -serines (PS; 46 C-atoms), triacylglycerols (TG; 46-60 C-atoms), cholesterol esters (CE; 20 C-atoms), ceramides (Cer; 20-36 C-atoms) and sphingomyelins (SM; 22-24 C-atoms) are detected in the brain. These lipids have high MWs and are therefore not found in plasma. Indeed, other phospholipids, especially glycerophosphocholines with various chains, are found in plasma in positive ion mode. As they possess lower MWs, they are accessible to the plasma. After analyzing the plasma lipid profile and comparing it to those in the brain, the results demonstrate that the plasma includes other lipids, especially fatty acids and glycerophoshplipids with lower MWs. Contrary, in the brain glycerophosphocholines, -ethanolamines, -serines, triacylglycerols, cholesterol esters, ceramides and sphingomyelins with much higher MWs are located.

The intensity of Modafinil in the desired concentration range of 50 µg/mL is in the lower limit of detection when applying HPLC measurements with absorbance detection and the peak is overlapped from the plasma peaks, hence a quantification is not possible. Thus, Diazepam was applied as an IS owing to its higher intensity and not overlapping with the peaks of the plasma. In order to increase sensitivity, LC-MS measurements are performed. CE-123 with Modafinil as IS is not successfully quantified with the HPLC due to baseline separation problems and the available column does not support these measurements. Thus, performing these measurements with LC-MS and another column will deliver better results. Due to these problems a quantification of CE-123 could not be accomplished.

6. Zusammenfassung

LC-MS wird heutzutage sehr gern verwendet, besonders im Bereich der *drug discovery* findet dieses analytische Verfahren Anwendung, um neu entdeckte Arzneistoffe zu identifizieren, nachweisen und quantifizieren. [1] [2]

Ziel meiner Arbeit ist es Plasma von Ratten zu untersuchen und dieses mit LC-MS zu vermessen. Einerseits werden *Lipidomics* analysiert, um die Lipidzusammensetzung des Plasmas zu bestimmen und in weiterer Folge die Zusammensetzungen von jungen und älteren Ratten zu vergleichen, um feststellen zu können inwiefern sich diese mit dem Alter ändern und wie die Zusammensetzung bei Vorhandensein des metabolischen Syndroms aussieht. Zunächst wird das Lipid-Profil vom Plasma mit dem vom Gehirn verglichen. Eine veränderte Komposition der Lipide im Gehirn weist auf Erkrankungen wie Alzheimer oder Psychose hin, ändert sie sich im Plasma, treten Erkrankungen wie Adipositas, Diabetes, Hyperlipidämien auf. [6] Andererseits wird ein vielversprechendes Modafinil-Derivat *CE-123* zunächst *via* HPLC, dann LC-MS im Plasma nachgewiesen und im Zuge der Quantifizierung eine Kalibriergerade erstellt. *CE-123* - das im Gegensatz zu Modafinil selektiver am DAT Rezeptor angreift – soll weniger Nebenwirkungen aufweisen und wird daher im Zuge von Pharmakokinetik Studien genauer untersucht. [8]

Ergebnisse meiner Arbeit zeigen, dass die Lipide, die in der Publikation von Smidak *et al.* 2017 [15] im Gehirn nachgewiesen wurden, nicht im Plasma aufzufinden sind, da sie viel größere Massen haben und daher dem Plasma schwer zugänglich sind. Im Plasma befinden sich eher Fettsäuren und kleinere Phospholipide (MG < 550). CE-123 kann erfolgreich im Plasma nachgewiesen werden, jedoch bereitet das Quantifizieren mit der HPLC mit der vorliegenden Säule und Modafinil als IS Probleme. Ein Schwanken der Werte liegt vor, daher wäre eine kleinere Säule mit kürzeren Trennzeiten empfehlenswerter und die LC-MS geeigneter. Der Modafinil Peak ist im Vergleich zum Derivaten Peak (gleiche Konzentrationen) viel kleiner, kann auch nicht von den Plasma Peaks abgetrennt werden und ist daher im Plasma nicht deutlich detektier- und quantifizierbar. Dazu empfiehlt sich Diazepam als IS zu nehmen, der Peak ist deutlicher sichtbar und wird zudem auch nicht von den Plasma Peaks überlappt.

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