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DIPLOMARBEIT

Characterization of recombinant Peroxidases in respect to efficiency and reaction characteristics for selected substrates

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Abstract

Peroxidases are oxidoreductases produced by a number of microorganisms and plants. They are heme proteins containing iron (III) protoporphyrin IX as the prosthetic group. Peroxidases have a molecular weight ranging from 30,000 to 150,000 Da and catalyze the reduction of peroxides, such as hydrogen peroxide but also oxidations of various organic and inorganic compounds. Peroxidase activity has been identified in plants, microorganisms and animals, where they play important roles. The potential of peroxidases is substantial and therefore they are the focus of interest of industries.

Eucodis Bioscience is interested in an industrial use of heme-peroxidases and therefore produces various types of that enzyme. To understand the effect of different peroxidases specific enzyme-substrate reactions were studied in this thesis using an unsaturated fatty acid and its methyl ester as substrate. An analytical strategy was developed to analyze reaction samples by means of thin layer chromatography (TLC) and an efficient staining protocol but also with GC coupled to mass spectrometry (GCMS). This method combination allowed to determine the conversion rates of the enzymes and subsequently by studying the conversion rates the enzymatic reaction conditions were systematically optimized by qualitative and semi-quantitative evaluation of reaction products. In this thesis several reaction products could be identified by GCMS.

Zusammenfassung

Peroxidasen sind Oxidoreduktasen welche von einigen Mikroorganismen und Pflanzen gebildet werden. Sie sind Häm-Proteine und enthalten Eisen (III) Protoporphyrin IX als prosthetische Gruppe. Das Molekulargewicht von Peroxidasen liegt zwischen 30.000 und 150.000 Da und sie katalysieren die Reduktion von Peroxiden, wie z.B. Wasserstoffperoxid aber auch die Oxidation von einigen organischen und anorganischen Verbindungen. Sowohl in Pflanzen, Mikroorganismen und Tieren konnte ihre Aktivität festgestellt werden, wo sie auch eine wichtige Rolle spielen. Wegen des erheblichen Potentials das in Peroxidasen steckt sind sie im industriellen Fokus.

Eucodis Bioscience ist an einer industriellen Verwendung von Häm-Peroxidasen interessiert und produziert deshalb bereits einige Typen dieser Enzyme. Um die Wirkung verschiedener Peroxidasen zu verstehen wurden innerhalb dieser Arbeit Enzym-Substrat Reaktionen mit ungesättigten Fettsäuremethylestern als Substrat untersucht. Eine analytische Strategie wurde entwickelt, um die Reaktionen mittels Dünnschichtchromatographie (DC) und einem effizienten Färbeprotokoll aber auch mittels Gaschromatographie gekoppelt mit Massenspektrometrie (GCMS) zu analysieren. Diese kombinierte Methode ermöglichte es die Umsatzraten der Enzyme zu ermitteln und nachfolgend durch das Studium der Umsatzraten die Reaktionsbedingungen durch qualitative und semi-quantitative Auswertung der Reaktions-produkte systematisch zu optimieren. In dieser Arbeit konnten einige Reaktionsprodukte mittels GCMS identifiziert werden.

Danksagung

An dieser Stelle möchte ich mich bei Professor Günther Allmaier recht herzlich für die Aufnahme in seiner Arbeitsgruppe bedanken.

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Table of Contents:

Eine gute wissenschaftliche Theorie sollte einer Barfrau erklärbar sein.

Ernest Rutherford (1871-1937)

1. Introduction

1.1. Eucodis Bioscience

Eucodis Bioscience (http://www.eucodis.com) is a highly specialized producer of industrial enzymes which are used in biopharmaceutical industries, fine chemical productions, cosmetic industry and many other industrial branches. Eucodis has a portfolio of about 50 innovative enzymes such as lipases, beta-lactamases, phospholipases, nitrile hydratases, peroxidases and others. Furthermore Eucodis also provides services regarding the production of recombinant proteins. These services include protein engineering, strain and bioprocess development, fermentations in pro- and eukaryotic host expression systems and routine industrial protein manufacturing.

Eucodis Bioscience was founded in Vienna, Austria, in 2007 and has a registered branch in Halle, Germany.

1.1.1. Peroxidases

The production of peroxidases is newly established within Eucodis and is aimed at biocatalytic oxidation reactions beyond known dehydrogenases, P450 enzymes and reductases. Eucodis offers a collection of more than 10 selected recombinant heme-peroxidases of bacterial, fungal and plant origin [1].

Peroxidases are enzymes which catalyze a reaction with the help of peroxides, such as hydrogen peroxide. Hydrogen peroxide gets reduced to water while an electron donor oxidized.

Heme-peroxidases in particular are enzymes with heme as prosthetic group and can be used in oxygenation reactions using hydrogen peroxide as co-substrate [1].

The aim of the thesis was to study the ability of heme-peroxidases to act as biocatalysts because of their following advantages and possibilities [1]:

- Highest activity, specificity and selectivity amongst oxidizing enzymes
- Chemoselective, regioselective and enantioselective synthesis of organic compounds
- Mild conditions in their reactions
- Minimized side reactions, in general reducing waste and the overall consumed process energy
- Replacing expensive cofactor regeneration systems as used e.g. in conventional P450-based catalysis
- Reduced effluents from conventional organo-metallic conversions

In Figure 1 typical peroxidase reactions of industrial relevance are visualized.

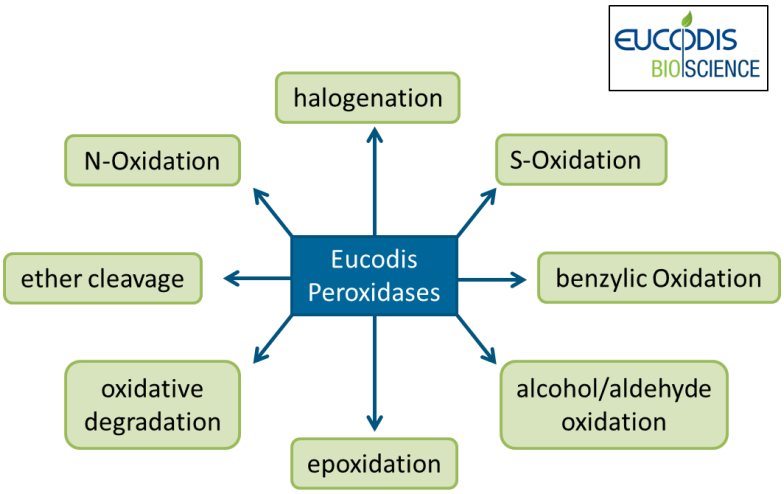


Figure 1: Typical peroxidase reactions of industrial relevance [adapted from Eucodis Bioscience]

1.2. Aim of this work

Eucodis Bioscience wants to push forward the production and sale of peroxidases for industrial use. In order to profit from these enzymes know-how on suitable substrates, reaction products and conversion rates has to be gained. The starting point of this project was to transform different substrates to their respective oxidized products with selected heme-peroxidases using peroxide as stabilization factor. Preliminary results were obtained from the conversion of soybean oil using a panel of peroxidases. Thin layer chromatography (TLC) was used to analyze the products. Figure 2 shows a TLC of such a reaction. At the beginning of this thesis the identity of the oxidation products and also conversion rates were not known.

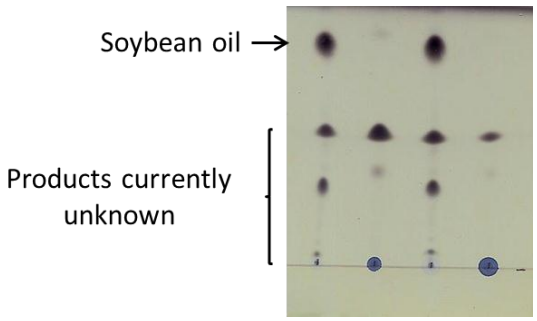


Figure 2: Thin layer chromatography of epoxidation reaction products of four different heme-peroxidases produced by Eucodis using soybean oil as substrate

The aim of this work was to develop an analytical method based on gas chromatography (GC) in combination with mass spectrometry (GCMS) to identify oxidation products originating from different peroxidases, different peroxide concentrations, yet from the same substrate.

1.3. Thin layer chromatography

Thin layer chromatography (TLC) is a simple however very useful and effective chromatographic method. The only things required for qualitative and semi-quantitative separations are a suitable, closed vessel containing a solvent (mobile phase) and a coated plate (stationary phase). The optimization of solvent system and stationary phase allows highly efficient separations and reproducible semi-quantification.

TLC is a very old method of analysis that has been well proven in practice occupying a prominent position for more than thirty years, especially for qualitative investigations. But the importance of TLC has been marginalized when high-performance liquid chromatography (HPLC) was introduced. Today TLC is mainly used for fast and quick checks of chemical reaction performances in organic synthesis but to a lesser extent in bioanalytics. This is also reflected in the marginalized presence and depth of theory taught for TLC in different institutions. In addition to that, there is a loss of specialized know-how in the use of TLC because of the restructuring of the chemical industry [2].

TLC uses a thin layer applied to a solid substrate as stationary phase on which the sample is applied at a distance of about one centimeter from the bottom (Figure 3). After placing the TLC plate into a closed vessel the mobile phase moves over the plate. The analyte has to be soluble in the mobile phase to be transported to the top of the plate. Different strengths of interactions between the analytes and the stationary phase leads to a separation of all analytes present in the applied sample.

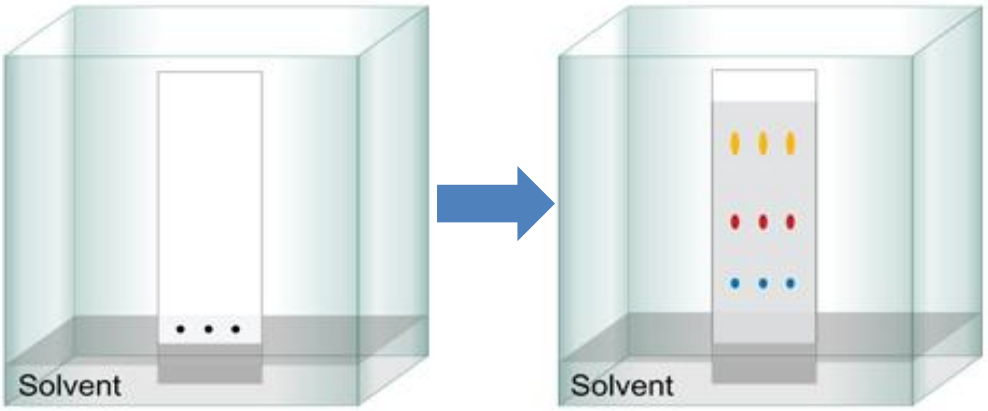


Figure 3: Typical setup for carrying out a thin layer chromatography. The spots on the plate are only schematic and do not represent a special sample. (Adapted from [3]).

TLC is used if

- a large number of samples has to be analyzed simultaneously, cost-effectively and within a short period of time,
- the used solvents would damage or irreversibly bind to the stationary phases of columns used in liquid chromatography (LC) or gas chromatography (GC) systems,
- the substances which have to be analyzed are not detectable after LC or GC separations

This list only gives a short overview why TLC is still important and used for analytical problems. It is always important to look at the present questions and to weigh the advantages and disadvantages of TLC in comparison to HPLC which is most of all more expensive in equipment acquisition.

In many different fields TLC is still in use, e.g. clinical chemistry, forensic chemistry, biochemistry, food analysis or environmental analysis [4].

1.3.1. Separation system – stationary and mobile phases

The stationary phase in TLC is a thin layer of diverse materials (modified or unmodified silica gel, aluminum oxide or cellulose) often immobilized on an aluminum plate. On this stationary phase the analytes are applied and then the plate is placed in the separation chamber containing the mobile phase. The mobile phase is a mixture of different solvents and the composition of the mobile phase is very important to get acceptable separations. Usually TLC's are developed vertical so that the solvent migrates from the bottom of the chamber/plate to the top (see Figure 3). Yet there are also methods available suggesting horizontal or descending developments. In this work twin trough chambers for vertical development were used.

There are many different stationary phases available. They all differ in use for different analytical problems. Adaption of pore diameters, coated layers and materials is important for proper method development. Also plates allowing the direct detection of the separated analytes are available. For this the detection reagent is incorporated in the stationary phase, e.g. a fluorescent indicator.

In this work unmodified high performance (HP) TLC plates with silica gel 60 on aluminum plates were used. The special silica gel on these plates has particle sizes of 5 to 6 µm with a pore size of 60 Å and this allows a higher fill factor as well as smoother surfaces and therefore reduced band diffusion [5].

1.3.2. Detection methods

There are several methods, which are used to detect the different analytes on a TLC plate. They can be classified into two main groups:

- Detection without derivatization
  - ✓ direct visual evaluation
  - ✓ direct optical evaluation using instruments
- Detection with derivatization

The first group of detection methods requests that the analytes are visible if illuminated by different light sources (e.g. daylight, UV lamp). The second group of detection methods needs derivatization of the analytes. There are many different possibilities how to visualize separated analytes. Some of them are:

- thermochemical reactions,
- irradiation with high-energy light,
- reaction with reagents applied by spraying, dipping, vapor treatment or coating to TLC plates.

In this work a solution of phosphomolybdic acid was sprayed onto the plate after separation or the plate was dipped into a developing reagent tank. After heating the plate with a heat gun blue/dark blue spots became visible on a yellow background. Phosphomolybdic acid is a good universal stain which is fairly sensitive to low concentrated solutions and it stains most functional groups. Yet it does not distinguish between different functional groups, e.g. by coloration of the spots on the TLC plate.

1.4. Gas chromatography

Gas chromatography (GC) is a separation method which uses the distribution of analytes between two non-miscible phases – the stationary and mobile phase. In GC systems the stationary phase can be solid or liquid and the mobile phase is gaseous. Already in 1941 Martin and Synge had the idea to use a gas as mobile phase for the separation of a substance mixture [6]. Martin and James implemented the method experimentally about ten years later [7]. Perkin-Elmer built the first commercial gas chromatograph in the middle of the 1950's.

Because of the technical simplicity of a gas chromatograph, GC is one of the most popular separation methods. It is applicable to all substances, which are vaporized intact directly or after derivatization. GC is used for many analytical problems, e.g.:

- permanent gases
- oil products
- food analysis
- oligosaccharides
- agricultural products
- doping and medical analysis
- drinking water
- …

Another point why GC gets a wide recognition is the fact that more than 80 % of all analytes on the so called black list (index of the most important organic harmful chemicals) of the European Union and the American Environmental Protection Agency can be analyzed by GC [8].

1.4.1. The gas chromatograph

Figure 4 shows schematically a typical gas chromatograph. The most important components are the carrier gas, the injector unit (nowadays usually an auto sampler), the stationary phase (column), the detector and a computer for data acquisition and evaluation.

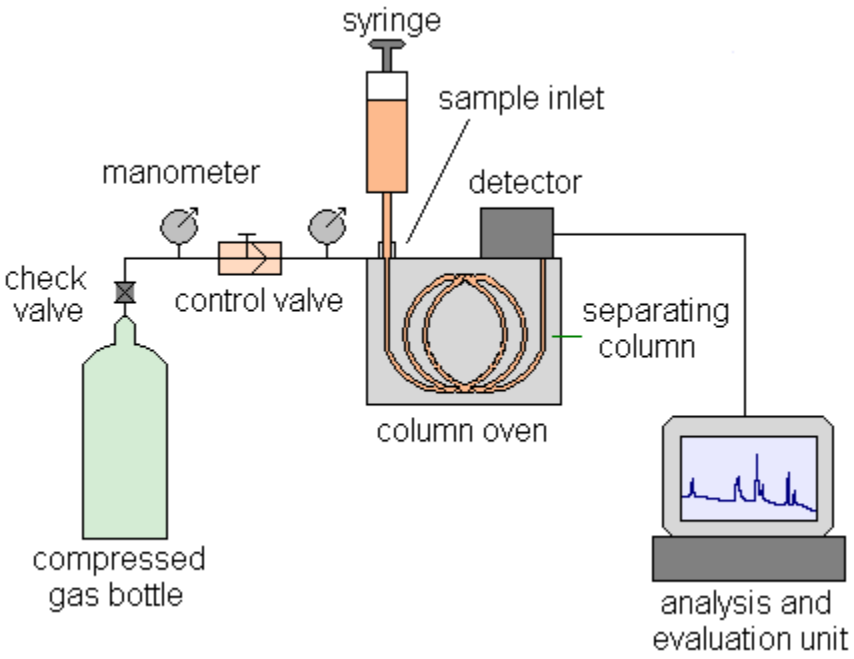


Figure 4: Scheme of a gas chromatograph [8].

In the next section the parts of a GC which have a crucial influence on the analysis are described in detail.

1.4.1.1. Sample inlet

The sample introduction can be carried out manually with a syringe or automatically with an autosampler. The latter one is far more reproducible and less labor intensive. The precision of the autosampler is very high but it is still advantageous to use an internal standard in order to be able to correct an error occurring during injection. In addition the possibility of automated analysis, e.g. overnight, is another reason why nowadays sample injection is usually carried out with an autosampler.

If the sample is already in gaseous form it can be applied directly into the carrier gas flow. A liquid sample has first to be evaporated in a small heated chamber. This chamber contains a vaporization tube made of quartz class, which is surrounded by a metal block that can be heated to specific temperatures (200 to 350°C) and is connected to the GC column. This chamber is the link between the carrier gas flow and the gas chromatographic separation column and is sealed from the outside with a septum made of silicon rubber.

There are several methods of sample injection. The most common methods are:

- on-column injection where the sample is applied directly onto the column,
- cold sample injection or programmed temperature vaporizing injection (PTV) to inject the sample with a special temperature gradient (also cooling is possible),
- direct injection with valve switching,
- or split/split less injection.

Split injection was used in this thesis. This type of sample application is very common and is suitable to inject very low amounts of sample to protect the column. The sample gets injected into the heated split-injector, gets vaporized and is mixed with the carrier gas. Through a defined ratio only a subset of the sample reaches the column whereas the rest leaves the split injector over the outlet (split). This ratio, also called split ratio is the ratio of the gas volume, which gets on the column and the total volume of the column.

Using split injection generates a high split flow which causes a higher carrier gas velocity and this allows an increased transport of the sample onto the separation column. This has the effect that only a narrow sample zone is applied on the column which leads to better peak profiles [9].

1.4.1.2. Sample separation

The separation system itself consists of the GC column, flushed by the mobile phase and coated with the stationary phase, and the GC oven, which allows applying an appropriate temperature or temperature profile to the column. A temperature gradient helps to pass analytes of different vapor pressure through the column while keeping good separation conditions (peak shape, short separation time, high number of theoretical plates).

There are two main types of columns used in GC:

- packed columns or
- fused silica open tubular columns (FSOT).

Packed columns consist of glass, steel or sometimes even Teflon tubing's with a diameter of three to eight mm and one to three m in length. At the inner surface there is an inert substrate (silica or Al_2O_3), which is coated with the stationary phase. The particle size of the carrier material determines the separating capacity of the column and increases with decreasing particle size. There are two advantages of this type of column: it is chemically and mechanically robust and allows the injection of relatively large amounts of sample. The disadvantage is the very low number of theoretical plates.

Fused silica columns are quartz capillaries with an inner diameter of 75 to 100 µm and about 30 to 50 m length. On the inner side there is the stationary phase, a coating made of high-boiling liquid or polymer, of one to only a few µm thickness. Using polymers as stationary phase, the temperature and chemical stability can be increased. In order to improve the mechanical stability of these columns the fused silica capillary is coated with a polyimide film on the outside. The big advantage of such columns is the high number of accessible theoretical plates. On the other hand this sort of column is not as resilient as the packed one.

In Figure 5 the two columns are compared and it is clearly visible that the separating capacity in the case of fused silica columns is much higher than in the case of packed columns.

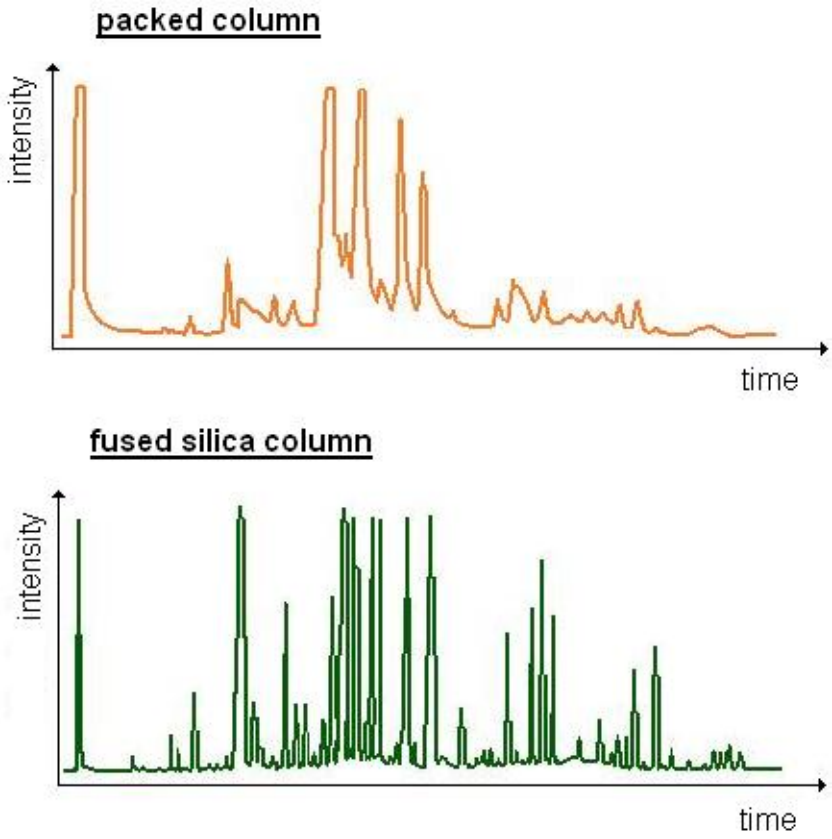
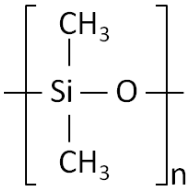


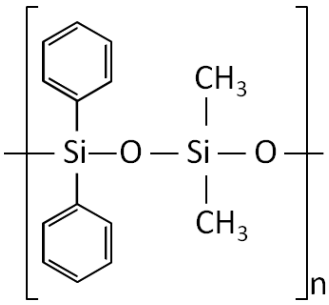
Figure 5: Comparison of a packed column and a fused silica column. In both cases the sample is an essential oil. Figure adapted from [8].

As mentioned above the stationary phase usually is a cross linked polymer. The high separation efficiency of GC makes it possible that there are only some major components used as stationary phase. Yet modifications of this polymer can increase the specificity of the column. In the list below the main types of stationary phases and their structures are mentioned.

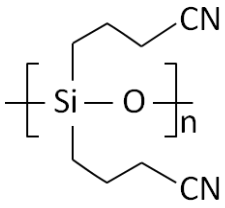
- methyl siloxane (MeSi)



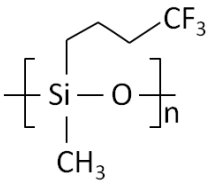
- methyl phenyl siloxane (MePhSi) with a low or high amount of phenyl siloxane



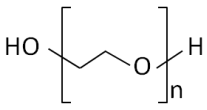
- cyanopropyl siloxane (CPSi) with middle or high amount of cyanopropyl siloxane



- methyl trifluoropropyl siloxane



- high molecular weight polyethylene glycol



In this work a modified polar stationary phase was used, polyethylene glycol treated with nitroterephthalic acid (BP21). This column is especially useful for the analysis of volatile free acids, fatty acid methyl esters, alcohols, aldehydes, acrylates and ketones.

In addition to the stationary phase also the mobile phase has a crucial influence on separation capacity. A high analytical separation capacity is defined through a high number of theoretical plates (N), which means that the height equivalent of the theoretical plates (HETP) decreases. Using Van Deemter-curves allows determining which gas and flow gives the best separation capacity. The Van Deemter-curve plots the HETP against the average flow rate and the minimum of the curves for each carrier gas gives the optimal adjustment relating to the flow rate. In Figure 6 the Van Deemter-curves for three very important carrier gases are presented. It is shown that hydrogen performs best (lowest HETP). In addition hydrogen provides a wide operation range where the HETP is very low in comparison with nitrogen and helium. Yet hydrogen is not compatible with many detectors and prone to explosion in case of leakages (oxygen). Because helium also has a broad range of flow rates providing low HETPs and because it works with a number of detectors it is the most common carrier gas used in GC despite the high price.

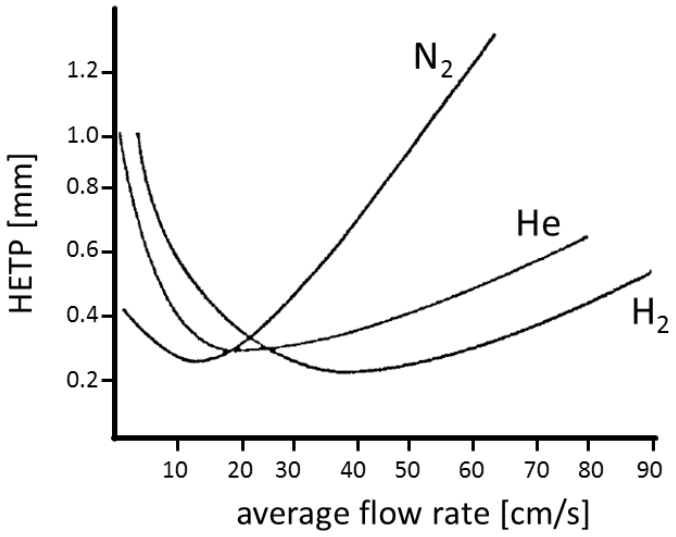


Figure 6: Van Deemter-curves for the carrier gases nitrogen, helium and hydrogen. The declared values belong to a standard column with a length of 30 m, an inner diameter of 0.25 mm and a film thickness of 0.25 µm. Figure adapted from [10].

1.4.1.3. Sample detection

In GC there are several detection methods and a lot of different detectors available. Detectors can be divided into concentration or mass flow dependent devices. Table 1 gives a short overview of the detectors usually used in GC.

Table 1: Overview of the most common detectors used in GC classified by concentration and mass flow dependence.

concentration dependent	mass flow dependent
thermal conductivity detector (TCD)	flame ionization detector (FID)
electron capture detector (ECD)	nitrogen-phosphorus detector (NPD)
	mass spectrometric detector (MSD)

In the case of concentration dependent detectors the produced signal is proportional to the concentration of substance in the detector volume. Mass flow dependent detectors generate a signal proportional to the mass flow, which means mass per time. The electric signal produced at the detector results in the chromatogram.

TCDs are those detectors used the longest in GC [11]. This type of detector uses a heated tungsten, nickel, platinum or alloy wire as active measurement element. The electric currents of these materials depend on the temperatures they reach [12]. The continuous measurement of the conductivity difference between a sample gas flow and a reference gas flow is the basic principle. This detector serves especially for the detection and quantification of permanent gases, carbon dioxide, sulfurous dioxide or inert gases [13].

The ECD consists of an ionization chamber containing a bent nickel sheet with a thin layer of ^{63}Ni as radioactive source. This beta emitter ionizes the carrier gas through the bombardment with electrons. The gas ions are moving to a collector electrode (anode) causing a basic current (residual current). If the sample is transported to the detector by the carrier gas, electron absorbing analyte molecules collect electrons and therefore reduce the residual current. The analyte concentration is thus proportional to the degree of electron capture. Because of the high sensitivity to halogens, organometallic compounds, nitriles or nitro compounds this detector is very important in environmental analysis, e.g. identification of polychlorinated biphenyls (PCBs) or organochloride pesticides [8,14].

The FID is the most widely spread detector used for GC because of low detection limits, wide linear ranges, an insignificant time constant and its robustness [15]. The analytes are burnt in a hydrogen flame and get partially ionized. This burning of the analyte also cleans the detector. Organic substances form positive carbocations which quantity is proportional to the amount of substance. This process releases electrons that are accelerated by a voltage applied between the cathode, the burner head, and the anode, a circular collector electrode. So the registered current is proportional to the amount of oxidized carbon atoms eluting per second [8].

A special detector is the NPD, which is a modified FID and consists of an alkali source (rubidium or cesium salt pearl) in order to identify special elements, preferentially nitrogen and phosphorus. A potential is applied between the bead and the anode and during normal operation, when only the carrier gas passes the column, the heated alkali bead (600-800°C) forms a plasma in the region of the bead emitting electrons that are collected at the anode producing a constant ion current. When a nitrogen or phosphorus containing solute is eluted from the column, the partially combusted nitrogen and phosphorus materials are reacting partially with the surface of the bead. The adsorbed material gets ionized and a small current from the plasma to the charged collector is formed increasing the current at the anode.

Because of the very low carrier gas flows used in capillary GC columns a direct coupling with a mass spectrometric detector is possible [16]. This type of detection was used in this work and a detailed explanation follows later (see chapter 1.5).

In addition to the above described detectors there are several detectors which are not so common:

- photoionization detector (PID)
- flame photometric detector (FPD)
- electrolytical conductivity detector (ELCD)
- thermo ionic detector (TID)
- Fourier transform infrared spectroscopy (FTIR)
- atomic emission detection (AED)

1.4.2. Quantitative gas chromatography

GC is a very powerful analytical method for the separation of samples, but the information on the separated analyte is rather low. A chromatogram visualizes different peaks and therefore the different substances which are present in the sample. Yet no information on the type and quantity of the analytes is available.

The quantitative analysis and evaluation is based on the comparison of the obtained sample peaks with the peak area or peak height of a specific reference/standard peak. This means that it is necessary to know which compounds are expected in the resulting chromatogram. This is unfortunately often not the case.

Consequently there is also the question of appropriate standards. They can be prepared in the laboratory or purchased as ready-made products. In general, physic-chemical properties of standards should be as similar to the sample as possible, in concentration and preparation. In addition to that the stability of the standards has to be taken to account [17].

For quantification the following common calibration procedures are used:

- External standardization: External standardization uses an external standard, which is present in the same matrix as the sample to be analyzed. Several concentration levels are prepared and measured in the same way as the unknown sample. The peaks of the unknown sample can then be directly referred to the peaks and peak areas of the standard. Through calibration curves a relation between peak area and concentration can be established. It is very important to operate at concentration levels in a range where the column is not overloaded and also the detector capacity not exceed. If the concentration is too high no linear correlation is detected.

- Internal normalization: This technique is carried out by mixing a standard containing all the components of the unknown sample. Through adding known weights of the pure components to each other the weight percentages can be calculated. After a chromatographic measurement the areas of these standard components are known and through dividing the weigh percentages by the areas of the peaks the concentration per unit area is known for each component. Setting one component as the reference (= response factor of 1) gives the response factors of the other components by dividing the concentration per unit area of these by the one of the reference.

  After a chromatographic analysis of the unknown sample the areas of the different components are known and multiplying them with the weight percent per unit area gives the raw weight percent in the unknown. Then these values have to be divided by the total weight percent (which is higher than 100 % because of usually unevenly injected sample sizes) to give the normalized weight percent. So with this technique the

variation of sample size can be corrected but the major disadvantage is that the standard has to be prepared and detected in the chromatographic system. Also the detector has to response uniformly to all components.

- Internal standardization: A defined amount of the internal standard is added to the unknown sample. This known substance then serves as reference value for the calculation of the response factors. In addition to that the internal standard also gets added to the calibration standard which also contains the substances which are present in the unknown sample. The determination of the response factor is then based on the correlation of the amounts of the substances to their normalized peak areas. With these calculations and correlations the amount/concentration of unknown sample can be calculated.

  This method is preferred as it avoids errors introduced during sample preparation, e.g. volume measuring errors or errors occurring during extraction or derivatization.

  In this work internal standards were used to determine and correct analyte extraction efficiencies and sample injection inaccuracies of the GC.

  There are some demands for an internal standard:

  - ✓ stability/inactivity during sample preparation/treatment
  - ✓ not present in the sample
  - ✓ no interference with other compounds in the GC
  - ✓ comparable behavior to the sample (sample preparation/analysis)

1.4.3. Sample derivatization

Because of the fact that there are many substances which are too polar or not thermo-stable enough or even not volatile enough, derivatization methods have to be used to allow GC analysis.

There are some prerequisites for derivatization reagents:

- Easy removal of excessive derivatization reagent or no interference of the reagent with the analysis.
- Avoidance of polar, not thermo-stable or hardly volatile derivatization side products.

There are several derivatization reagents, which can be used for GC analysis. The choice of reagent depends on the chemical properties of the analyte and the detector used for analysis.

Most commonly used derivatization reagents/methods:

- Diazomethane (CH_2N_2): converts acids and phenols to more stable compounds (methyl esters, methoxy substituted aromatic compounds). Very suitable for GC only the carcinogenicity of the reagent has to be taken into account.

$$R\text{-}COOH + CH_2N_2 \rightarrow R\text{-}COO\text{-}CH_3 + N_2$$

R-COOH…acids, phenols
$R\text{-}COO\text{-}CH_3$…derivatized acid/phenol

- Trimethylsilyl derivatives: Trimethylsilylether or -ester have an increased volatility and are less polar than the starting compounds (acids, sugars, alcohols). The problem with this type of derivatization is the probable formation of still non-volatile reaction by-products, which have to be removed before the GC analysis.

$$R_3Si\text{-}X + R'\text{-}H \rightarrow R_3Si\text{-}R' + HX$$

R…frequently methyl groups ($-CH_3$)
X…leaving group with low basicity
R'-H…acid, sugar, alcohol
R3Si-R'…derivatized acid/sugar/alcohol

- Extractive acylation: this method converts compounds soluble in water or bases into anions. After adding a lipophilic cation these anions can be extracted as ion pair into an organic phase. The organic phase contains the derivatization reagent which converts the solvated ion pairs into esters.

- Trimethyl sulfonium hydroxide (TMSH): This reagent was used in this thesis to convert free acids into methyl esters. TMSH was introduced in 1979 as a methylating agent for carboxylic acid compounds and since then it has proven to be a powerful derivatization reagent for GC analysis [18]. The derivatization reaction is very easy to handle and the reaction product is dissolved in a solution which can directly be injected into the GC. Usually the reaction works at room temperature, but heating can sometimes help to complete conversions. Compounds which can be derivatized are carboxylic acids, alcohols, thiols, phenols or N-heterocyclic compounds.

$$[(CH_3)_3S]^+OH^- + R'\text{-}COOH \rightarrow (CH_3)_2S + R'\text{-}COOCH_3 + H_2O$$

$[CH_3S]^+OH^-$…TMSH
R'-COOH…carboxylic acid, alcohol, thiol, phenol, N-heterocyclic compound
$(CH_3)_2S$…dimethyl sulfide

1.5. Mass spectrometry

Mass spectrometry (MS) is an analytical technique to separate charged molecules by their mass-to-charge ratio (m/z). The scheme of a typical mass spectrometer is shown in Figure 7.

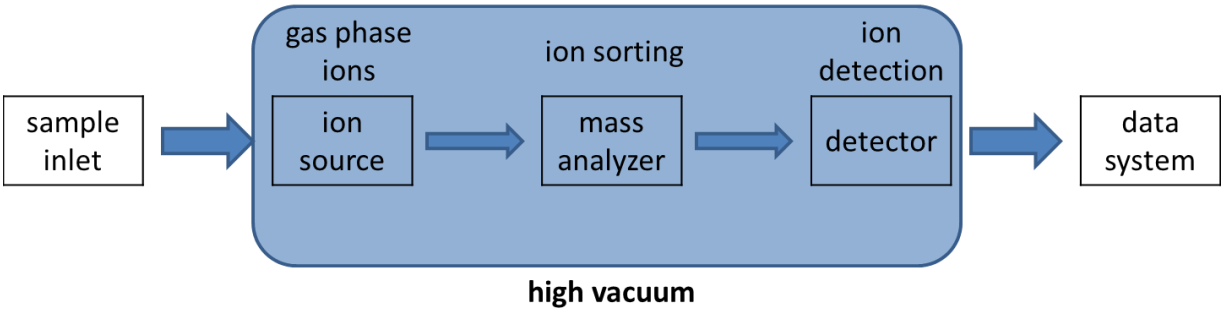


Figure 7: Parts of a typical mass spectrometer. The blue box indicates the area of the high vacuum.

In the first part of a mass spectrometer gas phase ions are generated. There are several ionization methods which can be used. The most important are listed here:

- chemical ionization (CI)
- electron ionization (EI)
- field ionization (FI)
- plasma desorption (PD)
- laser desorption (LD)
- matrix assisted laser desorption ionization (MALDI)
- thermospray ionization (TS)
- atmospheric pressure ionization (API)
- electrospray ionization (ESI)
- secondary ion mass spectrometry (SIMS)

For this thesis EI was used for the generation of gas phase ions and so only this method is explained in detail in the following section.

EI was first designed by Dempster and improved by Bleakney and Nier [19]. It is a method which is widely used especially in combination with GC. EI is a so-called “hard” ionization technique, destroying the actual analyte and therefore mostly fragment ions are observed. In Figure 8 a typical electron ionization unit is shown. The filament consisting of rhenium gets heated by passing through high currents. It releases electrons, which are accelerated towards an anode and collide with the gaseous sample molecules. These molecules get ionized and form ions. However very often positive ions are of higher interest, as it was also in this study. Therefore only positive ions are discussed.

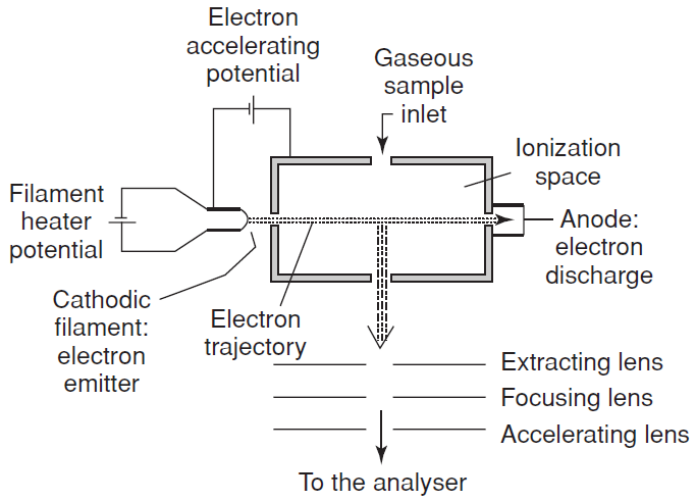


Figure 8: Scheme of an electron impact ionization module taken from [20].

Former instruments used 20 eV to accelerate the electrons providing decent fragmentation spectra of the analytes. Nowadays 70 eV are used because of more complete structure information and higher intensities of fragment ions.

The generated positive ions are accelerated towards the mass analyzer. There are several mass analyzers available showing different performances. Table 2 gives a comparison of the most important types of mass analyzers.

Table 2: Comparison of different mass analyzers; FWHM…full width at half maximum; TOF…time of flight; FTICR…Fourier transform ion cyclotron resonance

mass analyzer	mass limit	resolution (FWHM m/z 1000)	accuracy	energy collision
quadrupole	4000 Th	2000	100 ppm	low
ion trap	6000 Th	4000	100 ppm	low
TOF	>10^6 Th	5000	200 ppm	-
TOF reflectron	10^4 Th	20^4	10 ppm	low or high
magnetic	20^4 Th	10^5	<10 ppm	high
FTICR	30^4 Th	50^5	<5 ppm	low
orbitrap	50^4 Th	10^5	<5 ppm	-

In this work a quadrupole analyzer was used. The principle of a quadrupole was described by Paul and Steinwegen at Bonn University in 1953 [21]. A quadrupole analyzer is built of four rods (perfectly parallel) of circular or, ideally, hyperbolic shape. The applied electromagnetic field is built by applying a direct current (DC) and an alternating current (AC) to the rods. Only ions with a defined m/z can pass through the analyzer at a given DC/AC ratio. In the case of positively charged ions they will be drawn towards a negative rod when entering the space between the rods. If the potential changes sign before the ion discharges itself on this rod because of the applied AC, the ion will be accelerated in the other direction. The ion will be moved on a certain trajectory through the quadrupole analyzer (Figure 9).

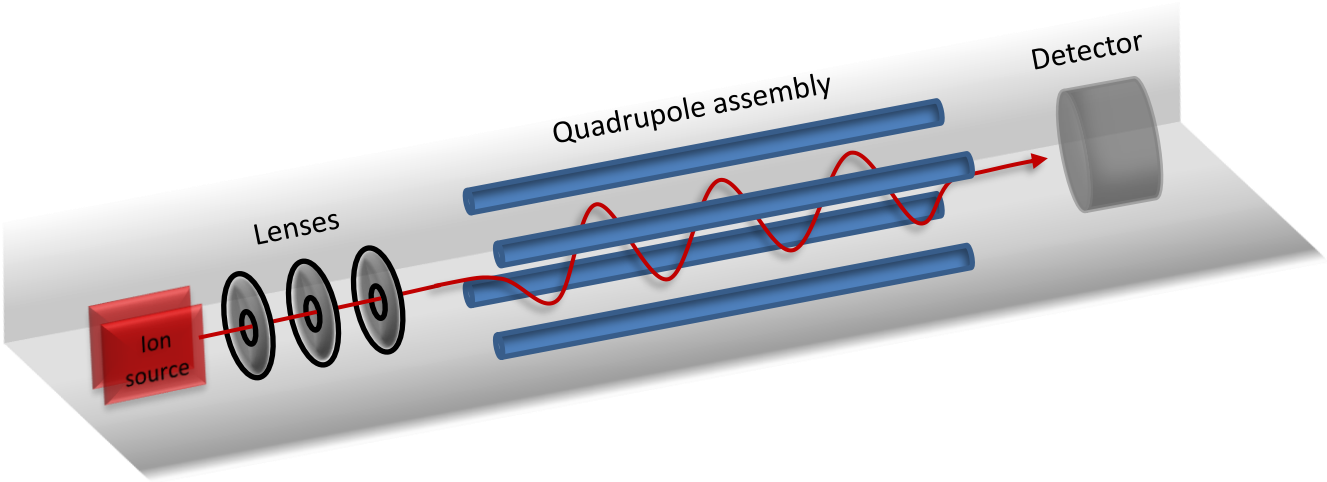


Figure 9: Quadrupole mass analyzer made up of the ion source, the focusing lenses, the quadrupole cylindrical rods and the detector.

Because of the fact that the DC/AC ratio changes very quick, ions with different m/z can be passed through the mass analyzer. So a predefined m/z range can be scanned in a few milliseconds and all the ions with m/z values within the selected range reach the detector, which is located at the end of the analyzer. The detector registers the ions and presents a useable signal. In the instrument used for this thesis an electron multiplier was installed as detector.

This type of detector accelerates the ions to a high velocity by holding an electrode at a high potential (opposite to the charge on the detected ions). When the ions hit the surface of the electrode a secondary emission of one to three electrons is induced. The produced electrons are then further amplified to produce a current by a cascade effect in the electron multiplier and the generated current is proportional to the abundance of the initial ions.

In Figure 10 two electron multipliers are shown. The left is a discrete dynode and the right is a continuous dynode electron multiplier. In the first one the electrons are accelerated to the next dynode because its potential is held at a lower level until they reach the detector end. The second one is also called a channeltron which is made of a lead-doped glass with a curved tube shape that has good secondary emission properties. An applied voltage between the two extremities of the tube produces a continuous accelerating field along its length. The generated cascade of electrons is collected finally by a metal anode at the detector exit and the current is measured.

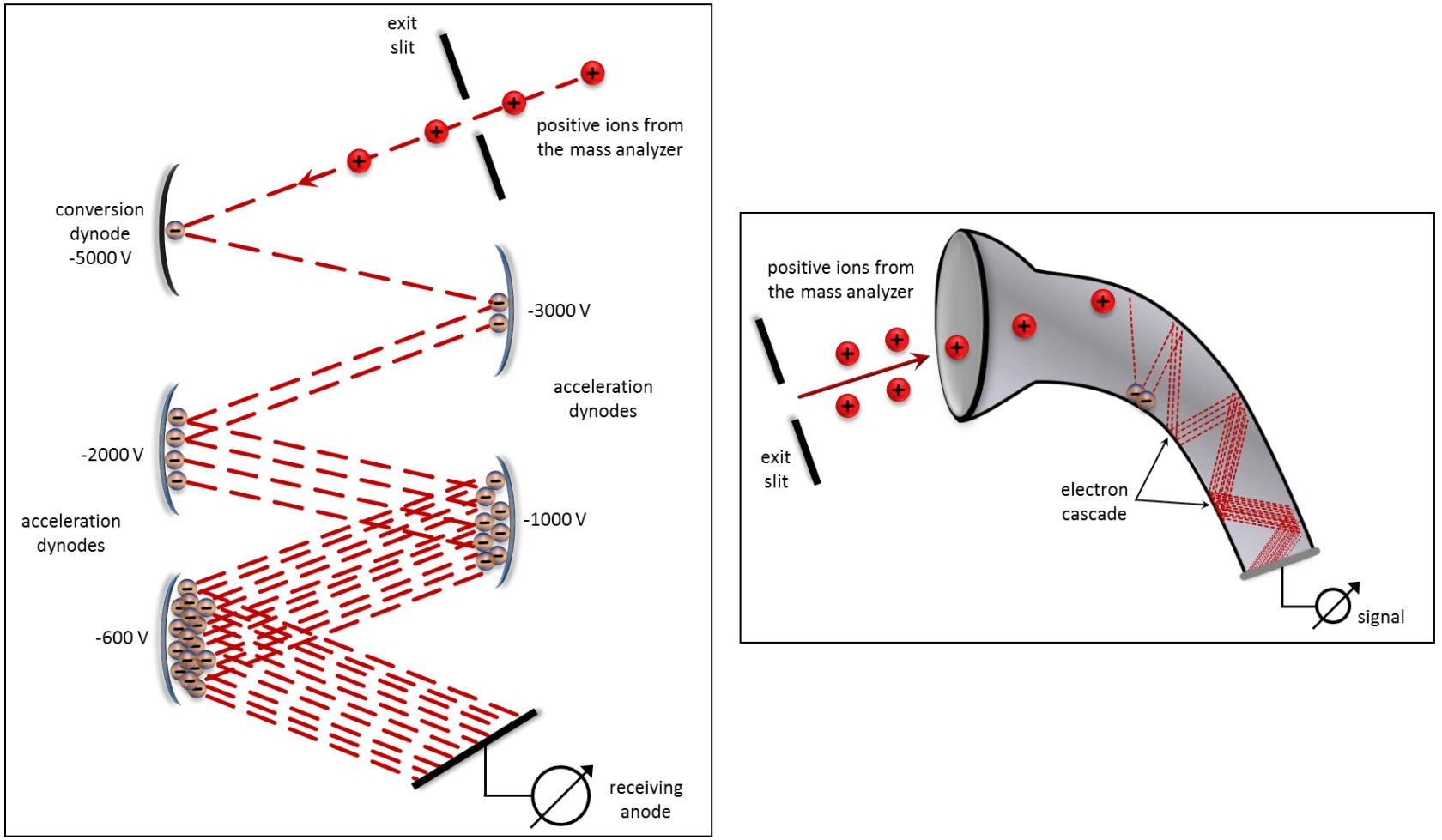


Figure 10: Comparison of a discrete dynode electron multiplier (left) and a continuous dynode electron multiplier (right).

1.5.1. Mass spectrometry as a detection system in GC instruments

As already mentioned in chapter 1.4.1.3 a mass spectrometer was used as detector after GC separation. This allows to obtain a mass spectrum for each eluted compound and therefore identification of the isolated product. Because of the fact that GC produces gas-phase molecules the use of EI as ionization method is very convenient. Furthermore the use of a quadrupole as mass analyzer makes the hyphenation with GC very cost efficient.

The most crucial point in GCMS hyphenation is the coupling of the two instruments. Packed columns are critical as they are run at higher gas flow rates than acceptable for MS instruments, which are working under high vacuum. Gas flows typically used with capillary columns are much better suited. There are two types of coupling interfaces: the open and the direct coupling.

In the case of an open coupling the column is placed into a tube which also holds the capillary leading to the MS source. This tube is vacuum sealed and reaches in a T-shaped tube in which a helium flow passes through in order to avoid any contact with atmospheric oxygen. This coupling does not enrich the sample and allows working at usual chromatographic conditions (carrier gas flow, one end of the column being under atmospheric pressure). The realization is very easy and requires no further setting.

The direct coupling means that the capillary column directly enters the MS ion source through a set of vacuum-sealed joints. Compared with the open coupling this type has the disadvantage of difficult changes of the column.

1.5.2. Mass spectrometry for structure elucidation

GCMS is a very powerful method in order to separate the constituents of a complex sample and furthermore to identify the separated analytes. GCMS analysis gives chromatograms in which mass spectra exist for each time point. With the help of software the mass spectra can be assigned to certain compounds by comparing the measured mass spectrum with entries in a database. In this thesis NIST08, consolidated by the National Institute of Standards and Technology, was used as database containing over 200 000 spectra of over 190 000 unique compounds. Although this can help to identify unknowns in a sample several other tools are available which can be helpful for structure elucidation, e.g. isotopic pattern, nitrogen rule, more specific algorithms, double bond equivalents or special fragmentation pathways and fragment losses respectively.

To determine a structure from a mass spectrum certain rules can be followed. The analysis and evaluation of mass spectra starts at the determination of the molecular ion, which can be the most intense peak. Because of the fact that EI is a hard ionization method the molecule ion can be obtained hardly in the spectra.

The next step is to calculate mass differences between the molecular ion peak and the other obtained peaks. The differences then represent neutral fragment losses, which can help to assemble the initial structure. Typical neutral losses are: H_2, CH_4, H_2O, C_2H_4, CO, NO, CH_3OH, H_2S, HCl, CH_2=C=O, CO_2. The remaining fragment carries the charge and will be detected. Clearly defined eliminations such as alpha-, benzyl and allyl or alkyl fragmentation can further help during the process of compound/structure identification. Furthermore the nitrogen-rule is very important. With this the number of nitrogen atoms in a molecule can be determined by looking at the number of the molecular mass of the molecular ion or any fragment.

A clear structure elucidation is not easy and takes up some time and effort but there are many tools and possibilities to be successful at some point. For EI spectra structure elucidation remains very often critical or not successful because of the fact that the molecule ion is not present or hardly present.

Es spielt keine Rolle, wie großartig deine Theorie ist, es spielt auch keine Rolle wie klug du bist – wenn es sich nicht durch Experimente belegen lässt, dann ist es einfach falsch.

Richard Feynman (1918-1988)

2. Experimental

2.1. Materials

2.1.1. Chemicals and Enzymes

Table 3 gives an overview of all chemicals used in this thesis.

Table 3: List of the used chemicals and their purities as well where they were purchased or obtained

product	purity/quality	company
9,10-epoxyoctadecanoic acid	100 %	Sigma-Aldrich, St. Louis, USA
acetic acid	≥ 99.8 %	Sigma-Aldrich, St. Louis, USA
acetone	for analysis	Merck, Darmstadt, Germany
cis-11,14,17-Eicosatrienoic acid methyl ester (Lot # SLBD1623V)	≥ 98 %	Sigma-Aldrich, St. Louis, USA
diethyl ether	min. 99.5 %	Lab-Scan analytical sciences, Dublin, Ireland
di-sodium hydrogen phosphate	≥ 99.5 %	Fluka Chemie AG Buchs, Switzerland
ethanol (EtOH)	min. 99.8 %	Merck, Darmstadt, Germany
gum arabic	-	Sigma-Aldrich, St. Louis, USA
Luperox TBH70X, tert-butyl hydroperoxide solution	70 wt. % in H_2O	Sigma-Aldrich, St. Louis, USA
methyl linolenate (Lot # SLBH2722V)	≥ 99 %	Sigma-Aldrich, St. Louis, USA
methyl oleate	≥ 99.0 %	Sigma-Aldrich, St. Louis, USA
n-hexane	min. 99 %	VWR, Radnor, Pennsylvania, USA
oleic acid (Lot # SLBD7659V)	≥ 99 %	Sigma-Aldrich, St. Louis, USA
petroleum ether	-	Institute of Applied Synthetic Chemistry, TU Vienna
10 % phosphomolybdic acid solution	-	Sigma-Aldrich, St. Louis, USA
phosphoric acid (H_3PO_4)	≥ 85 wt. % in H_2O	Sigma-Aldrich, St. Louis, USA
sodium dihydrogen phosphate monohydrate	≥ 99.5 %	Fluka Chemie AG Buchs, Switzerland
sodium hydroxide (NaOH)	min. 99 %	Merck, Darmstadt, Germany
Supelco 37 component FAME mix (Lot # LC04806V)	certified reference material; TraceCERT®	Sigma-Aldrich, St. Louis, USA
Luperox® TBH70X, tert-Butyl hydroperoxide (t-BHP) (Lot # BCBL0887V)	70 wt. % in H_2O	Sigma-Aldrich, St. Louis, USA
tert-Butyl methyl ether (MTBE)	≥ 99.5 %	Fluka Chemie AG Buchs, Switzerland
trimethyl sulfonium hydroxide (TMSH)	for GC derivatization	Fluka Chemie AG Buchs, Switzerland
Triton X-100	extra pure	Scharlau Chemie, Spain
ultrapure water (ultra-high quality, UHQ)	specific resistivity ≥ 18 MΩ*cm	water purification system from Millipore Simplicity

The used peroxidases were obtained directly from Eucodis either in liquid form (Per15 dissolved in a 50 mM phosphate buffer, PBS) or as powder (lyophilized; Per1, Per14, Per15).

2.1.2. Materials and Instrumentation

- Microsyringes (10 µl & 250 µl) from Innovative Labor Systeme GmbH, Stützerbach, Germany
- Twin trough chamber from CAMAG, Muttenz, Switzerland
- High performance TLC (HPTLC) plates from Merck, Darmstadt, Germany:

  Silica gel on aluminum foils with a pore size of 60 Å and a particle size of 5-6 µm; dimension: 5x7.5 cm;
- BP21 (FFAP) column terephthalic acid (TPA) treated polyethylene glycol from SGE analytical science, Melbourne, Australia

  Dimension: 25 m x 0.22 cm; inner diameter: 0.25 µm
- Ultrasonic bath (USC200TH) from VWR, Radnor, Pennsylvania, USA
- Laboratory centrifuge from Sigma-Aldrich, St. Louis, USA
- Reagent sprayer from VWR, Radnor, Pennsylvania, USA
- Oven (UM 100) distributed by Memmert, Schwabach, Germany
- Heat gun from Starke, Müllnern, Austria
- Thermomixer comfort by Eppendorf AG (Hamburg, Germany)
- pH meter MP220 from Mettler Toledo, Schwarzenbach, Switzerland
- GCMS-QP2010 Plus running GCMSsolution v2.72 for setting up the instrument and GCMS postrun solution v2.70 for data analysis. All from Shimadzu Biotech Kratos Analytical, Manchester, UK

2.1.3. Solutions

All enzymatic reactions were set up in a buffer system. Two different buffers were used: a 50 mM Tris-HCl (pH 7.5) buffer and a 50 mM phosphate buffer (pH 7.5). The Tris-HCl buffer was prepared and obtained from Eucodis whereas the phosphate buffer was prepared using the Henderson-Hasselbalch equation:

$$pH = pka + log\frac{[A^-]}{[HA]}$$

pH…desired pH of the buffer (7.6)
pka…logarithmic acid dissociation constant ($H_2PO_4^-$; 7.21)
A^-…molar concentration of the acid's conjugate base
HA…molar concentration of the undissociated weak base

With the ratio of $[A^-]/[HA]$ which is 0.05 M (to get a 50 mM phosphate buffer) the molarities and finally the initial amount of A^- (Na_2HPO_4) and HA ($NaH_2PO_4xH_2O$) could be calculated:

A^- = 0.0355 M HA = 0.0145 M

A^- = 5.0434 g/l HA = 1.9971 g/l

A^- = 0.5043 g/100 ml HA = 0.1997 g/100 ml

Finally 0.5047 g of Na_2HPO_4 and 0.1999 g of $NaH_2PO_4xH_2O$ were dissolved in 100 ml of UHQ. The pH was adjusted to 7.5 with 0.05 M NaOH and 0.05 M H_3PO_4 using the pH meter.

2.2. Thin layer chromatography

2.2.1. Sample preparation

The enzymatic reactions were performed in an aqueous solution and so the analytes, fatty acids and fatty acid esters, were extracted with n-hexane, a non-polar solvent. After centrifugation the organic layer could be removed and used for TLC.

- volume of n-hexane: total reaction volume:
  200 µl aqueous buffer containing enzymes, the fatty acid and the peroxide was extracted with n-hexane;
  Various volumes: 100 µl, 200 µl; 200 µL performed best
- number of extractions: one
- aqueous layer was kept at 4°C until the analysis of the sample was finished
- extracts were kept at 4°C until further analysis and later stored at minus 20°C

2.2.2. Separation system

The following solvent mixtures were used as mobile phases:

Solvent 1: n-hexane/MTBE/EtOH/acetic acid (60/40/1/0.1) (v/v/v/v)

Solvent 2: petroleum ether/diethyl ether/acetic acid (80/20/2) (v/v/v)

Solvent 1 [25] was used for the separations in the beginning. Later solvent 2 was used as it provided better separation efficiencies. Before starting the separation the solvent was filled into the HPTLC chamber and it the gas phase was saturated with solvent vapor for one hour. Then the separation was started.

The samples were applied on the plates about 1 cm from the bottom. After letting them dry the HPTLC plate was put into the HPTLC chamber. When the solvent front was one cm away from the top (after about 20 minutes) the separation was stopped by removing the plates. The HPTLC plates were dried under ambient conditions before detecting the analytes. In Figure 11 the used HPTLC chamber with two HPTLC plates is shown.

Figure 11: Twin trough chamber from CAMAG used for HPTLC analysis.

2.2.3. Detection method

For the detection of analytes on the HPTLC plates two methods were used.

Method A: A reagent sprayer (Figure 12) was used to spray a ready-to-use solution of 10% phosphomolybdic acid onto the plates.

Method B: The plates were dipped into the same solution (Figure 13) for 10 sec as deep that the whole separation distance was covered with the stain. This allowed a better and homogenous staining of the plates.

In both cases the HPTLC plates were dried for one minute and subsequently heated with a heat gun until bands were visible (approx.. 0.5-1 min).

Figure 12: Reagent sprayer used for staining the HPTLC plates and detecting the sample bands [22].

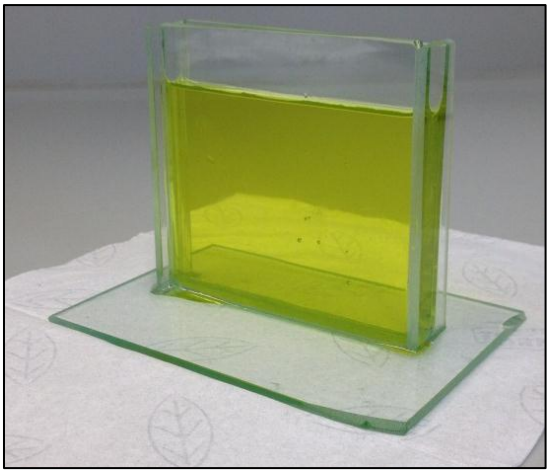


Figure 13: Glass chamber used for dipping the HPTLC plates into the staining solution.

2.3. Gas chromatography-mass spectrometry

Figure 14 shows the GCMS instrument all analyses were run on. Helium was used as mobile phase and the stationary phase was a BP21 column as described in chapter 1.4.1.2.

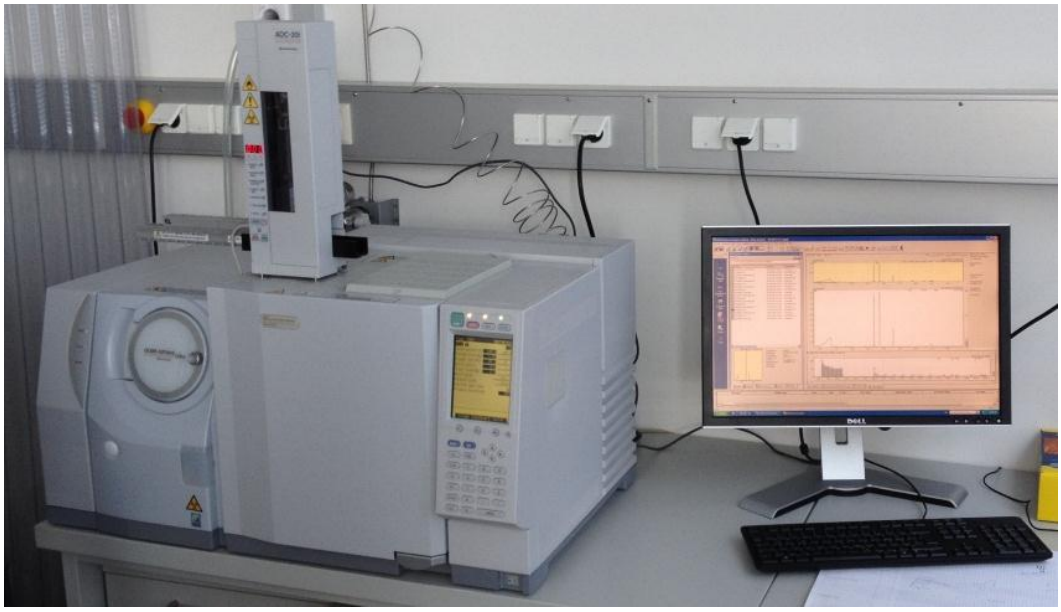


Figure 14: Picture of the GCMS from Shimadzu and the connected PC

2.3.1. Sample preparation

In order to analyze the samples of the enzymatic reaction the analytes had to be extracted with n-hexane. The n-hexane extracts were used directly for GCMS analyses, provided that there was no need of derivatization. The enzymatic reactions with methyl oleate did not need to be derivatized because the esters are stable and volatile enough for GCMS. On the other hand oleic acid needed derivatization before analysis.

The derivatization was performed with TMSH. The derivatization procedure was carried out as described by Dron, J. and R. Linke et al [23]. Briefly, in a GC vial 10 µl of the sample in n-hexane were added to 90 µl chloroform and subsequently 50 µl TMSH were added. The reaction mixture was mixed sufficiently and put into an oven for 30 min at 45°C.

This vial was directly used for analysis without further sample preparation.

2.3.2. GCMS separation

In order to establish a suitable method for the GCMS analysis of the epoxidation reactions of fatty acid methyl esters (FAMEs) a mix with 37 different FAMEs was used (chain lengths: C4 to C24).

Table 4 lists the initially applied GCMS settings.

Table 4: GCMS parameters used for the separation of the 37 component FAME mix

parameter	value	unit
column oven temperature	32	°C
injection temperature	220	°C
injection mode	split	-
carrier gas pressure	125.00	Pa
total flow	19.90	ml/min
column flow	2.82	ml/min
linear velocity	66.00	cm/sec
purge flow	3.00	ml/min
split ratio	5.0	-
ion source temperature	200	°C
interface temperature	220	°C
solvent cut time	2.30	min
mass range	50-500	m/z
scan speed	1666	amu/sec
event time	0.3	sec

Depending on the split ratio the flow and linear velocity values given in Table 4 may vary slightly. For control the carrier gas pressure was used and so this value was fixed.

The temperature program for GC separation (Table 5, Figure 15) was adjusted in a way to allow a separation of all the FAME mix components.

Table 5: GC temperature program for the separation of the FAME mix

rate [min^{-1}]	final temperature [°C]	hold time [min]
-	32.0	3.00
35.00	150	0.00
2.20	170	8.00
1.80	220	15.00

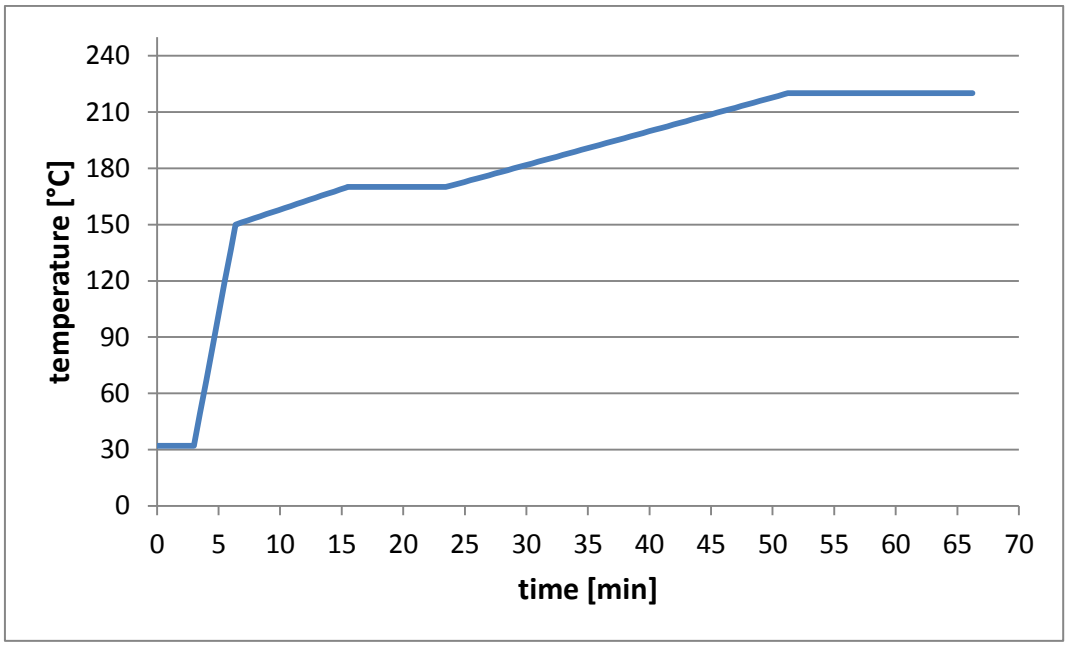


Figure 15: GC temperature program for the separation of 37 FAME mix components

Because the performed enzymatic reactions did contain only one FAME or one fatty acid (FA) a simpler and shorter temperature program was used (Table 4).

Table 6: GC temperature program used for the performed reactions

rate [min^{-1}]	final temperature [°C]	hold time [min]
-	32.0	3.00
20	220	15.00

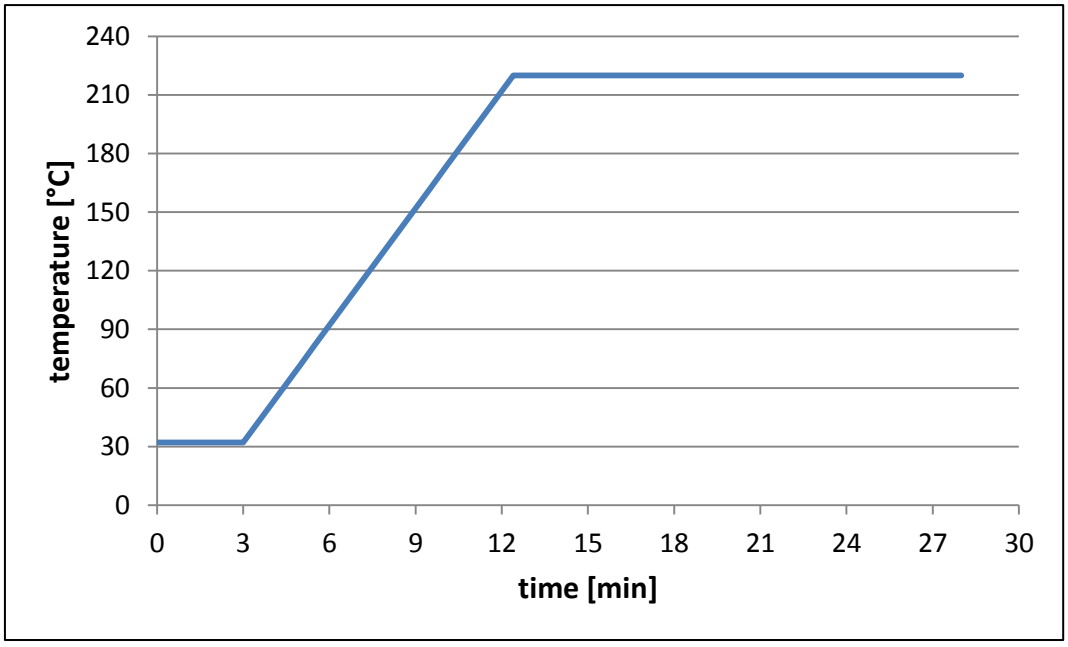


Figure 16: GC temperature program used for the performed enzymatic reactions

Jeder junge Wissenschaftler sollte stets die Möglichkeit im Auge behalten, dass ein vermeintlich irritierendes Versagen von Labortechnik, das zu inkonsistenten Ergebnissen führt, ein- oder zweimal im Leben auch ein Hinweis auf großartige Entdeckungen sein könnte.

Patrick Blackett (1897-1974)

3. Results and Discussion

3.1. GCMS method development

As already mentioned in the experimental part the GCMS method development was carried out with a FAME mix and different individual FAMEs. A predefined method was used to separate the different fatty acid esters, which was then adapted in order to get an optimized method for the analysis. In addition to that parameters investigated by Eder, K. [24] were taken into considerations for method development.

3.1.1. FAME measurements

The split injection was used because it is the classical injection mode for the analysis of FAMEs and has the advantage that samples with a relatively high concentration of FAME's can be injected without the risk of overloading the stationary phase, causing a degradation of the column performance. The only problem which has to be considered and can have serious consequences is the discrimination between high- and low-boiling compounds in the sample [24].

Also the stationary phase is very important for the separation of FAMEs. A very polar column was chosen for analysis because of the high resolving power for FAME mixtures. Yet it has to be mentioned that the lifetime and the thermal stability of these columns are not so good [24].

In Figure 17 the chromatogram of the FAME mix using the GCMS method given in Table 4 and Figure 15 is shown. The peaks are marked with numbers (1 to 37) and the names of the FAME mix compounds are mentioned in Table 12 in the appendix. It is clearly visible that nearly all methyl esters of the mix are separated. Only the peaks 17 and 18 are badly separated showing only one peak with a shoulder for both components. These two peaks represent the two isomeric forms of 9-Octadecenoic acid methyl ester and are therefore difficult to separate under these conditions. The last two peaks (36 and 37) are not baseline separated as well but it is clearly visible that there are components present.

These measurements were carried out to investigate the behavior of the different FAMEs on the used stationary phase. Furthermore the measurements helped to understand the software and to learn which parameters have to be adjusted to obtain suitable separations and resolutions.

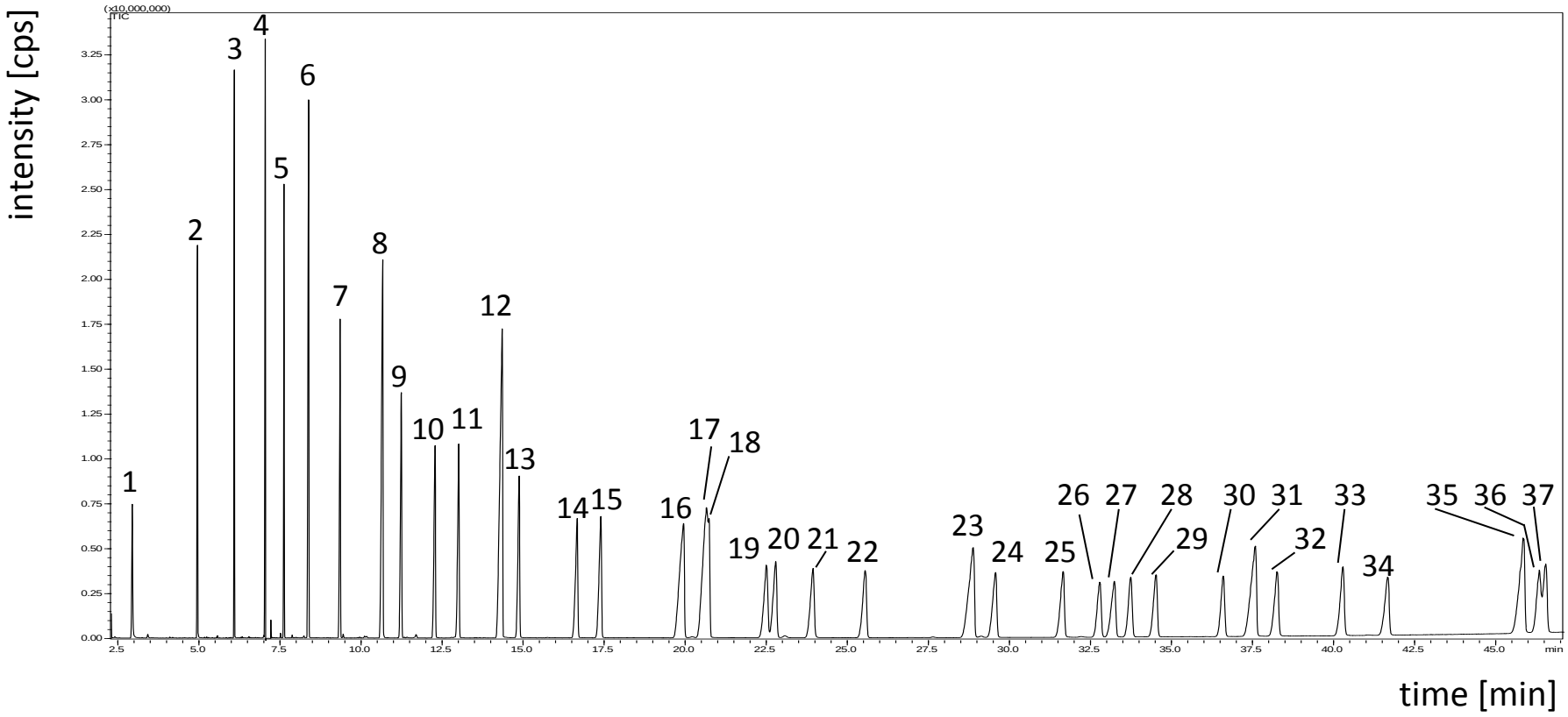


Figure 17: Chromatogram of the 37 FAMEs. The peaks are numbered from 1 to 37 sorted by retention time. The names of the methyl esters are listed in the appendix in Table 12.

3.1.2. Derivatization

The derivatization of the samples and especially the fatty acids (see chapter 2.3.1) was a very quick and simple procedure. The advantage of the method with TMSH is that the derivatization solution could be injected directly into the GCMS and further sample preparation was not necessary.

In order to study the efficiency of the reaction and to see if the derivatization can be performed as described in [18], oleic acid (see Figure 18) was derivatized with TMSH. Oleic acid is furthermore one of the substrates used for the enzymatic reactions. TMSH esterifies the acid to the methyl ester (methyl oleate in the case of oleic acid). The ester itself also was used as substrate for the enzymatic reaction and was therefore available as standard for comparison. In Figure 20 the chromatogram of methyl oleate is shown, exhibiting a retention time of 13.37 min and in Figure 21 the MS spectrum is visualized.

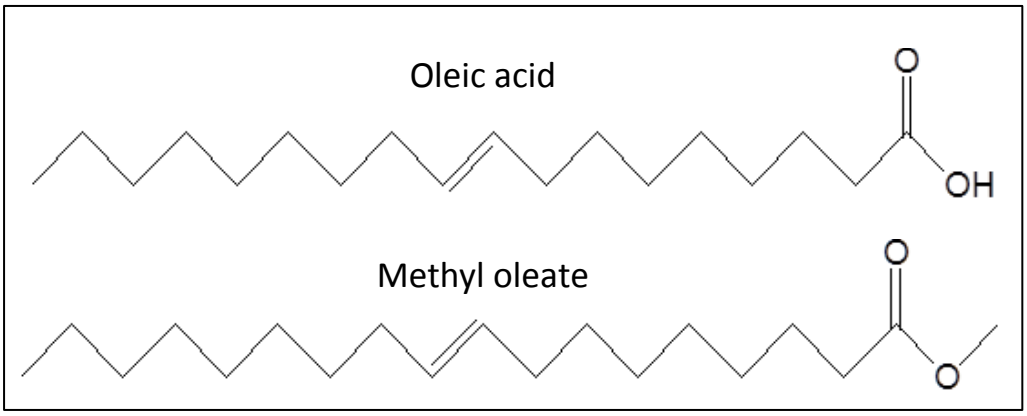


Figure 18: Molecular structures of oleic acid (cis-9-Octadecenoic acid) and methyl oleate (Methyl cis-9-octadecenoate)

Figure 22 shows the chromatogram of methyl oleate derived from the derivatization reaction carried out in our laboratory according to the protocol described in chapter 2.3.1. It is clearly visible that the peak in this chromatogram has the same retention time (13.37 min) as the methyl oleate standard. Also the MS spectra can be compared (Figure 21). Therefore it was confirmed that the derivatization method was carried out properly. The identity of the substance was also confirmed through automatic database search in the NIST library (more about that in 3.2.6). This moreover confirmed the compatibility of the instrument and the MS performance with available, commercial EI databases which was a substantial information for further work, where the identification of unknown reaction products was essential. However it has to be mentioned that a side product of the derivatization reaction was detected at 7.38 min, which could not be identified by the obtained mass spectrum and database search. Figure 23 shows the mass spectrum of the unknown.

A second fatty acid, namely 9,10-epoxyoctadecanoic acid (Figure 19), was used to prove the concept for the chosen derivatization procedure. Because 9,10-epoxyoctadecanoic acid was obtained as a powder, an n-hexane solution had to be prepared in order to allow the derivatization reaction. However it was not possible to completely dissolve the substance and so after centrifugation the supernatant of unknown concentration was used for derivatization. Figure 24 shows the chromatogram of the derivatization product and the structure (9,10-epoxyoctadecanoic acid methyl ester). The MS spectrum can be seen in Figure 25.

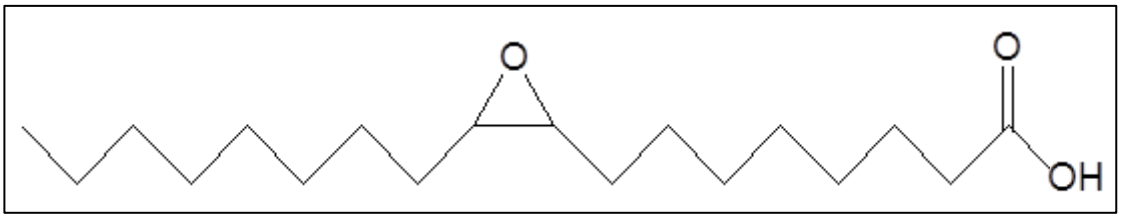


Figure 19: Molecular structure of 9,10-epoxyoctadecanoic acid

All compounds eluting between four and 11.5 min are derivatization reaction related peaks. The peaks at about 12.1 and 13.2 min are very likely impurities of the 9,10-epoxyoctadecanoic acid itself. The most intensive peak at 17.15 min is the derivatization product itself, 9,10-epoxyoctadecanoic acid methyl ester. The identification of the product was performed by database search using the NIST08 database. The similarity score of the mass spectrum was 94 which is a very high similarity score, representing therefore identity. Details about the identification are found in chapter 3.2.6.

Based on chromatographic results and mass spectral information it is clearly visible that the derivatization protocol also works for this analyte.

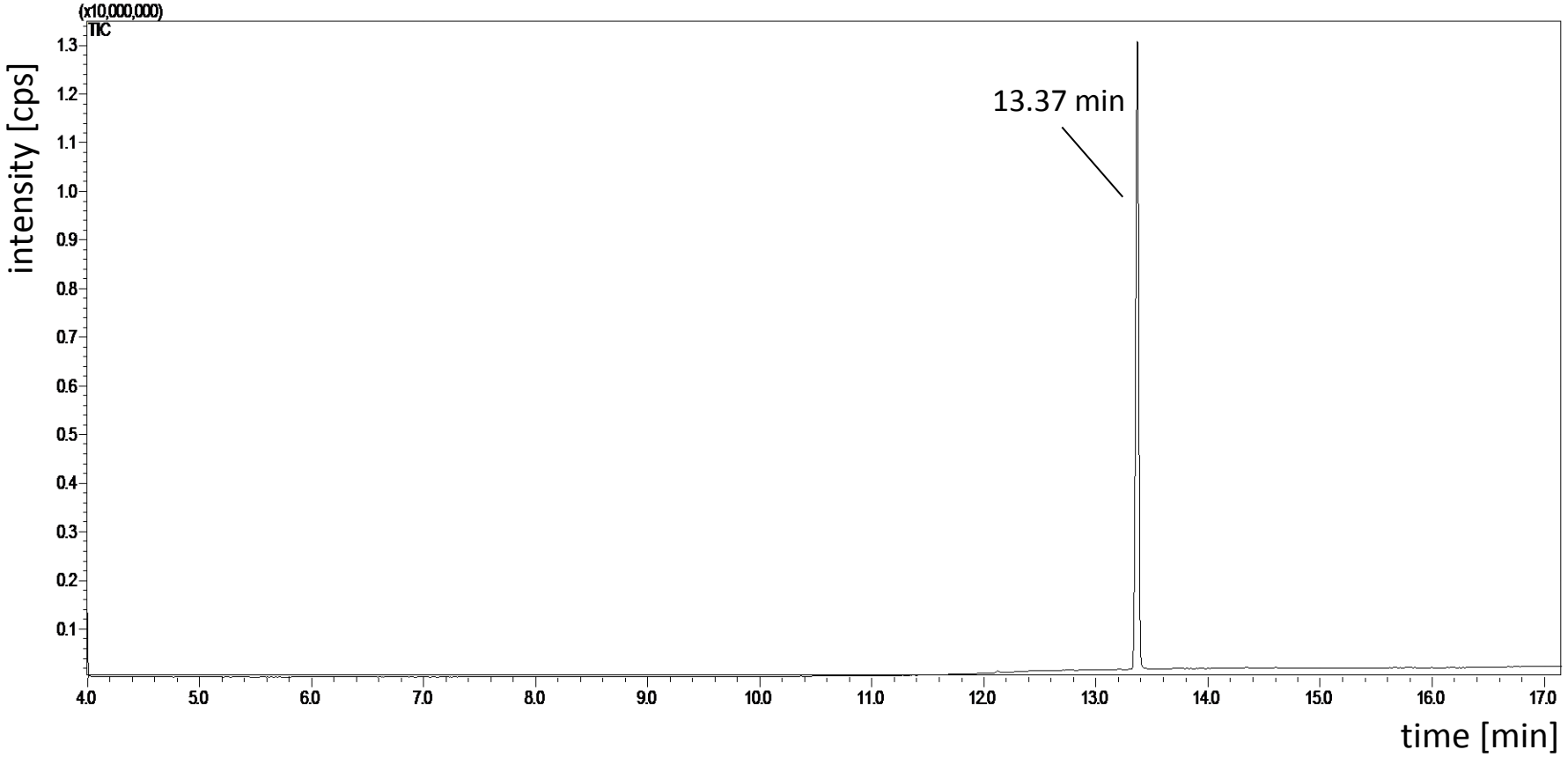


Figure 20: Chromatogram of methyl oleate (3 mM) with a retention time of 13.37 min. The sample was injected with a split ratio of 1/100 in order to avoid overloading of the column at higher concentrations.

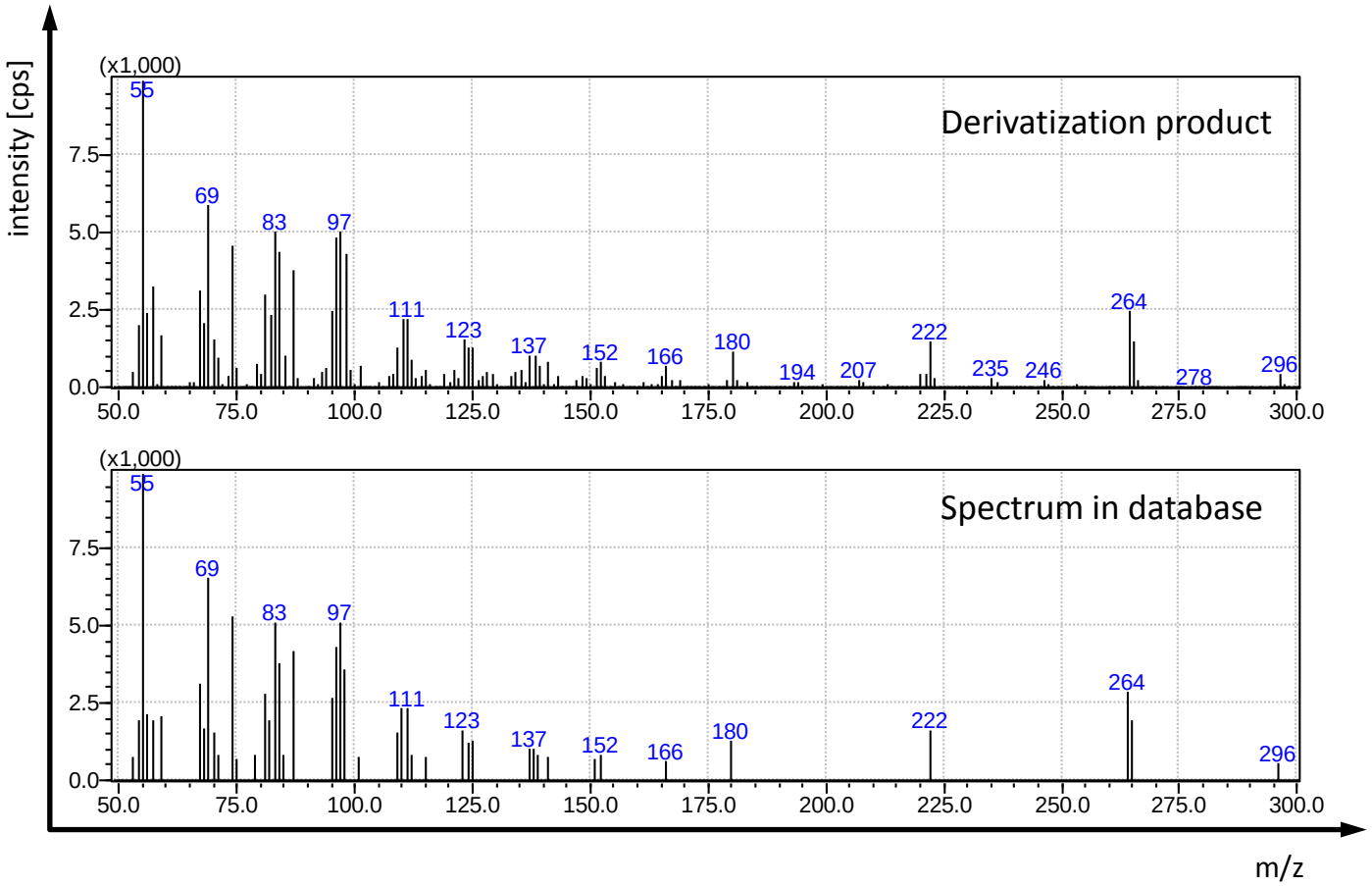


Figure 21: MS spectrum of methyl oleate. The top spectrum shows the fragment ion spectrum of the derivatization product of oleic acid and the spectrum at the bottom shows the database entry in NIST for methyl oleate.

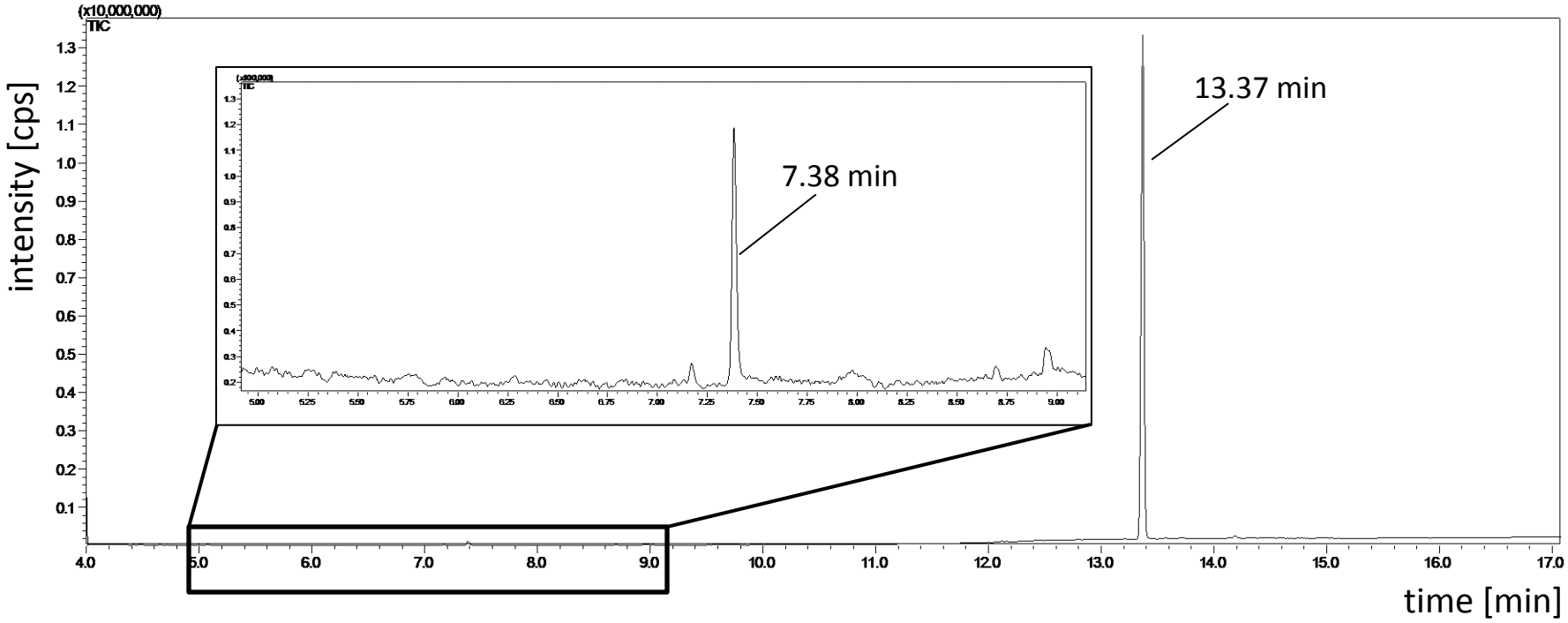


Figure 22: Chromatogram of 3 mM oleic acid derivatized with TMSH. The sample was injected with a split ratio of 1/100 in order to avoid overloading of the column at higher concentrations. The peak at 13.37 min corresponds to methyl oleate. The peak at 7.38 min could not be identified and is a side product of the derivatization reaction.

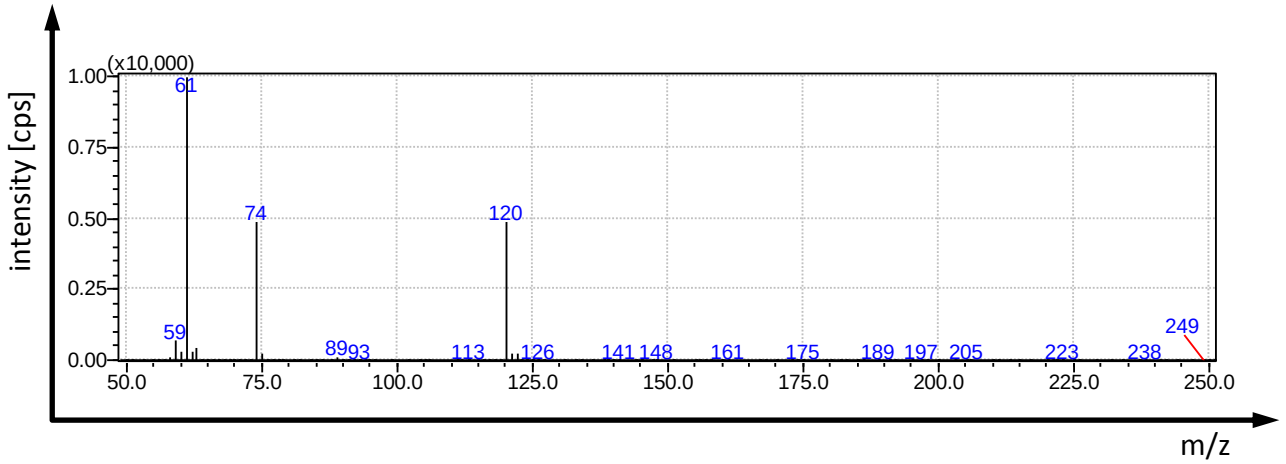


Figure 23: MS spectrum of the unknown substance (7.38 min) being a result of the derivatization

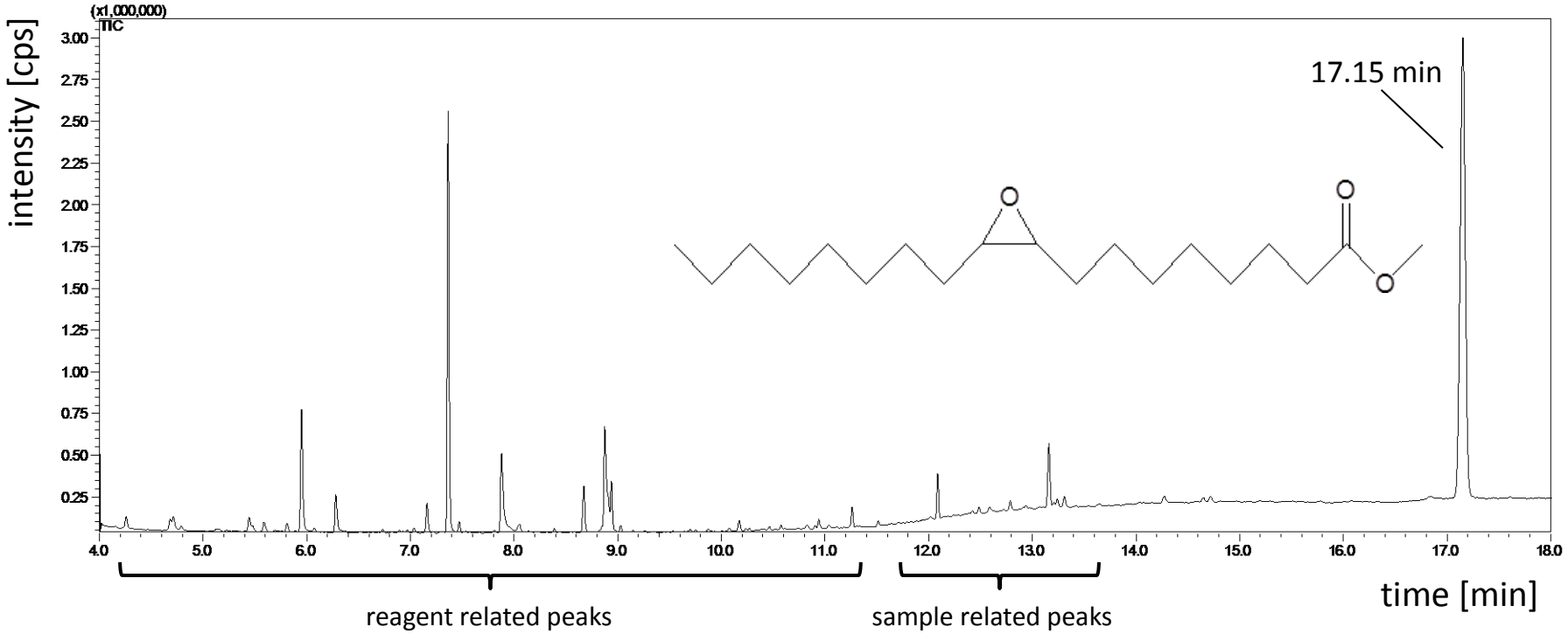


Figure 24: Chromatogram of 9,10-epoxyoctadecanoic acid after esterification with TMSH. Reagent related signals, unspecific signals from the sample and the derivatization product eluting at 17.15 min are indicated.

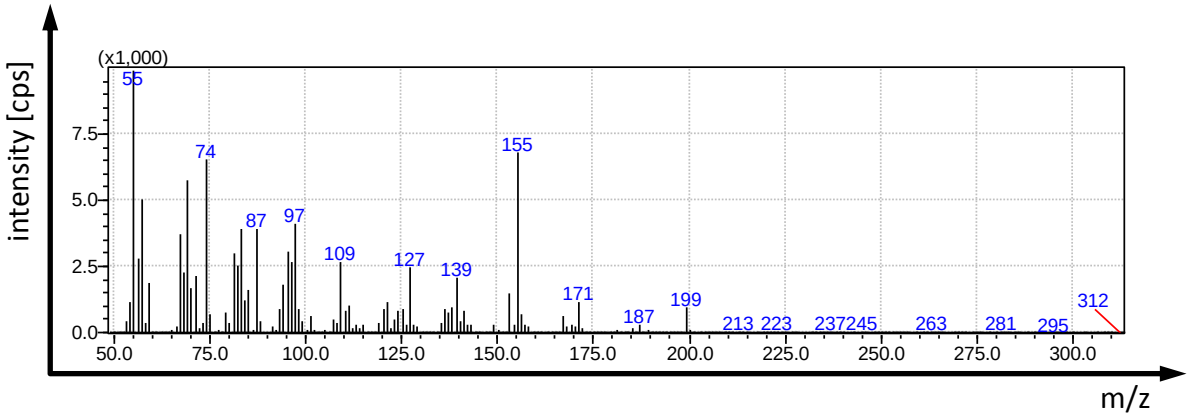


Figure 25: MS spectrum of 9,10-epoxyoctadecanoic acid methyl ester

3.2. Fatty acid and fatty acid methyl ester epoxidation reactions

Methyl oleate was used to investigate the activity of peroxidases produced at Eucodis. The FAME was incubated with a peroxidase and peroxide. The different reaction parameter and the use of different additives were studied to obtain a total conversion or at least a very high conversion of the ester. In addition to that the reaction products were desired to be identified by GCMS.

In addition to the fatty acid methyl ester also oleic acid was used for the reactions, the reaction products were derivatized and the GCMS data were compared to the conversion of methyl oleate.

3.2.1. Reaction conditions

If not stated otherwise the enzymatic reactions were performed in the thermomixer at 25°C at 650 rpm. The reaction time was 24 hours.

The reaction solution always contained the substrate (oleic acid or methyl oleate), the peroxidase and t-BHP (as peroxide) in an aqueous solution (buffer). Because of the fact that the substrates are not totally soluble in water or in buffer it was investigated if detergents influence the solubility on the one hand and the reaction itself on the other hand. Therefore Triton X-100 and gum arabic was used to allow a better solubility of the substrate by forming more and smaller droplets of the substrates.

The concentration of the peroxidase was 10 or 100 mg/ml of the lyophilized enzyme. The concentration was increased from 10 to 100 mg during the study to improve the performance of the conversion.

The concentration of the substrate was between 1 and 5 mM referred to the total reaction volume. Stocks with 1 M or 100 mM of the substrates were prepared in n-hexane.

The necessary concentration of the peroxide was not really clear at the beginning. A series of concentrations were tested in order to get a good conversion of the substrate to corresponding products.

Figure 26 shows a TLC plate after development with phosphomolybdic acid for different enzymatic reactions. Lane A and F show negative control reactions (reactions without an enzyme). Two different enzymes were tested, namely Per14 and Per15. Lane B (Per15) and C (Per14) were carried out with oleic acid whereas D (Per15) and E (Per14) were carried out with methyl oleate as substrate. The visualized substrates are marked with red circles in the negative controls.

The different conversion efficiencies of the different peroxidases are discussed later in chapter 3.2.4. What can be seen in Figure 26 is that there are a lot of products when using methyl oleate

compared with the use of oleic acid. The reason therefore can either be the fact that products formed from oleic acid are not visible on the plate or that the concentration of products is not high enough. But GCMS measurements confirmed that the same products were formed from the two different substrates. Because of that and the fact that methyl oleate has a better solubility than oleic acid, the ester was used for further enzymatic reactions.

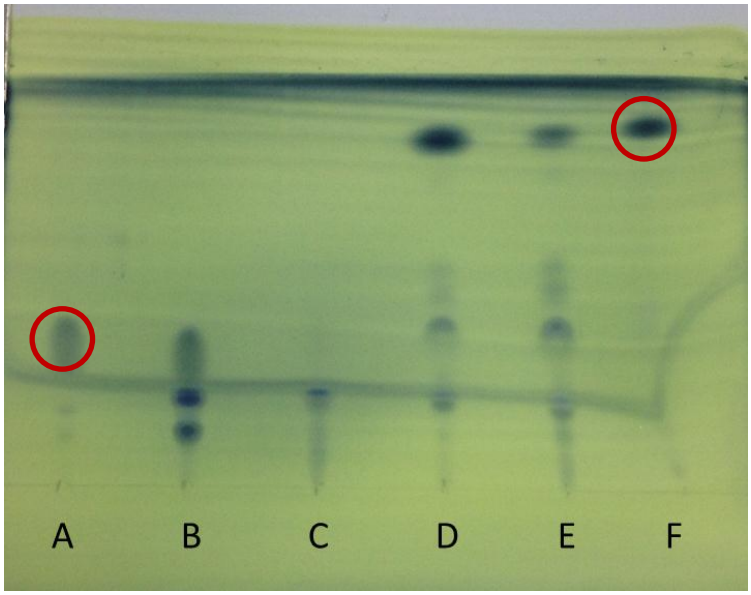


Figure 26: HPTLC of enzymatic reactions using oleic acid and methyl oleate as substrate for peroxidase Per14 and Per15. Lanes A and F show the migration of the not conversed substrates (A … oleic acid, F … methyl oleate). Lane B shows the reaction products for Per15/oleic acid, lane C for Per14/oleic acid, lane D for Per15/methyl oleate and lane E Per14/methyl oleate. For the separation solvent 2 (see chapter 2.2.2) and detection by dipping (see 2.2.3) was used.

3.2.2. Optimization of thin layer chromatography

At the beginning solvent 1, n-hexane/MTBE/EtOH/acetic acid (see also chapter 2.2.2), was used for HPTLC separation [25]. This mobile phase allowed good separation of the different analytes. The detection method used in the beginning facilitated phosphomolybdic acid solution, which was sprayed onto the plates. Because of the fact that the formed aerosol also contained larger aerosol droplets it was hardly possible to apply the detection solution homogenously on the whole plate. This means that the intensity of the coloring is not all the time representative for the amount of analyte and it is not possible to compare spots from the same substance on different TLC plates. This consequently means that it was hardly possible to make statements about the conversion efficiency of the enzymatic reactions. In Figure 27 this staining artifact can clearly be seen. On this plate the same sample was applied three times and stained by spraying the staining solution on top (the second lane was covered to prevent staining). It is clearly visible that there is a difference in the intensities between lane one and three.

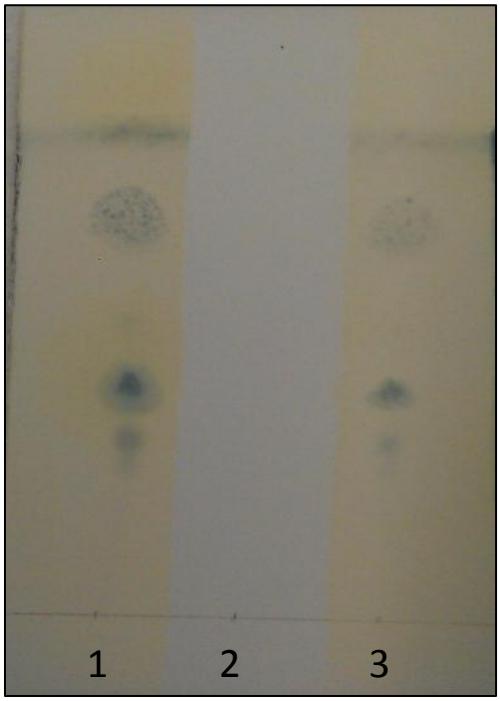


Figure 27: HPTLC plate stained with an aerosol of phosphomolybdic acid solution applied with a TLC sprayer. On this plate the reaction products of an enzymatic reaction were applied three times (lane 1, 2 and 3). Lane 2 was covered to prevent staining (separated, unstained analytes were used for further analysis).

To improve the staining method the plates were no longer developed by spraying the staining solution onto the plates, but the plates were dipped into the phosphomolybdic acid solution. By this it was possible to get a homogenous coloring and to compare spot intensities in respect to relative quantities. The already discussed HPTLC plate in Figure 26 was visualized by this method.

After dipping the plates in the staining solution and letting them dry under ambient conditions the plates were heated with a heat gun and the spots appeared. Yet excessive heating led to an intensive coloring of the whole plate. Not only the analytes became darker but also the background and so the detection of very weakly stained analytes was very hard. Subsequently heating the plate for a long time was avoided. It was very difficult to find the optimal heating time, because also other parameters like distance of the heat gun to the plate were influencing the staining efficiency.

As a consequence the separation solvents were changed. Solvent 2, petroleum ether/diethyl ether/acetic acid (see also chapter 2.2.2), was tested. The change of the amount of ether and the removal of n-hexane and EtOH turned out to be beneficial as the intensive background coloring was omitted. Furthermore no influence of the heating time was observed. The use of solvent 2 did not change the separation performance.

Figure 28 shows a comparison of the two used solvent mixtures used for the same samples. At first glance it is obvious that the background of the plate on the left is much darker than the background of the plate on the right. Furthermore an analyte, which is marked with a red circle, can be seen on the plate treated with solvent 2 but not on the other plate.

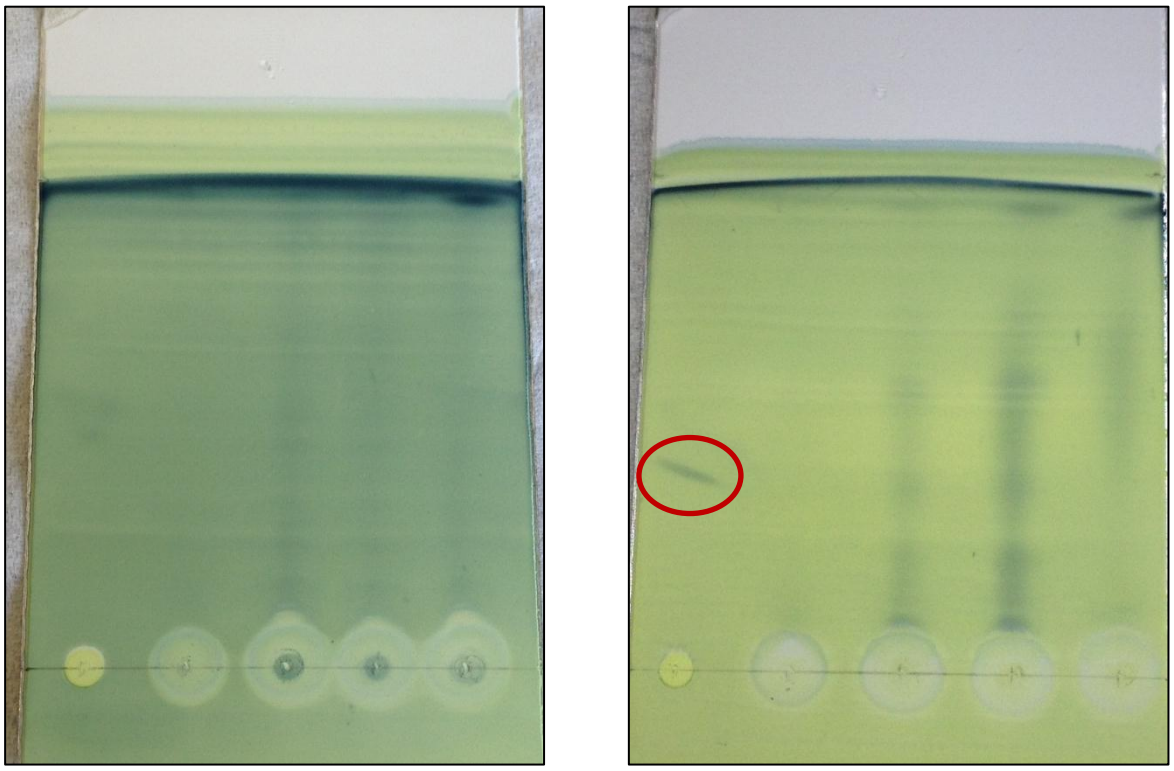


Figure 28: Comparison of solvent 1 (n-hexane/MTBE/EtOH/acetic acid, left) and solvent 2 (petroleum ether/diethyl ether/acetic acid, right) for HPTLC analysis with consecutive developing of the plate by tipping the plates in a phosphomolybdic acid solution and drying with a heat gun. Both plates were loaded with 9,10-epoxyoctadecanoic acid and reaction products from different enzymatic conversions.

It has to be mentioned that method development for HPTLC separation and optimizing reaction conditions for the enzymatic conversions were carried out in parallel. Consequently several HPTLC plates presented were developed with solvent 1 and a sprayed detection solution. Later the second solvent system and the dipping method were used for interpretation of the reactions. All Figures presenting results from solvent 1 and spraying of the detection reagent will from this point on be marked with “(Method 1)”. All results from solvent 2 and dipping into the detection solution will be marked with “(Method 2)” accordingly.

3.2.3. Optimization of t-BHP concentration

t-BHP was used as co-substrate for the enzymatic reactions to stabilize the enzyme in order to allow the conversion of the substrate. Because of the fact that the optimal t-BHP concentration was unknown, it was investigated how much t-BHP has to be used for sufficient conversion rates. t-BHP was used at different concentration levels (10, 50, 100, 200, 400, 1200 mM) while all other reaction conditions kept constant.

In order to determine the extraction efficiency and sample losses an internal standard (extraction standard) was used. Methyl linolenate (molecular structure see Figure 40) was used as sample preparation standard and will be abbreviated with IS_2 in the further work. In addition to that also an injection standard was used to compensate errors during sample injection into the GCMS. Therefore cis-11,14,17-Eicosatrienoic acid methyl ester was used, it will be abbreviated as IS_1. The molecular structure is shown in Figure 39. A standard solution containing methyl oleate and the two standards was used to compare the results of performed reactions.

Reaction conditions:

- substrate: 5 mM methyl oleate
- peroxidase: 1.2 µM Per14
- 20 % (v/v) acetone
- buffer: 50 mM Tris HCl pH7.5
- reaction volume: 200 µl
- 24 h at 25°C and 650 RPM

After adding 5 mM of IS_2 (the same as the substrate) the performed reactions were extracted with 200 µl n-hexane and TLC was carried out (Figure 29).

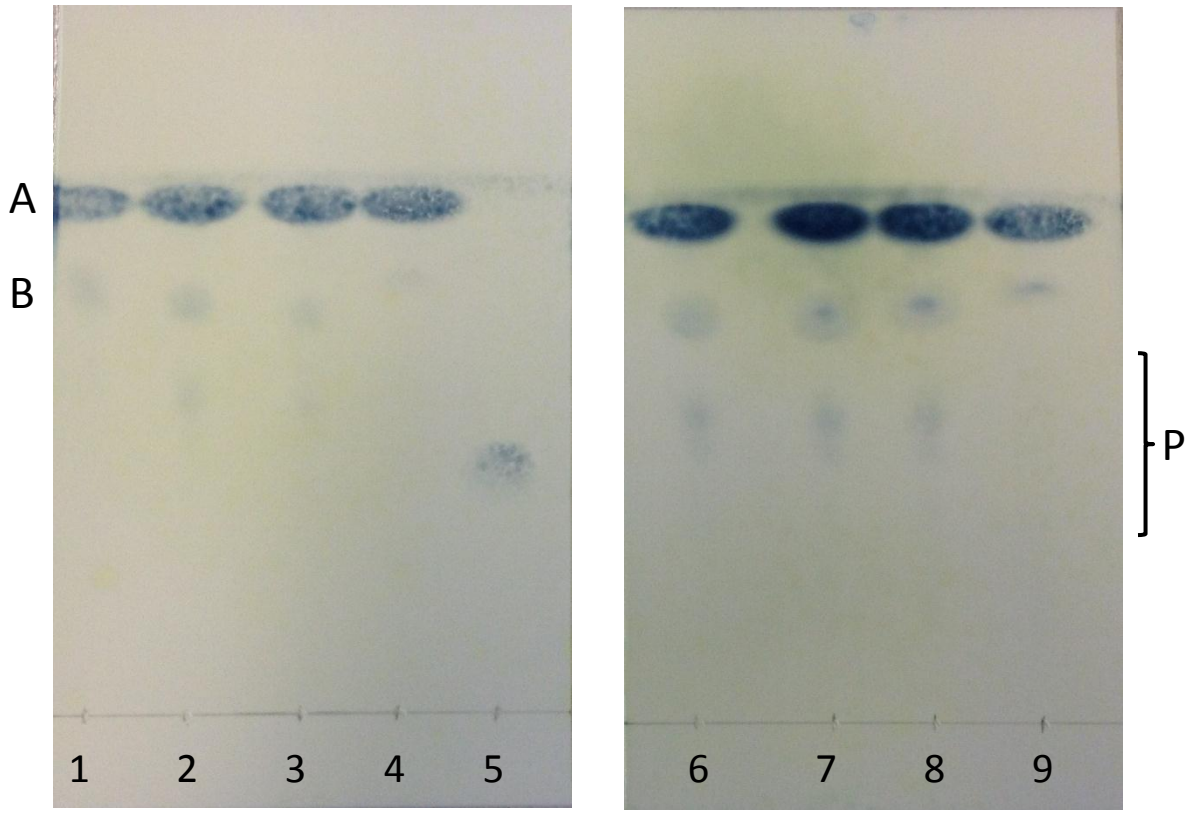


Figure 29: HPTLC of different enzymatic reactions. Lane 1: Standard solution containing methyl oleate, IS_1 (cis-11,14,17-Eicosatrienoic acid methyl ester) and IS_2 (methyl linolenate); Lane 2: Reaction solution containing methyl oleate, Per14, 10 mM t-BHP and IS_2; Lane 3: reaction solution without t-BHP (negative control 1); Lane 4: reaction solution without enzyme (Per14) and 400 mM t-BHP (negative control 2); Lane 5: 9,10-epoxyoctadecanoic acid methyl ester; reaction solutions after 24 hours of reaction containing 50 mM (Lane 6), 100 mM (Lane 7), 200 mM (Lane 8) and 400 mM t-BHP (Lane 9) ; A…migration distance for the substrate; B…migration distance for IS_2; P… products; (Method 1)

In Figure 29 it is clearly visible that the conversion rate increases with increasing peroxide concentration. With increasing concentration the spot intensities of the products also increase. The exception is lane 9 because there the intensity is lower compared to the other reactions. It seems that a concentration of 400 mM is too high having an aversive effect on the reaction. After GCMS analysis it became clear that this lower intensity is not a result of a lower conversion rate but a result of still inhomogeneous staining of the HPTLC plate (discussed below). The need for more accurate GCMS analysis became very clear at this point.

In Figure 29, lane five, the derivatized epoxide was applied in order to see if this compound is a product of the enzymatic reaction. It was expected that the methyl oleate is peroxidized at the double bond resulting in 9,10-epoxyoctadecanoic acid methyl ester. HPTLC results suggest that this is not the case and that other products are formed.

Lane three and four represent the negative controls containing either no peroxide (lane three) or no peroxidase (lane four) respectively. No reaction products are observed for the enzyme free solution and only hardly visible spots are present for the peroxide free solution suggesting at least some conversion reaction.

The markings A and B show the migration distance of the substrate (methyl oleate, A) and the internal standard IS_2 (methyl linolenate, B). Because all reactions contain these two esters the spots are visible in all reactions.

In addition to the HPTLC analysis also GCMS analysis was carried out. Therefore 90 µl of the n-hexane extracts were mixed with 10 µl of IS_1 giving a final concentration of 0.65 mM (similar to the substrate). Using already esters for the conversion reaction makes the derivatization step obsolete for these analyses. The temperature program mentioned in chapter 2.3.2 (Table 4, Table 6 and Figure 16) was used for separation. In Figure 30 the interesting parts of the chromatograms are shown. The interesting peaks are obviously of low intensity. The substrate peaks and the peaks of the internal standards are labeled (S…substrate).

Details on the results referring to the internal standards are discussed later in chapter 3.2.5. Based on the comparison of the results for the reactions using different t-BHP concentrations with the negative control, containing no enzyme (upper panel in Figure 30), the following statement can be made: The enzymatic reaction has the best conversion rate if 400 mM t-BHP are used. This assumption is especially taken from the fact that the unresolved peak between 22.0 and 23.0 min increases in intensity with increasing peroxide concentrations. Also other peaks eluting earlier from the column (e.g. 9.04, 9.70, 11.50 min) are increasing in intensity and peak area with increasing t-BHP concentration.

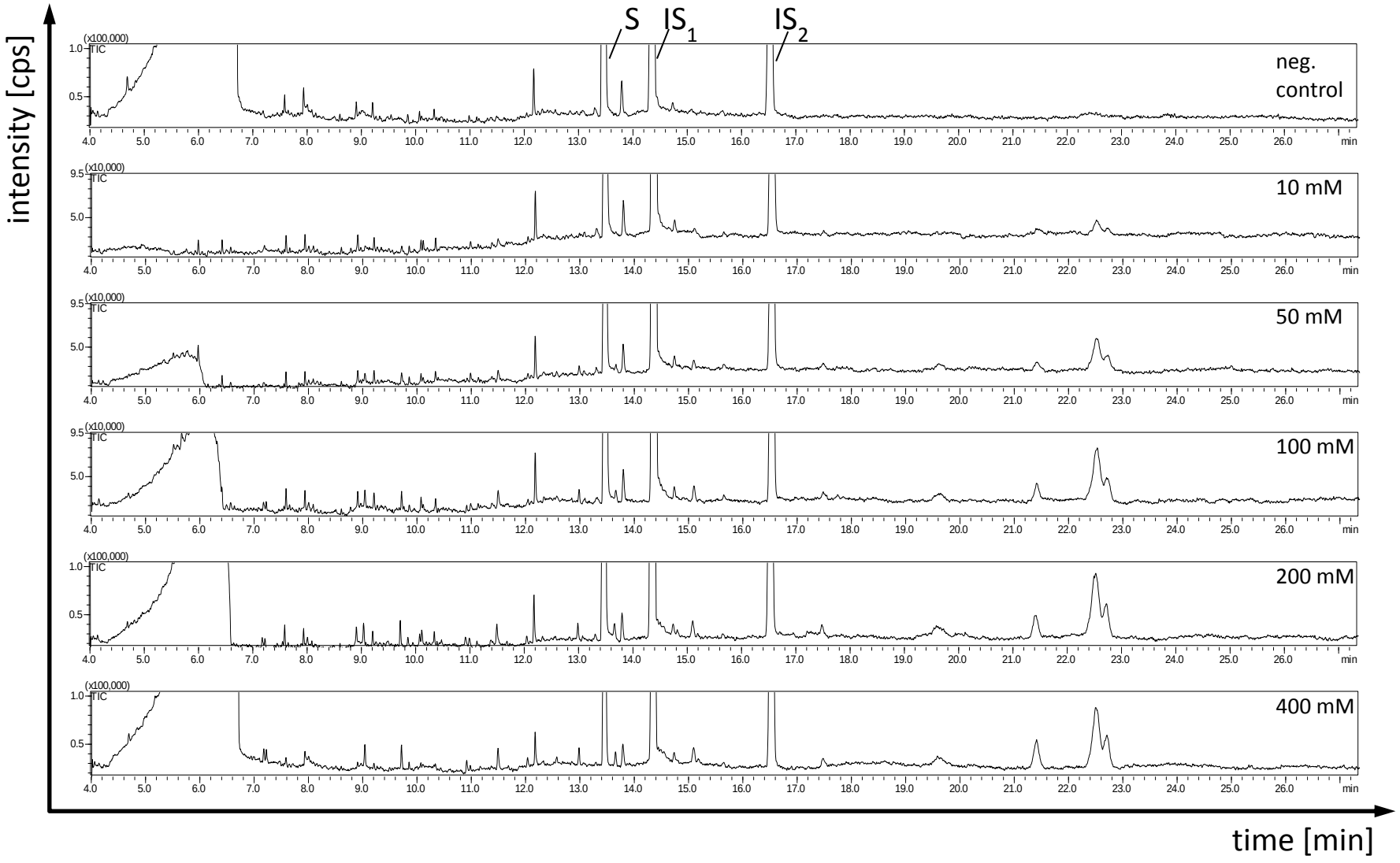


Figure 30: Chromatograms of enzymatic reactions performed with different peroxide concentrations. S…substrate (methyl oleate); IS_1 and IS_2…internal standards (1…cis-11,14,17-Eicosatrienoic acid methyl ester, 2…Methyl linolenate);

Furthermore it was found that a peroxide concentration of 1.2 M seems to be too high. Comparing this high concentration level with the reaction containing only 400 mM peroxide showed no clearly defined spots on the HPTLC plate. In Figure 31 the area of the methyl oleate is especially marked and it is clearly visible that the spot in the right lane (1.2 M t-BHP) is more intense than the one on the left (400 mM t-BHP).

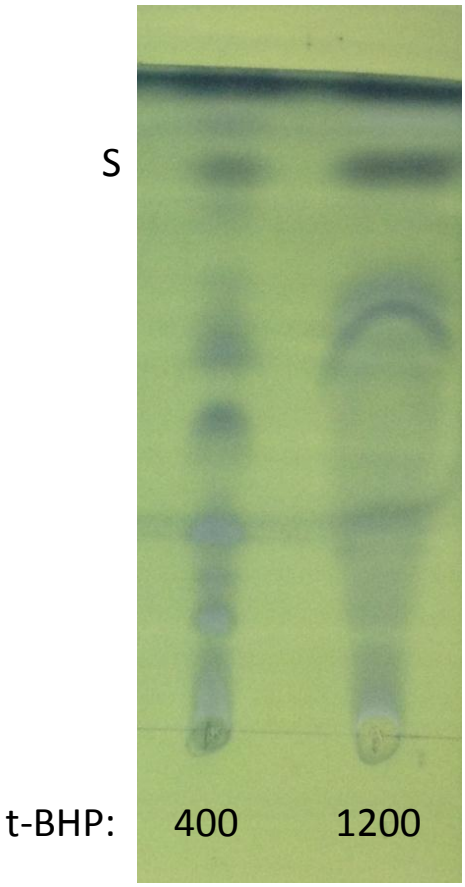


Figure 31: TLC of two enzymatic reactions. The left was performed with 400 mM t-BHP whereas the right was performed with 1200 mM; S…substrate (methyl oleate) (Method 2)

3.2.4. Use of different peroxidases

As mentioned in the introduction more than 10 selected recombinant heme-peroxidases are produced by our collaborator. Several of these peroxidases were used for the enzymatic reactions with a special focus on those having the highest activity according to the collaborators enzyme assays (details not shown). The enzymatic reactions were performed with three different peroxidases (Per1, Per14 and Per15) with a concentration of 1.2 µM. The other reaction conditions were kept constant for all reactions.

Reaction conditions:

- substrate: 5 mM methyl oleate
- peroxidase: 1.2 µM
- t-BHP: 400 mM
- 20 % (v/v) acetone
- buffer: 50 mM Tris HCl pH7.5
- reaction volume: 100 µl
- 24 h at 25°C and 650 RPM

The following peroxidases were used: Per1, Per14 and Per15

After adding IS_2 to have a final concentration of 5 mM (the same as the substrate) the performed reactions were extracted with 100 µl n-hexane and reaction products were separated by TLC (Figure 32).

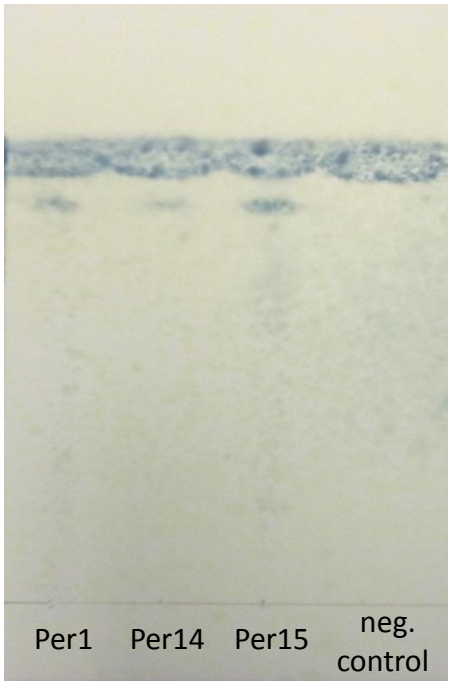


Figure 32: Three exemplary reactions with three different enzymes separated by HPTLC. For each sample 1 µL extraction solution was applied 15 times. (Method 1)

In Figure 32 only the substrate and again the internal standard are visible. On the rest of the plate only in lane three additional spots seem to be present. However it is not possible to make statements about the reactions efficiency. As mentioned in chapter 3.2.3 the quality of the TLC separation is good however staining efficiency and reproducibility is very low for Method 1.

In order to evaluate the conversion rate of the different peroxidases GCMS measurements were carried out. For this 10 µl of the injection standard (final concentration 0.65 mM) were added to 90 µl sample and the solution was injected directly into the GCMS. The chromatograms of the extracts from the negative control (without peroxidase) and the three reactions are shown in Figure 33.

Compared to the negative control the time range from 17.0 to 23.0 min of the enzymatic reaction with Per14 (also marked in the chromatogram with a red box) is remarkable. There are several peaks, which are not visible in the negative control. These peaks indicate that there was indeed conversion of the substrate to yet unknown products. The same was observed for signals detected between 7 and 12 min. After these results it can be said that Per14 converts the substrate methyl oleate very well leading to several products.

In addition to that a comparison of the conversion performance of the different peroxidases used makes it clear that the conversion rate is much higher for Per14 than for Per1 or Per15. Although it is indeed possible to detect almost all peaks in all three enzymatic reactions, the intensities of the signals differ and are much higher for Per14. Some signals cannot be found for Per15 (i.e. signals at 17.4 or 19.5 min; marked in the chromatogram with two blue boxes).

Detailed statements about the quantification and the used internal standards are made in the next section (3.2.5).

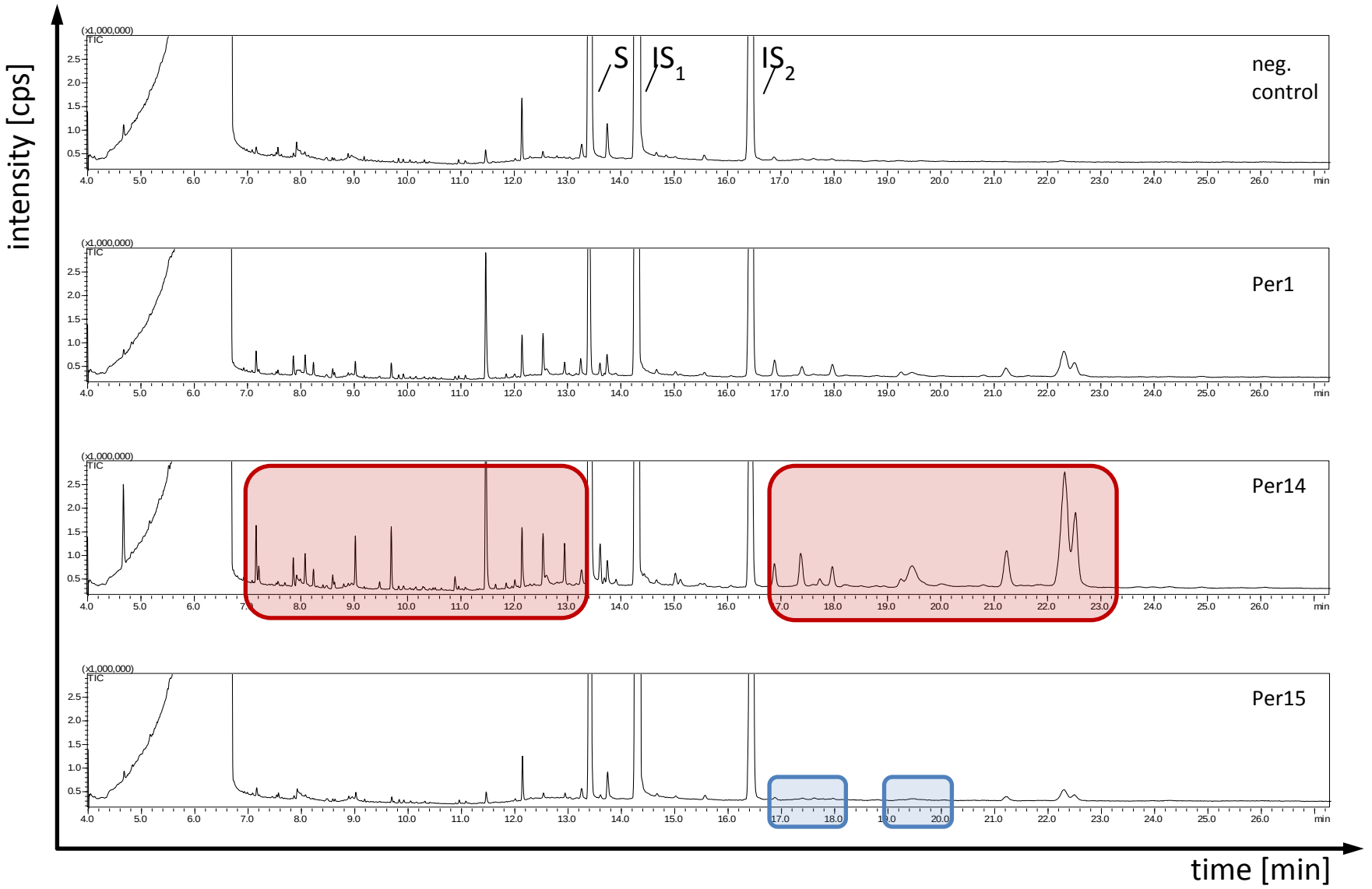


Figure 33: GCMS chromatogram of the extracts taken from enzymatic reactions performed with Per1, Per14 and Per15 and no peroxidase as a negative control. S…substrate (methyl oleate); IS_1 and IS_2…internal standards;

3.2.5. Quantification – internal standards

In order to semi-quantify the final products but also to quantify the conversion rate for the substrate, internal standards were employed to allow a comparison of the performed enzymatic reactions. Two FAMEs, namely 11,14,17-eicosatrienoic acid methyl ester and methyl linolenate (Figure 39 and Figure 40), were selected because of their similarity to the studied substrate (methyl oleate). The detailed requirements and demands were already discussed in chapter 1.4.2.

11,14,17-eicosatrienoic acid methyl ester was used as a standard correcting eventual differences between several GCMS injections, taking therefore the instrumental variation into account. Methyl linolenate was used to correct sample preparations variations and to correct the results with respect to incomplete extraction from the aqueous reaction solution. This standard therefore takes the extraction efficiency of n-hexane for fatty acids from the enzymatic reaction into consideration. Figure 34 represents a chromatogram of an n-hexane mixture of the studied substrate, methyl oleate, and the two internal standards. Signals are not overlapping with the analyte (i.e. the substrate) which is essential for this study. In the course of the project it was found that the internal standards also do not overlap with products originated from the performed enzymatic reactions.

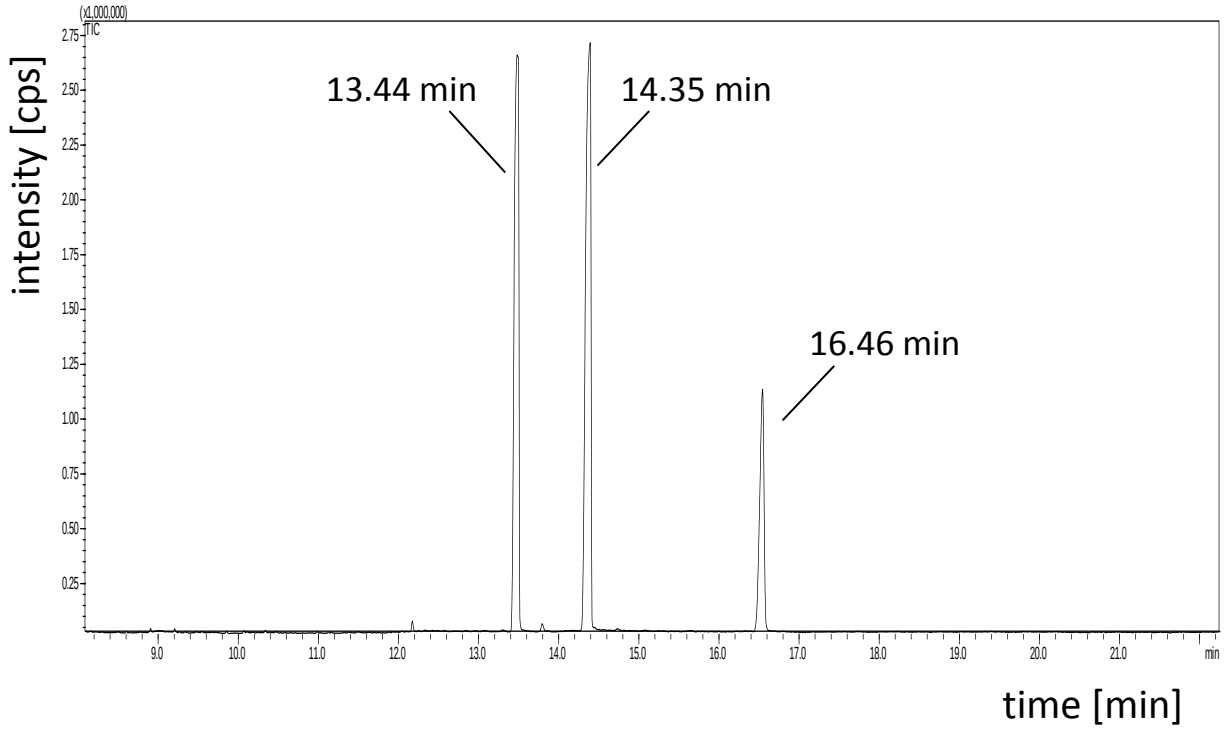


Figure 34: Excerpt of a GCMS chromatogram of a n-hexane solution containing 5 mM the substrate methyl oleate (13.44 min), the internal standard methyl linolenate (14.35 min) correcting sample preparation inaccuracies and the second internal standard 11,14,17-eicosatrienoic acid methyl ester (16.46 min) correcting the instrumental variations.

The following equation was used to consider extraction losses and injection differences:

$$area_{sample}(target) = \frac{area_{ex}(target) * area_{sample}(actual) * area_{inj}(target)}{area_{ex}(actual) * area_{inj}(actual)} \quad \textbf{Equation 1}$$

$area_{sample}$ (target)…correct peak area of the analyzed sample
$area_{sample}$ (actual)…actual peak area of the analyzed sample
$area_{ex}$ (target)…theoretical peak area of IS_2
$area_{ex}$ (actual)…actual peak area of IS_2
$area_{inj}$ (target)…theoretical peak area of IS_1
$area_{inj}$ (actual)…actual peak area of IS_1

The theoretical peak area of IS_1 was obtained from a signal derived from a chromatogram gained after adding 10 µl of IS_1 (6.55 mM in n-hexane) to 90 µl n-hexane whereas the theoretical peak area of IS_2 was obtained from a signal derived from a chromatogram gained after adding 10 µl of IS_2 (55 mM in n-hexane) to 100 µl n-hexane.

All standard addition steps were performed with microsyringes to obtain exact volumes.

The following enzymatic reactions were carried out for relative quantification:

Reaction setup one (reaction volume: 200 µl):

- substrate: 5 mM methyl oleate
- peroxidase: 11.7 µM Per14
- t-BHP: 10, 50, 100, 200 and 400 mM (five reactions)
- 20 % (v/v) acetone
- buffer: 50 mM Tris HCl pH7.5
- 24 h at 25°C and 650 RPM

Reaction setup two (reaction volume: 100 µl):

- substrate: 5 mM methyl oleate
- peroxidase: 11.7 µM Per1, Per14 & Per15 (three reactions)
- t-BHP: 400 mM
- 20 % (v/v) acetone
- buffer: 50 mM Tris HCl pH7.5
- 24 h at 25°C and 650 RPM

These reactions are the same as already mentioned in chapter 3.2.3 (Optimization of t-BHP concentration) and 3.2.4 (Use of different peroxidases). In all cases negative controls were performed, i.e. reactions without peroxidases. In Figure 35 and Figure 36 the results are shown. These are the same chromatograms as in Figure 30 and Figure 33 but not zoomed in to allow the comparison of the substrate peak with the two peaks of the internal standards.

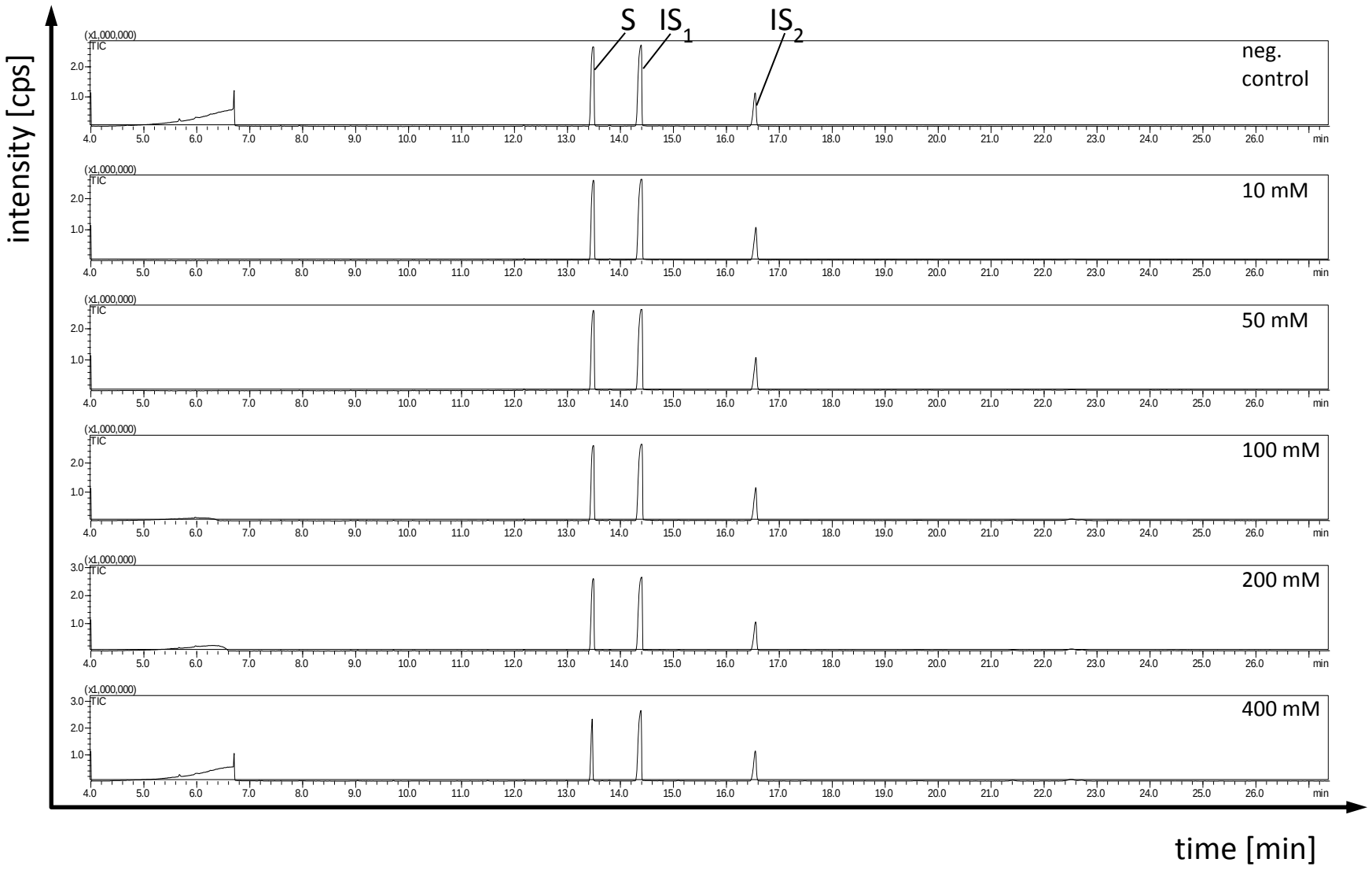


Figure 35: GCMS chromatograms of enzymatic reactions using Per14 as peroxidase but different concentrations of peroxide (10, 50, 100, 200 and 400 mM t-BHP). S…substrate (methyl oleate); IS_1 and IS_2…internal standards (1…cis-11,14,17-Eicosatrienoic acid methyl ester, 2…Methyl linolenate);

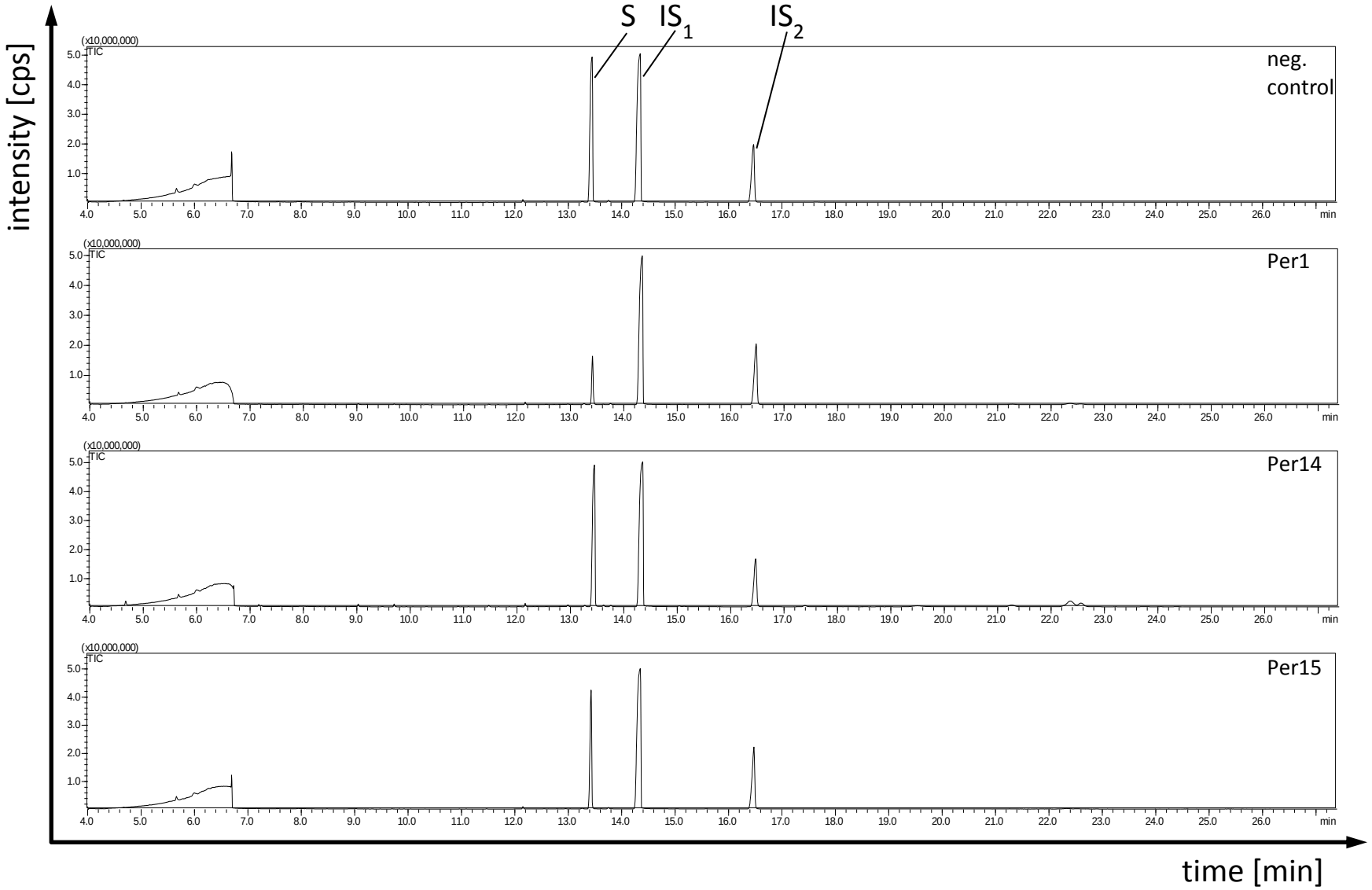


Figure 36: GCMS chromatograms of enzymatic reactions using different peroxidases (Per1, Per14 and Per15). S…substrate (methyl oleate); IS_1 and IS_2…internal standards (1…cis-11,14,17-Eicosatrienoic acid methyl ester, 2…Methyl linolenate);

In Figure 35 and Figure 36 it is clearly demonstrated that the substrate peak elutes at 13.4 min and that the intensity of this peak decreases in comparison with the corresponding peak in the negative control, which contains no enzyme. This decrease in intensity and most importantly peak area corresponds to the successful conversion of the methyl oleate to its yet unknown products. In Table 7 and Table 8 the peak areas of all relevant peaks (substrate, IS_1, IS_2) are given.

Table 7: Peak areas of the substrate peak and the peaks of the two internal standards IS_1 and IS_2 from reactions performed with different peroxide concentrations and Per14 as enzyme

reaction	peak area [a.u.]		
	substrate (13.4 min)	IS_1 (14.3 min)	IS_2 (16.5 min)
negative control	1.03E+07	1.21E+07	4.03E+06
10 mM t-BHP	9.49E+06	1.26E+07	3.76E+06
50 mM t-BHP	8.07E+06	1.18E+07	3.89E+06
100 mM t-BHP	9.48E+06	1.31E+07	4.03E+06
200 mM t-BHP	9.09E+06	1.25E+07	3.82E+06
400 mM t-BHP	5.32E+06	1.08E+07	3.97E+06

Table 8: Peak areas of the substrate peak and the peaks of the two internal standards IS_1 and IS_2 from reactions of reaction performed with different enzymes and 400 mM t-BHP

reaction	peak area [a.u.]		
	substrate (13.4 min)	IS_1 (14.3 min)	IS_2 (16.5 min)
negative control	1.64E+08	2.37E+08	7.40E+07
Per1	3.09E+07	2.18E+08	7.55E+07
Per14	1.32E+08	2.38E+08	6.16E+07
Per15	9.61E+07	2.37E+08	7.73E+07

With the values in Table 7 and Table 8, Equation 1 and the theoretical peak area of IS_2 the corrected peak areas and furthermore the conversion rates for the different reactions were calculated and compared to the negative control (Table 9 and Table 10).

Table 9: Corrected peak areas (based on Equation 1) of the substrate methyl oleate and calculated conversion rates

reaction	corrected peak area [a.u.]	conversion rate [%]
negative control	1.76E+07	-
10 mM t-BHP	1.67 E+07	5.38
50 mM t-BHP	1.46 E+07	17.01
100 mM t-BHP	1.50 E+07	14.95
200 mM t-BHP	1.59 E+07	9.92
400 mM t-BHP	1.04 E+06	41.14

Table 10: Corrected peak areas (based on Equation 1) of the substrate methyl oleate and conversion rates

reaction	corrected peak area [a.u.]	conversion rate [%]
negative control	8.40E+05	-
Per1	1.69E+05	79.92
Per14	8.07E+05	3.93
Per15	4.71E+05	43.95

The conversion rates in Table 9 show that at 400 mM t-BHP 41 % of the substrate is converted. It is very interesting that there is a higher conversion rate when using 50 mM t-BHP instead of 10, 100 or 200 mM. If this increase is biologically significant cannot be confirmed as these experiments were performed only once because of time and cost efficiency. It can also not be stated that the increase from approx. 15 % (10 – 200 mM) to more than 40 % (400 mM) is reproducible or maybe a false positive result.

However taking a closer look at the chromatograms in Figure 30 it can be confirmed that also the analytes eluting at 22.5 min show peak with higher intensities. These findings were true for also other, maybe not so prominent peaks. Possibilities for false positive results (i.e. increase in conversion rate) can be a less effective extraction for the substrate.

Using different peroxidases the conversion rates seem to be best for Per1 followed by Per15 and Per14. Yet it has to be mentioned that several rather small peaks were detected for Per14 which have signal-to-noise ratios smaller than two or are not present at all in the other reactions. So again no clear conclusion could be drawn for the correlation between conversion rates and the different enzymes.

After these experiments it was not possible to give a statement about the efficiency of the conversion. More experiments are necessary to assess the reason for this finding. Sample pretreatment, the addition of the internal standard and the extraction process have to be investigated in detail. Because of the limited time spent on this topic of the project quantification was not the focus of subsequent work.

3.2.6. Identification of unknown reaction products

The obtained gas chromatographic peaks from the different enzymatic reactions were tried to be identified using the NIST08 database as already mentioned in chapter 1.5.2. In the case of identification of possible products, reaction pathways for the enzymatic reaction were considered.

The most interesting product identified was 9,10-epoxyoctadecanoic acid or 9,10-epoxyoctadecanoic acid methyl ester and its cleavage products. This product was expected to be identified. Besides this, several other peaks could be identified in all chromatograms, some of them only after derivatization. In Table 11 the identified compounds, their molecular weights and formulas are listed. The molecular structures, MS spectra (database vs. unknown) of the products and the substrate are shown in Figure 41 to Figure 52 in chapter 6.3. Furthermore Figure 38 shows two chromatograms, one before and one after derivatization, where all identified peaks are marked with numbers. These numbers are sorted by their retention time in Table 11. In addition to that, derivatization reagent related peaks are marked in the chromatogram with a red arrow. These peaks mainly come from Triton X-100 and the peroxide.

Reaction conditions:

- substrate: 23.69 mM methyl oleate
- peroxidase: 55.5 µM Per14
- t-BHP: 378.6 mM
- 0.05 % Triton X-100
- 0.25 % gum arabic
- buffer: 100 mM phosphate pH 7.0
- reaction volume: 211.1 µl
- 24 h at 25°C and 650 RPM

In this experimental setup the reaction conditions contained high amounts of peroxide to guarantee good enzymatic conversion and were further improved by adding Triton X-100 and gum arabic to get a better emulsification of the hydrophobic methyl oleate in the aqueous buffer solution. It was the main goal of this study to identify as many reaction products as possible.

In Figure 37 a HPTLC plate with separated reaction products is shown. The dark spots migrating almost with the solvent front are again the substrate (methyl oleate). Lane F is the negative control for the enzymatic reaction employing detergents. Significant background signals are observed. But it can also be seen that the addition of detergents leads to a higher conversion rate for the substrate when comparing lane A with lanes B and C. This fact was also observed after GCMS analysis where the intensities of the signals resulting from the products were much higher in the reactions with the detergents than in the reaction without them.

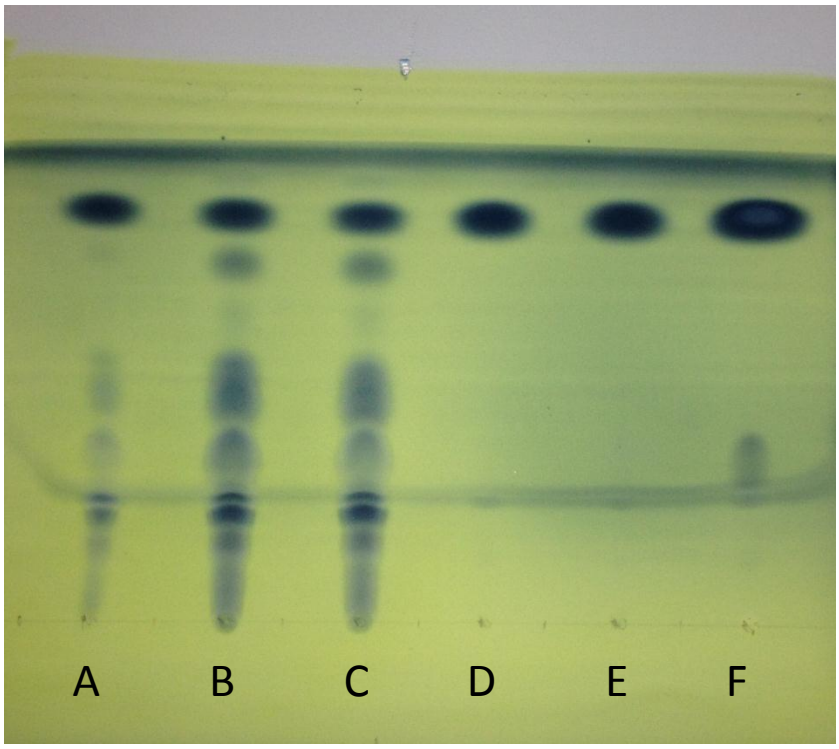


Figure 37: HPTLC of the performed enzymatic reactions. Lane A presents reaction products from enzymatic reactions without detergents, lane B with gum arabic and lane C with gum arabic and Triton X-100. Lane D is the negative control for Lane A, Lane E for lane B and lane F for lane C. (Method 2)

Table 11: The table shows the search results after submitting mass spectra of detected compounds to the NIST08 database. Additionally to search result similarity results describing the statistical significance of identification are listed beside chromatographic retention times, molecular weights and sum formulas. Furthermore, information on the necessity of derivatization for detection is listed.

peak number	retention time [min]	similarity score [%]	compound name	molecular weight [g/mol]	formula	derivatization necessary
1	7.11	97	Octanoic acid, methyl ester	158	$C_9H_{18}O_2$	no
2	7.16	95	Nonanal	142	$C_9H_{18}O$	no
3	7.88	95	Nonanoic acid, methyl ester	172	$C_{10}H_{20}O_2$	yes
4	*8.84*	*97*	*Dimethyl Sulfoxide (DMSO)*	*78*	C_2H_6OS	*yes*
5	8.97	94	2-Decenal	154	$C_{10}H_{18}O$	no
		89	2-Nonenal	140	$C_9H_{16}O$	
6	9.64	87	2-Decenal	154	$C_{10}H_{18}O$	no
		87	2-Nonenal	140	$C_9H_{16}O$	
7	10.67	92	Heptanedioic acid, dimethyl ester	188	$C_9H_{16}O_4$	yes
8	10.84	92	Methyl 8-oxooctanoate	172	$C_9H_{16}O_3$	no
9	11.25	96	Octanedioic acid, dimethyl ester	202	$C_{10}H_{18}O_4$	yes
10	11.41	82	9-oxo-nonanoic acid, methyl ester	186	$C_{10}H_{18}O_3$	no
11	11.79	95	Nonanedioic acid, dimethyl ester	216	$C_{11}H_{20}O_4$	yes
12	11.94	95	Nonanoic acid	158	$C_9H_{18}O_2$	no
13	12.88	90	Methyl 10-oxo-8decenoate	198	$C_{11}H_{18}O_3$	no
14	13.32	96	9-Octadecenoic acid, methyl ester	296	$C_{19}H_{36}O_2$	no
15	17.14	93	9,10-epoxyoctadecanoic acid methyl ester	312	$C_{19}H_{36}O_3$	no
16	17.49	93	9,10-epoxyoctadecanoic acid methyl ester	312	$C_{19}H_{36}O_3$	no

Three compounds in Table 11, namely 2-decenal, 2-nonenal and 9,10-epoxyoctadecanoic acid methyl ester, can exist in cis and trans configurations and it is not possible to distinguish between these two isomers by MS. Because of that two different retention times are listed for each compound.

2-Decenal and 2-nonenal are both products very likely to be formed during enzymatic treatment by degradation of the formed epoxide product or the substrate. As no pure standard was available to determine retention times for these two products it was not possible to decide which one was actually formed, especially as the similarity scores are quite good for both compounds.

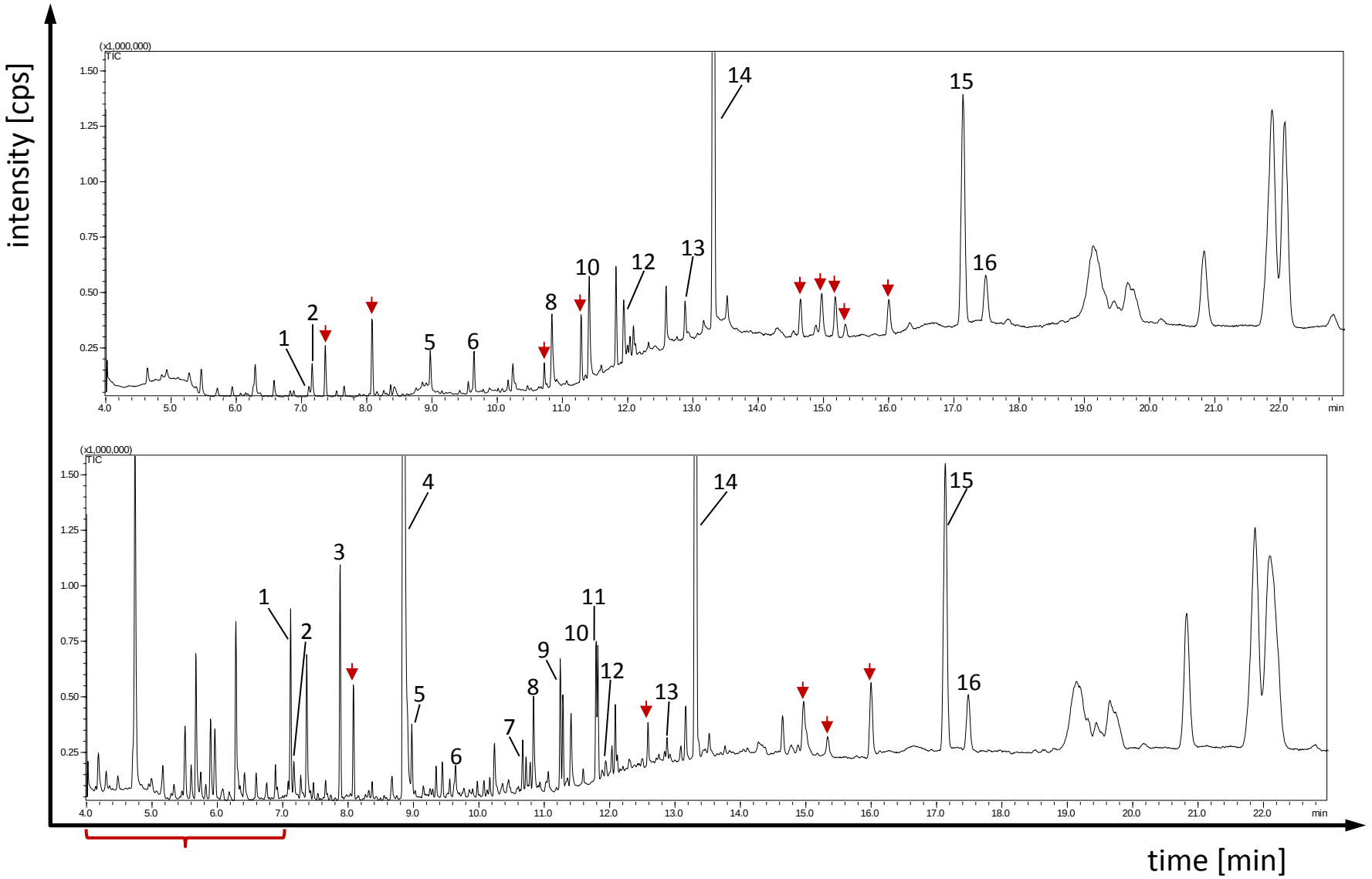


Figure 38: GCMS chromatograms of the very same enzymatic reaction with (upper panel) and without (lower panel) derivatization. The peaks are marked with numbers, and the identified components are listed in Table 11. The red bracket and arrows mark reagent related peaks, mainly from Triton X-100 and the peroxide. In addition to that the first minutes (4.0 to 6.9 min) also contain only reagent related peaks.

Table 11 shows several interesting products formed in the enzymatic reactions. DMSO is only a side product of the derivatization, which originates from dimethyl sulfide after getting oxidized by the present peroxide. Dimethyl sulfide is the product of the esterification where one of the three methyl groups of TMSH binds to the acids and dimethyl sulfide remains. The formation of DMSO is not really critical for the chromatographic separation of the other products, because the peak does not overlap with other signals. Figure 38 shows that the peak of DMSO (peak 4) is coeluting with 2-decenal/2-nonenal (peak 5) at 8.97 min. So by similarity search peak 5 could not be identified. Because the peak is also present in samples which were not derivatized it was assumed that the peak at 8.97 represents 2-decenal or 2-nonenal respectively.

Table 11 also points out that four peaks are not present in underivatized samples. One of them is nonanoic acid methyl ester. Derivatization of the respective acid forms the methyl ester. However the fatty acid form is present in both chromatograms at 11.94 min. Yet, after derivatization this peak decreases by a factor of 2.2 and the methyl ester becomes detectable (decreased retention time because of increased volatility). The fact that there are still traces of nonanoic acid left in the sample points to the fact that the derivatization is not as complete as supposed. Compounds 3, 7, 9 and 11 are only visible after derivatization and were all identified as dimethyl esters. The dicarboxylic forms are not volatile enough to be separate by GCMS and can therefore not be detected. These dicarboxylic acids are very interesting and have a potential use in white biotechnology.

At 17.14 and 17.49 min, 9,10-epoxyoctadecanoic acid methyl ester was identified which was the most expected product. The identity of the epoxide was furthermore confirmed by comparison to GCMS chromatograms of the available standard. The chromatogram is presented in Figure 24 showing a retention time of 17.15 min for the methyl ester. Potential degradation of this epoxide in the reaction solution can lead to the detection of nonanal and 9-oxo-nonanoic acid methyl ester, both potential products were detected. Besides many other possibilities of degradation products, 9-oxo-nonanoic acid methyl ester is again a potential product arising high interest in white biotechnology. One promising application of bifunctional derivatives is the use as building blocks for polymers [26].

Peaks not marked in Figure 38 could not be identified using the NIST08 database. The similarity scores for the mass spectra were lower than 70 % pointing out that the database entry differs significantly from the detected mass spectrum. Substances eluting between 18.5 and 22.5 min could also not be identified although the peak intensities are quite high and the underlying compounds seem to be prominent products of the enzymatic reactions.

Am Ende wird alles gut. Und wenn es nicht gut wird, ist es noch nicht das Ende.

Oscar Wilde (1854-1900)

4. Conclusion

Peroxidases have a substantial potential for industrial use as biocatalysts. They can be used for several enzymatic reactions avoiding the use of chemicals which have several disadvantages like side reactions, harsh reaction conditions or waste management issues. As a result it is possible to synthetize chemoselective, regioselective and enantioselective organic compounds in a more convenient way.

The analytical strategy developed in this thesis allowed investigating the reaction conditions of the conversion of methyl oleate with several heme-peroxidases produced by Eucodis Bioscience. It was possible to use TLC as a first indicator whether the conversion of the substrate took place or not. Furthermore it could be shown that the amount of t-BHP, the peroxide, is a crucial parameter for the enzymatic reaction. It is stabilizing the peroxidase itself but if the concentration is too high (1.2 M) the enzyme gets again inactivated [27], as shown by decreasing conversion rates. It seems that 400 mM t-BHP is a suitable concentration to allow efficient conversion rates. Yet these experiments were carried out only once and have to be repeated for confirmation.

It seems that the fermentation or the genetic design of the enzymes is very crucial. This conclusion was drawn from the fact that different peroxidases lead to different conversion rates and the formation of different amounts of reaction products. This finding has again to be confirmed.

Using methyl oleate (23.69 mM), t-BHP (378.6 mM), Per14 (55.5 mM), the addition of additives and 24 hours of mixing the solution at 650 RPM on the thermomixer, allowed the identification of 9,10-epoxyoxtadecanoic acid methyl ester as one reaction product which was expected already at the beginning. 9,10-Epoxyoxtadecanoic acid methyl ester is the most likely oxidation product of methyl oleate. Furthermore it was found that this reaction product degrades into several further compounds. After sample derivatization it was possible to detect dimethyl esters derived from dicarboxylic reaction products originating from the epoxide. These dimethyl esters can be used as raw material for the preparation of perfumes, polymers, adhesives or macrolide antibiotics. The majority of the production of dicarboxylic acids is still based on a variety of chemical conversions. However the production of long-chain dicarboxylic acids is accompanied with the formation of numerous unwanted by-products. Additionally, production costs increase enormously. Because of that the microbial approach compared to the chemical synthesis is very interesting and a promising field [26].

Finally in addition to the epoxidized methyl oleate and the dicarboxylic acids also several other products could be identified. The determination of the real reaction pathways for the products was not the scope of this thesis. However the identified aldehydes, methyl esters and their bifunctional counterparts have to be degradation products of the formed epoxide or the methyl oleate.

In summary it can be stated that the large number of different products shows that the reaction conditions should be further improved to allow the formation of specific products.

5. Outlook

Amongst the information gathered from the analysis, there are still unclear points which require further investigation. On the one hand there was not enough time to focus on the quantification of the enzymatic reactions. In order to make clearer statements about the conversion rates and the efficiency of the peroxidases it would be very important to quantify conversion rates.

There is also the possibility that some formed products could not be identified by GCMS. If there are products which are not volatile enough although the samples were derivatized, it is not possible to detect them. Other analytical techniques can help in that case. One possibility would be liquid chromatography-mass spectrometry (LCMS). With LCMS it is possible to avoid the loss of information about products which are not volatile or thermo stable enough or too polar for GCMS analysis.

Furthermore many different substrates exist for the conversion with the given peroxidases. During the outline of the project the decision was made to further focus on the epoxidation of fatty acid methyl esters, so it was not possible to investigate other substrates.

Finally also the fermentation of peroxidases can be further optimized to get even better and more efficient enzymes allowing higher conversion rates and the formation of larger quantities of products.

This work was a good starting point in the analysis of peroxidases and a lot of information could be collected, but since it is only a starting point there is still high potential for a better understanding of the behavior of these very interesting enzymes.

6. Appendix

6.1. Supelco 37 component FAME mix

In Table 12 all 37 components of the FAME mix are shown sorted by their retention times.

Table 12: List of the 37 components FAME mix sorted by retention times obtained from GCMS analysis

peak no.	ret. time [min]	compound name	formula	MW [g/mol]
1	2.94	Butanoic acid, methyl ester	$C_5H_{10}O_2$	102.13
2	4.95	Hexanoic acid, methyl ester	$C_7H_{14}O_2$	130.18
3	6.09	Octanoic acid, methyl ester	$C_9H_{18}O_2$	158.24
4	7.05	Decanoic acid, methyl ester	$C_{11}H_{22}O_2$	186.29
5	7.62	Undecanoic acid, methyl ester	$C_{12}H_{24}O_2$	200.32
6	8.38	Dodecanoic acid, methyl ester	$C_{13}H_{26}O_2$	214.34
7	9.36	Tridecanoic acid, methyl ester	$C_{14}H_{28}O_2$	228.37
8	10.67	Tetradecanoic acid, methyl ester	$C_{15}H_{30}O_2$	242.39
9	11.24	9-Tetradecenoic acid, methyl ester	$C_{15}H_{28}O_2$	240.38
10	12.29	Pentadecanoic acid, methyl ester	$C_{16}H_{32}O_2$	256.42
11	13.01	10-Pentadecenoic acid, methyl ester	$C_{16}H_{30}O_2$	254.41
12	14.35	Hexadecanoic acid, methyl ester	$C_{17}H_{34}O_2$	270.45
13	14.88	9-Hexadecenoic acid, methyl ester, (Z)-	$C_{17}H_{32}O_2$	268.43
14	16.67	Heptadecanoic acid, methyl ester	$C_{18}H_{36}O_2$	284.48
15	17.40	cis-10-Heptadecenoic acid, methyl ester	$C_{18}H_{34}O_2$	282.46
16	19.95	Octadecanoic acid, methyl ester	$C_{19}H_{38}O_2$	298.50
17	20.66	9-Octadecenoic acid (Z)-, methyl ester	$C_{19}H_{36}O_2$	296.49
18	20.74	9-Octadecenoic acid, methyl ester, (E)-	$C_{19}H_{36}O_2$	296.49
19	22.50	9,12-Octadecadienoic acid, methyl ester	$C_{19}H_{34}O_2$	294.47
20	22.79	9,12-Octadecadienoic acid (Z,Z)-, methyl ester	$C_{19}H_{34}O_2$	294.47
21	23.95	6,9,12-Octadecatrienoic acid, methyl ester	$C_{19}H_{32}O_2$	292.46
22	25.55	9,12,15-Octadecatrienoic acid, methyl ester, (Z,Z,Z)-	$C_{19}H_{32}O_2$	292.46
23	28.88	Eicosanoic acid, methyl ester	$C_{21}H_{42}O_2$	326.56
24	29.58	cis-11-Eicosenoic acid, methyl ester	$C_{21}H_{40}O_2$	324.54
25	31.66	cis-11,14-Eicosadienoic acid, methyl ester	$C_{21}H_{38}O_2$	322.53
26	32.79	Methyl 8,11,14-eicosatrienoate	$C_{21}H_{38}O_2$	320.51
27	33.24	Heneicosanoic acid, methyl ester	$C_{22}H_{44}O_2$	340.58
28	33.74	5,8,11,14-Eicosatetraenoic acid, methyl ester, (all-Z)-	$C_{21}H_{36}O_2$	318.49
29	34.53	11,14,17-eicosatrienoic acid, methyl ester	$C_{21}H_{36}O_2$	320.51
30	36.60	cis-5,8,11,14,17-Eicosapentaenoic acid, methyl ester	$C_{21}H_{32}O_2$	316.48
31	37.59	Docosanoic acid, methyl ester	$C_{23}H_{46}O_2$	354.61
32	38.25	13-Docosenoic acid, methyl ester, (Z)-	$C_{23}H_{44}O_2$	352.59
33	40.29	cis-13,16-Docasadienoic acid, methyl ester	$C_{23}H_{42}O_2$	350.58
34	41.67	Tricosanoic acid, methyl ester	$C_{24}H_{48}O_2$	368.64
35	45.85	Tetracosanoic acid, methyl ester	$C_{25}H_{50}O_2$	382.66
36	46.35	4,7,10,13,16,19-Docosahexaenoic acid, methyl ester, (all-Z)-	$C_{23}H_{34}O_2$	342.51
37	46.55	15-Tetracosenoic acid, methyl ester, (Z)-	$C_{25}H_{48}O_2$	380.65

6.2. Molecular structures of the internal standards

In the following figures the molecular structure of the used internal standards can be seen. In Figure 39 the molecular structure of 11,14,17-Eicosatrienoic acid methyl ester and in Figure 40 of methyl linolenate are visualized.

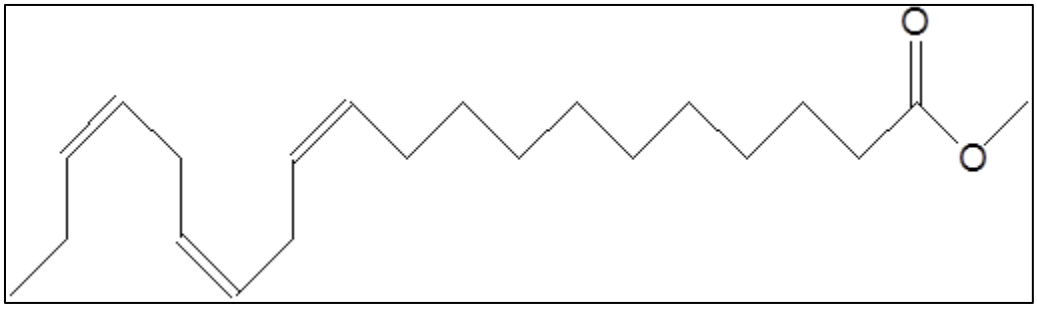


Figure 39: Molecular structure of 11,14,17-Eicosatrienoic acid methyl ester used as internal standard in order to correct injection differences

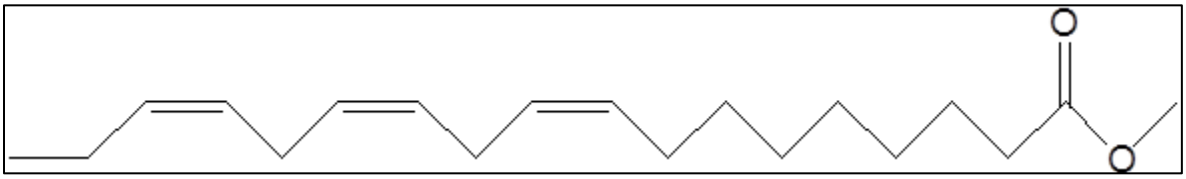


Figure 40: Molecular structure of methyl linolenate used as internal standard in order to correct the extraction sample preparation efficiency

6.3. MS spectra of the identified compounds

In the following figures the MS spectra of the identified compounds compared to the MS spectra of the database are shown. In addition to that the molecular structures also are shown.

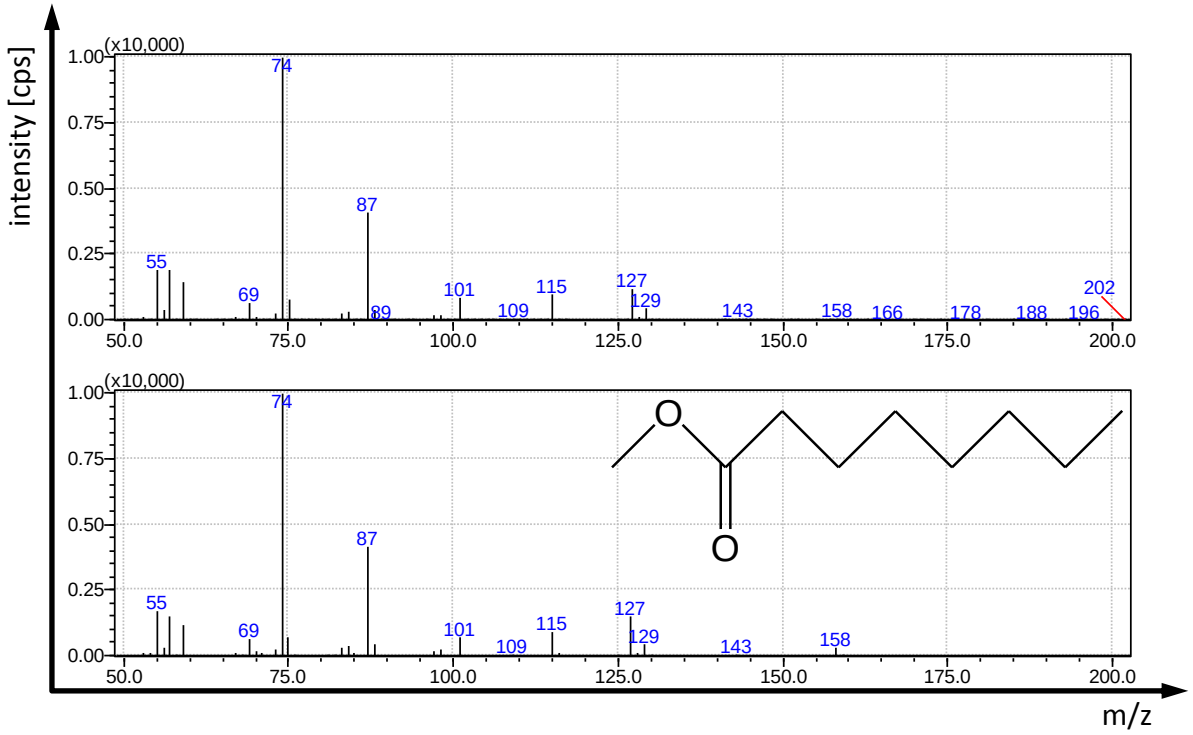


Figure 41: MS spectrum of octanoic acid methyl ester (7.11 min). The top spectrum is the spectrum of the sample and at the bottom the database spectrum is presented. In the database spectrum the molecular structure is shown.

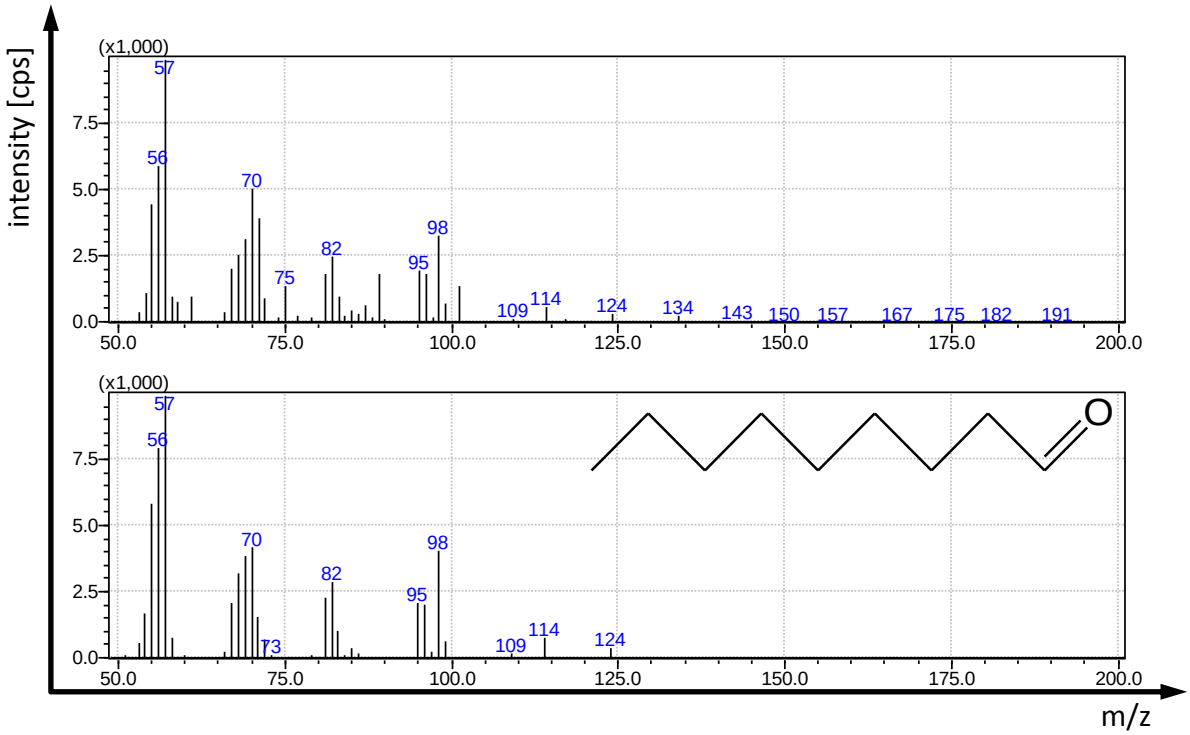


Figure 42: MS spectrum of nonanal (7.16 min). The top spectrum is the spectrum of the sample and at the bottom the database spectrum is presented. In the database spectrum the molecular structure is shown.

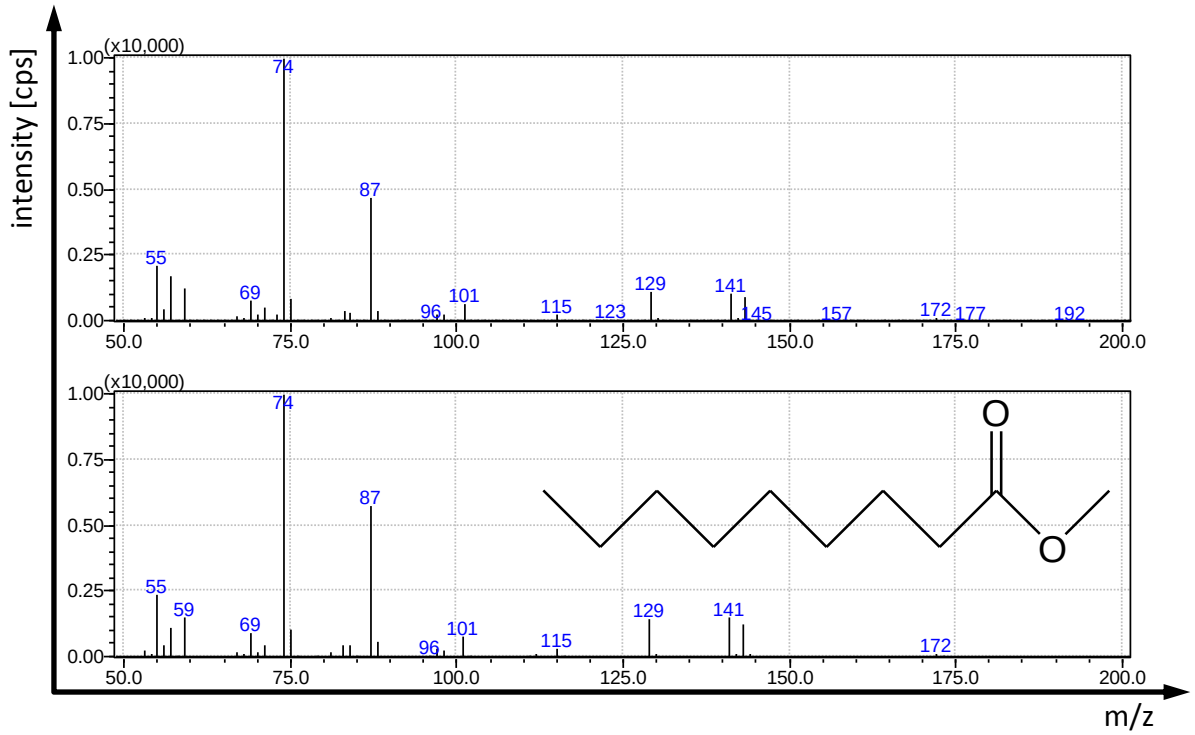


Figure 43: MS spectrum of nonanoic acid methyl ester (7.88 min). The top spectrum is the spectrum of the sample and at the bottom the database spectrum is presented. In the database spectrum the molecular structure is shown.

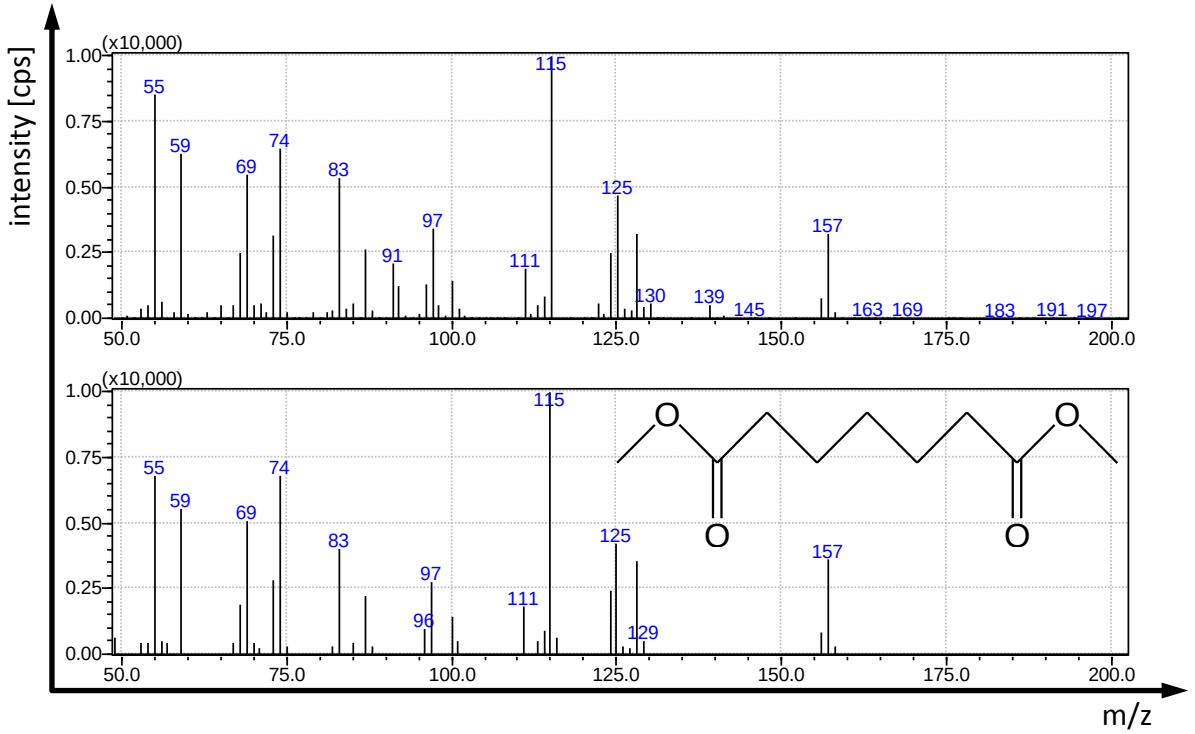


Figure 44: MS spectrum of heptanedioic acid dimethyl ester (10.67 min). The top spectrum is the spectrum of the sample and at the bottom the database spectrum is presented. In the database spectrum the molecular structure is shown.

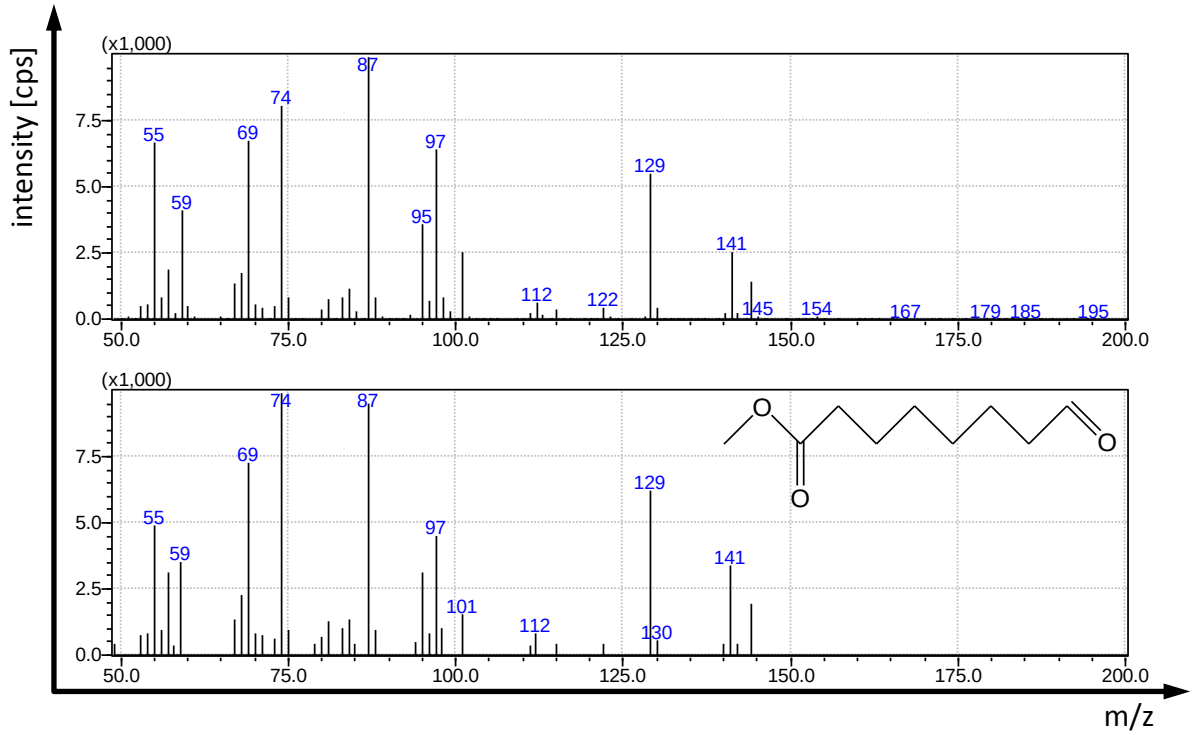


Figure 45: MS spectrum of methyl 8-oxooctanoate (10.84 min). The top spectrum is the spectrum of the sample and at the bottom the database spectrum is presented. In the database spectrum the molecular structure is shown.

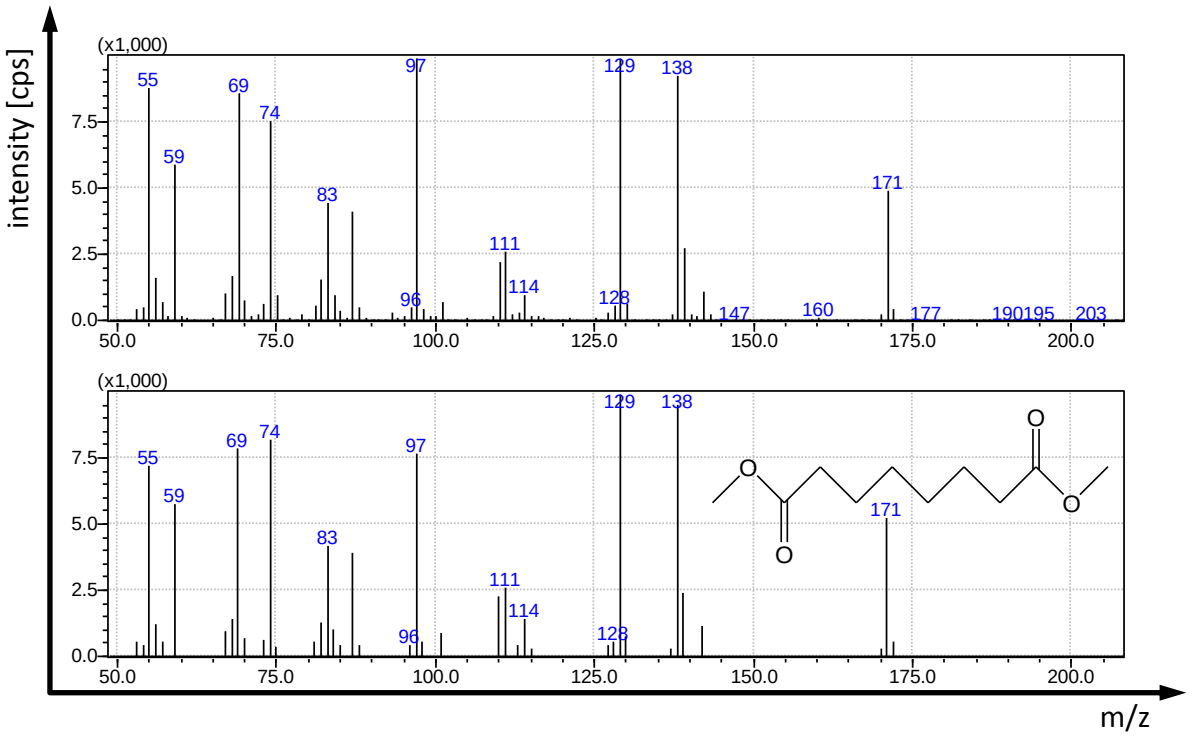


Figure 46: MS spectrum of octanedioic acid dimethyl ester (11.25 min). The top spectrum is the spectrum of the sample and at the bottom the database spectrum is presented. In the database spectrum the molecular structure is shown.

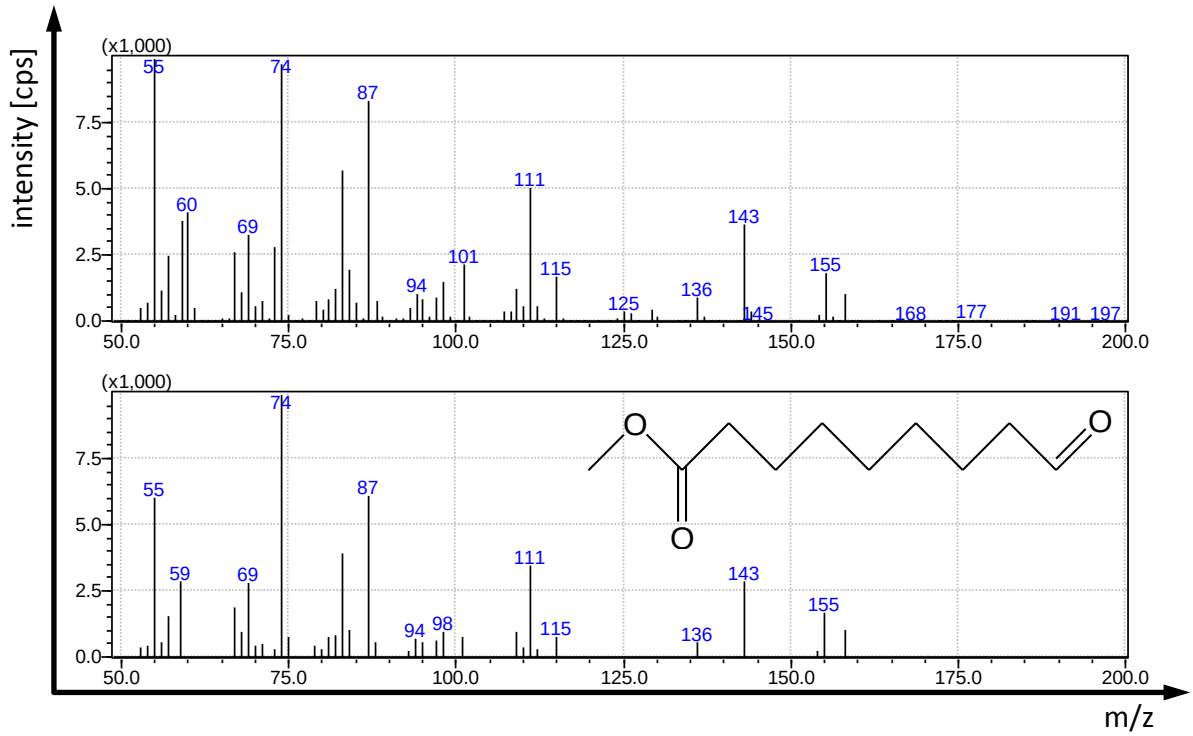


Figure 47: MS spectrum of 9-oxo-nonanoic acid methyl ester (11.41 min). The top spectrum is the spectrum of the sample and at the bottom the database spectrum is presented. In the database spectrum the molecular structure is shown.

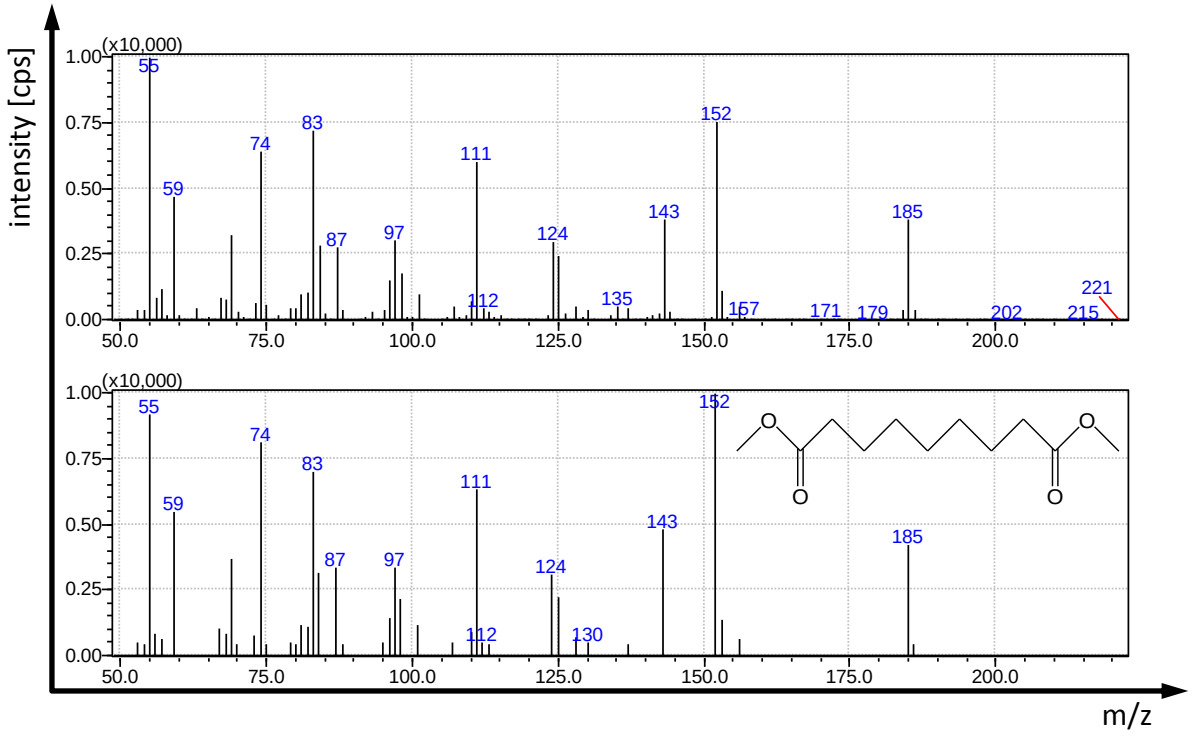


Figure 48: MS spectrum of nonanedioic acid dimethyl ester (11.79 min). The top spectrum is the spectrum of the sample and at the bottom the database spectrum is presented. In the database spectrum the molecular structure is shown.

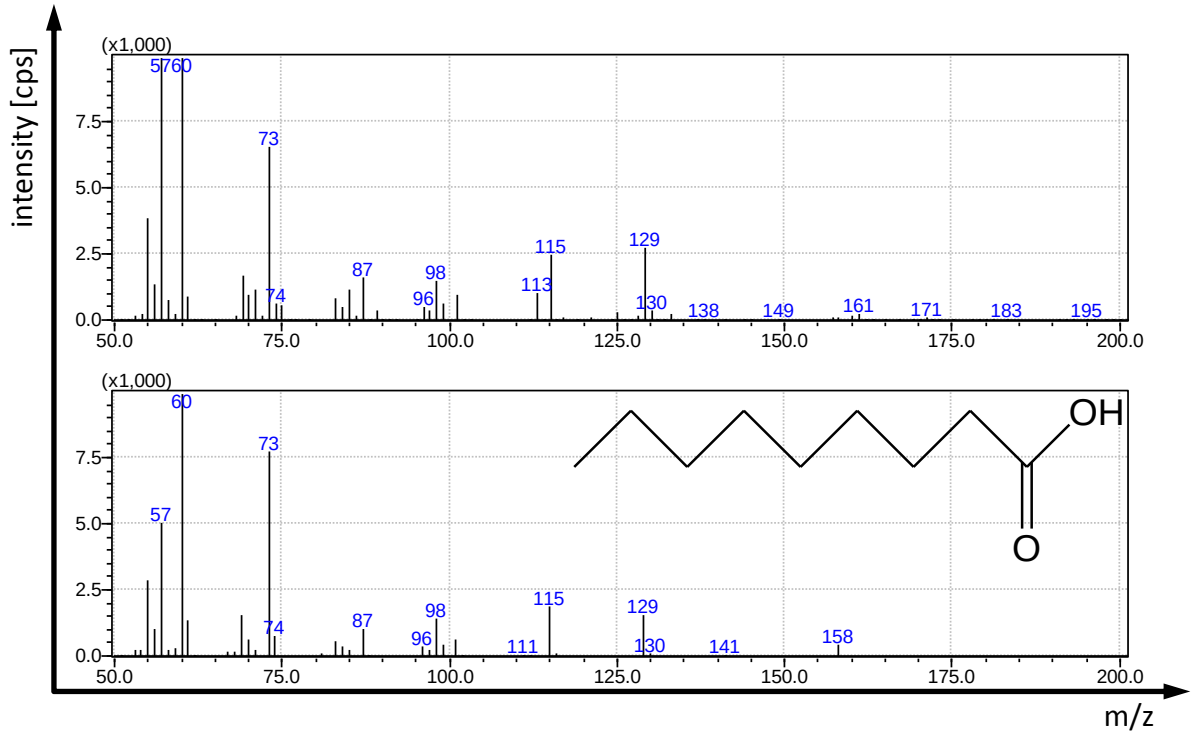


Figure 49: MS spectrum of nonanoic acid (11.94 min). The top spectrum is the spectrum of the sample and at the bottom the database spectrum is presented. In the database spectrum the molecular structure is shown.

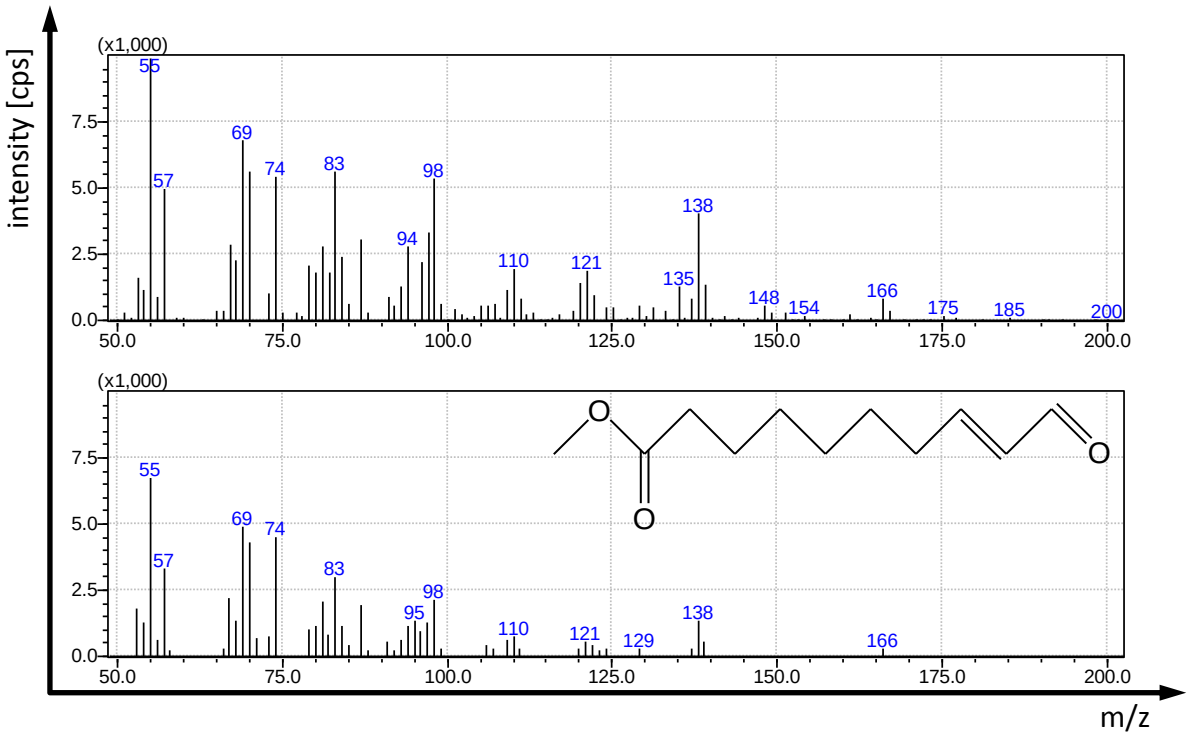


Figure 50: MS spectrum of methyl 10-oxo-8-decenoate (12.88 min). The top spectrum is the spectrum of the sample and at the bottom the database spectrum is presented. In the database spectrum the molecular structure is shown.

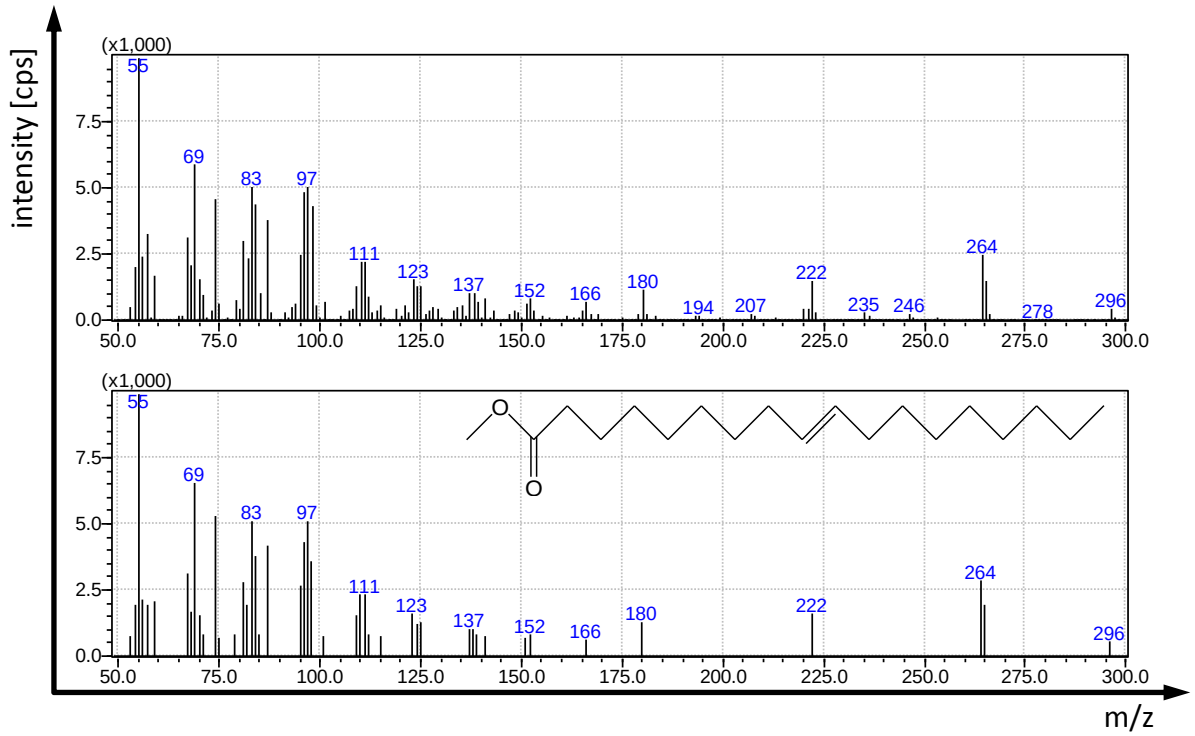


Figure 51: MS spectrum of 9-octadecenoic acid methyl ester (13.32 min). The top spectrum is the spectrum of the sample and at the bottom the database spectrum is presented. In the database spectrum the molecular structure is shown.

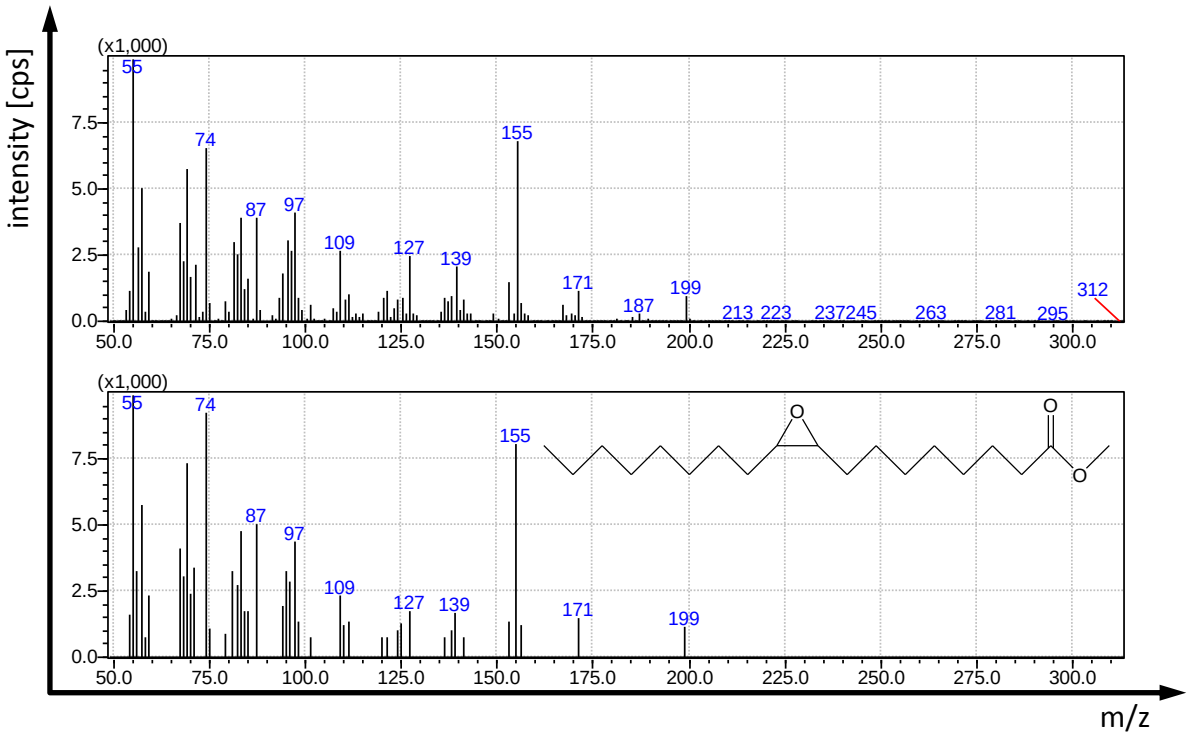


Figure 52: MS spectrum of 9,10-epoxyoctadecanoic acid methyl ester (17.14/17.49 min). The top spectrum is the spectrum of the sample and at the bottom the database spectrum is presented. In the database spectrum the molecular structure is shown.

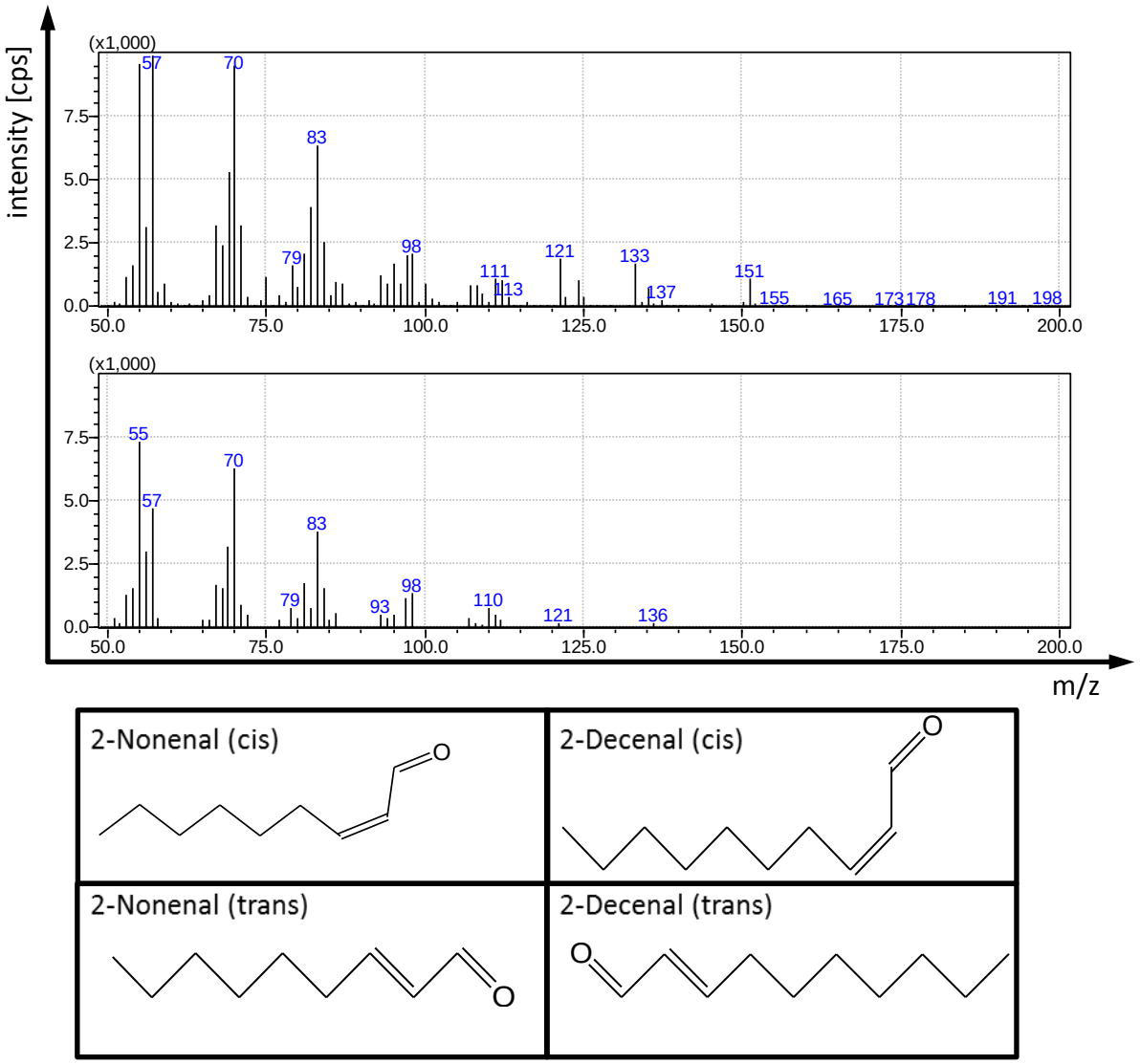


Figure 53: MS spectrum of 2-decenal/2-nonenal (8.97/9.64 min). The top spectrum is the spectrum of the sample and at the bottom the database spectrum is presented. The different molecular structures are shown below the spectra.

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