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DIPLOMARBEIT / DIPLOMA THESIS

Titel der Diplomarbeit / Title of the Diploma Thesis

Preparations for NMR-based biochemometrics of the fungi *Fomitopsis pinicola* and *Hygrocybe conica*

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List of Abbreviations

ACN	Acetonitrile
C	Carbone (atom)
CAD	Charged Aerosol Detector
CAS-Nr.	Chemical Abstract Number
DCM	Dichloromethane
DMSO	Dimethyl sulfoxide
ELSD	Evaporative Light Scattering Detector
ER	Estrogen Receptor
EtOAc	Ethyl acetate
EtOH	Ethanol
FA	Formic Acid
FDP	Farnesyl diphosphate
FSH	Follicle-stimulating Hormone
FP	*Fomitopsis pinicola*
HC	*Hygrocybe conica*
HEMWat	Hexane-Ethyl Acetate-Methanol-Water
HPCCC	High Performance Counter Current Chromatography
HPLC	High Pressure Liquid Chromatography
HPLC-MS	High Pressure Liquid Chromatography – mass spectrometry
H_2O	Water
LC-MS	Liquid chromatography – mass spectrometry
LH	Luteinizing Hormone
MeOH	Methanol
MeOD	Methanol deuterium
MS	Mass spectrometry
NMR	Nuclear Magnetic Resonance
O	Oxygen (atom)
PgR	Progesterone Receptor
RP	Reversed Phase
SS	Schwärzender Saftling
STS	Steroid sulfatase
TLC	Thin Layer Chromatography
UV/VIS	Ultraviolet visible spectroscopy
WHO	World Health Organisation

Acknowledgment

I would like to thank my supervisor Univ.-Prof. Mag. pharm. Dr. Judith Maria Rollinger for her unconditional support and for this special opportunity of being part of the Department of Pharmacognosy. The whole Department was not only very kind, but also very supportive. I am grateful for the opportunity of doing my diploma thesis in such a warm environment.

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Thank you all so much!

Abstract

Natural products have always been a significant source for the treatment of various diseases. In this diploma thesis I investigated the fruit body extracts of two different fungi. The extract of *Fomitopsis pinicola* (=FP) has recently shown an inhibition of steroid sulfatase (STS), an enzyme involved in hormone-dependent cancers like breast cancer or prostate cancer. At this point it was not clear which bioactive components of FP are responsible for the STS inhibition. Starting from the bioactive FP extract, I herein describe the development of a sample preparation protocol for a successful biochemometric processing of the fungal material to determine its active constituents.

Different techniques were used for chemical investigations, such as High-Performance Counter Current Separation (HPCCC) and Flash Chromatography. The monitoring was performed by Thin Layer Chromatography (TLC) and analytical High-Performance Liquid Chromatography (HPLC).

During this diploma thesis 32 microfractions (FP01_01 to FP01_32) were collected from the most active FP extract. These fractions were further examined by NMR spectroscopy as well as STS inhibition testing.

The second topic of this diploma thesis was the chemical investigation of *Hygrocybe conica*. This diploma thesis covers the extraction of the fungal material, , the development of suitable HPLC and TLC methods as well as a LC-MS based dereplication of its constituents.

Zusammenfassung

Naturprodukte waren immer eine bedeutende Quelle für die Behandlung verschiedener Krankheiten. In dieser Diplomarbeit wurden die Fruchtkörper von zwei verschiedenen Pilzen untersucht. Zuvor wurde für den Extrakt des Pilzes *Fomitopsis pinicola* (FP) eine STS-Hemmung ermittelt. Substanzen, die eine STS-Hemmung aufweisen, können möglicherweise zur Behandlung von hormonell bedingten Krebsarten wie Brustkrebs oder Prostatakrebs eingesetzt werden. Zu diesem Zeitpunkt war nicht klar, welche bioaktiven Komponenten von FP für diese STS-Hemmung verantwortlich sind. Die Substanz-spezifische Auftrennung eines Vielstoffgemischs/Extraktes, sowie die Bestimmung der für die observierte Wirkung verantwortlichen Inhaltsstoffe ist ein zentrales Thema in der Naturstoffforschung. Um dieses Ziel zu erreichen beschreibe ich in dieser Arbeit die Entwicklung eines Protokolls für die Probenvorbereitung für eine erfolgreiche biochemometrische Verarbeitung.

Die mykochemischen Untersuchungen wurden durch unterschiedliche Methoden durchgeführt. Es wurden Techniken wie die Hochleistungs-Gegenstrommtrennung (HPCCC), sowie Flash Chromatographie herangezogen. Zur Überprüfung der Ergebnisse wurde sowohl die Dünnschichtchromatopraphie (TLC), als auch die Hochleistungschromatographie (HPLC) herangezogen.

Während der vorliegenden Diplomarbeit wurden 32 Mikrofraktionen (FP01_01 to FP01_32) gesammelt. Diese gesammelten Fraktionen wurden auf ihre STS inhibierende Aktivität getestet und zusätzlich mittels ^{1}H NMR Spektroskopie untersucht.

Das zweite Thema dieser Diplomarbeit befasst sich mit der Untersuchung des Pilzes *Hygrocybe conica* (HC). In diesem Teil werden die Extraktion des Pilzes HC, die Entwicklung von geeigneten HPLC- und TLC-Methoden sowie eine LC-MS-basierte Dereplikation beschrieben.

1. Aim of Work

As it is difficult to identify bioactive compounds out of a complex multicomponent mixture, the aim was to apply statistical methods to correlate a complex chemical profile (e.g. MS or NMR data) to an associated bioactivity (= biochemometry). This should facilitate the process of drug discovery from natural sources.

Thus, the aim of my work was to establish a sample preparation protocol for a successful biochemometric processing.

In one part of my thesis, I worked on the development of a TLC and HPLC method for the ethanolic extract of the fruiting bodies from the fungus *Hygrocybe conica* (HC) to enable a dereplication of this fungal extract. This information provides valuable information about already known constituents and potentially new compounds worth to isolate.

In this respect, the task was to develop suitable analytical methods (TLC and HPLC) and a fractionation method for the bioactive crude extract to simplify it.

In another part of my thesis, the task was to develop a TLC and HPLC method for the STS inhibiting ethanolic extract (Fompin00442c) of the fungus *Fomitopsis pinicola*. Here, the dereplication was one goal, but another aim was to separate this bioactive extract into microfractions to be subjected to NMR and MS measurement as well as STS inhibition testing.

2. Introduction

2.1. Biochemometrics

Centuries ago, the use of natural products was one of the only therapeutic options for various diseases. Nowadays, drug discovery from natural sources is an independent field of research of ever-increasing importance. By identifying individual ingredients of plants and researching their effects, many new therapies for various diseases have been discovered (Newman et al., 2016).
One of the main difficulties in drug discovery from nature is the often very high complexity of the bioactive crude extract. Thus, the overall goal is to identify an active constituent at the earliest possible stage and to avoid wasted efforts on fractions that do not contain promising hit compounds. Moreover, to avoid the re-discovery of already known or undesired compounds (e.g. assay interfering compounds), an imperative step in accelerating natural product drug discovery is dereplication which has become increasingly important in the past years (Gaudencio et al., 2015).

To get even more information, statistical methods for the correlations of biological with chemical data have been established. Martens and co-workers first introduced the term "biochemometrics" for the process of applying statistical methods to correlate a complex chemical profile (e.g. MS or NMR data) to an associated bioactivity (Martens et al., 2006).

Recently, a great deal of interest among the scientific community in the application of such methods is reflected in the increasing number of publications in this field (e.g. Aligiannis, 2016 or Britton, 2018). Generally, the rather new term "biochemometrics" describes a comprehensive workflow integrating chemical and biological data using multivariate statistics as powerful alternative to classical bioassay-guided fractionation.
In this diploma thesis, the key technique for the biochemometric approach was NMR (complemented by information gathered from HRMS).
My goal was to establish a sample preparation protocol for a successful biochemometric processing. In this respect, the task was to develop suitable analytical methods (TLC and HPLC) and a fractionation method for the bioactive crude extract to simplify it. The obtained microfractions were then prepared for NMR, MS, and bioactivity testing.

2.2. *Hygrocybe conica*

Hygrocybe conica (Schaeff.) P. Kumm. is a colourful member of the genus *Hygrocybe* and is also known as the witch's hat. It was first described in 1774 by Jacob Christian Schäffer, who named it *Agaricus conicus.* In 1871 it was transferred to the genus *Hygrocybe* by Paul Kummer.

H. conica grows as a single fungus but also in smaller groups besides paths, in cemeteries, in parks and in mixed forests. It can be found across northern Europe and North America.
H. conica is a small red-yellow, cone-shaped fungus up to 5 diameters, with yellowish lamellae and yellow-red stem, which turns black as the fungus grows older. *H. conica* belongs to the family *Tricholomataceae* of Basidiomycetes.

Figure 1 Fruit bodies of *Hygrocybe conica* (©Andreas Kunze)

H. conica is mainly used for consumption by indigenous people in Peninsiular Malaysia (Chong et al., 2014). During my research I found some scientific literature about the fungus, mainly on its phenolic and possible antioxidant properties. There is quite a bit of literature about the mush-room before the 21st century, but during the last ten years more and more studies have been published about *H. conica* covering its antioxidative effects, one of the most recent study I found was published 2014 by Chong EL et al..

The fungus is largely made up of water-soluble material, indicating that *H. conica* mainly contains of semi-polar phenolic compounds such as 2,3- and 4,5-secodopa, muscaflavin (see Figure 2), tannic acid (which is described as the main bioactive compound in *H. conica*, see Figure 3) and other semi-polar to non-polar antioxidants (Chong et al., 2014).

Muscaflavin (see Figure 2) is enzymatically synthesized from the dopamine precursor L-DOPA (Steglich & Preuss et al., 1975).

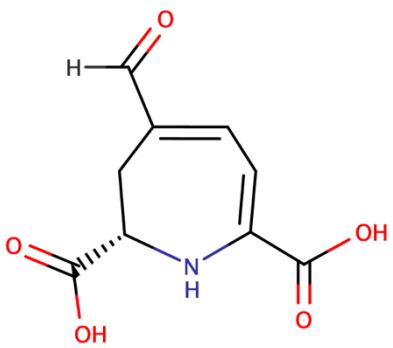


Figure 2 Muscaflavin

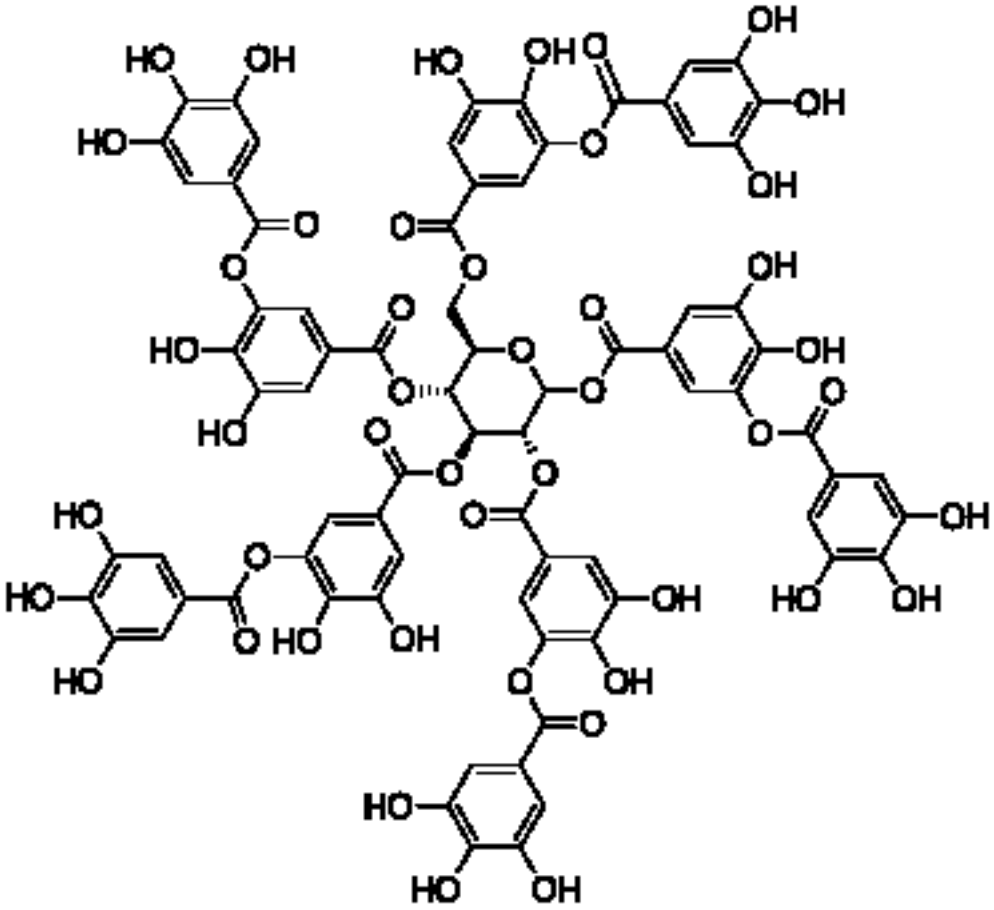


Figure 3 Tannic acid (https://upload.wikimedia.org/wikipedia/commons/thumb/6/65/Tannic_acid.svg/1920px-Tannic_acid.svg.png)

The recent study by Chong et al. (2014), indicates an antioxidative potential of *H. conica* by fractionating a crude water extract of *H. conica* using open column chromatography. Further the possible antioxidant activities were tested by using the DPPH (2,2-diphenyl-1-picryl-hydrazyl-hydrate) assay, ABTS assay (2,2'-Azino-di(3-ethylbenzthiazolin-6-sulfonic acid, antioxidants measuring) and FRAP (ferric reducing antioxidant power) assay, in addition the Folin-Ciocalteau reagent assay was used to evaluate the total phenolic content. The detection was performed by HPLC and TLC.

Chong et al., were able to prove an antioxidant effect due to the presence of phenolic constituents, like the tannic acid, which was also determined as the main bioactive compound of *H. conica*.

The fungus material for my diploma thesis has been provided by Dr. med. Harro Danninger, who claimed that he eats a few milligrams of the dried mushroom to treat his headache and was convinced of its pain-relieving ability. During my research I did not find any information about a

possible analgesic activity of the fungus, so there is still a lack of information which makes further investigation interesting.

2.3.*Fomitopsis pinicola*

Fomitopsis pinicola (Sw. Ex Fr.) Karst. is a polypore fungus which belongs to the family *Fomitopsidaceae* of the division Basidiomycetes (https://www.first-nature.com/fungi/fomitopsis-pinicola.php, accessed December 2017).

Polypores are a morphological group of Basidiomycetes, which comprise a large group of various fungi. These captivating types of mushrooms typically grow on live or dead trees, are very tough, leathery to woody, relatively large and its spores are held in tubes. The purpose of the basidiocarp is to produce the basidiospores, which are essential for long distance spread of the fungus (http://www.svims.ca/council/Polypo.htm, accessed December 2017).

Especially polypores have been used for over 5000 years, as a traditional remedy for various applications ranging from food or tinder material to medicinal use. Therefore, they offer an interesting field of research. In particular, five traditionally used polypore species (*Laetiporus sulphureus*, *Fomes fomentarius*, *Fomitopsis pinicola*, *Piptoporus betulinus*, and *Laricifomes officinalis*) have raised scientific interest. They were traditionally used for the treatment of various diseases, like dysmenorrhoea, pyretic diseases, treatment of coughs and cancer (Grienke et al., 2014).
In addition to the medicinal importance of this group, the ability of assisting in the decomposition of dead wood is also characteristic. In some cases, polypores are even considered as pathogens on living wood (https://www.mushroomexpert.com/polyporales.html, accessed December 2017).

In approximately 1810, *F. pinicola* has first been described by the Swedish botanist Olof Schwartz as *Boletus pinicola*. In 1881, the species was then transferred to the new genus *Fomitopsis* by the Finnish mycologist Petter Adolf Karsten.

This fungus grows on deciduous and coniferous trees and is one of the most common wood rotting fungi. *F. pinicola* is widely distributed in many countries in the world, from central Europe to Scandinavia (Wang et al., 2014).
Not only because it is non-toxic, but also because of its reported anti-inflammatory, antimicrobial, anti-fungal and anti-cancer effect, the medical importance of *F. pinicola* has increased over the years (Wang et al., 2014).

Figure 4 Fruit bodies of *F. pinicola* growing on a dead tree. (http://www.svims.ca/council/illust/Fomitopsis%20pinicola%205%20Michael%20Beug.htm)

F. pinicola has a unique coloration which makes it almost unmistakable. Young fruit bodies are pure white and are capable of excreting aqueous drops. As the fungus grows older, the fruit body develops an orange to red waxy crust on the upper surface (see Figure 5) which will further turn into a blue to dark grey colour. There is also a characteristic red edge which is remaining as the fungus gets older. The fruit bodies are able to reach a width up to 30 cm (Rösecke, 2000).

Figure 5 The fruit body of *Fomitopsis pinicola* (upper surface) (https://www.siegigerstner.at/naturfuehrer/reich-der-pilze/basidienpilze/staenderpilze/hutpilze/porenpilze/rotrandiger-porling/)

Figure 6 The fruit body of *Fomitopsis pinicola* (bottom surface) (https://www.siegigerstner.at/naturfueh-rer/reich-der-pilze/basidienpilze/staenderpilze/hutpilze/porenpilze/rotrandiger-porling/)

Scientific investigations of Basidiomycetes in general showed a wide range of bioactive compounds with promising activities, providing potential drug candidates (Gomes et al., 2019). Compound classes of Basidiomycetes with demonstrated antitumoral potential include sesquiterpenes, sesterpenoids, lactones, and polyoxygenated steroids (Gomes et al., 2019).

Mycochemical analyses of *F. pinicola* have proven the presence of lanostane triterpenoids, such as ergosterol and ergosterol derivatives as major structure class (Wang et al, 2014).

Terpenes are chemical substances consisting of isoprene units with the molecular formula C_5H_8 (see Figure 7). The structure of terpenes is based on different numbers of C_5–units linked via C-C bonding.

Most terpenes are natural substances of plant origin.
A distinction is made according to the number of contained C_5-units:

Monoterpene → C_{10} (2 C_5-units)
Sesquiterpene → C_{15} (3 C_5-units)
Diterpene → C_{20} (4 C_5-units)
Triterpene → C_{30} (6 C_5-units)
Tetraterpene → C_{40} (8 C_5-units)
Polyterpene → C_n

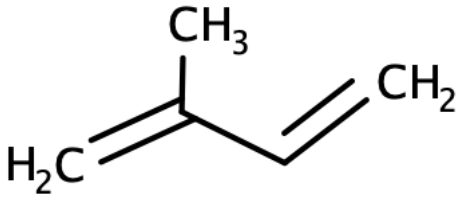


Figure 7 Isoprene unit

Monoterpenes, for instance, are components of essential oils. They are volatile and smell characteristically, mostly aromatic. Chemically, they are often very labile and photosensitive.

Triterpenes are members of the group of isoprenoids with the molecular formula $C_{30}H_{48}$. Mainly found in plants, fungi, bacteria and even in animals, triterpenes are originated from the C_{30} precursor squalene.

Squalene (see Figure 8) is a polyunsaturated hydrocarbon derived from two molecules of farnesyl diphosphate (FDP) which is the precursor of sesquiterpenes and is primarily known because of its importance in cholesterol synthesis.

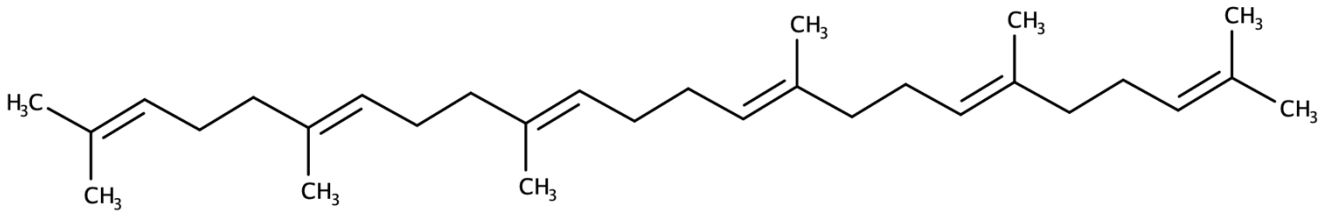


Figure 8 Squalene

Squalene is contained in shark liver oil which is the richest source of this terpene. Recently, several studies showed anticancer, antioxidant, drug carrier, skin hydrating and emollient bioactivities for squalene and squalane (Kim et al., 2012).
(3 S)-2,3-oxidosqualene plays a key role in sterol synthesis in plants, fungi and animals.
Regarding the cyclization of 2,3-oxisqualene there is a big difference in the biosynthesis of sterols between plants and animals. A majority of triterpenes which can be found in nature are cyclic triterpenes with mostly 4-5 ring systems (Kushiro et al., 2010).

In animals, the lanosterol synthase (LAS) converts the intermediate in lanosterol. In plants, the cycloartenol synthase (CAS) converts the intermediate in cycloartenol (see Figure 9) (Sawai et al., 2006).

Cyclic triterpenes are the precursor of various metabolites including steroids, sterols and saponins. These metabolites play an important physiological role in the cells (Kushiro et al., 2010).

Phytosterols are essential elements for forming plasma membranes, especially CAS protein has a vital role in sterol biosynthesis, which is essential for plant cell viability (Jin et al., 2017).

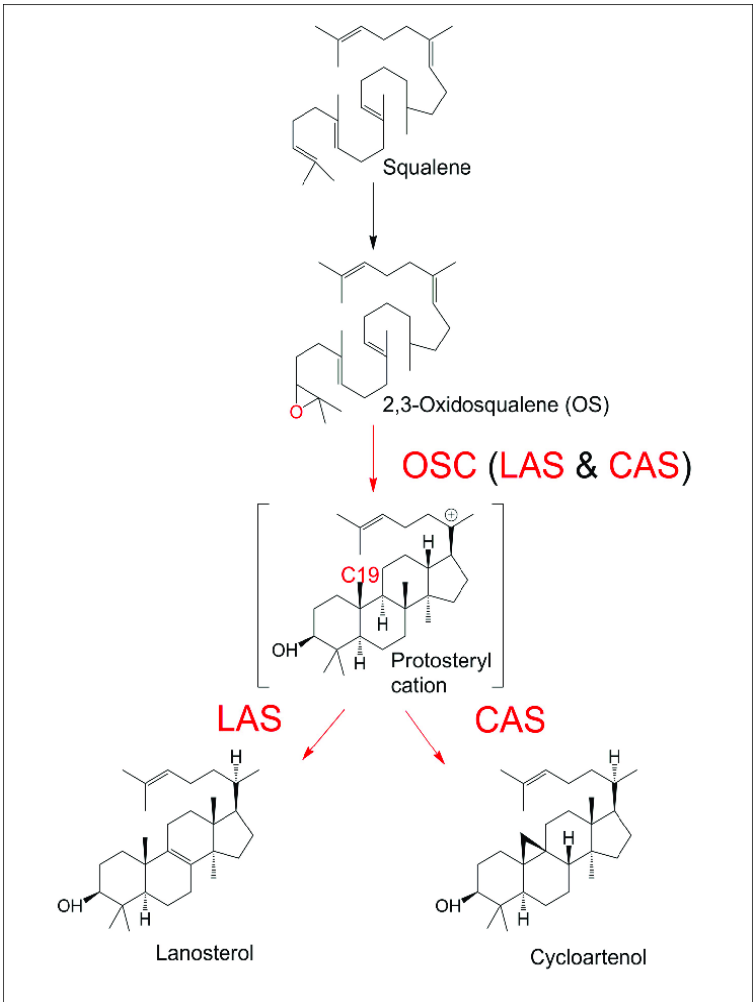


Figure 9 Sterol synthesis, LAS and CAS (Dahlin et al., 2016)

The research for usable evidence for my diploma thesis was easier for *F. pinicola* than it was for *H. conica*. *F. pinicola* is the subject of several studies and is well described in scientific literature. During my research I found quite a lot of studies about secondary metabolites regarding Basidiomycetes or polypores.

By the end of the 1990s, only ten triterpenes and steroids had been isolated and described in the scientific literature, but in 2000 Rösecke was able to determine the presence of far more ingredients by HPLC and also TLC (Rösecke, 2000). *F. pinicola* consists of more than 100 chemically proven components. The volatile components include more than 40 substances, such as aliphatic alcohols, ketones, aldehydes, terpenes and aromatic compounds. In the following paragraphs the most characteristic constituents are described.

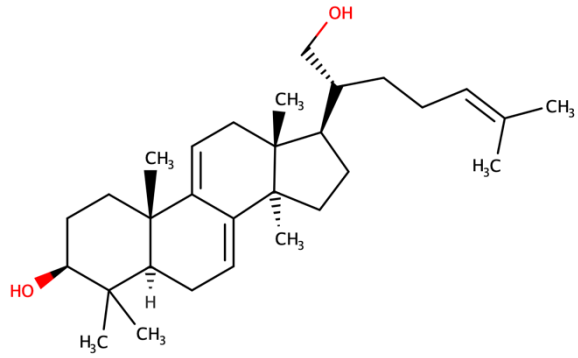


Figure 11 lanosta-7,9(11),24 -trien-3β,21-diol

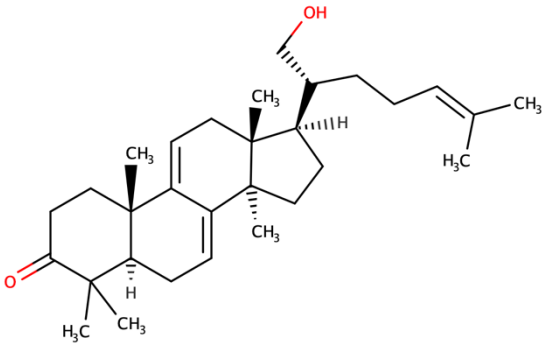


Figure 10 21-hydroxylanosta-7,9 (11),24-trien- 3-on

Keller et al. (1996) observed an antimicrobial activity against *Bacillus subtilis* for five isolated *F. pinicola* triterpenes (3α-acetoxylanosta-8,24-dien-21-oic acid, pinicolic acid A (see Figure 12), trametenolic acid B, polyporenic acid and ergosta-7,22-dien-3β-ol using a TLC bioassay (Rösecke, 2000).

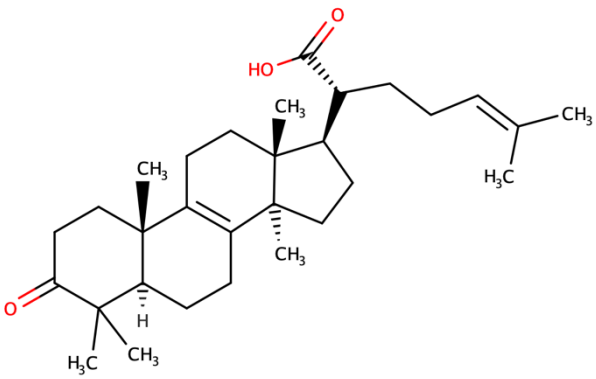


Figure 12 Pinicolic acid

Recent studies showed that trametenolic acid B (see Figure 13) has two important mechanisms: not only a H+/K+-ATPase inhibition activity (Zhang et al., 2013), but also an antitumor effect through regulating the expression of P-glycoprotein (Zhang et al., 2014).

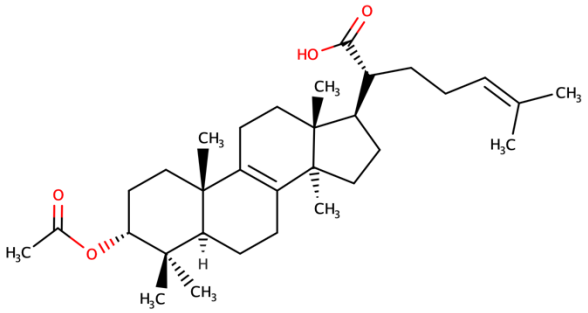


Figure 13 Trametenolic acid B

Another compound which deserves attention, is polyporenic acid (see Figure 14). Research proved its capability of reducing cell proliferation via induction of apoptosis (Ling H. et al., 2009).

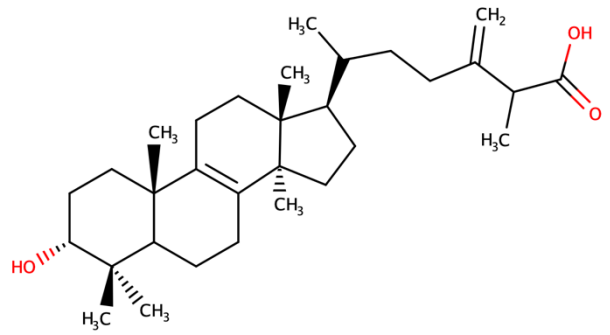


Figure 14 Polyporenic acid

In 2014, ergosta-7,22-dien-3β-ol (see Figure 15) was tested regarding its contribution in the CHOP (CCAAT – enhancer – binding protein homologous protein) pathway mediated ER (endoplasmatic reticulum) - stress in inflammatory processes, which induces apoptosis. Ergosta-7,33-dien-3β-ol was the most active compound during testing. After incubating cells in the presence of ergosterol the expression of CHOP was similar to untreated cells (Pereira et al., 2014). Ergosterol produces all important functions as a growth factor and can therefore be defined as primary metabolite (Rösecke, 2000).

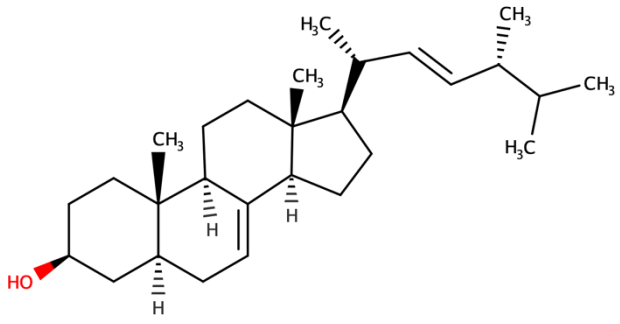


Figure 15 Ergosterol (Ergosta-7,22-dien-3β-ol)

Besides triterpenes, the fruit body of *F. pinicola* contains heterogalactanes (Usui et al., 1981) and the typical mushroom alcohols (R)- and (S)-oct-1-en-3-ol (see Figure 16), octan-3-one, some sesquiterpenes, and hydrocarbons (mainly β-barbatane) (Fäldt et al., 1999).

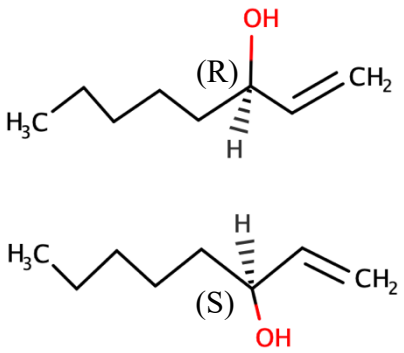


Figure 16 (R)/(S)- oct-1-en-3-ol

2.4. Cancer – an overview

One of the biggest medical challenges of our time is tumor treatment. Globally seen, cancer is the second leading cause of death. (WHO website, September 2018).
Cancer is not just one disease, there are many different cancers, making treatment a major challenge. The 5 most common types are cancers of the lung, breast, colorectal, prostate, and stomach (https://www.wcrf.org/dietandcancer/cancer-trends/worldwide-cancer-data, accessed March 2019).

2.4.1. Causes of cancer development

As already mentioned, cancer is not just one disease, it is a complex group of diseases with many different causes. It is actually not possible to define why one person develops cancer and the other one doesn't, but research showed that there are risk factors, which are able to increase the chances of developing cancer.

Epidemiological evidence indicates a combination of a person's diet, nutrition and physical activity level, as well as control of infections and genetic factors to influence the risk of cancer development. External factors, which are said to have a possible carcinogenic impact are intense sun exposure, alcohol and tobacco consumption and red meat (Ames et al., 1995).

2.4.2. Cancerogenesis

Cancer develops through the change of a normal cell into a tumor cell. They multiply uncontrollably and grow into healthy tissue → a tumor **develops** and **spreads.**

The cell cycle describes the proliferation of cells and is decisive to cancer development. During this cycle, ordinary cells can be damaged and turn into tumor cells. Our immune system is able to repair damaged DNA fragments or to eliminate them. A very important role during this process is given to tumor suppressor proteins, like p53.

The main task of p53 is to eliminate the damaged cell by inducing programmed cell death. Cancer develops because of a damaged tumor suppressor protein, due to its missing control function (Müller-Esterl et al., 2011).

Tumor development is divided into three phases, **tumor initiation, tumor promotion** and **tumor progression.** During **tumor initiation**, cell proliferation occurs as a result of mutation, clonal expansion and selection. **Tumor promotion** is characterised by an increased proliferation of tumor cells. **Tumor progression** is the last phase and leads to an increased growth speed and invasiveness of damaged cells (see Figure 17).
The final step is known as **metastasis**, which contains the spread of tumor cells through the bloodstream or the lymph system (Aktories et al., 2013).

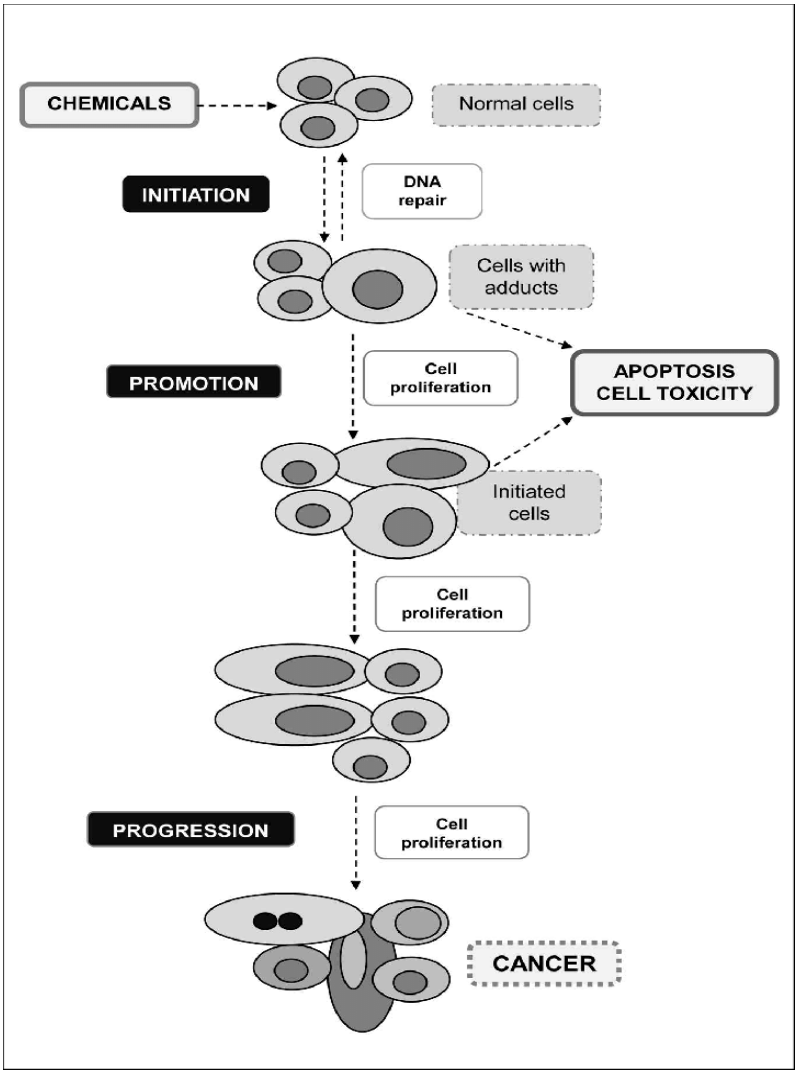


Figure 17 Cancerogenesis Stages (https://www.researchgate.net/figure/Chemical-carcinogenesis-stages-and-the-occurrences-involved-in-each-one-modified-from_fig7_298444949)

2.4.3. Therapy

The aim of tumor chemotherapy is the elimination of tumor cells. For this purpose, chemotherapeutic agents are used, which are also referred to as cytostatics. Despite considerable progress in tumor chemotherapy, a cure by using cytostatics **(curative therapy)** works only for a few tumors, like Morbus Hodgkin or lymphomas.

The chemotherapy has great importance for further therapy, after a successful surgical removal of the tumor or after radioptherapy **(adjuvant therapy)**.

The use of cytostatics prior to surgery is referred as **neoadjuvant therapy.** This is done with the aim of reducing the size of a tumor through prior chemotherapy, in order to improve the chances of a complete surgical removal.

Often, however, only partial remission, relief of tumor-related symptoms and a very limited extension of lifetime is achieved by chemotherapy (Aktories et al., 2013).

2.4.3.1. Anti-hormonal therapy

Special attention must be given to hormone-related cancers, like prostate cancer, breast cancer and ovarian cancer. Hormones are able to promote cancer growth and possibly act as co-carcinogenes during proliferation (Mutschler, 2007).

Recently, studies were able to proof a relation between breast cancer growth and high hormone levels (Schüler-Toprak, 2017). The risk of breast cancer increases by an excessive exposure to estrogen, such as estradiol (Wen et al., 2017). Particularly, estrogen metabolites could potentially initiate the cancer development by binding to DNA and furthermore forming them into depurinating adducts. Said adducts may induce mutations that provide cancer (Wen et al., 2017).

Anti-hormonal therapy is used for cancer types that are sensitive to hormones. If the hormone receptor status of a patient is positive (ER+, PR+), an anti-tumoral therapy will be the first-choice therapy method. The most frequent form of hormone therapy of breast cancer works by blocking hormones from docking to receptors on tumorous cells, performed by drugs like **Tamoxifen** or **Toremifene**, which belong to the group of Selective Estrogen Receptor Modulators (**SERMS**).

Another common form of therapy is the reduction of the hormone levels in the body. The production of estrogen is catalysed by an enzyme called **aromatase**, which is found in fat tissues. Therefore, aromatase-inhibitors are common drugs in anti-tumoral therapy, but they are mainly prescribed for post-menopausal women (Cancer.org, accessed June 2017).

The influence from sex hormones on hormone-dependent tumors are also described by a high concentration of gonadorelin (**GnRH**). GnRH is responsible for the secretion of LH and FSH, which are capable of converting estrogenes and testosterones. Especially, for the treatment of systemic hormone-sensitive prostate cancer, ganodorelin antagonists or agonists are used (Parihar et al., 2016).

2.4.3.2. Inhibition of steroid sulfatase

Steroid sulfatase (**STS**) is an enzyme, which cleavs the sulfonate function on steroids. STS catalyses the formation and hydrolysis of aryl (**estrone sulfate (E1S)**) and alkyl (**dehydroepiandrosterone (DHEA)**) steroid sulfates to active steroids, which can stimulate tumor growth, especially of hormone-dependent tumors, like breast and prostate cancer.
A lot of estrogens that are produced can be converted to estrogen sulfates by sulfotransferase (SULT). Estrogens can be changed from hydrophobic to hydrophilic molecules through SULT. Furthermore, estrogen sulfates are unable to bind to the estrogen receptor (ER), therefore they are biologically inactive (Reed et al., 2005).

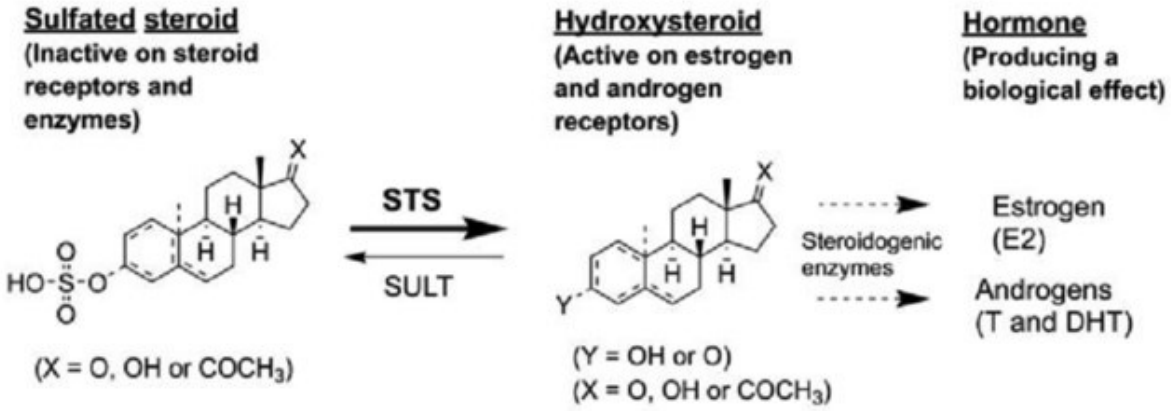


Figure 18 Key role of steroid sulfatase (STS) and sulfotransferase (SULT) in the synthesis of estrogens like estradiol (E2) and androgens like testosterone (T) and dihydrotestosterone (DHT) (https://www.researchgate.net/figure/Fig-1-A-Key-role-of-steroid-sulfatase-STS-in-the-synthesis-of-estrogen-estradiol_fig1_280063068)

The steroid sulfatase enzyme is a monomer with a molecular mass of 63 kDa. It consists of an N-terminal signal peptide of 21 to 23 amino acids, with four potential and two functional glycosylation sites (Reed et al., 2005).
The role of STS in hormone-dependent cancer growth makes it an important target for research. The tertiary structure is shaped like a mushroom, with 2 anti-parallel α-helices forming a binding pocket by emerging from the globular domain directly to the substrate-binding cleft (Demydchuk et al., 2017) (see Figure 19).

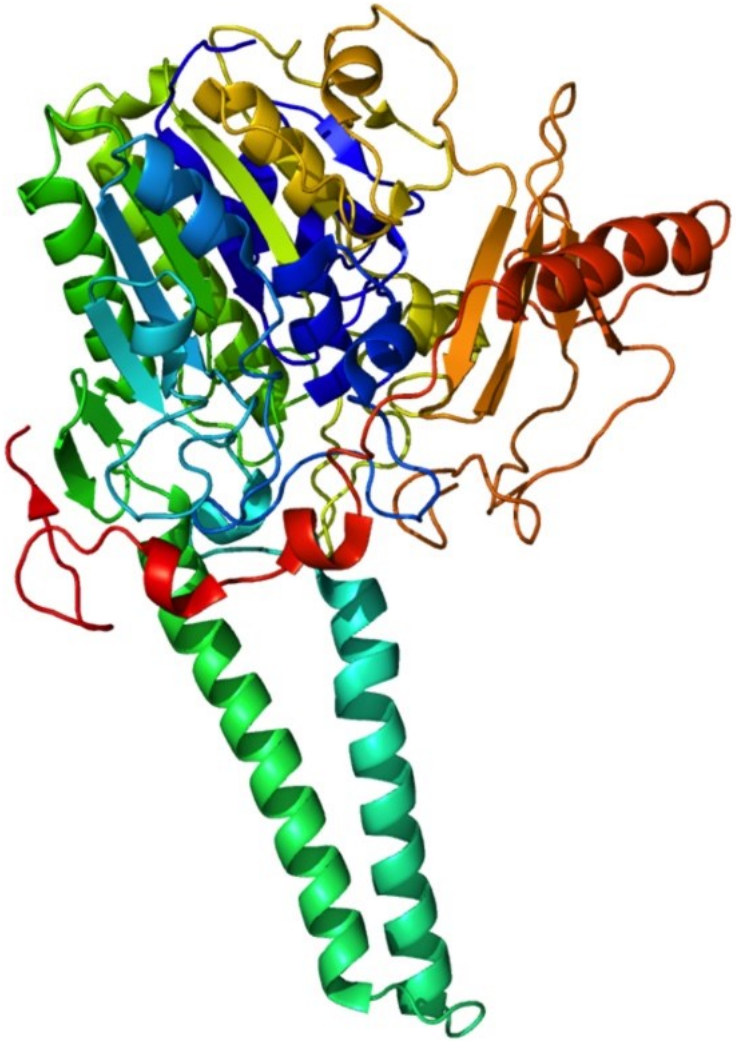


Figure 19 Tertiary structure of STS (https://upload.wiki-media.org/wikipedia/commons/e/ed/Protein_STS_PDB_1p49.png)

3. Results

3.1. *Hygrocybe conica*

3.1.1. Extraction

To prepare an extract of *H. conica,* 150,26 mg of the dried fruit body was filled into a centrifugation tube and 15 ml of EtOH 96% were added. The tube was covered with aluminium foil and sonicated for 20 minutes at room temperature. After centrifugation for 5 minutes (3.500 rpm) the extract was filtered through a pasteur pipette (which was stuffed with cotton wool) and collected in a pear shape flask. The fungal material was washed with 10 ml of EtOH and again filtered. The EtOH extract was dried and then transferred to a pre-labeled and pre-weighed glass vial for further work. The extract which yielded 12,69 mg, was named "SS_extract", referring to the German name of the fungus – i.e. "Schwärzender Saftling".

3.1.2. TLC system optimization and analysis

The optimization of a thin layer chromatography (TLC) system was an important part for further work. It was performed on Silica gel 60 F_{254} aluminium sheets. A part of the extract was dissolved in MeOH and then applied (200 µl) on several small TLC sheets. Each sheet was developed with a different solvent system. After a few tests the combination of dichloromethane, methanol and formic acid (FA) (10:0,25:0,25) performed without chamber saturation, turned out to be the best one regarding the separation of contained extract compounds. Derivatisation was performed by spraying the TLC plate with methanolic sulfuric acid 5% and methanolic vanillin 1%. Then the plate was dried at 80°C for 8 min and inspected at daylight.

Although a good solvent system was used, there was a complex big blue spot visible at R_f=0,76, other interesting spots were found at R_f= 0,58, R_f= 0,63 and R_f= 0,95 (see Figure 20).

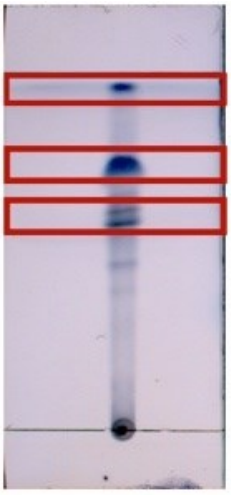


Figure 20 TLC analysis of the *H. conica extract (SS_extract)*

3.1.3. HPLC system optimization and analysis

To get more detailed insights into the composition of the *H. conica* extract, an HPLC system was developed. The initial trials were performed with the Agilent Zorbax SB-C18 column using a mobile phase system of ACN/Acidic H_2O. The main challenge of this task was the unknown complexity of the extract.

The first run started with 15% ACN at a flow rate of 1.000 ml/min and increased continuously to 30% (at 6 min), 60% (12 min), 70% (22 min), and 98% (29 min). After a plateau phase with 98% ACN for 12 min the gradient went down again to 15% at 43 minutes. After re-equilibration, the run stopped at 50 minutes.
With this method, the separation of the extract components was insufficient, therefore the gradient as well as the run time were optimized.

Figure 21 shows the chromatogram of the extract obtained with the final optimized HPLC method, with distinctly separated peaks at RT = 1 min, 15 min, 17 min, 18 min and 19 min.

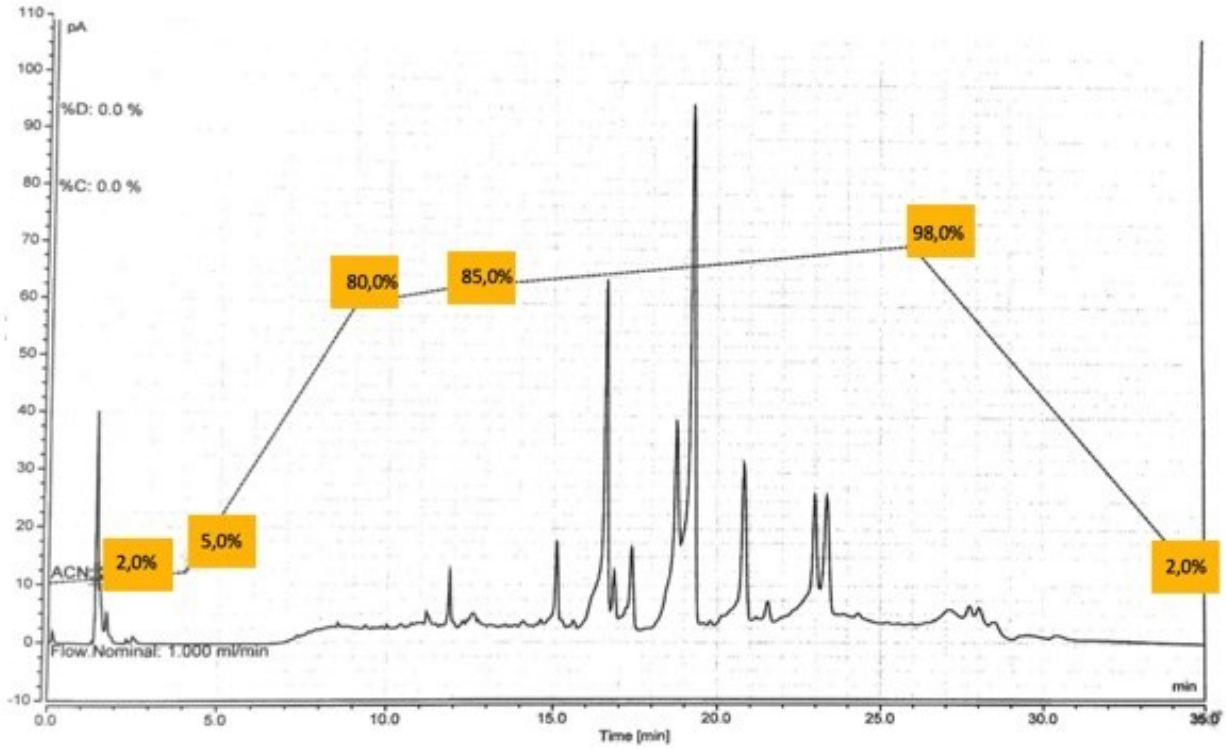


Figure 21 HPLC Analysis of *H. conica* extract with the optimized HPLC method, Detector: CAD

3.1.4. Dereplication of *H. conica* extract components using HPLC-MS

After HPLC-CAD analysis, HPLC-MS was performed to determine the molecular mass of contained compounds. Over the entire range of the chromatogram a few distinct peaks were discovered (see Figure 22). Most of the observed molecular masses are not described in literature for constituents of this fungus, therefore an identification or dereplication was not yet possible. Only three known constituents were identified, possibly fatty acids (see Table 2).

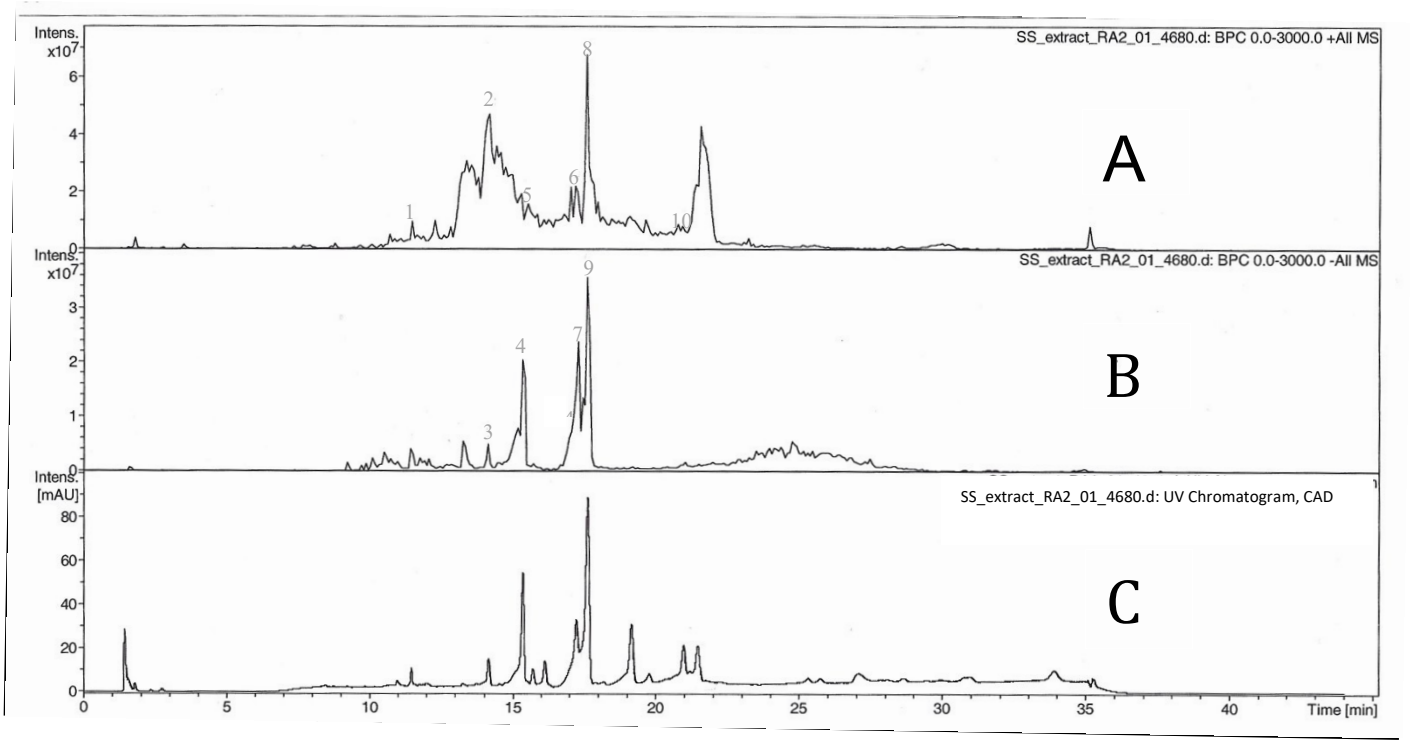


Figure 22 (A) MS Chromatogram of *H. conica* (SS_extract), Base peak chromatogram in negative mode; (B) Base peak positive mode (C) HPL-chromatogram detected with CAD

Table 1 Proposed structures, peaks, retention time, LC-mass data of SS_extract
n.a. = not available

Peak	Retention Time (min)	LC-MS mass number (m/z)		Molar mass estimated	Proposed Structures
		Positive mode	Negative mode		
1	11,4	760,6	n.a.	760	
		557,5	n.a.	558	
2+3	14,1	498,4.	n.a.	498	
		520,3	n.a.	521	
		n.a.	271,1	271	
		n.a.	760,6	760	
4	15,3	498,4	n.a.	500	
		520,4	n.a.	520	
5	15,6	760,6	n.a.	760	
		736,6	n.a.	737	
6+7	17,2	n.a.	281,1	281	9,12-Octadecadienoic acid (9Z,12Z)- (fatty acid molecular weight: 280,45)

		282,2 (563,2 Dimer)	281,1	281	9,12-Octadecadienoic acid (9Z,12Z)
		498,4	n.a.	500	
		520,3	n.a.	520	
		736,6	n.a.	736	
		760,6	n.a.	760	
8+9	17,6	657,6	n.a.	658	
		565,4	n.a.	656	
		587,5	n.a.	588	
		283	281,1	281	9,12-Octadecadienoic acid (9Z,12Z)- (fatty acid molecular weight: 280,45)
10	21,2	571,5	n.a.	571	
		527,4	n.a.	527	
		615,5	n.a.	615	
		483,4	n.a.	483	
		659,5	n.a.	660	
		703,5	n.a.	703	
		760,6	n.a.	760	
		439,3	n.a.	440	
		310,3	n.a.	310	
		284,3	n.a.	284	

Due to the small amount of sample, a fractionation of the SS_extract for further investigations was not possible. Most of the masses did not correspond to any of the known constituents of *H. conica*. This fact makes this fungus very interesting for further investigations, once more starting material can be obtained.

3.2.*Fomitopsis pinicola*

The main topic of this diploma thesis was to establish a sample preparation protocol for following biochemometric analyses. The complexity of natural product extracts is an immense challenge in finding bioactive compounds. Therefore, it was important to find a system which separates/simplifies the bioactive crude extract into microfractions. The aim was to obtain different concentrations of constituents over consecutive fractions (see Figure 23). All microfractions should then be tested for their bioactivity and their ^{1}H NMR and LC-HRMS data should be measured.
Through correlation of NMR and bioactivity data, bioactive compounds should be visible prior to isolation.

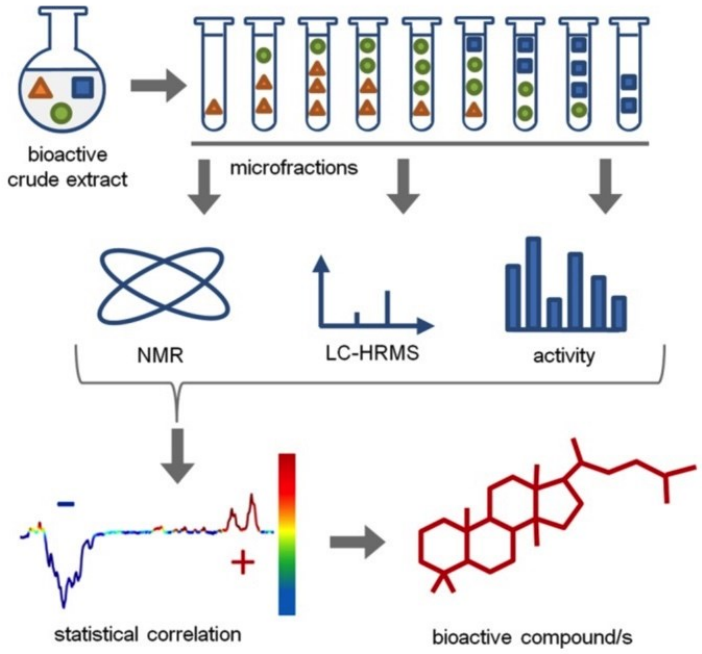


Figure 23 Schematic overview of the proposed workflow (Grienke et al., 2019)

3.2.1. HPCCC

In a first attempt to fractionate the STS inhibiting extract of *F. pinicola* (Fompin00442c), high performance counter current chromatography (HPCCC) was chosen as a technique which works according to the liquid-liquid separation principle (see Figure 24).

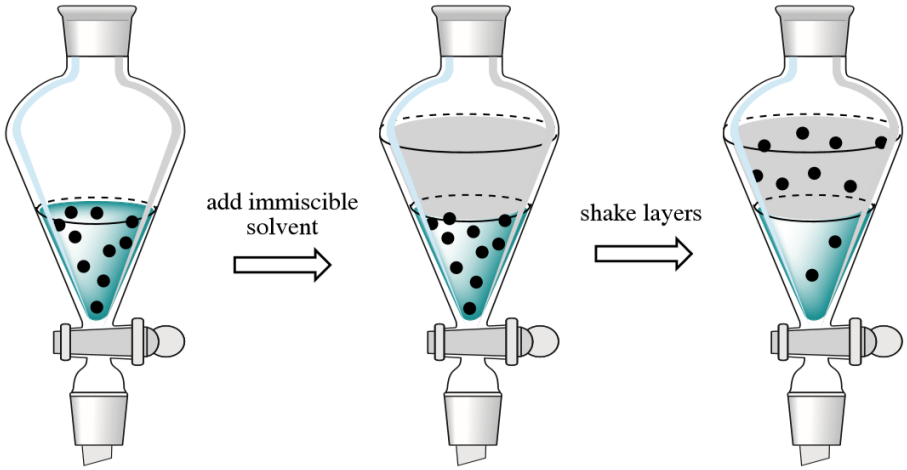


Figure 24 Principle of liquid - liquid separation (https://chem.libretexts.org/Bookshelves/Organic_Chemistry/Book%3A_Organic_Chemistry_Lab_Techniques_(Nichols)/4%3A_Extraction/4.1%3A_Overview_of_Extraction)

The liquid-liquid separation is a very common technique in extracting natural products. During this process a solution with dissolved components is placed in a separatory funnel and an immiscible solvent will additionally be added. This creates two layers, which are shaken together. Mostly one of the layers contains of an aqueous solvent and the other of an organic one. The Components then move from one layer to the other, which is defined as the separation part of the process. Depending on its polarity, a certain proportion of the dissolved components is included in one of the phases, which makes it very easy to obtain them (see Figure 24).

As with classic chromatographic techniques, a HPCCC system also consists of a stationary phase and a mobile phase, which can be defined depending on the task. Various defined solvent systems for HPCCC can be found in the HEMWat Table (see 5.2.3, “High Performance Counter Current Separation”, Page 48). For each system there is a defined composition for the stationary and for the mobile phase.

As already explained, the separation is achieved by a distribution of the sample between two immiscible solvents. This method is a very important chromatography technique for separating natural products, because it provides scalable analytical capabilities (see Chapter 5.2.2, “High Performance Liquid Chromatography”, Page 47).

3.2.2. HPCCC system development

3.2.2.1. TLC monitoring for HPCCC system

First I started to find a suitable HPCCC solvent system, by monitoring each used system by TLC. Since it was not clear at the beginning which solvent system would work best, I decided to start testing system 17, which is known for working fine with MeOH extracts and is defined as the entry point. In addition to system 17, I tested the more polar solvent systems 14-16. The exact composition of the systems can be found in the HEMWat table (see Chapter 5 “Materials and

Methods", page 48). I started by dissolving 2,5 mg of the *F. pinicola* extract (Fompin00442c) in 6,5 ml MeOH than added the required amount of the other solvents according to the HEMWat Table. Each system was tested successively. The upper and lower phase was then separated (1 ml per tube) and evaporated. The residue was dissolved in 1 ml HPLC quality methanol. 200 µl were transferred to inserts for the HPLC analysis and 200 µl were used for the TLC monitoring (see Chapter 5.3.3 "TLC system optimization", Page 49). This procedure was repeated for every tested system. The goal was to find a system that would provide the best separation of the sample. Overall, I tested solvent system 14 to 23. According to TLC monitoring, systems **20**, **21** and **22** (see Figure 25,26,27) showed the most promising results.

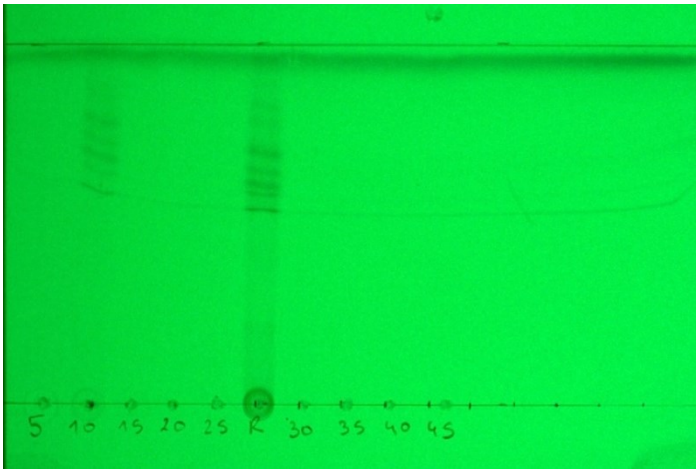


Figure 25 HEMWat System 20, every fifth tube was applied on the plate, the TLC plate was dried and derivatized with 5% H_2SO_4 in MeOH and 1%Vanillin in MeOH, R= Reference (*F. pinicola* extract), detected at 254 nm

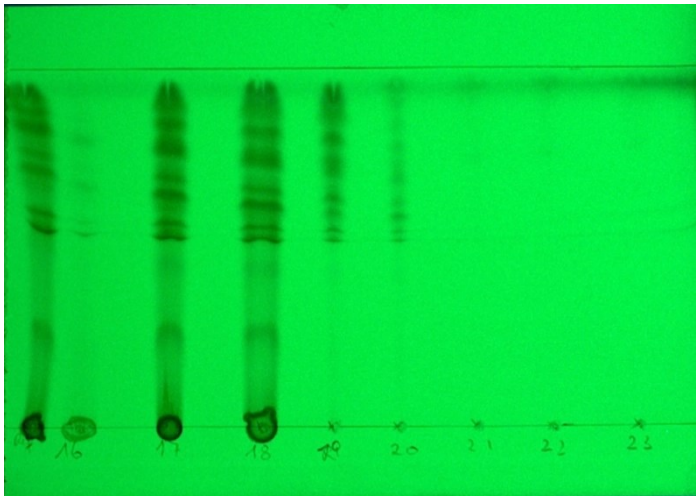


Figure 26 HEMWat System 21, tubes number 15 to 23 were tested, the TLC plate was dried and derivatized with 5% H_2SO_4in MeOH and 1%Vanillin in MeOH, R= Reference (*F. pinicola* extract) detected by 254nm

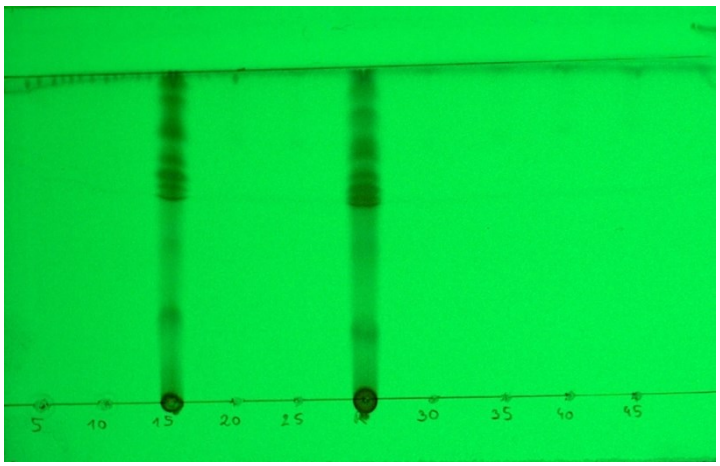


Figure 27 HEMWat System 22, every fith tube was tested, the TLC plate was dried and derivatized with 5% H_2SO_4 in MeOH and 1%Vanillin in MeOH, R= Reference (*F. pinicola* extract) detected by 254nm

3.2.2.2. HPLC development

After finding promising results by TLC monitoring, I started with the HPLC system optimization for analysing the different solvent systems. After a few attempts the proper HPLC system was established (see Chapter 5.4.3, "HPLC system optimization and analysis" Page 49). My plan was to integrate each received chromatogram and to calculate the partition coefficient between upper and lower phase according to the area under the curve for each peak.

The partition coefficient is calculated by using following formula:

Partition coefficient for each peak: $K = \frac{\text{area upper layer}}{\text{area lower layer}}$

To compare the partition coefficients you need two values for each peak (peak area of the upper phase and peak area of the lower phase). An ideal partition coefficient, would be between 0.5 – 4, which would indicate a proper separation. The HPLC result was not that optimal as expected, unfortunately the chromatogram showed no baseline separation, so it was not possible to do any calculation. Therefore, I decided to focus on the TLC results and planned to run the HPCCC according to promising TLC results with the systems 20, 21 and 22.

I was able to test 2 systems (20 and 21) via the analytical separation mode by HPCCC (small scale separation). Therefore, I prepared aliquots of the stationary phase (lower phase) and mobile phase (upper phase). The exact compositions of the phases are described in the HEMWat Table (see Chapter 5.4.4.1 "Separation by analytical HPCCC", Page 51) . First the HPCCC device was rinsed with a MeOH/H_2O mixture (1:1). After the pump was turned on, I purged the device to get rid of remaining air. Further, the flow rate was set to 2 ml/min at a pressure of 87 psi. The HPCCC column holds 25 ml, therefore I prepared twice the amount of solvent (50 ml lower phase and

50 ml upper phase) to ensure that the column is sufficiently filled. I decided to run the separation by normal phase. Both solvent systems were tested one after the other. The following described process was used for solvent system 20 as well as system 21, just the compositions of the stationary phase and the mobile phase were different (see Chapter 5.4.4.1, "Separation by analytical HPCCC", Page 51).

Step 1: stationary phase (=lower phase)
First I took two column volumes of stationary phase to fill the device. So I prepared 50 ml, this ensures that 25 ml are contained in the column. The stationary phase was than pumped through the machine to ensure that the washing solvent was pushed out of the device. The rotation was set to 200 rpm at a pressure of 101 psi by 2 ml/min.

Step 2: mobile phase (=upper phase)
Since the device is very sensitive, the pump was purged before every solvent change. The mobile phase was pumped through the device at a rotation of 1600 rpm and a pressure of 145 psi at 1 ml/min.

After these steps the next goal was to establish an **equilibrium**. After switching to the mobile phase, a new measuring cylinder was needed to collect the outflow. At some point the stationary phase stopped dropping out and the mobile phase was coming out (visually monitored – two phases visible). As the mobile phase was dropping out I stopped the pump, but kept the rotation running.

Now the dissolved sample (2,5 mg) was transferred to the machine, via an injection needle and the outflow was collected in tubes.

Shortly before the end of the process an elution extrusion was made. This means to pump out the stationary phase to ensure no sample was lost.

The obtained fractions were monitored by TLC (used parameters see Chapter 5.4.2.1, "TLC system for *Fomitopsis pinicola*", Page 50) , but unfortunately both systems showed no satisfying separation. No further testing of other systems was possible to perform due to technical problems of the HPCCC device.

At this point other alternative methods had to be chosen for proper separation.

3.2.3. Seperation by PuriFlash

Another solution for the separation of the FP extract had to be found, therefore the PuriFlash system has proven to be the device of choice, because of its great separation ability, especially for natural products.
Time-based microfractionation via reversed phase flash chromatography was used. We started with a C_{18} HQ column as stationary phase and a gradient system of ACN/H_2O as mobile phase.

Further information regarding this process can be found in Chapter 5 “Material and Methods” on page 44.

As already mentioned, the mobile phase was a gradient system starting with a low percentage of ACN 5% with a higher amount of water and then increasing ACN to 98 % and lowering water to 2%. In total, 125 tubes (15 ml each) for further investigation were collected.

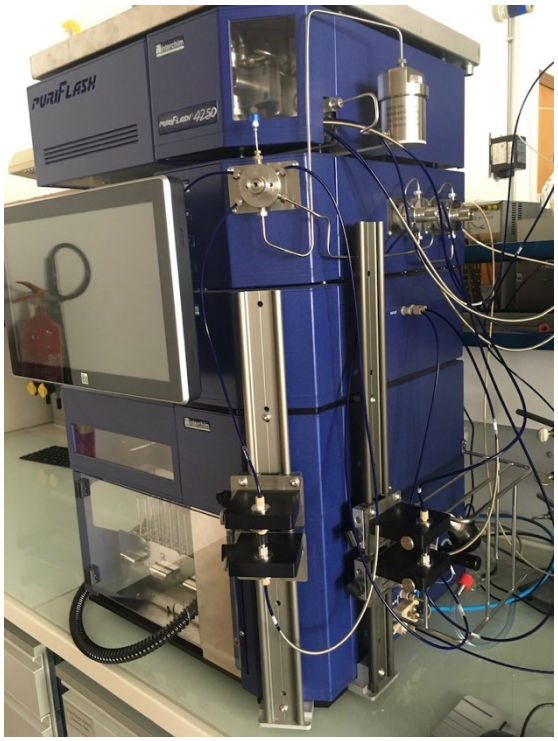


Figure 28 PuriFlash Interchim

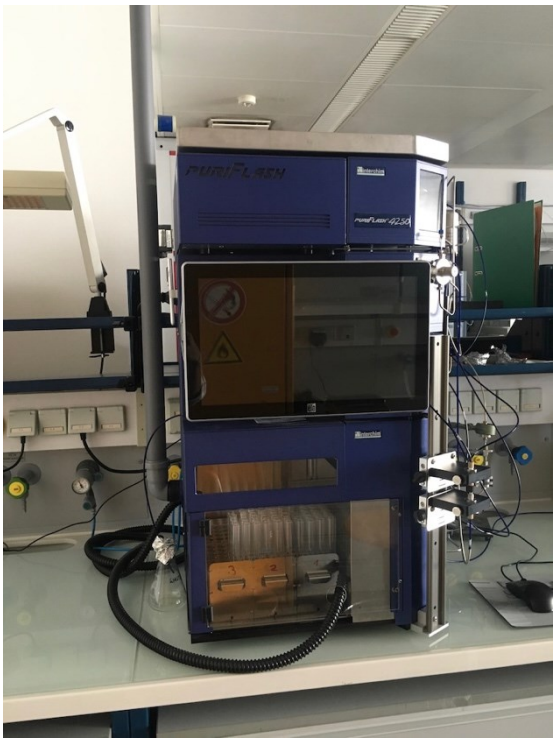


Figure 29 PuriFlash Interchim

3.2.4. Monitoring by TLC

Afterwards, each of the 125 tubes were monitored by TLC, to see if there was a proper separation of constituents. I decided to use the photographed TLC pattern on which the constituents are best shown. The following figures (30-38) show the TLC pattern detected by 254 nm, 366 nm and some by VIS, but always after derivatization (see Chapter 5.3.3, “TLC System optimization”, Page 48). Despite different detection I choose the following pictures because the results were the most clearly visible here.

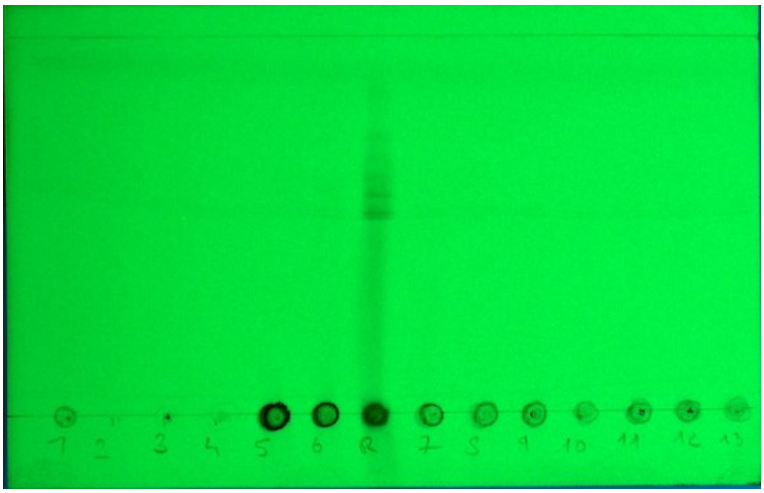


Figure 30 Fp00442c_01_01 to _01_13, 254nm, after derivatization

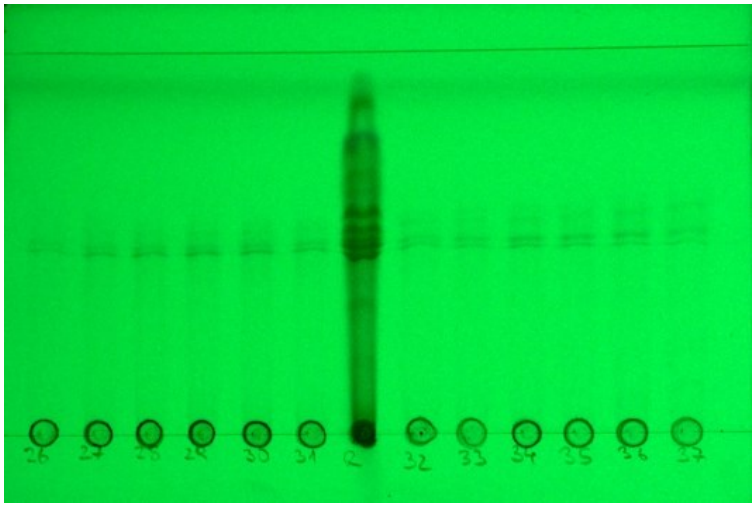


Figure 31 Fp00442c_01_26 to _01_37, 254nm, after derivatization

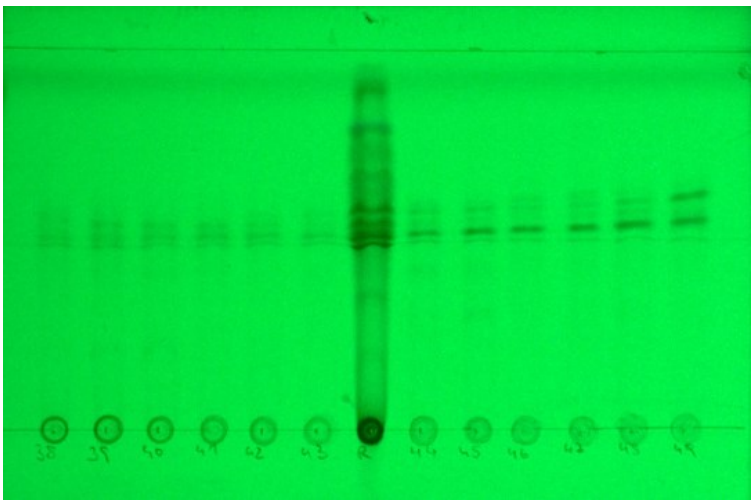


Figure 32 Fp00442c_01_38 to _01_49, 254nm, after derivatization

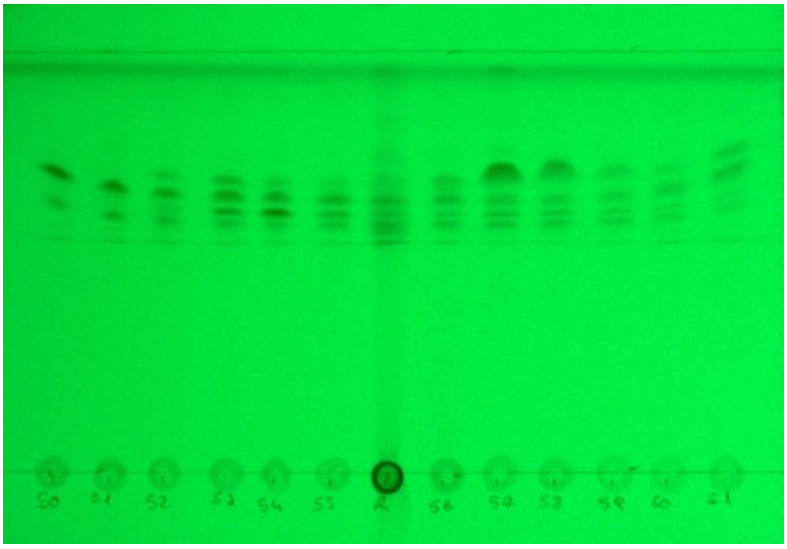


Figure 33 Fp00442c_01_50 to 01_61, 254nm, after derivatization

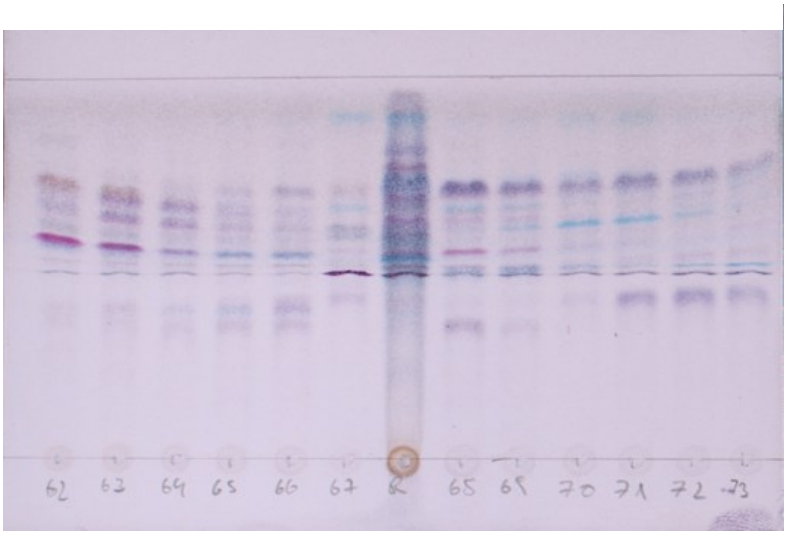


Figure 34 Fp00442c_01_62 to _01_73, VIS, after derivatization

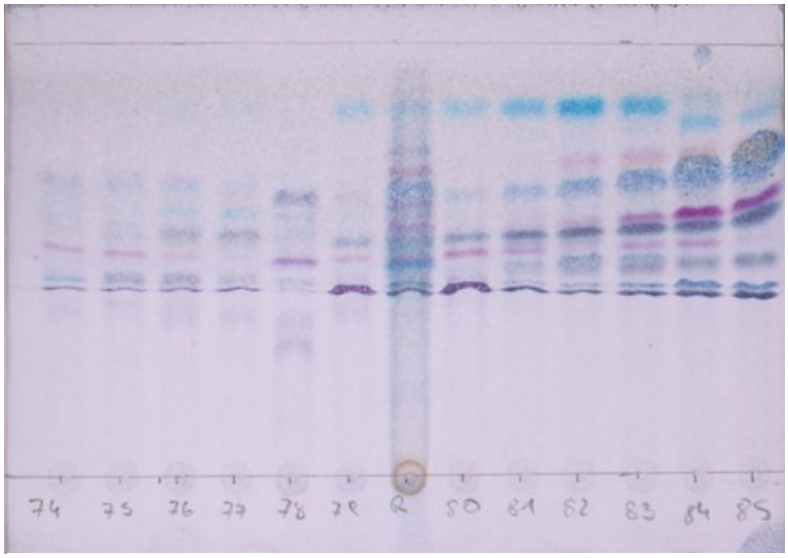


Figure 35 Fp00442c_01_74 to _01_85 VIS, after derivatization

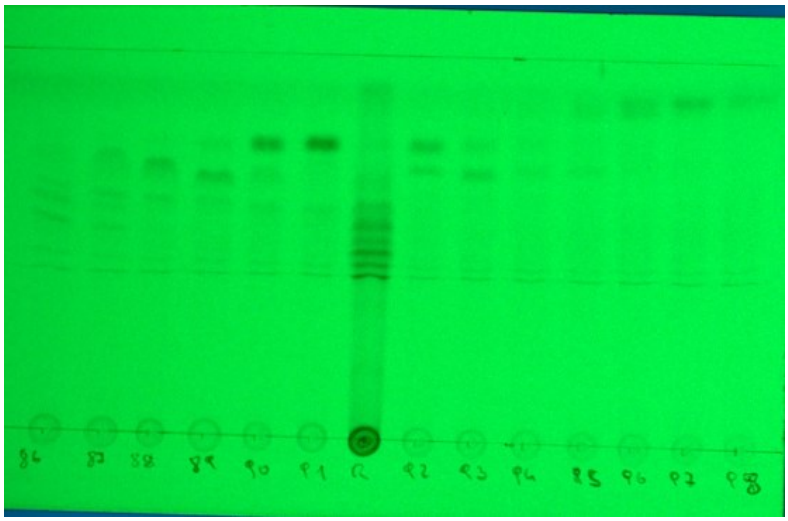


Figure 36 Fp00442c_01_86 to _01_98, 254nm, after derivatization

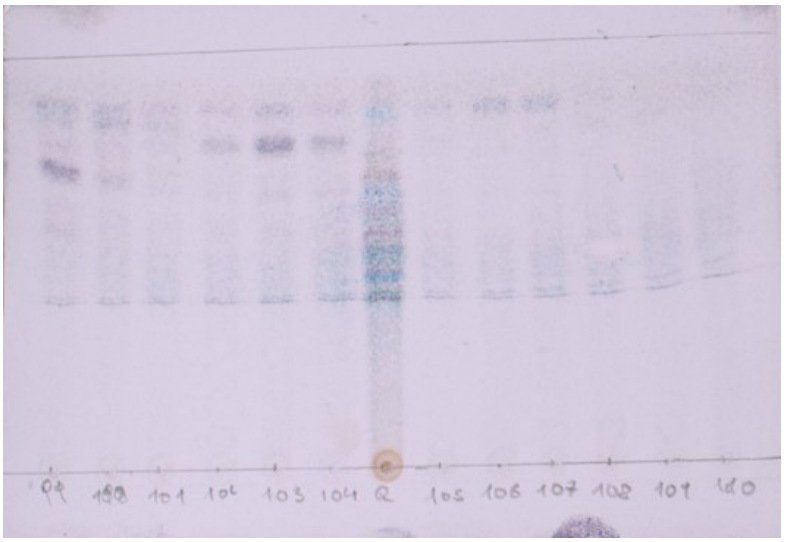


Figure 37 Fp00442c_01_99 to _01_110, VIS, after derivatization

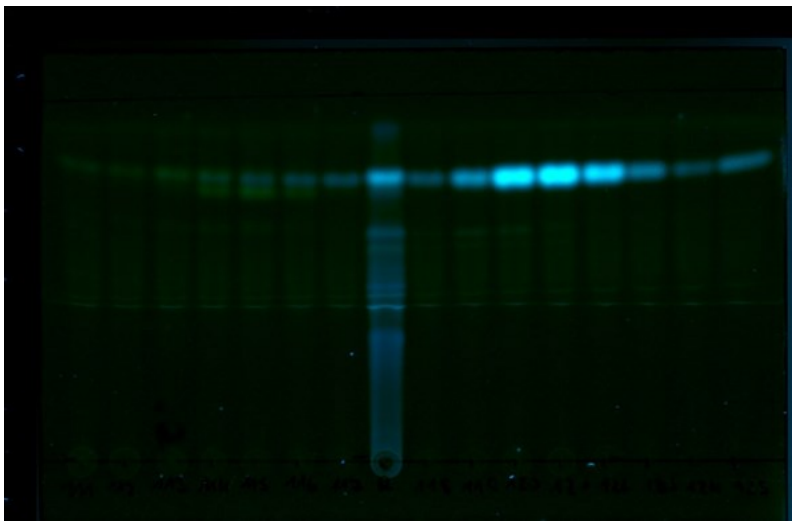


Figure 38 Fp00442c_01_111 to _01_125, 366nm, after derivatization

According to the results of the TLC monitoring we decided to discard the first two fractions due to lack of compounds and combined the tubes, according to their TLC pattern. We pooled 125 tubes to 41 fractions, which were transferred to pre-labelled and pre-weighed pear-shaped flasks. The fractions were dried by rotary evaporation and the dry weight was determined. We decided to combine the fractions in such a way to ensure a minimum dry weight of 3.5 mg per fraction (see Table 1).

Table 1 125 tubes pooled to the 32 microfractions

Sample	Tube number	Sample	Tube number
Fompin00442_01_**01**	3,4,5	Fompin00442_01_**17**	63,64,65
Fompin00442_01_**02**	6,7,8	Fompin00442_01_**18**	66,67,68
Fompin00442_01_**03**	9,10,11	Fompin00442_01_**19**	69,70,71
Fompin00442_01_**04**	12- 24	Fompin00442_01_**20**	72,73,74
Fompin00442_01_**05**	25,26	Fompin00442_01_**21**	75,76,77
Fompin00442_01_**06**	27,28,29	Fompin00442_01_**22**	78,79,80
Fompin00442_01_**07**	30,31,32	Fompin00442_01_**23**	81,82,83
Fompin00442_01_**08**	33,34,35	Fompin00442_01_**24**	84,85,86
Fompin00442_01_**09**	36,37,38	Fompin00442_01_**25**	87,88,89
Fompin00442_01_**10**	39,40,41 + half of 42,43,44	Fompin00442_01_**26**	90,91,92
Fompin00442_01_**11**	45,46,47 + half of 42,43,44	Fompin00442_01_**27**	93,94,95
Fompin00442_01_**12**	48,49,50	Fompin00442_01_**28**	96,97,98
Fompin00442_01_**13**	51,52,53	Fompin00442_01_**29**	99,100,101
Fompin00442_01_**14**	54,55,56	Fompin00442_01_**30**	102,103,104
Fompin00442_01_**15**	57,58,59	Fompin00442_01_**31**	105-125
Fompin00442_01_**16**	60,61,62	Fompin00442_01_**32**	NL → Overrun

Figure 39 32 obtained microfractions

Therefore, I dissolved the dry fractions with MeOH and combined them in new **32** pre-labelled and pre-weighed glass vials. They were then dried by a sample concentrator and the weight was checked.

3.2.5. Sample preparation for STS inhibition testing

For bioactivity screenings, HPLC quality methanol was added to transfer aliquots of approximately 1 mg of each fraction to 32 pre-labelled and pre-weighed Eppendorf tubes. 1000 µL MeOH (HPLC) was added and the required µL to remove 1 mg was calculated and adjusted for each fraction according to its yield (see Table 14, Page 53). Afterwards the tubes were put into the centrifuge (3000 rpm/5min), then dried by a sample concentrator to determine the weight and to possibly correct the weight if needed.

The tubes were send to Dr. Paul Foster (University of Birmingham, United Kingdom) for further STS inhibition testing at a concentration of 50 µg/mL.

The inhibition effect of each fraction was shown as percentage of STS activity in comparison to the positive control STX64 (Irosustat) which was set to 0 % STS activity. The lowest STS activities, and therefore the highest STS inhibition were observed for fractions FP01_15, FP01_16 and FP01_17 (see Figure 41).

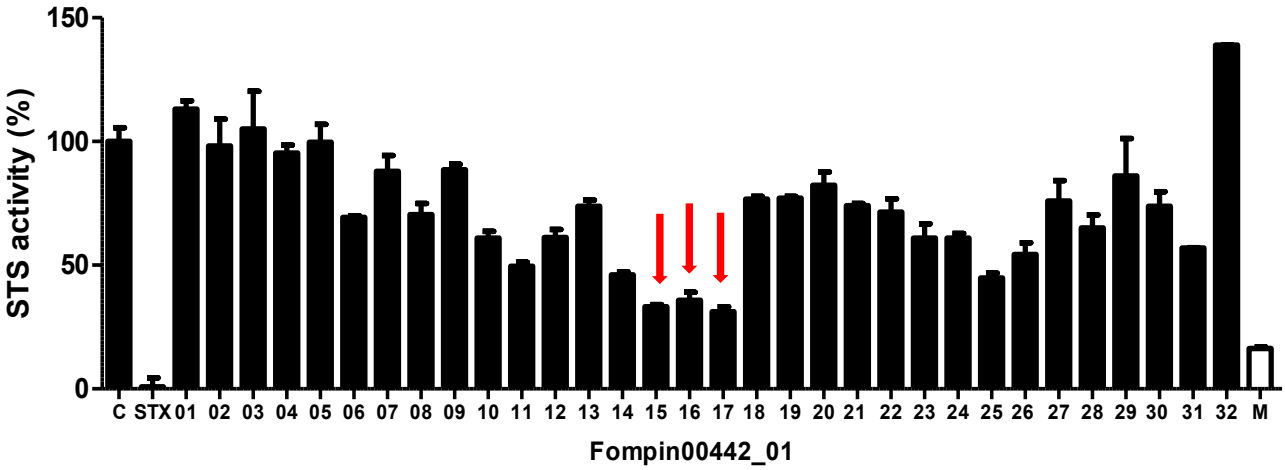


Figure 40 STS activity testing results

3.2.6. Sample preparation for ^{1}H NMR analysis

For ^{1}H NMR sample preparation the aim was to transfer an aliquot of approximately 2,8 mg of each fraction to pre-labelled and pre-weighed glass vials closed by a lid. Therefore 1000 µL HPLC quality methanol was added to each fraction and the required µL to remove approximately 2,8 mg was calculated and adjusted for each fraction according to its yield (see Table 14, Page 53). The 32 glass vials were dried over night by using a sample concentrator (for the final yield of each fraction see Table 16) and were stored in the desiccator.

To begin with the sample preparation I labelled the NMR tubes with a permanent marker and cleaned the outside of the tubes with a paper cloth and EtOH and put it in the NMR stand.

Our goal was to obtain a concentration of approximately 2.8 mg / 900 µL for each fraction. According to the desired concentration, the following Table 2 describes the yield in each glass vial and the needed amount of solvent (deuterated MeOH).

Table 2 needed amount of solvent for NMR measurement, Fompin00442_01_05 (marked in green) has the lowest yield and therefore the lowest amount of needed solvent, the same amount of solvent (645 µL) was used for all fractions to ensure a uniform concentration.

Sample	Yield [mg]	Amount solvent needed [µL]
Fompin00442_01_01	3,13	1006
Fompin00442_01_02	2,17	698
Fompin00442_01_03	2,21	710
Fompin00442_01_04	2,48	797
Fompin00442_01_05	**2,02**	**649**
Fompin00442_01_06	2,22	714
Fompin00442_01_07	2,22	714
Fompin00442_01_08	2,44	784
Fompin00442_01_09	2,69	865
Fompin00442_01_10	2,77	890
Fompin00442_01_11	3,33	1070
Fompin00442_01_12	2,84	913
Fompin00442_01_13	2,54	816
Fompin00442_01_14	2,77	890
Fompin00442_01_15	2,27	730
Fompin00442_01_16	3,29	1058
Fompin00442_01_17	3,11	1000
Fompin00442_01_18	3,11	1000
Fompin00442_01_19	2,80	900
Fompin00442_01_20	3,02	971
Fompin00442_01_21	3,02	971
Fompin00442_01_22	3,04	977
Fompin00442_01_23	2,91	935
Fompin00442_01_24	3,52	1131
Fompin00442_01_25	2,32	746
Fompin00442_01_26	3,18	1022
Fompin00442_01_27	2,76	887
Fompin00442_01_28	2,75	884

Fompin00442_01_29	3,13	1006
Fompin00442_01_30	2,90	932
Fompin00442_01_31	3,45	1109
Fompin00442_01_32	3,39	1090

Therefore I used the MeOD-only Hamilton syringe to add 900 µL of methanol-d4 into the glass vials. It was important to remove the lid of the glass vials shortly before adding the solvent, to ensure no humidity to come in and no evaporation.

Further I took the Eppendorf pipette and set the volume to 900 µL.
900 µL was taken out of each glass vial and put into pre-labelled and pre-weighed Eppendorf tubes. Afterwards the tubes were put into the centrifuge (3000 rpm/5 min). After the centrifugation, I recognized a precipitation inside of some Eppendorf tubes. So I tried to change the used Eppendorf tubes (company VWR) to Eppendorf tubes from the manufacturer **Sarstedt**. The affected fractions were newly prepared as already described. No precipitation occurred on the second attempt with Eppendorf tubes from **Sarstedt**.

Since Fompin00442_01_**05** has the lowest concentration of 2.02 mg, the same amount of solvent (645 µL) was used for all fractions to ensure a uniform concentration. After centrifugation, 645 µL of each Eppendorf tube was transferred to the corresponding NMR tube, which was then closed.

Unfortunately, one fraction (Fompin00442_01_**06**) contained more precipitation than expected, so it was impossible for me to collect 645 µL. After consulting Prof. Dr. Emmanuel Mikros about the problem, I tried to carefully collect as much as possible (see Figure 42). The remaining rest of the Eppendorf tubes were poured back to the original glass vials. Further, I washed the Eppendorf tubes twice with HPLC quality methanol, to ensure that nothing of the remaining precipitate was lost.

I transferred the rest of the tubes back to the original glass vials and handed the prepared tubes to Univ.-Prof. Mag. Dr. Ernst Urban (University of Vienna) for ^{1}H NMR measurement.

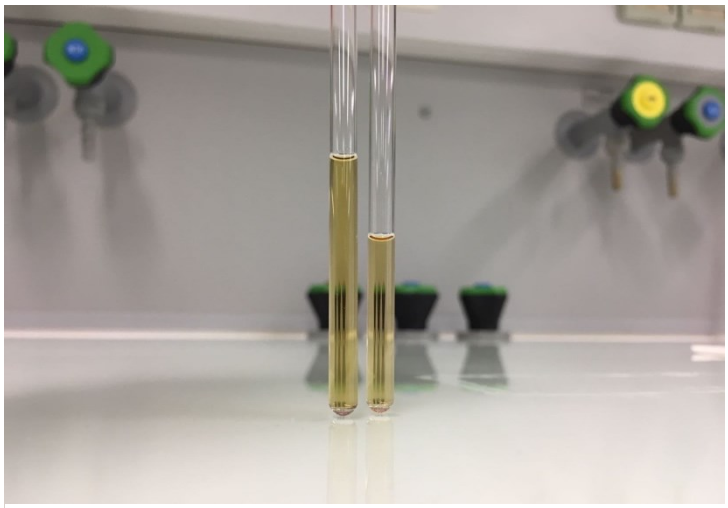


Figure 41 The NMR tube of Fompin00442_01_06 contained less than the other fractions

3.2.6.1. HPLC sample preparation for further HPLC-MS analysis

After ^{1}H NMR testing we decided to analyse the prepared NMR samples by HPLC-MS and to perform TLC monitoring. The NMR tubes were opened and the content transferred back to the 32 pre-labelled and pre-weighed glass vials and washed with 500 µL HPLC quality methanol, to ensure nothing was lost. The glass vials were then dried by a sample concentrator for 2 days.

645 µL HPLC quality methanol were added to each glass vial, 50 µL were transferred to HPLC vials and were filled up to 200 µL by adding 150 µL HPLC quality methanol. All fractions were prepared with the same procedures except fraction Fompin00442_01_15. In this fraction 10 µL were unintentionally previously taken and discarded. Therefore 635 µL instead of 645 µL were added to the glass vial, further 50 µL were transferred to HPLC vials and filled up to 200 µL with HPLC quality methanol. For further HPLC-MS analysis I handed the vials to Dr. Ammar Tahir, University of Vienna, Department of Pharmacognosy.

3.2.6.2. Additional TLC analysis of NMR samples

In addition to the preparation of the samples for HPLC-MS analysis, I monitored the content of the 32 glass vials that contained the NMR samples. I used the same TLC system and parameters then in the previous TLC monitoring (see Chapter 5.3.3 “TLC system optimization and analysis”, Page 48). The TLC pattern shows how well the separation worked.

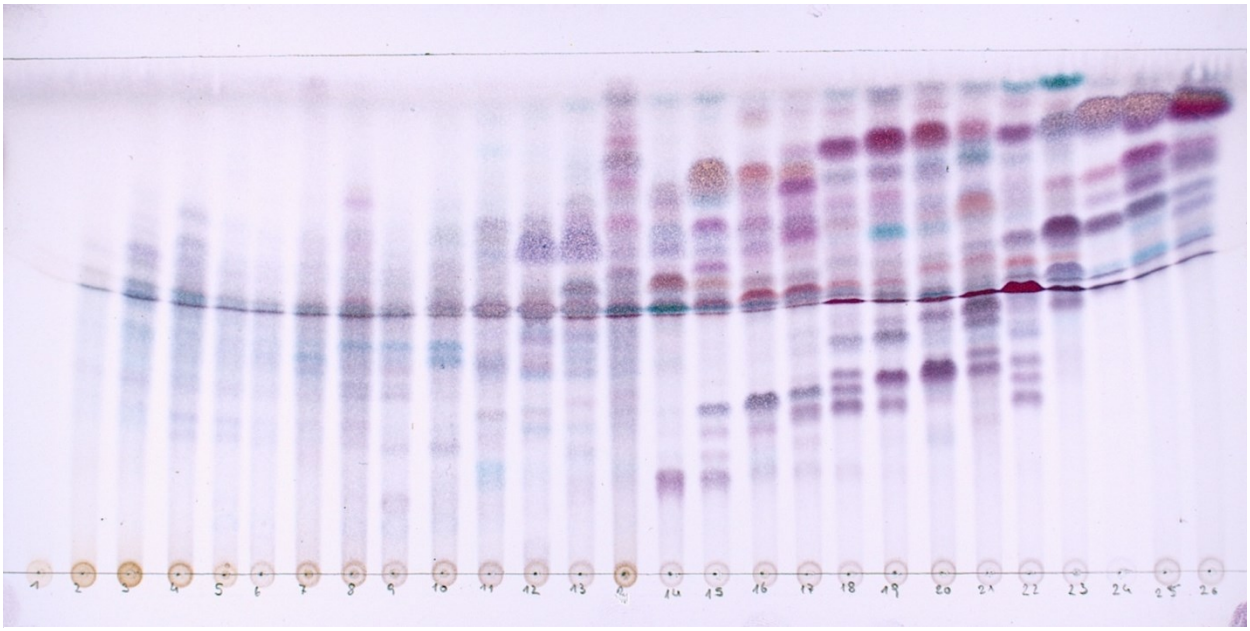


Figure 42 TLC monitoring of 32 NMR samples, Number 1-26, after derivatization, UV/VIS

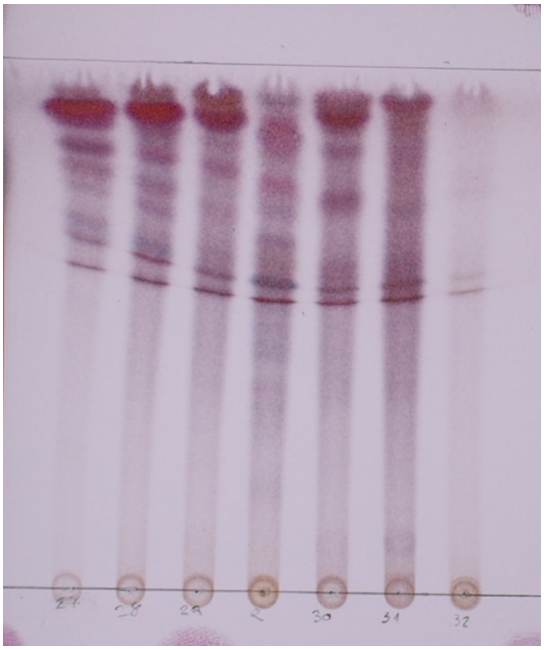


Figure 43 TLC monitoring of 32 NMR samples, Number 27-32, after derivatization, UV/VIS

4. Discussion and Conclusion

4.1. Mycochemical investigation of *Hygrocybe conica*

This part of my diploma thesis dealt with the chemical investigation of *H. conica*. This thesis covers the development of a TLC and HPLC system for the analysis of contained constituents. In particular, the focus was to identify known secondary metabolites. *H. conica* is not thoroughly described in literature. It seems that a large part of its constituents has not been identified yet, which suggests that this fungus remains an interesting research area.

Despite the limited amount of sample, it was still possible to obtain some data through MS analysis and dereplication. However, many detected masses could not be compared with known compounds. This either hints to new unknown natural compounds or derivates of known structures of *H. conica*. In sum, *H. conica* provides a big potential for further investigations.

4.2. NMR based heterocovariance analysis of *Fomitopsis pinicola* fractions

The main part of my diploma thesis was to separate a bioactive extract of *Fomitopsis pinicola* into microfractions, to use biochemometric tools to identify the responsible bioactive compounds prior to isolation.

Investigations were done by using different chromatographic techniques, such as TLC, HPCCC and flash chromatography. Due to technical problems with the HPCCC instrument, we were forced to find another proper separation method, therefore the flash chromatographic system PuriFlash was chosen. The extract Fp00442c was separated and collected into 125 tubes (15 ml). The monitoring of obtained fractions was performed by TLC.

The tubes were combined due to their TLC pattern into 32 microfractions and investigated further by ^{1}H NMR. It was a challenge to establish a sample preparation protocol for a successful biochemometric processing. Some obstacles had to be overcome, but at the end of my work I developed a well-functioning NMR sample preparation protocol.

Further research concerning the isolation and identification of the active compounds of *F. pinicola* (FP00442c) were ongoing at the time of finishing my practical work at the department (Grienke et al, 2019).

5. Material and Methods

This chapter includes details of all used materials and methods which were mentioned in chapter 3 “Results”.

5.1. Instruments, Solvents and Reagents

5.1.1. Instruments

Table 3 Instruments

Device	Technical Features
Analytical Balance	Sartorius BP210D
CAD	Charged Aerosol Detector, Corona Ultra RS
Centrifuge	Mini spin for Eppendorf vials
Centrifuge	Laborfuge 400, Heraeus function line
Hot Air Blower	HG 2000 E, Steinel
HPLC	Dionex Ultimate 3000, Software: Chromeleon 7
Flash Chromatography	Interchim PuriFlash 4250, Software: Interchim soft
Mass Spectrometer	Bruker Daltonics Esquire 3000 plus Ion Trap
NMR	Bruker NMR 500 MHz
Rotary Evaporator	Rotavapor R-210, Büchi
TLC Spray Cabinet	CAMAG® TLC Spray Cabinet II
TLC Visualizer	CAMAG®
Ultrasonic Bath	Transsonic T460, Elma
UV Lamp	CAMAG®
Vacuum Pump	V-710, Büchi
Vortex	Genius 3, IKA

5.1.2. Solvents and Reagents

Table 4 Solvents/ Reagents

Solvent/ Reagents	Description
Acidic Water	99% H_2O, 0,9% AA, 0,1% FA
Acetic Acid	Carl Roth GmbH, 99%
Acetone	Rectapur, ÖAB, distilled
Acetonitrile for HPLC	HiPerSolv Chromanorm
DMSO	Emsure, Merck
Dichlormethane	Rectapur, ÖAB, distilled
Ethanol 96%	ÖAB, distilled
Ethyl acetate	Rectapur, ÖAB, distilled
Formic Acid	99%
H_2O for HPLC	Purified by exchanger
Methanol	ÖAB, distilled
Methanol for HPLC	HiPerSolv Chromanorm
n-Hexane	Normapur
Sulfuric Acid	ACS Reagent, 95.0-98.0, Sigma-Aldrich
Vanillin	Reagent Plus, 99% Sigma-Aldrich

5.1.3. Column Material

- Interchim prepacked column, C_{18} HQ Column (15 µm, 35 g)
- Silica gel 60 (0.040-0.063 mm) Merck

5.1.4. Spraying Reagents for TLC

- Solution 1: 5% H_2SO_4 in MeOH
- Solution 2: 1% Vanillin in MeOH

5.1.5. TLC plates

- TLC Silica gel 60 F_{254},
    - Aluminium sheets 20x20 cm, 0.2

5.2.Chromatographic techniques

5.2.1. Thin Layer Chromatography

Thin Layer chromatography (TLC) is a very simple chromatography technique and gives a first indication of possible constituents of a sample. Aluminium sheets are used as TLC plates and are coated with an adsorbent, which represents the stationary phase. First, the sample will be applied at a starting point next to a reference sample, then the plate will be placed in a vessel filled with solvents, which represents the mobile phase. The solvent s start slowly running upwards along the plate as sharp front from one edge to the opposite edge of the plate. During this process the constituents are separated in terms of their solubility/polarity and adsorption to the stationary phase. The finished chromatogram is then visually inspected at daylight or UV light. Derivatisation with e.g. vanillin-sulfuric acid reagent can improve the visibility of components especially when they are not visible in UV or daylight (Kandyoti et al., 2017).

5.2.2. High Performance Liquid Chromatography

HPLC is a very important and commonly used chromatography technique for the separation and analysis of mixtures. A column filled with adsorbent material represents the stationary phase and the mobile phase is formed by liquids. The sample elutes with the mobile phase continuously flowing through the column. The individual components of the sample migrate unequally fast and interact differently with the adsorbent material. This leads to a proper separation of the constituents as they elute differently out of the column. At the end of a HPLC run, a chromatogram is received. The individual signals are called peaks.

5.2.3. High Performance Counter Current Chromatography

High Performance Counter Current Chromatography is based on the liquid-liquid separation principle of two immiscible phases. For this technique a liquid stationary phase, a liquid mobile phase and a centrifugal force field is used for a proper separation. Due to the centrifugal force field the liquid stationary phase is retaining, while the mobile phase is pushed through. The aim is to effectively distribute the components between the two phases.

As with classic column technology, there is a stationary phase and a mobile phase, which can be defined depending on the task.

According to the HEMWat Table, it is possible to test different solvent systems to find a suitable one depending on the task.

Table 5 HEMWat Table

HEMWat System No.		Hexane	Ethyl Acetate	Methanol	Water	Butanol
1	More Polar ↑	0	0	0	5	5
2		0	1	0	5	4
3		0	2	0	5	3
4		0	3	0	5	2
5		0	4	0	5	1
6		0	1	0	1	0
7		1	19	1	19	0
8		1	9	1	9	0
9		1	6	1	6	0
10		1	5	1	5	0
11		1	4	1	4	0
12		1	3	1	3	0
13		2	5	2	5	0
14		1	2	1	2	0
15		2	3	2	3	0
16		5	6	5	6	0
17	Entry point	1	1	1	1	0
18	↓ Less Polar	6	5	6	5	0
19		3	2	3	2	0
20		2	1	2	1	0
21		5	2	5	2	0
22		3	1	3	1	0
23		4	1	4	1	0
24		5	1	5	1	0
25		6	1	6	1	0
26		9	1	9	1	0
27		19	1	19	1	0
28		1	0	1	0	0

5.2.4. Flash Chromatography

Flash chromatography is an alternative to slow and inefficient chromatography techniques. In flash chromatography, organic substance mixtures are separated using low pressure. The mobile phase (liquid) is pushed by pressure, through the solid stationary phase, which is represented by e.g. a silica adsorbent packed column (Andrews R. S. et al., 1986). During this process the sample is eluting downward based on its affinity to the solvent and the sorbent. The eluent is then collected in numerous fractions. Nowadays, an evaporative light-scattering detector (ELSD) or photodiode array (PDA) detectors are added to the flash system for better detection (flash-purification.com, accessed August 2019).

5.3. *Hygrocybe conica*

5.3.1. Fungal material

The fungus material for my diploma thesis has been provided by Dr. med. Harro Danninger. The dried and powdered sample is from Tyrol (Austria).

5.3.2. Extract preparation

To prepare the extract of *H. conica* 150,26 mg of the dried mushroom was filled into glass centrifugation tubes and 15 ml of EtOH 96% were added. The tubes were than covered with aluminium foil and subsequently put into a sonic bath for 20 minutes at room temperature. Further the tubes were centrifugated for 5 minutes at 3.500 rpm, the supernatant fluid was filtered through a pasteur pipette (stuffed with a cotton woo)l and directly collected in a pear shaped flask. The *H. conica* material was washed with 10 ml EtOH. On the rotary evaporator the EtOH extract was reduced and transferred to prepared glass vials (pre-weighed, pre-labelled). The amount obtained is 12,69 mg.

5.3.3. TLC system optimization and analysis

The used TLC system consists of a combination of dichloromethane, methanol and FA (10:0,25:0,25) and was performed without chamber saturation. The detection was performed with using the spraying reagents methanolic sulfuric acid 5% and methanolic vanillin 1%, then the TLC plates were dried for 8 min at 80°C.

Table 6 TLC system parameters

Stationary Phase	TLC Silica Gel 60 F_{254}
Mobile Phase	DCM: MeOH : FA (10:0,25:0,25)
Spraying reagents	5% methanolic sulfuric acid, 1% vanillin in MeOH
Detection	UV_{254}, UV_{366}, VIS

5.3.4. HPLC system optimization and analysis

The following tables (Table 7 and 8) describe the parameters of the developed HPLC method for the investigation of *H. conica* extract.

Table 7 Parameters of developed HPLC-method

HPLC Device	Stationary Phase	Temperature in Cº	Flow rate (ml/min)	Injection volume (µL)	Detection	Detection wavelength
Dionex Ultimate 3000 RS	Zorbax (4.6 x 150 nm, 3-5 Micron)	35	1.0	10	CAD, UV	210

Table 8 Parameters for optimized method

Time (min)	Mobile Phase A (%) = Acidic Water (H_2O + 0,9% FA + 0,1% AA)	Mobile Phase B (%) = Acetonitrile
0.00	98	2
4.00	95	5
9.00	20	80
12.0	15	85
26.0	2	98
35	98	2

5.4. *Fomitopsis pinicola*

5.4.1. Fungal material

The fruit bodies of *Fomitopsis pinicola* were collected in August 2013 at 850 m in Viggartal, Ellbögen, Austria and identified by U. Peintner (Institute of Microbiology, University of Innsbruck, Austria).

5.4.2. HPCCC system development

5.4.2.1. TLC System for *Fomitopsis pinicola*

At the beginning of my diploma thesis, a separation of the crude extract Fompin00442c by HPCCC was envisaged. Therefore, a TLC system was implemented to guide the establishment of a proper HPCCC separation system using the HEMWat Table (see Table 5). The following table (Table 9) describes the parameters of used TLC system. The TLC was performed without chamber saturation.
The detection was performed with methanolic sulfuric acid 5% and methanolic vanillin 1%, then the TLC plates were dried for 8 min at 80°C.

Table 9 Parameters of used TLC system

Stationary Phase	TLC Silica Gel 60 F_{254}
Mobile Phase	DCM: MeOH: H_2O (10:1:0,25)
Spraying reagents	5% Methanolic sulfuric acid, 1% Vanillin in MeOH
Detection	UV_{254}, UV_{366}, VIS

5.4.3. HPLC analysis of *Fomitopsis pinicola*

After finding a proper solvent system for HPCCC by using the HEMWat Table, a HPLC analysis was performed. Therefore, aliquotes of the upper and the lower phase of each HEMWat test system used were injected into the HPLC device (see Chapter 7 “Appendix”).

Table 10 Parameters of developed HPLC-method: Fompin_03_17

HPLC Device	Stationary Phase	Temperature in Cº	Flow rate (ml/min)	Injection volume (µL)	Detection	Detection wavelength
Dionex Ultimate 3000 RS	Zorbax (4.6 x 150 nm, 3-5 Micron)	35	1.0	10	CAD, UV	210

Table 11 Parameters for optimized method

Time (min)	Mobile Phase A (%) = Acidic Water (H_2O + 0,9% FA + 0,1% AA)	Mobile Phase B (%) = Acetonitrile
0.00	95	5
2.00	55	45

11.00	35	65
21.00	30	70
30.00	10	90
40.00	2	98
41.00	95	5

5.4.4. Establishing an HPCCC system

First the column must be filled with stationary phase, therefore the mixture MeOH : H_2O (1:1) which is used for storage has to be removed from the tubings. The machine is then filled with 2 column volumes (=50 ml) of stationary phase, while rotating at 200 rpm. Afterwards, a centrifugal force (by rotating at 1600 rpm) is implemented and the mobile phase is pumped into the system. During this step, a dynamic equilibrium between stationary and mobile phase is established.

5.4.4.1. Separation by analytical HPCCC

Composition of System 20		
Solvent	**100 ml Stationary phase** (= lower phase)	**100 ml Mobile phase** (= upper phase)
Hexane	0,73 ml	84,77 ml
Ethyl Acetate	18,04 ml	14,13 ml
Methanol	53,94 ml	1,03 ml
H_2O	27,29 ml	0,06 ml

Applied sample quantity: 50 mg
Column volume: 25 ml
Volume of sample loop: 3 ml
Flow Rate: 1 ml/min
Pressure: 145 psi
Rotation speed: 1600 rpm
Pumping direction: upper phase = mobile phase (normal phase)
60 fractions collected at 2 ml/tube

Composition of System 21		
Solvent	**100ml Stationary phase** (= lower phase)	**100ml Mobile phase** (= upper phase)
Heptane	1,07 ml	89,01 ml
Ethyl Acetate	16,224 ml	9,98 ml
Methanol	59,32 ml	0,97 ml
H_2O	23,40 ml	0,04 ml

Applied sample quantity: 50 mg
Column volume: 25 ml
Volume of sample loop: 3 ml
Flow Rate: 1 ml/min
Pressure: 145 psi
Rotation speed: 1600 rpm
Pumping direction: upper phase = mobile phase (normal phase)
70 fractions collected at 2 ml/tube

5.5. PuriFlash Microfractionation

The device used for this diploma thesis is the Interchim puriFlash© 4250 (see Figures 28, 29 and Table 4). The stationary phase was represented by a PURIFLASH COLUMN 15 C 18 HQ 35G – 35.0 g (22 bar) and a gradient system of ACN/H_2O as mobile phase. The different parameters and solvent gradients are shown in Table 12 and Table 13. The sample (740 mg) was dissolved in MeOH and then carefully mixed with silica gel. The excess solvent was then removed by evaporation. The sample/silica mixture was then applied to the dry load cartridge. Furthermore, the cartridge was then inserted into the device. As detectors served an evaporative light scattering detector (ELSD) and a photo diode array (PDA) detector.

Flash chromatography of the sample Fompin00442c

Table 12 Flash chromatography paramters

Column	PuriFlash Column 15 C 18 HQ 35G – 35.0 g (22 bar)
Applied amount	740.00 mg
Method name	Fompin_02
Flow rate	15 ml/min

Table 13 mobile phase and gradient

Time (min)	% A (H_2O)	% B (Acetonitrile)
00 s	95	05
15:00	95	05
30:00	55	45
50:00	35	65
01:10:00	30	70
01:30:00	10	90
01:50:00	02	98

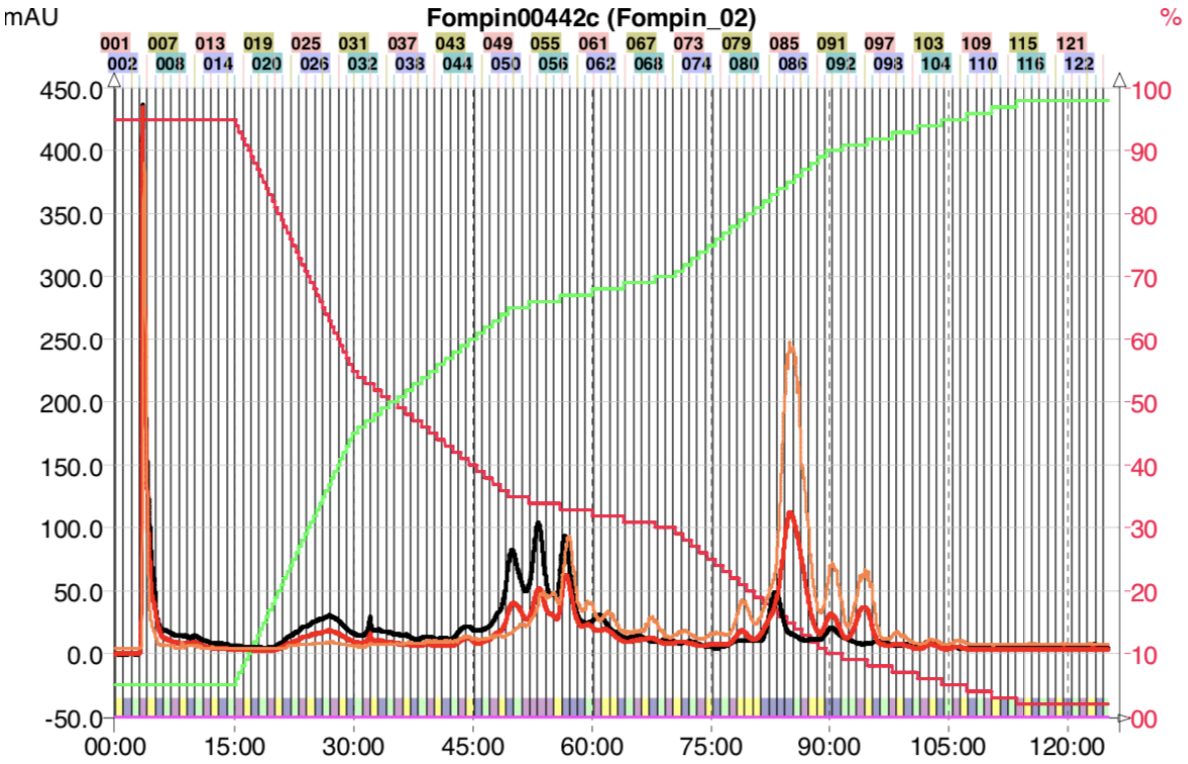


Figure 44 Flash chromatogram; Method: Fompin_02, solvents: Water (red), ACN (green), Detection: ELSD (orange), UV_{245nm} (black)

Table 14 Overview of the 125 collected fractions

Sample	Collected Fractions	Yield [mg]
Fompin00442c_01_01	3,4,5	70,11
Fompin00442c_01_02	6,7,8	22,54
Fompin00442c_01_03	9,10,11	11,18
Fompin00442c_01_04	12- 24	14,94
Fompin00442c_01_05	25,26	3,59
Fompin00442c_01_06	27,28,29	5,62
Fompin00442c_01_07	30,31,32	10,40
Fompin00442c_01_08	33,34,35	4,08
Fompin00442c_01_09	36,37,38	3,50
Fompin00442c_01_10	39,40,41 (42,43,44)	6,92
Fompin00442c_01_11	45,46,47 (42,43,44)	6,93
Fompin00442c_01_12	48,49,50	9,07
Fompin00442c_01_13	51,52,53	16,00
Fompin00442c_01_14	54,55,56	22,94
Fompin00442c_01_15	57,58,59	30,31
Fompin00442c_01_16	60,61,62	11,10

Fompin00442c_01_17	63,64,65	5,22
Fompin00442c_01_18	66,67,68	9,31
Fompin00442c_01_19	69,70,71	5,45
Fompin00442c_01_20	72,73,74	4,86
Fompin00442c_01_21	75,76,77	3,83
Fompin00442c_01_22	78,79,80	11,38
Fompin00442c_01_23	81,82,83	14,50
Fompin00442c_01_24	84,85,86	65,08
Fompin00442c_01_25	87,88,89	30,63
Fompin00442c_01_26	90,91,92	22,58
Fompin00442c_01_27	93,94,95	19,36
Fompin00442c_01_28	96,97,98	5,29
Fompin00442c_01_29	99,100,101	3,58
Fompin00442c_01_30	102,103,104	3,61
Fompin00442c_01_31	105-125	18,15
Fompin00442c_01_32	NL	6,88

5.5.1. Prepared fractions for STS inhibition testing

Table 15 Fractions for STS inhibition testing

Sample	Amount (mg)	Sample	Amount (mg)
Fompin00442c_01_01	2,41	Fompin00442c_01_17	1,25
Fompin00442c_01_02	1,21	Fompin00442c_01_18	1,18
Fompin00442c_01_03	1,15	Fompin00442c_01_19	1,20
Fompin00442c_01_04	1,34	Fompin00442c_01_20	1,10
Fompin00442c_01_05	1,02	Fompin00442c_01_21	1,48
Fompin00442c_01_06	1,48	Fompin00442c_01_22	1,31
Fompin00442c_01_07	1,09	Fompin00442c_01_23	1,40
Fompin00442c_01_08	1,21	Fompin00442c_01_24	1,36
Fompin00442c_01_09	1,17	Fompin00442c_01_25	1,10
Fompin00442c_01_10	1,62	Fompin00442c_01_26	1,45
Fompin00442c_01_11	1,60	Fompin00442c_01_27	1,18
Fompin00442c_01_12	1,35	Fompin00442c_01_28	1,20
Fompin00442c_01_13	1,25	Fompin00442c_01_29	1,24
Fompin00442c_01_14	1,20	Fompin00442c_01_30	1,02
Fompin00442c_01_15	1,20	Fompin00442c_01_31	1,20
Fompin00442c_01_16	1,70	Fompin00442c_01_32	1,00

5.5.2. Prepared fractions for NMR measurements

Table 16 Prepared fractions for NMR measurements

Sample	Amount (mg)	Sample	Amount (mg)
Fompin00442c_01_01	3,13	Fompin00442c_01_17	3,11
Fompin00442c_01_02	2,17	Fompin00442c_01_18	3,11
Fompin00442c_01_03	2,21	Fompin00442c_01_19	2,80
Fompin00442c_01_04	2,48	Fompin00442c_01_20	3,02
Fompin00442c_01_05	2,02	Fompin00442c_01_21	3,02
Fompin00442c_01_06	2,22	Fompin00442c_01_22	3,04
Fompin00442c_01_07	2,22	Fompin00442c_01_23	2,91
Fompin00442c_01_08	2,44	Fompin00442c_01_24	3,52
Fompin00442c_01_09	2,69	Fompin00442c_01_25	2,32
Fompin00442c_01_10	2,77	Fompin00442c_01_26	3,18
Fompin00442c_01_11	3,33	Fompin00442c_01_27	2,76
Fompin00442c_01_12	2,84	Fompin00442c_01_28	2,75
Fompin00442c_01_13	2,54	Fompin00442c_01_29	3,13
Fompin00442c_01_14	2,77	Fompin00442c_01_30	2,90
Fompin00442c_01_15	2,27	Fompin00442c_01_31	3,45
Fompin00442c_01_16	3,29	Fompin00442c_01_32	3,39

6. References

Books

Aktories, Förstermann, Hoffmann (2013), Allgemeine und spezielle Pharmakologie und Toxikologie 11. Auflage, Urban & Fischer, München: 884-964

Müller-Esterl W., (2011) Biochemie – Eine Einführung für Mediziner und Naturwissenschaftler, 2. Auflage, Springer, Frankfurt: 511-514

Mutschler et al., (2007) Anatomie Physiologie Pathophysiologie des Menschen 6. Auflage, Wissenschaftliche Vertragsgesellschaft, Deutschland, 115-123

Pahlow M., (2015) Das große Buch der Heilpflanzen, Nikol, Hamburg: 2013, 9-13

Strauss D., (2000) Chemie für die pharmazeutische Praxis 6. Auflage, Deutscher Apotheker Verlag, Stuttgart: 460-467

Diploma Thesis

Salzer D. (2017) Analysis and chemical Investigation of the bryozoan *Flustra foliaceae* and generation of a seperation protocol for polar and non-polar triterpenes from *Gloeophyllum odoratum*, University of Vienna

Ibrahim M. (2017) Separation of lanostane triterpenes from *Gloeophyllum odoratum*, University of Vienna

Patent

Andrews R. S. et al., (1986), Flash Chromatography, Patent Number: 4,591,442 *United States Patent,* Date of Patent: May 27, 1986

Dissertation

Rösecke J., (2000) Isolierung und Identifizierung von Inhaltsstoffen aus Holz zersetzenden Basidiomyceten, Dissertation, Universität Hamburg

Papers

Aligiannis, N.; Halabalaki, M.; Chaita, E.; Kouloura, E.; Argyropoulou, A.; Benaki, D.; Kalpoutzakis, E.; Angelis, A.; Stathopoulou, K.; Antoniou, S.; Sani, M.; Krauth, V.; Werz, O.; Schutz, B.; Schafer, H.; Spraul, M.; Mikros, E.; Skaltsounis, L. A., Heterocovariance based metabolomics as a powerful tool accelerating bioactive natural product identification. ChemistrySelect 2016, 1, (10), 2531-2535

Anastasiadi Z., Lianos GD., Ignatiadou E., Harissis HV., Mitsis M., (2017) Breast cancer in young women: an overview, *Updates in Surgery*, Vol 69, Issue 3, 313-317

Ames B. N., Gold L. S., Willett W. C., (1995) The causes and prevention of cancer, *PNAS*, Vol 92, Issue 12

Ardenne R., Döpp H., Musso H., Steglich W., (1974), Über das Vorkommen von Muscaflavin bei Hygrocybe (Agricales) und seine Dihydroazepin-Struktur, *Zeitschrift für Naurforschung,* Vol 29, 637-639

Britton, E. R.; Kellogg, J. J.; Kvalheim, O. M.; Cech, N. B., Biochemometrics to identify synergists and additives from botanical medicines: A case study with Hydrastis canadensis (goldenseal). J. Nat. Prod. 2018, 81, (3), 484-493

Chong E. L., Khoo H. E., et al., (2014), Antioxidative properties of an extract of *Hygrocybe conica*, a wild edible mushroom, *Malaysian Journal of Nutrition*, Vol 20, Issue 1, 101-111

Dahlin P., Srivastava V., Bulone V., McKee L. S., (09. Nov.2016), The Oxidosqualene Cyclase from the Oomycete Saprolegnia parasiticaSynthesizes Lanosterol as a Single Product; Front. Microbiol.

Demydchuk M., Hill C. H., Zhou A., et al., (2017), Insights into Hunter syndrome from the structure of iduronate-2-sulfatase, *Nature Communications*, Vol 8, Article number 15786

Dutta S., MahalanobishS., Saha S., Gosh S., Sill P. C., (2019), Natural products: An upcoming therapeutic approach to cancer, *Food and Chemical Toxicology*, Vol 128, 240-255

Grienke U., Zöll M., Peintner U., Rollinger J. M., (2014), European medicinal polypores – A modern view on traditional uses. *Journal of Ethnopharmacology*, Vol 154 Issue 3, 564-583

Grienke U, Foster PA, Zwirchmayr J, Tahir A, Rollinger JM, Mikros E, (2019) ^{1}H NMR-MS-based heterocovariance as a drug discovery tool for fishing bioactive compounds out of a complex mixture of structural analogues. *Scientific Reports*; 9: 11113

Gaudencio, S. P.; Pereira, F., Dereplication: racing to speed up the natural products discovery process. Nat. Prod. Rep. 2015, 32, (6), 779-810

Fäldt J., Jonsell M., Nordlander G., Borg-Karlson A., (1999), Volatiles of Bracket Fungi *Fomitopsis pinicola* and *Fomes fomentarius* and Their Functions as Insect Attractants, *Journal of Chemical Ecology*, Vol. 25, Issue 3, 567-590

Jin M. L., Lee W. M., Kim O. T., (2017), Two Cycloartenol Synthases for Phytosterol Biosynthesis in *Polygala tenuifolia* Willd, International Journal of molecular Science, Vol 18, Issue 11, 2426

Kandyoti R., Herod A., Morgan T., et al., (2017) Analytical techniques for low mass materials, Solid Fuels and Heavy Hydrocarbon Liquids (second Edition), 472

Keller A. C., Maillard M. P., Hostettmann K., (1996), Antimicrobial steroids from the fungus *Fomitopsis pinicola*, *Phytochemistry*, Vol 41, Issue 4, 1041-1046

Kushiro T., Ebizuka Y., (2010), Triterpenes, *Comprehensive Natural Products II*, Vol. 1, 673-708

Ling H., Zhou L., Jia X., Gapter LA, Agarwal R., Ng KY., (2009) Polyporenic acid C induces caspase-8-mediated apoptosis in human lung cancer A549 cells., *Mol Carcinog.,* Vol 48, issue 6: 498-508

Spencer K., Chard T., (2013) Pregnancy, *The Immunoassey Handbook* (Fourth Edition) 757-776

Striegler S., Haslinger E.,(1996), Cerebrosides from fomitopsis pinicola (Sw. Ex Fr.) Karst., Chemical Monthly, Vol. 127, 755-761

Usui T., Hosokawa S., Mizuno T. et al., (1981) Investigation of the Heterogeneity of Heterogalactan from the Fruit Bofird of Fomitpsis pinicola, by employing Concanavalin A-Sepharose affinity Chromatography, The Journal of Biochemistry, Vol 89, Issue 4, 1029-1037

Woolston C., (2015) Breast cancer, *Nature* volume 527, Page 101

Martens, H.; Bruun, S. W.; Adt, I.; Sockalingum, G. D.; Kohler, A., Pre-processing in biochemometrics: correction for path-length and temperature effects of water in FTIR bio-spectroscopy by EMSC. J. Chemom. 2006, 20, (8-10), 402-417

Merino Bonilla J. A., Torres Tabanera M., Ros Mendoza L. H. (ocotber -september 2017) Breast cancer in the 21st century: From early detection to new therapies, *Radiologia (English version)*, Vol. 59, Issue 5, 368-379

Newman, D. J.; Cragg, G. M., Natural products as sources of new drugs from 1981 to 2014. J. Nat. Prod. 2016, 79, (3), 629-661

Parihar J. S., Kim I. Y., (2016) Chapter 57 - Second-Line Hormonal for Castrate-Resistant Prostate Cancer, *Prostate Cancer (second edition)*, 533-540

Pereira DM., Correia-da-Silva G., Valentao P., Teixeira N., Andrade PB., (2014), Anti-inflammatory effect of unsaturated fatty acids and Ergosta-7,22,dien-3-ol from Marthasterias glacialis: prevention of CHOP-mediated ER-stress and NF-$_k$B activation. *PLos One,* Vol. 9, Issue 2

Poirier D., Roy J., Maltais R., Ayan D., (2015), A Potent Inhibitor of Steroid Sulfatase (EM-1913) Blocks Tumor Growth in Nude Mice (MCF-7 Xenograft), Current Enzym Inhibition 11 (1)

Reed M. J., Purohit A., Woo L. W. L., Newman S. P., Potter B. V. L., (2005) Steroid Sulfatase: Molecular, Biology, Regulation and Inhibition, *Endocrine Reviews* Vol. 26, Issue 2 171-202

Webb P., Jordan S., (2017) Epidemiology of epithelial ovarian cancer, Best Practice & Research *Clinical Obstetrics & Gynaecology*, Vol. 41, 3-14

Sariwati A., Purnomo AS., Kamei I., (2017), Abilities of Co-cultures of Brown-Rot Fungus Fomitopsis pinicola and Bacillus subtilis on Biodegradation of DDT., Current Microbiology, Vol. 74, Issue 9, 1068–1075

Sawai S., Akashi T., Sakurai N., et al., (2006) Plant Lanosterol Synthase: Divergence of the Sterol and Triterpene Biosynthetic Pathways in Eukaryotes, *Plant and Cell Physiology*, Vol 47, Issue 5, 673-677

Stanway S. J., Delavault P., Purohit A., Woo L. W. L., Thurieau C., Potter B. V. L., Reed M. J. (2007), Steroid Sulfatase: A new Target for Endocrine Therapy of Breast Cancer, *The Oncologist,* Vol 12 no. 4, 370-374

Schüler-Toprak S., Treeck O., Ortmann O., (2017), Human Chorionic Gonadotropin and Breast Cancer, *International Journal of molecular Science*, Vol 18, Issue 7, 1587

Kim S.-J. , Karadeniz F., (2012), Chapter 14 – Biological Importance and Applications of Squalene and Squalane, *Advances in Food and Nutrition Research*, Vol 65, 223-233

Shi Z.T., Bao H.Y., Feng S., (2017) Anti tumor activity and structure-activity relationship of seven lanostane-type triterpenes from Fomitopsis pinicola and F. officinalis, *Chinese Pharmaceutical Association*

Steglich W., Preuss R., (1975), L-3,4-dihydroxyphenylalanine from carpophores of *Hygrocybe conica* and *H. ovina*, *Phytochem.,* Vol 14, Issue 4, 1119

Strauss J. F., FitzGerald G.A., (2019) Chapter 4 – Steroid Hormones and Other Lipid Molecules Involved in Human Reproduction, Reproductive Endocrinology (Eighth Edition), 75-114.e7

Terradas F., Wyler H., (1991), The secodopas, natural pigments in *Hygrocybe conica* and *Amanita muscaria*, *Phytochem.,* Vol 30, Issue 10, 3251-3253

Vieira D. C., Gomes M., Vinicius M., Barrosde O., et al. (2019), Antioxidant, anti-inflamatory and cytotoxic/ antitumoral bioactives from the phylum Basidiomycota and their possible mechanism of action, *Biomedicine & Pharmacotherapy*, Vol. 112, 108-643

Wang Y., Cheng X., Wang P., Wang L., Fan J., Wang X., Liu Q., (2014). Investigating Migration Inhibition and Apoptotic Effects of *Fomitopsis Pinicola* Chloroform Extract on Human Colorectal Cancer SW-480 Cells, *PLOS ONE 9 (7)*

Wen C., Wu L., Fu L., Wang B., Zhou H., (2017), Unifying mechanism in the initiation of breast cancer by metabolism of estrogen , *Journal of molecular medicine*, Vol. 16, Issue 2, 1001-1006

Wong Jin Yi, Fook Yee Chye, (2009), Antioxidant properties of selected tropical wild edible mushrooms, Journal of Food Composition and Analysis, Vol. 22, 269-277

Zhang Q., Huang N., Wang J., Luo H., He H., Ding M., et al., (2013) The H+/K+-ATPase inhibitory activites of Trametenolic acid B from *Trametes lactinea* (Berk.) Pat, and its effects on gastric cancer cells, *Fitoterapia,* Vol. 89, 210-217

Zhang Q., Wang J., He H., Liu H., Yan X., Zou K., (2014) Trametenolic acid B reverses multi drug resistance in breast cancer cells through regulating the expression level of P-glycoprotein. *Phytother Res*, Vol 28, Issue 7, 1037-1044

Websites

http://www.svims.ca/council/illust/Fomitopsis%20pinicola%205%20Michael%20Beug.htm, (accessed December 2017)

https://www.siegigerstner.at/naturfuehrer/reich-der-pilze/basidienpilze/staenderpilze/hut-pilze/porenpilze/rotrandiger-porling/) (accessed March 2018)

https://www.who.int/en/news-room/fact-sheets/detail/cancer (accsessed August 2019)

https://de.wikipedia.org/wiki/Terpene#/media/Datei:Isoprene_Structural_Formulae_V.1.svg (accessed March 2019)

https://de.wikipedia.org/wiki/Squalen#/media/Datei:Squalen_Strukturformel.svg (accessed October 2019)

https://www.researchgate.net/figure/Enzymatic-cyclisation-of-2-3-oxidosqualene-OS-to-lanosterol-LA-and-cycloartenol_fig6_309741817 (accessed August 2019)

https://www.wcrf.org/dietandcancer/cancer-trends/worldwide-cancer-data (accessed March 2019)

https://www.cancer.gov/about-cancer/causes-prevention/risk (accessed March 2019)

https://www.researchgate.net/figure/Carcinogenesis-phases-initiation-promotion-progression-and-metastasis-A-Initiation_fig2_279304092 (accessed August 2019)

https://www.researchgate.net/figure/Chemical-carcinogenesis-stages-and-the-occurrences-involved-in-each-one-modified-from_fig7_298444949 (accessed August 2019)

https://www.cancer.gov/about-cancer/causes-prevention/risk accessed in march 20193 (accessed in August 2019)

https://www.cancer.org/cancer/breast-cancer/treatment/hormone-therapy-for-breast-cancer.html (accessed September 2019)

https://www.frontiersin.org/files/Articles/179132/fphar-07-00030-HTML/image_m/fphar-07-00030-g001.jpg (accessed September 2019)

https://www.researchgate.net/figure/Fig-1-A-Key-role-of-steroid-sulfatase-STS-in-the-synthesis-of-estrogen-estradiol_fig1_280063068 (accessed September 2019)

https://www.natur-in-nrw.de/HTML/Pilze/Agaricales/PA-332.html (accessed March 2019)

https://en.wikipedia.org/wiki/Hygrocybe_conica (accessed September 2017)

http://www.erc-hplc.de/lc-systeme/verteilungschromatographie-hpccc.html (accessed August 2019)

https://www.med4you.at/laborbefunde/techniken/chromatographie/lbef_chromatographie_hplc_gc.htm (accessed August 2019)

https://patentimages.storage.googleapis.com/20/5e/af/6231b813f9b26a/US4591442.pdf (accessed August 2019)

http://www.flash-purification.com/detecting-the-undetectable-in-flash-column-chromatography-part-2/ (accessed August 2019)

https://patentimages.storage.googleapis.com/20/5e/af/6231b813f9b26a/US4591442.pdf (accessed October 2019)

https://www.mushroomexpert.com/polyporales.html, (accessed December 2017)

http://www.svims.ca/council/Polypo.htm, (accessed December 2017)

7. Appendix

7.1.HPLC Chromatograms of *Hygrocybe conica*

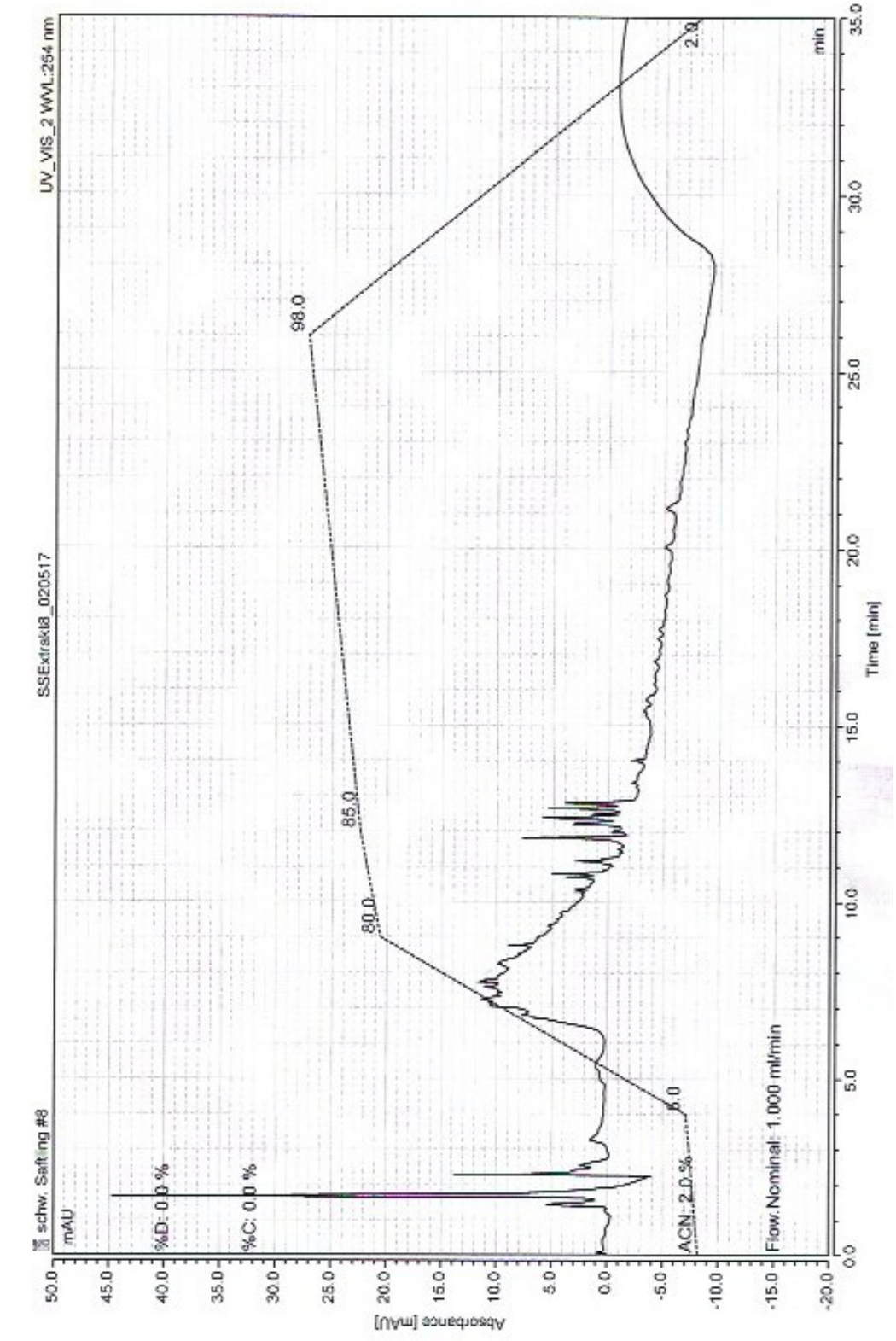


Figure 45 HPLC method optimization process

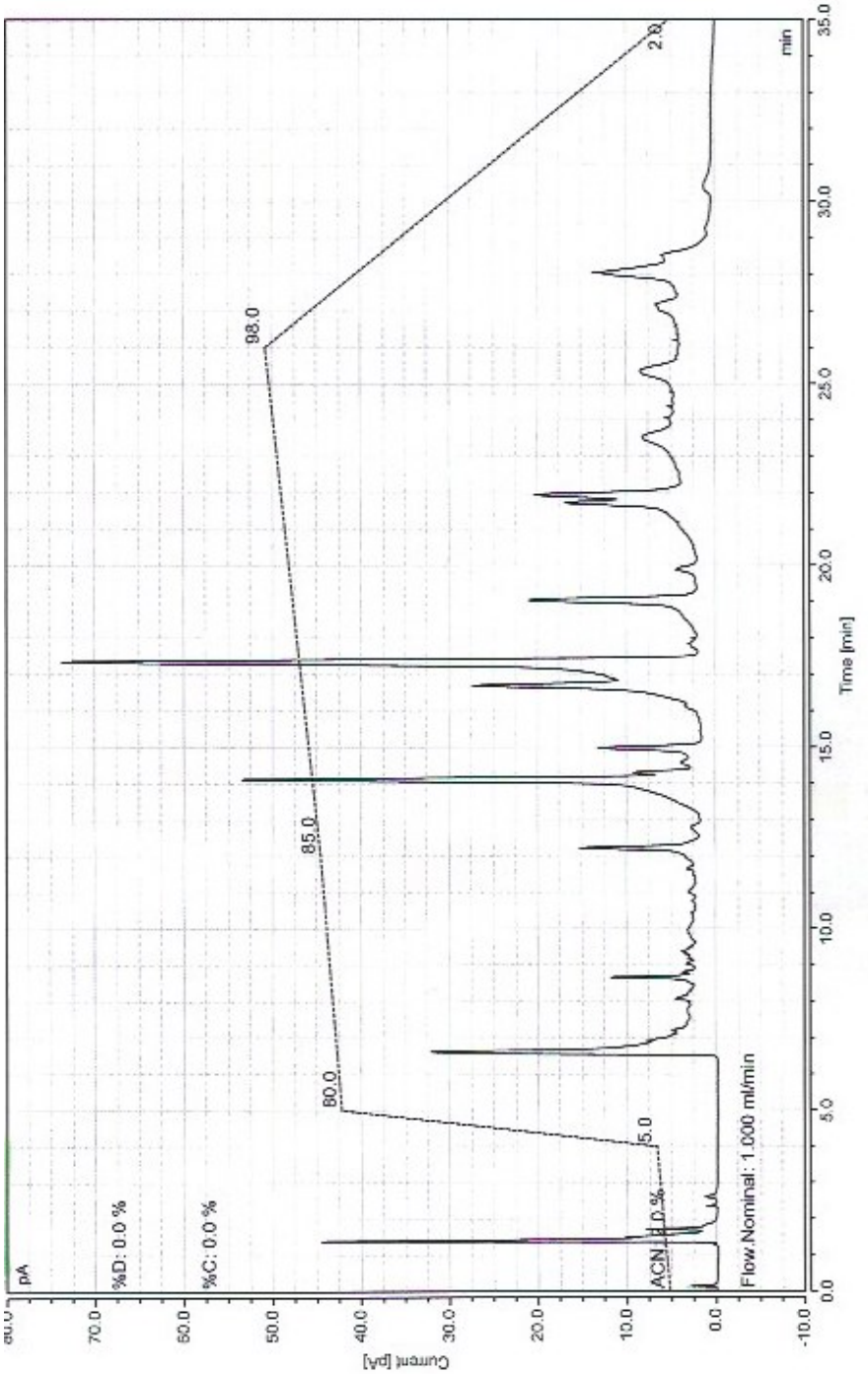


Figure 46 HPLC method optimization process, UV/VIS, 254nm

7.2.MS Data *Hygrocybe conica*

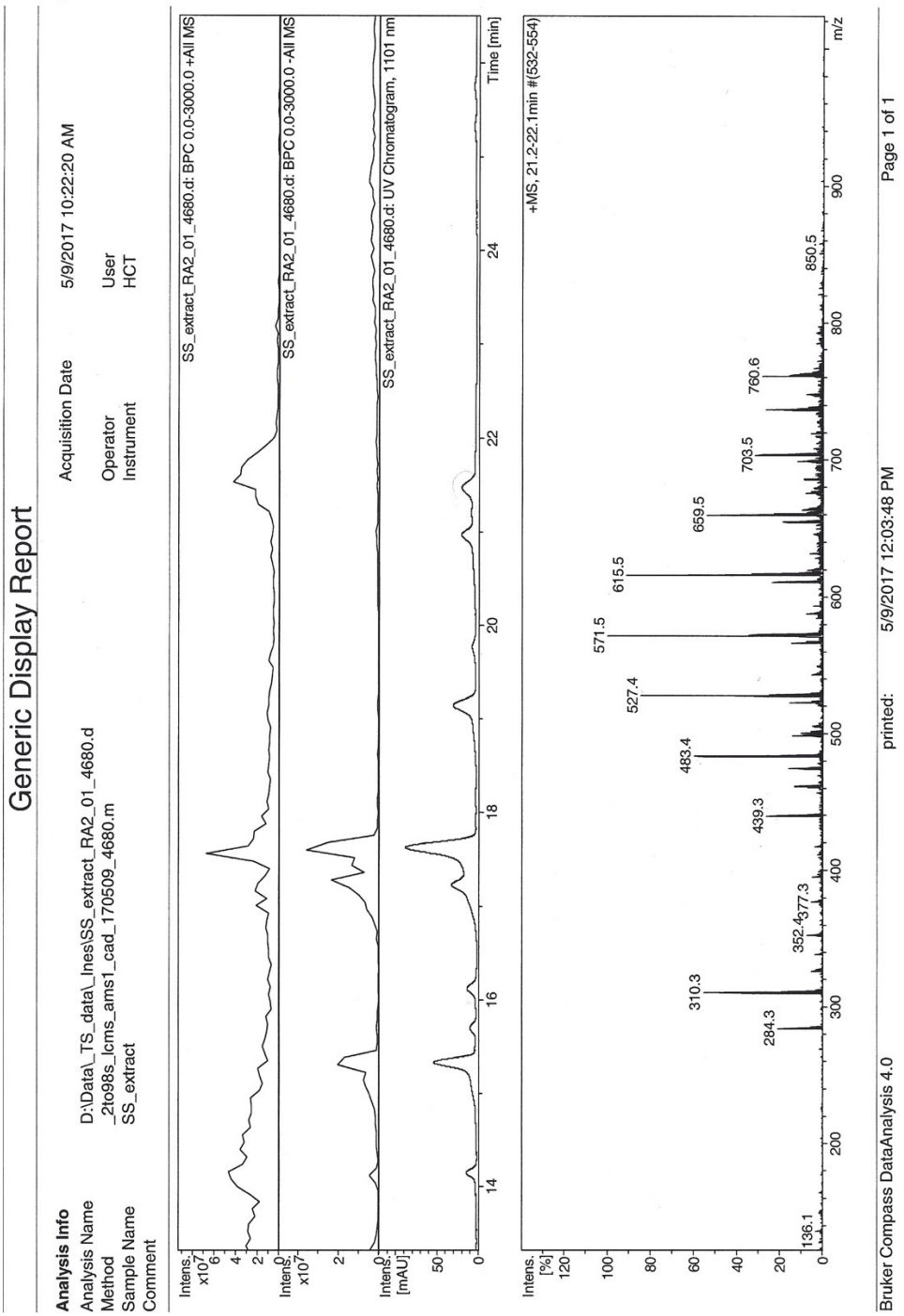


Figure 47 MS ss_extract

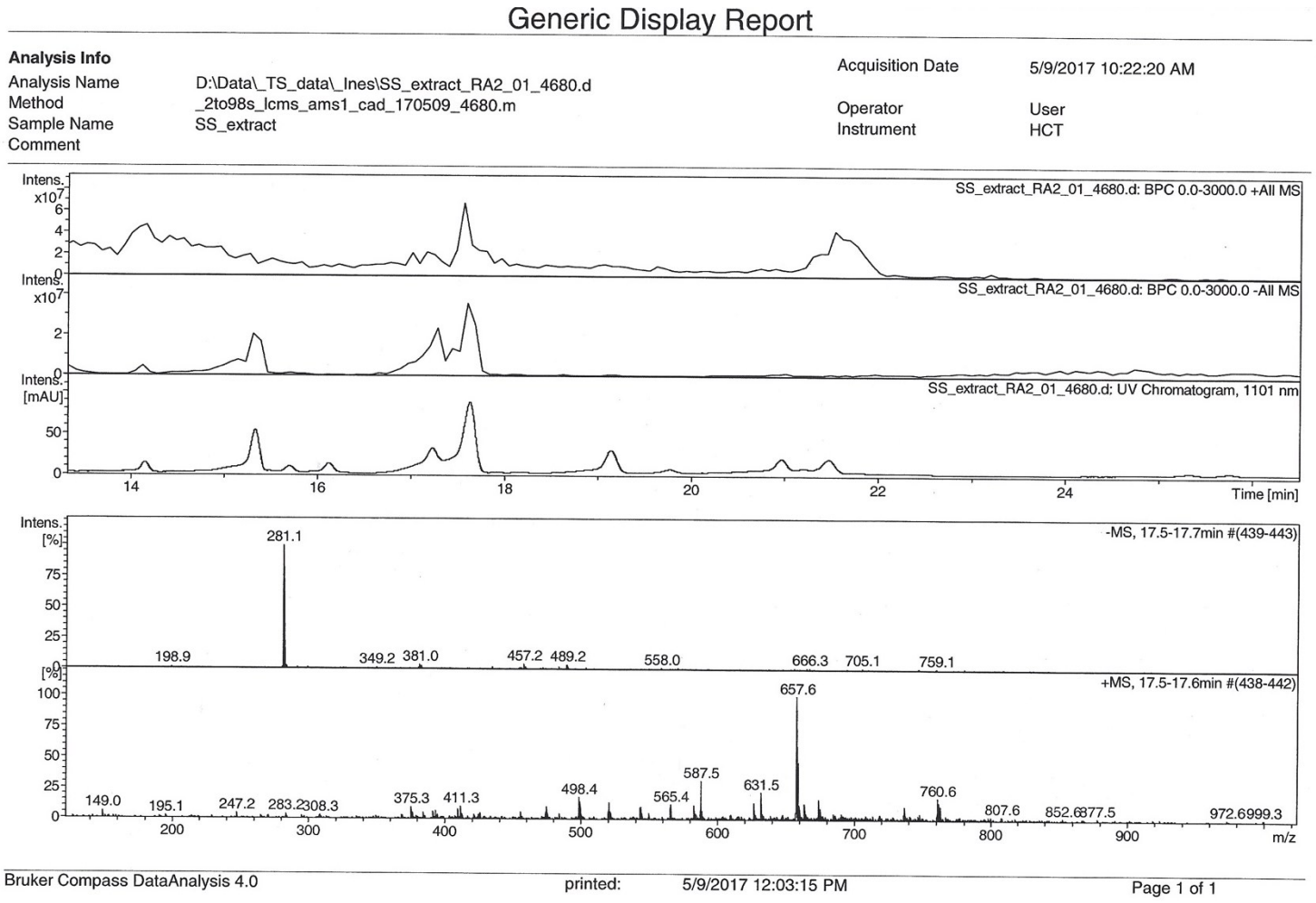


Figure 48 MS SS_extract

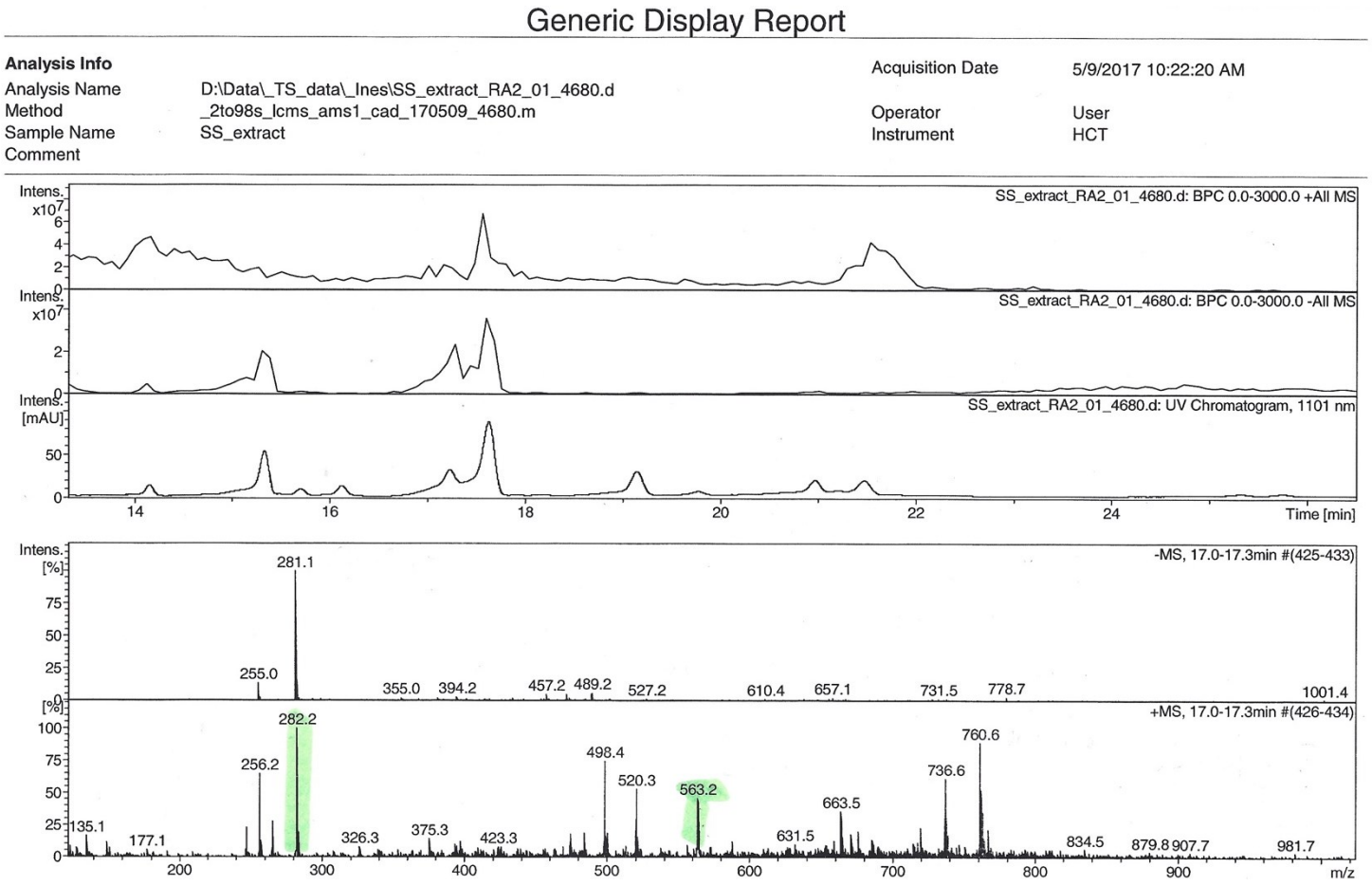


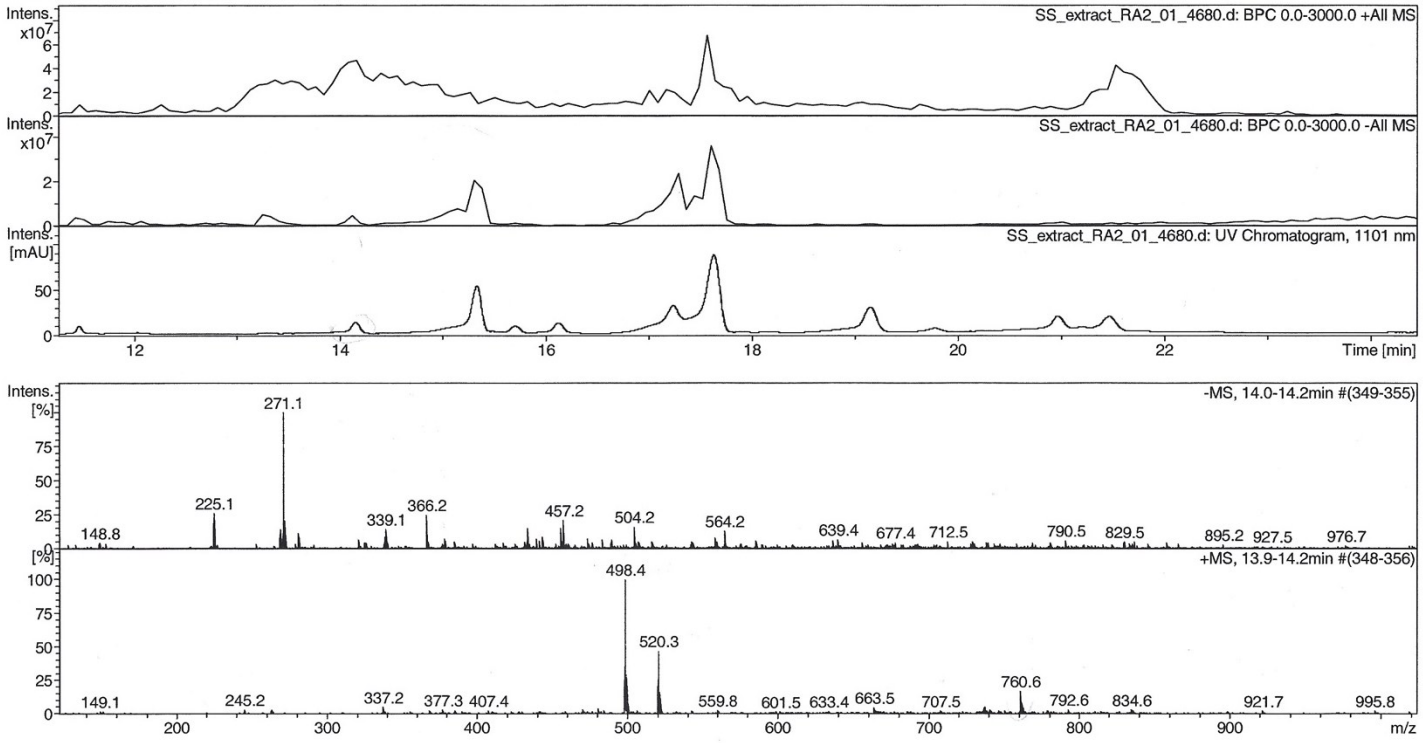
Figure 49 MS SS_extract

Figure 50 MS SS_extract

Generic Display Report

Analysis Info

Analysis Name	D:\Data_TS_data_Ines\SS_extract_RA2_01_4680.d	Acquisition Date	5/9/2017 10:22:20 AM
Method	_2to98s_lcms_ams1_cad_170509_4680.m	Operator	User
Sample Name	SS_extract	Instrument	HCT
Comment			



Bruker Compass DataAnalysis 4.0 printed: 5/9/2017 12:01:22 PM Page 1 of 1

Figure 51 MS SS_extract

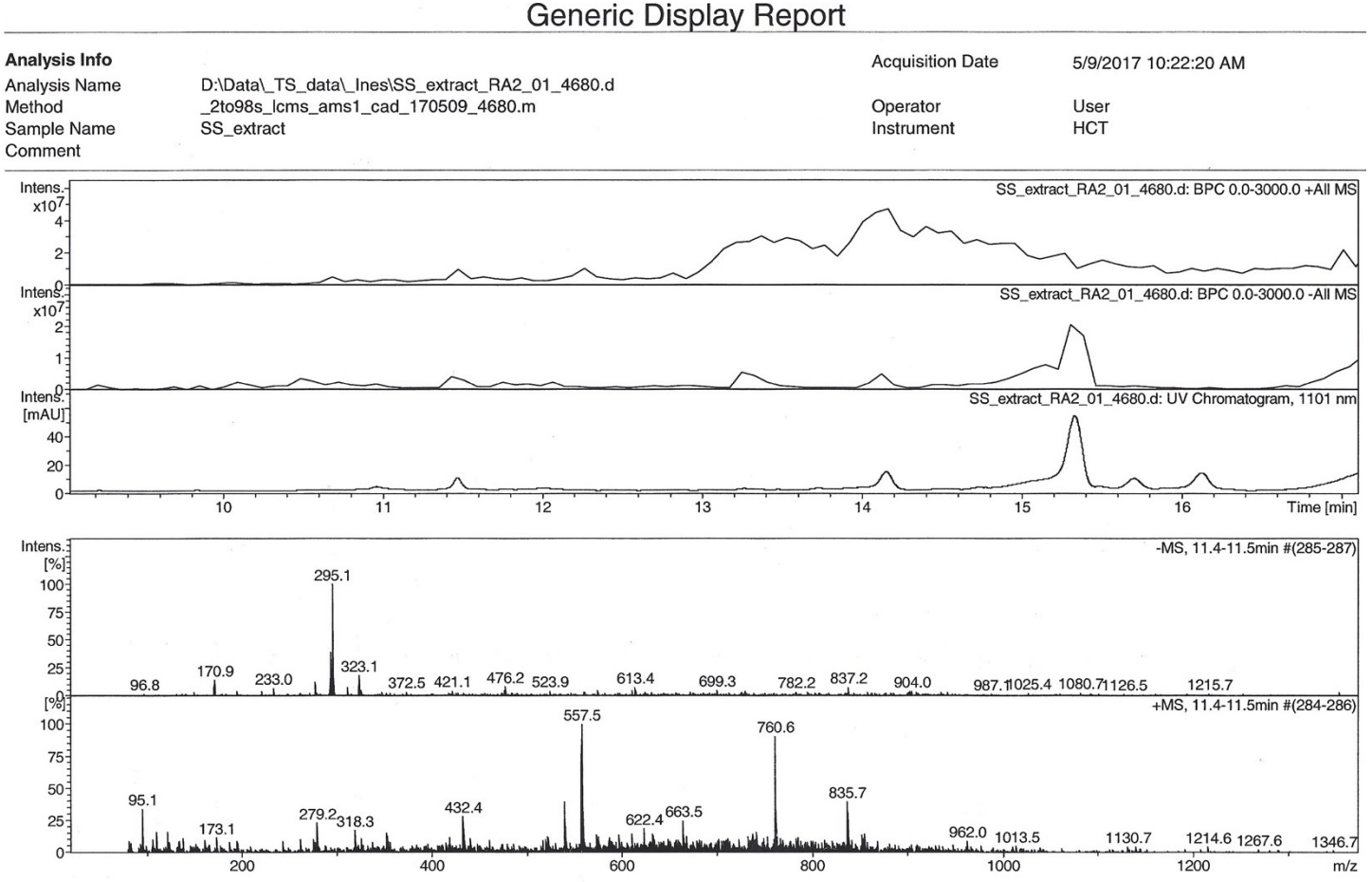


Figure 52 MS SS_extract

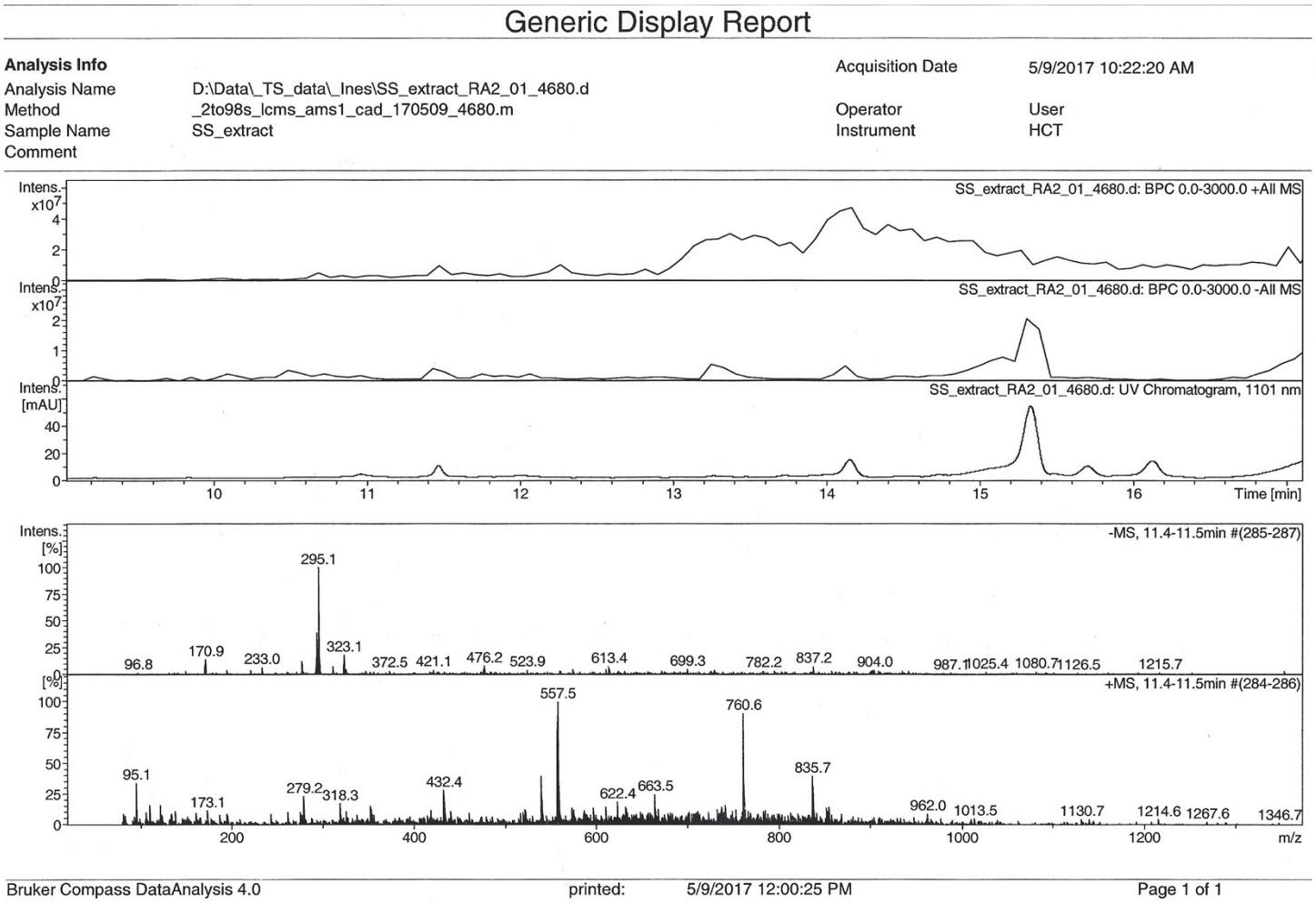


Figure 53 MS SS_extract

7.3.HPLC – optimization *Fomitopsis pinicola*

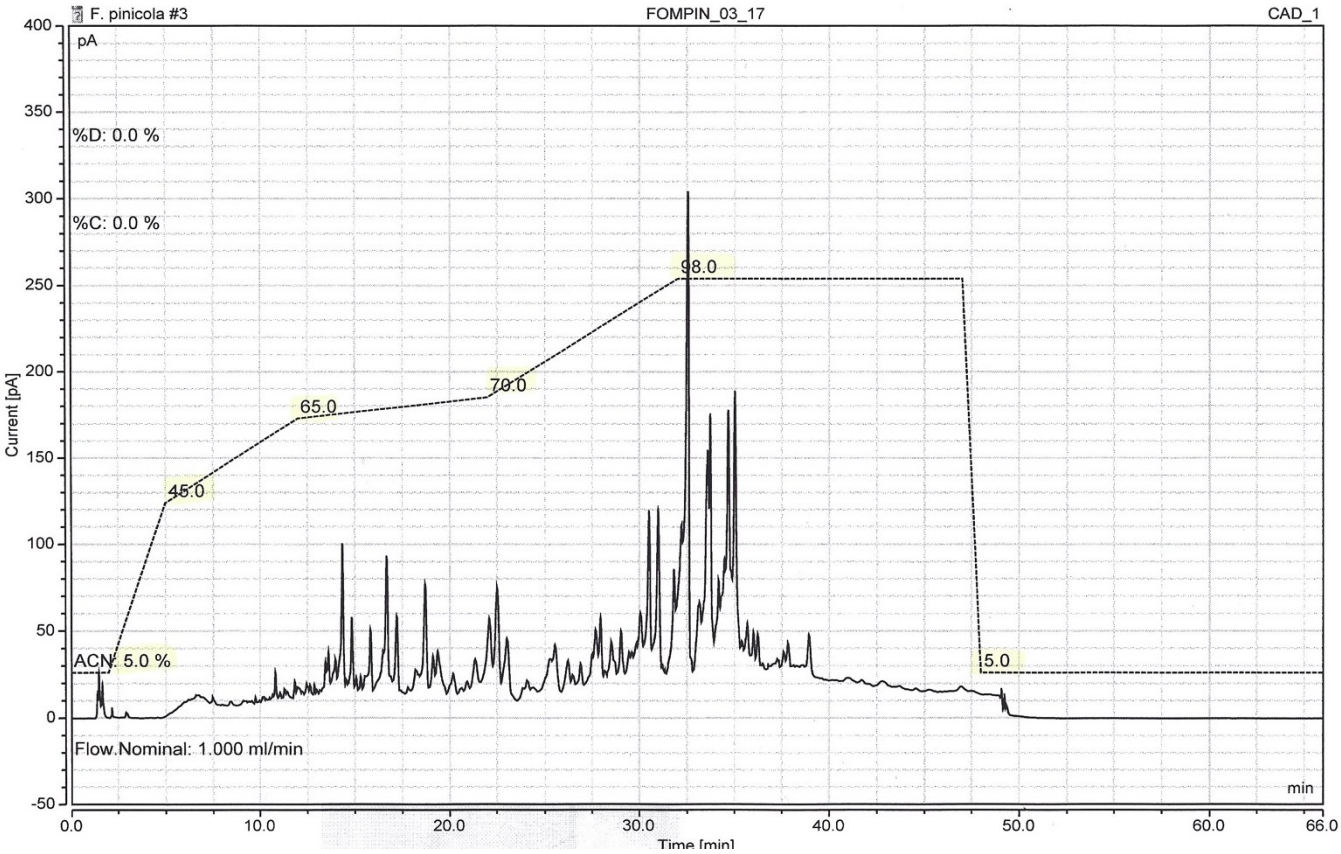


Figure 54 used HPLC method for FP

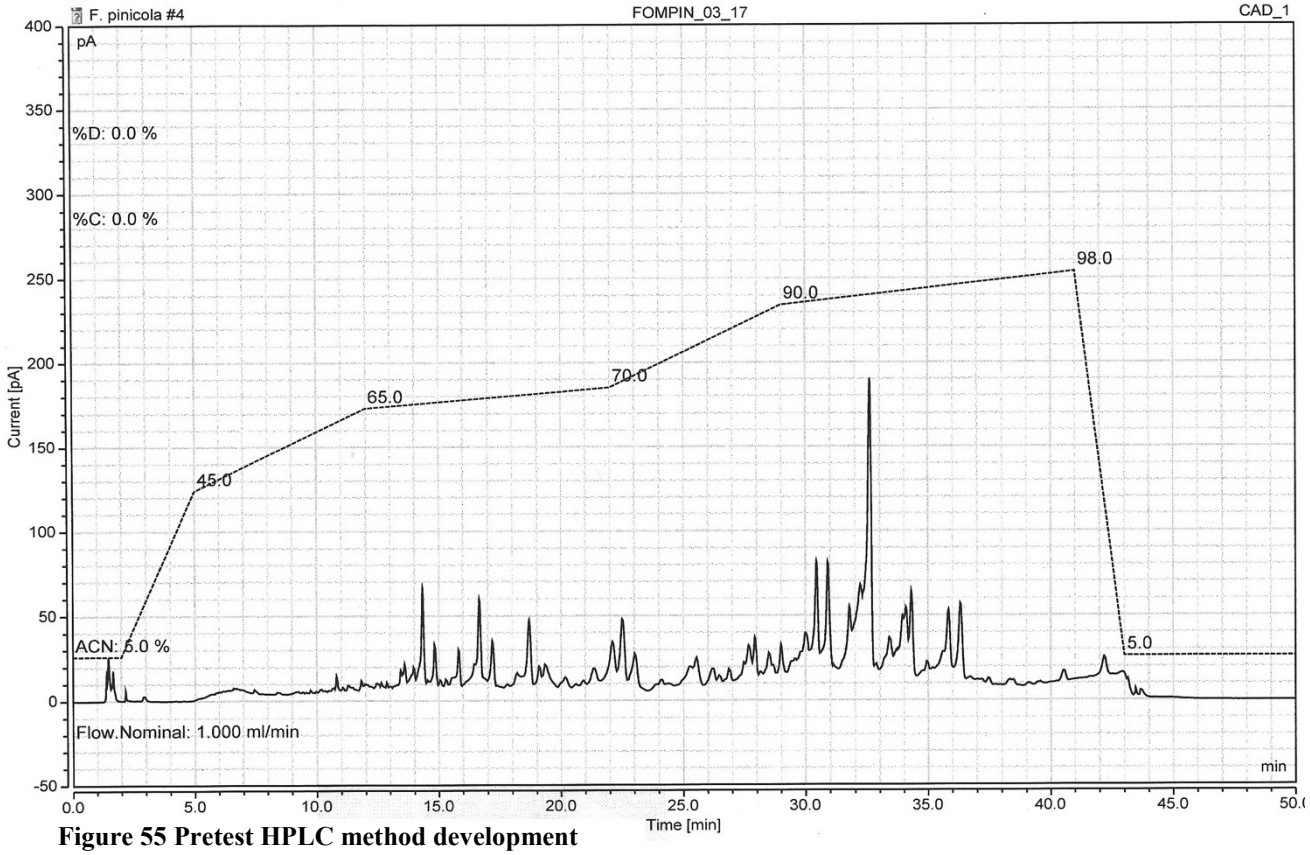


Figure 55 Pretest HPLC method development

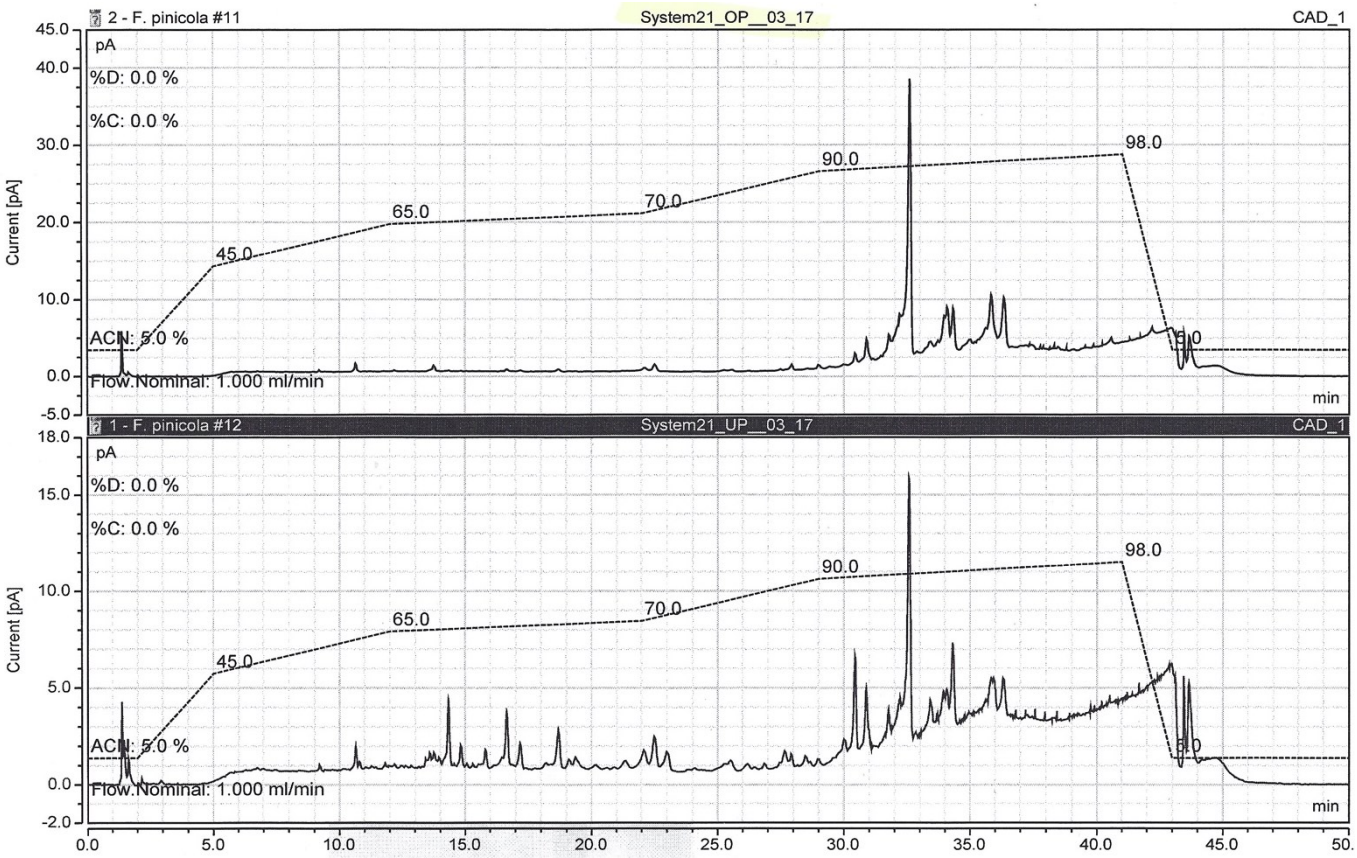


Figure 57 HEMWat System 21 upper (OP) and lower (UP) phase, HPLC-CAD

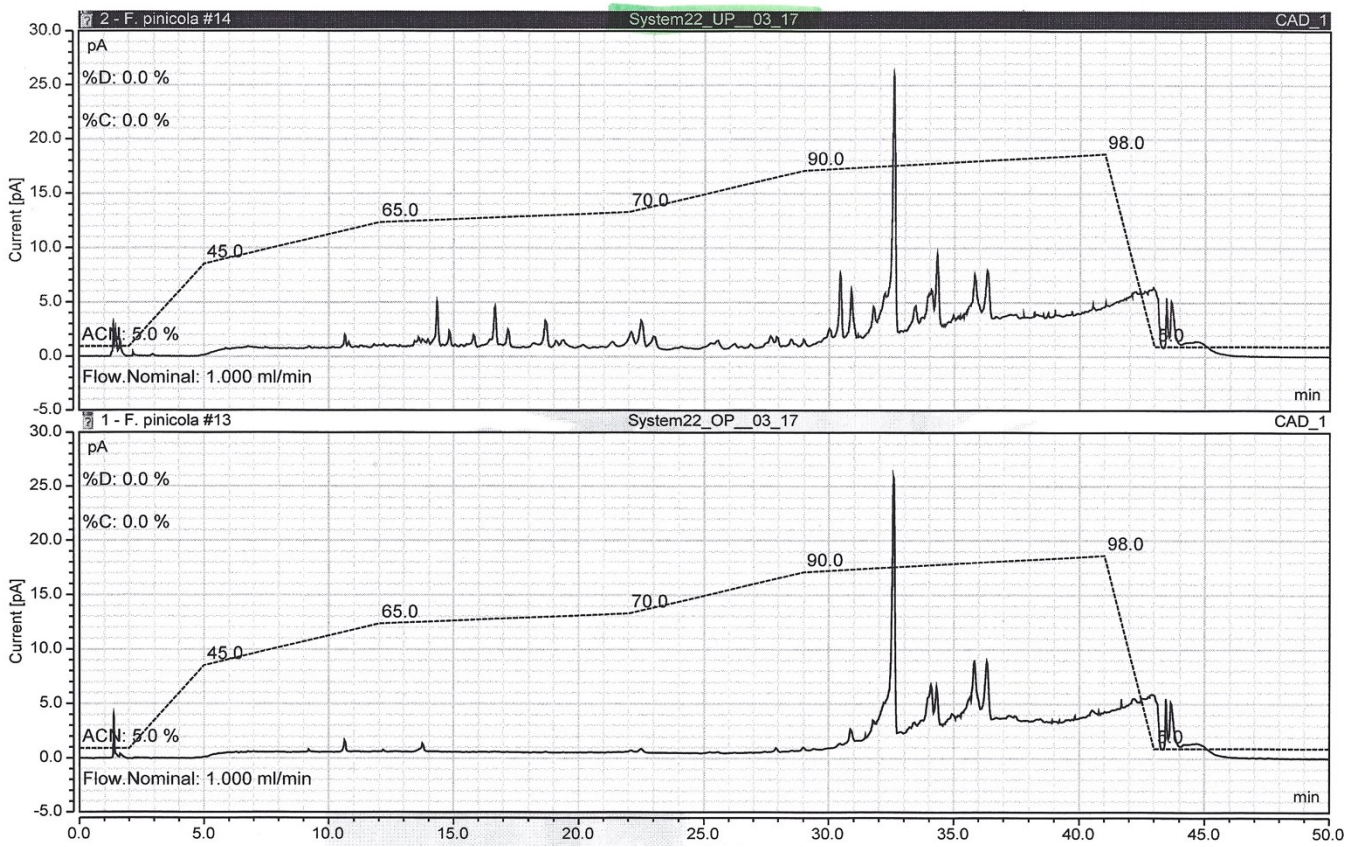


Figure 56 HEMWat System 22 upper (OP) and lower (UP) phase, HPLC-CAD

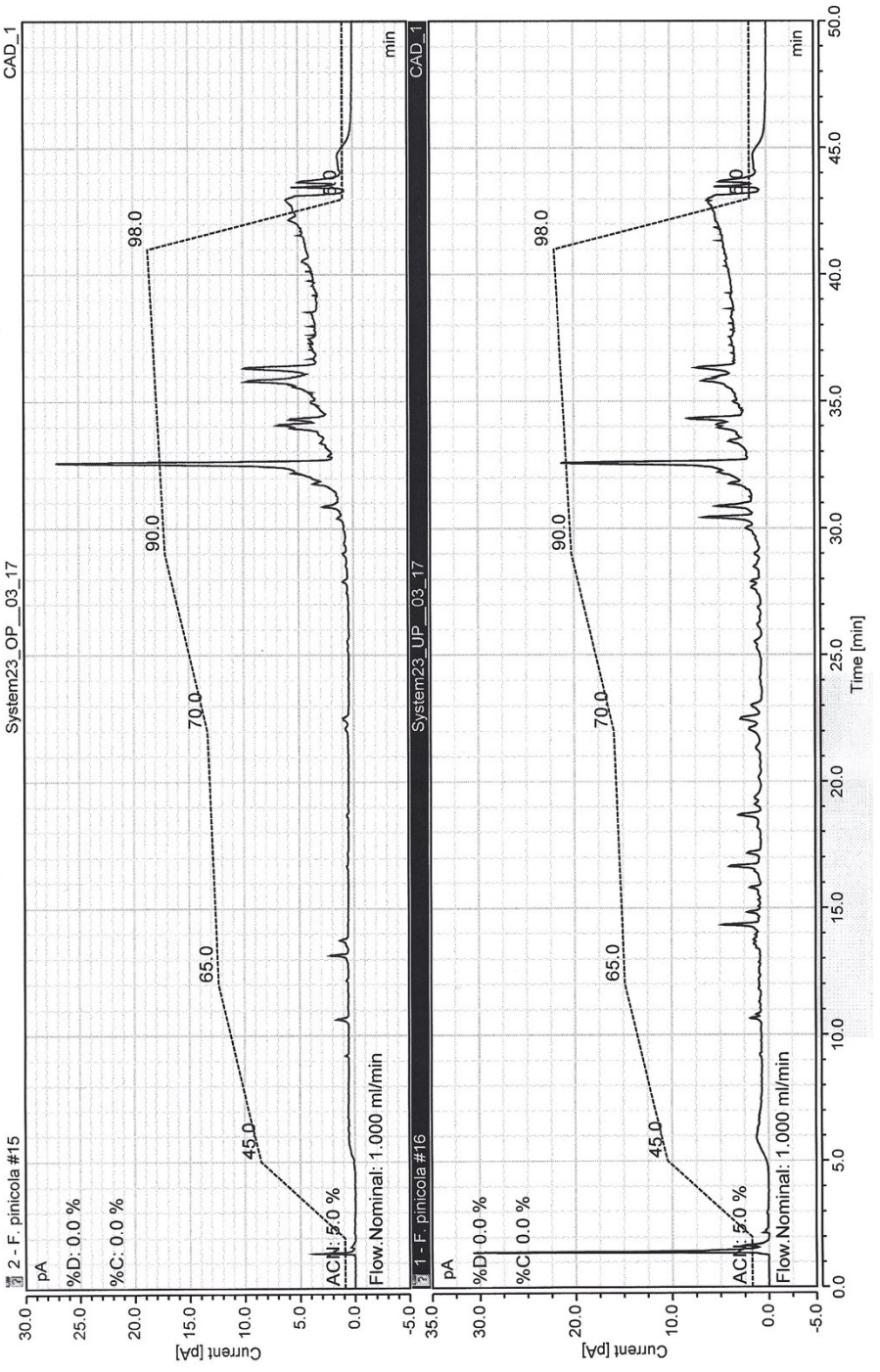


Figure 58 HEMWAt system 23 upper (OP) and lower (UP) phase, HPLC-CAD

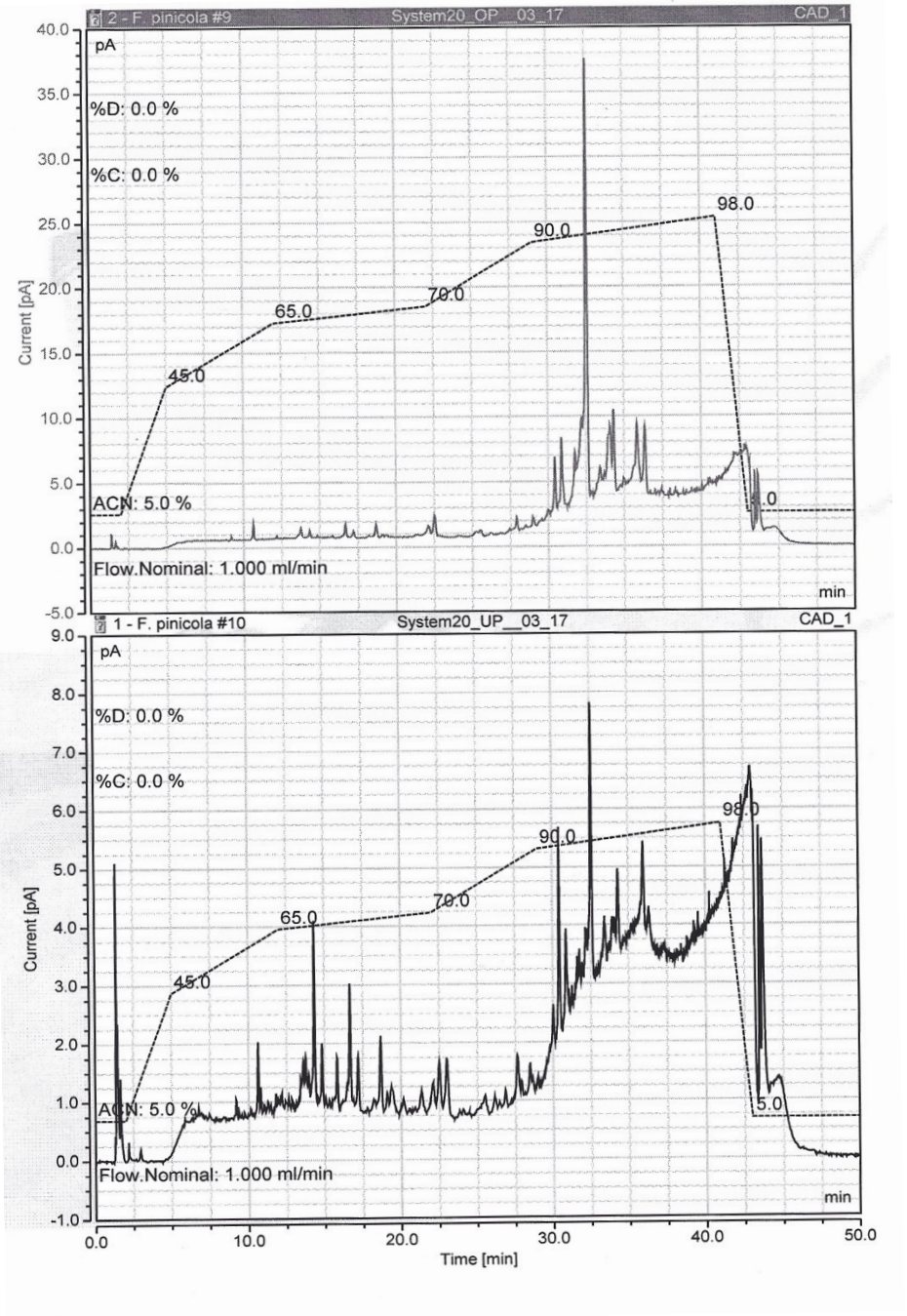


Figure 59 HEMWat solvent system 20 upper(OP) and lower phase (UP), HPLC-CAD

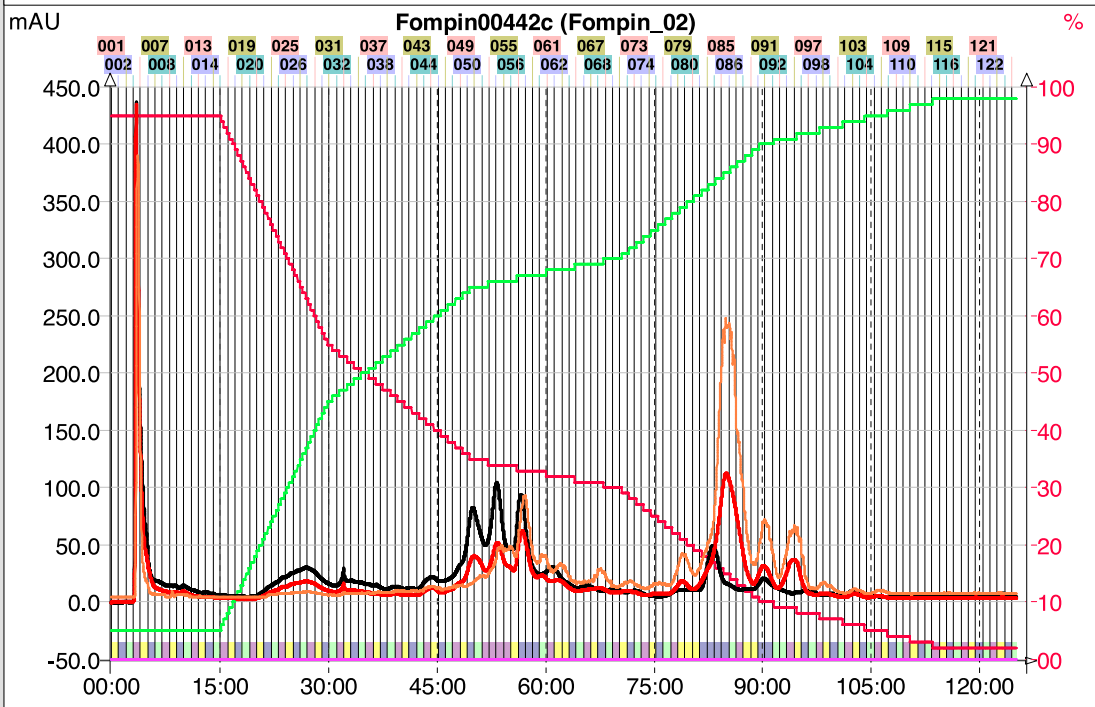
7.4.PuriFlash – Protocol

Data Time : 7/12/2017 11:56:49 AM

170712-1145-Fompin00442c.Flash

Printing Time : 7/12/2017 3:12:27 PM

Title : Fompin_02	Author : Ines T.
Sample : Fompin00442c	Column : PURIFLASH COLUMN 15 C18 HQ 35G - 35.0 g (22 bar)
Solvent A : H2O	Solvent C :
Solvent B : Acetonitril	Solvent D :
Channel 1 : UV600:SIG1 ==> 245 nm	Channel 2 : UV600:SCAN ==> 200-600
Channel 3 : ELSD ==> 49°-4	
Equil+Inject Mode : Equil. (Simple) + Valve Inj. + Wait	Stop Mode : Pause
RUN Time : 7/12/2017 11:45:57 AM	



Peak Tracking

22 mL				22 mL				22 mL			
111	110	089		088	067	066	045	044	023	022	001
112	109	090		087	068	065	046	043	024	021	002
113	108	091		086	069	064	047	042	025	020	003
114	107	092		085	070	063	048	041	026	019	004
115	106	093		084	071	062	049	040	027	018	005
116	105	094		083	072	061	050	039	028	017	006
117	104	095		082	073	060	051	038	029	016	007
125	118	103	096	081	074	059	052	037	030	015	008
124	119	102	097	080	075	058	053	036	031	014	009
123	120	101	098	079	076	057	054	035	032	013	010
122	121	100	099	078	077	056	055	034	033	012	011

<< RackSet #1 >>

<< RackSet #2 >>

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