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„Analysis and chemical investigation of the bryozoan *Flustra foliacea* and generation of a separation protocol for polar and non-polar triterpenes from *Gloeophyllum odoratum*“

verfasst von / submitted by

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Table of Contents

Acknowledgement ..... **I**

Table of Contents ..... **II**

1. List of Abbreviation **1**

2. Abstract **3**

3. Aim of the Work **5**

3.1. Analysis and chemical investigation of the bryozoan *Flustra foliacea* 5

3.2. Generation of a separation protocol for a selected fraction of polar and non-polar triterpenes of *Gloeophyllum odoratum* 5

4. Introduction **6**

4.1. The bryozoan *Flustra foliacea* L. 6

4.1.1. The ocean as habitat 6

4.1.2. Description of bryozoans 6

4.1.3. Role of bryozoans 7

4.1.4. The "animal" *Flustra foliacea* (hornwrack) 8

4.1.5. Indole alkaloids of the physostigmine type 9

4.1.6. Chemical profile of *Flustra foliacea* 9

4.1.7. Biological activity 12

4.2. The fungus *Gloeophyllum odoratum* 14

4.2.1. Historical use of mushrooms 14

4.2.2. Description of fungi and their environmental role 14

4.2.3. *Gloeophyllum odoratum* 15

4.2.4. Investigated compounds of *Gloeophyllum odoratum* 16

4.2.4.1. Volatile compounds 16

4.2.4.2. Non-volatile compounds 16

4.2.5. Role of hormone dependent cancers 17

4.2.5.1. Tumour development 17

4.2.5.2. Hormone dependent cancers 17

4.2.5.3. Therapy 18

4.2.6. Steroid sulfatase 18

4.2.6.1. Structure properties 19

5. Results and Discussion 21

5.1. Analysis and chemical investigation of the marine bryozoan *Flustra foliacea* 21

5.1.1. TLC analysis 21

5.1.2. HPLC analysis of FlufolADMUVIE_1 22

5.1.3. Dereplication of FlufolADMUVIE_1 using HPLC-MS 23

5.2. Generation of a separation protocol for a selected fraction for polar and non-polar triterpenes in *Gloeophyllum odoratum* 29

5.2.1. Preliminary tests with GOEF 30

5.2.2. GOEF01 30

5.2.3. GOEF02 30

5.2.4. GOEF03 30

5.2.5. STS inhibition by GOEF fractions 34

5.2.6. Purification of GOEF03_17 35

6. Conclusion 37

6.1. Analysis and chemical investigation of the bryozoan *Flustra foliacea* 37

6.2. Generation of a separation protocol for a selected fraction of polar and non-polar triterpenes in *Gloeophyllum odoratum* 38

7. Materials and Methods 40

7.1. Chromatographic techniques 40

7.1.1. Thin Layer Chromatography 40

7.1.2. High Performance Liquid Chromatography 40

7.1.3. Flash Column Chromatography 40

7.2. Analysis and chemical investigation of the marine bryozoan *Flustra foliacea* 41

7.2.1. Animal material 41

7.2.2. Labelling *Flustra foliacea´s* extract 41

7.2.3. Generation of FlufolADMUVIE_1 41

7.2.4. TLC system for FlufolADMUVIE_1 42

7.2.5. HPLC optimization 42

7.2.6. Mass spectrometer parameters 43

7.3. Generation of a separation protocol for a selected fraction of polar and non-polar triterpenes in *Gloeophyllum odoratum* 44
7.3.1. Origin of GOEF 44
7.3.2. TLC system for GOEF 44
7.3.3. Flash chromatography of GOEF03, GOEF04 and GOEF05 45
7.3.3.1. Applied amount of the samples GOEF01, GOEF02, GOEF03, GOEF04 and GOEF05 46
7.3.3.2. Fraction yields of GOEF03, GOEF04 and GOEF05 47
7.3.4. HPLC-CAD of GOEF03, GOEF04 and GOEF05 49
7.3.5. Prepared fractions sent to Dr. Paul Foster for testing on STS inhibition 50
7.3.6. Preliminary tests for further purification steps of GOEF03_17 51
7.4. Instruments, solvents and reagents 53
7.4.1. Instruments 53
7.4.2. Solvents and reagents 54
7.4.3. HPLC column for investigation of *F. foliacea* 54
7.4.4. HPLC column for HPLC-CAD 54
7.4.5. Flash Chromatography columns 54
7.4.6. Spraying reagents for TLC 55
7.4.7. TLC plates 55
8. List of Figures 56
9. References 57
10. Appendix 61
10.1. TLC fractions of GOEF03 after Flash Chromatography 61
10.2. Chromatograms of GOE, GOED, GOEF, GOEP and GOEW 63
10.3. Chromatograms of GOEF03_01-17 by HPLC-CAD 66

1. List of Abbreviation

ACN	Acetonitrile
CAD	Charged Aerosol Detector
$CHCl_3$	Chloroform
DCM	Dichloromethane
Det. Wavel.	Detection Wavelength
DHEA	Dehydroepiandrosterone
DHEAS	Dehydroepiandrosterone Sulfate
E1	Estrone
E1S	Estrone Sulfate
ELSD	Evaporative Light Scattering Detector
ER	Endoplasmic Reticulum
EtOAc	Ethyl acetate
EtOH	Ethanol
F_{254}	Fluorescent indicator at 254 nm
FSH	Follicle Stimulating Hormone
GnRH	Gonadotropin-Releasing Hormone
GOE	ethanolic extract of *Gloeophyllum odoratum*
GOED	dichloromethane fraction of *Gloeophyllum odoratum*
GOEF	non-soluble fraction of *Gloeophyllum odoratum*
GOEP	petroleum ether fraction of *Gloeophyllum odoratum*
GOEW	aqueous fraction of *Gloeophyllum odoratum*

HPLC	High Performance Liquid Chromatography
H_2O	Water
Inj. Vol.	Injection Volume
LC	Liquid Chromatography
LH	Luteinizing Hormone
MeOH	Methanol
MS	Mass Spectrometry
NA	Neuraminidase
n.c.	no correlation
NMR	Nuclear Magnetic Resonance
rpm	rounds per minute
SERM	Selective Estrogen Receptor Modulator
STS	Steroid Sulfatase
SULT	Sulfotransferase
TCM	Traditional Chinese Medicine
Temp.	Temperature
TGF_α	Transforming Growth Factor α
TLC	Thin Layer Chromatography
UK	United Kingdom
UV	Ultra Violet
VIS	Visible

2. Abstract

Within the framework of this diploma thesis two organisms originating from totally different habitats were investigated. On the one hand a marine organism *Flustra foliacea* L., which settles shallow areas of cold waters in the northern hemisphere, and on the other hand *Gloeophyllum odoratum* (Wulfen) Imazeki, a mushroom mainly found on stumps of spruces. This thesis covers an analysis of the secondary metabolite profile of the bryozoan *Flustra foliacea* with subsequent comparison to already reported compounds. The aim was to see if there was any difference in the composition of the main constituents (flustramines). Therefore, a HPLC-MS analysis of the extract was performed. Most of the detected ion fragments could be assigned to compounds described in literature. Due to the very small amount of starting material further preparative separations were not possible.

The second aim of this thesis was to generate a separation protocol for polar and non-polar triterpenes of a selected fraction (GOEF) of the fungus *Gloeophyllum odoratum* for the subsequent testing for the inhibition of steroid sulfatase (STS), an enzyme which converts inactive hormones in their biological active forms playing an important role for the growth of hormone dependent cancers such as breast cancer, gynaecological tumours, or prostate cancer. GOEF, a non-soluble fraction obtained after liquid-liquid separation of the ethanolic extract of *Gloeophyllum odoratum*, was fractionated by flash chromatography (FC) and monitored by thin layer chromatography (TLC) and high performance liquid chromatography – charged aerosol detector (HPLC-CAD). The obtained fractions GOEF03_01-17 were sent to a collaboration partner (Dr. Paul Foster, University of Birmingham, UK) for STS inhibition assays. Some of the fractions showed a significant inhibition of STS. Particularly GOEF03_17 was identified as the most potent fraction which showed 55% STS inhibition.

Zusammenfassung

Im Rahmen dieser Diplomarbeit wurden zwei Organismen mit unterschiedlichen Lebensräumen untersucht. Einerseits *Flustra foliacea* L., ein mariner Organismus, bestehend aus zahlreichen einzelnen Moostierchen, die kalte Gewässer der nördlichen Hemisphäre bevorzugen und andererseits der Pilz *Gloeophyllum odoratum* (Wulfen) Imazeki, der hauptsächlich auf Fichtenstümpfen anzutreffen ist.

Ein Teil dieser Arbeit umfasst die Analyse sekundärer Metaboliten des Moostierchens *Flustra foliacea* mit anschließendem Abgleich der erhobenen Daten und den in der Literatur bereits beschriebenen Strukturen. In Folge dessen, galt es herauszufinden, ob Abweichungen in der Zusammensetzung der Hauptinhaltsstoffe, den sogenannten Flustraminen, festzustellen sind. Der Extrakt wurde mit HPLC-MS untersucht, woraufhin eine grobe Übersicht erstellt wurde und die erhaltenen Ionenfragmente den in der Literatur beschriebenen Inhaltsstoffen zugeordnet werden konnten. Aufgrund der verschwindend kleinen Ausgangsmenge war es nicht möglich weitere Schritte bezüglich Fraktionierung und Isolierung zu unternehmen.

Das zweite Hauptaugenmerk dieser Arbeit lag auf einer geeigneten Trennung polarer und apolarer Triterpene einer bestimmten Fraktion (GOEF) des Pilzes *Gloeophyllum odoratum* und der anschließenden Testung auf Inhibierung des Enzyms Steroidsulfatase (STS), das die Umwandlung von Hormonvorstufen in ihre biologisch aktive Form katalysiert. STS spielt besonders in der Entstehung hormonabhängiger Tumore, wie Brustkrebs, gynäkologischer Tumore und Prostatakrebs eine bedeutende Rolle.

Der nicht-lösliche Teil GOEF, der nach einer flüssig-flüssig Verteilung des ethanolischen Extrakts erhalten wurde, wurde mittels Flash Chromatographie (FC) fraktioniert und in weiterer Folge mithilfe von Dünnschichtchromatographie (DC) und der Hochleistungsflüssigkeitschromatographie, gekoppelt mit einem Charged Aerosol Detector, (HPLC-CAD) kontrolliert. Die dadurch erhaltenen Fraktionen GOEF03_01-17 wurden anschließend zur Testung auf STS-Inhibierung an einen Kollaborationspartner (Dr. Paul Foster, University of Birmingham, UK) geschickt. Einige dieser Fraktionen zeigten eine eindeutige biologische Aktivität. Besonders GOEF03_17 stellte sich als aktivste Fraktion, die STS zu 55% inhibieren konnte, heraus.

3. Aim of the Work

3.1. Analysis and chemical investigation of the bryozoan *Flustra foliacea*

The aim of this part of my diploma thesis was to investigate the chemical composition of the bryozoan *Flustra foliacea* L. by comparing the obtained information to already known compounds reported in the literature. In particular, the focus was to identify, whether there is any diversity in the quantity and quality of constituents of *Flustra foliacea.*

3.2. Generation of a separation protocol for a selected fraction of polar and non-polar triterpenes of *Gloeophyllum odoratum*

The main part of my thesis dealt with the generation of a separation protocol of the fraction GOEF which was provided by a former diploma student (Stefanie Pucher, 2014) who also worked on the mushroom *Gloeophyllum odoratum* (Wulfen) Imazeki. The aim was particularly to find a method to separate the polar from the non-polar compounds using flash chromatography. Furthermore, from previous studies it was known that this fungus showed bioactivity on the enzyme steroid sulfatase (STS). Thus, this was an interesting point finding a possible bioactivity in the further separated fractions of GOEF.

4. Introduction

4.1. The bryozoan *Flustra foliacea* L.

4.1.1. The ocean as habitat

More than 70 percent of the earth´s surface is covered with water but still big parts of the world´s oceans are not explored, especially the deep sea. This is the largest habitat for microorganisms, plants and animals and of major importance for climate and life on earth (Meeresbürger, accessed in December 2017).

As the largest of all habitats on earth the ocean has a remarkable diversity of species and a unique ecosystem in which plants and animals have adjusted to survive. Some places or habitats differ from “movement of water, amount of light, temperature, water pressure, nutrients, availability of food, and saltiness of water” (National Park Service, accessed in December 2017).

4.1.2. Description of bryozoans

Bryozoans (= Ectoprocta) belong to the so called Tentaculata, which are also called Lophophorata. These animals are classified due to the occurrence of a lophophore, a specialised feeding organ, surrounding the mouth with ciliated tentacles. While the cilia are providing a water flow towards the mouth, the tentacles are catching floating nutritious particles and transport them to the mouth opening. Unlike pseudocoelomate rotifers, their coelom is covered with mesodermal epithelia (Campbell & Reece, 2009).

Due to the U-shaped gut of bryozoans, their mouth and anus are very close to each other. The anus is not located on the terminal side of the zooid but below the lophophore (University of California Museum of Paleontology, accessed in December 2017).

Bryozoans are tiny and colonial organisms that can be crusty or arboreal. They are composed of several to millions of individuals, the zooids (Fig. 1) (Smithsonian Marine Station at Fort Pierce, accessed in December 2017). Due to the many zooids in the colony there are several different types of individuals, each one with a particular function. Each zooid is protected by a solid “skeleton of calcium carbonate”, the zooecium.

The phenotypes of bryozoans are more than different. They can be encrusting and spreading over the ground. Others grow upwards and can be firm, foliaceous or treelike, which has been more frequently in the Paleozoic. Today, most of the bryozoans are crusty types (University of California Museum of Paleontology, accessed in December 2017).

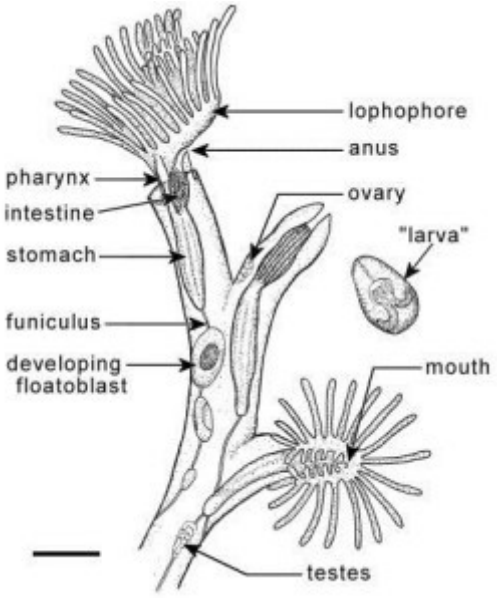


Figure 1: Animation of a single zooid
(sleloinvasives.org; accessed in December 2017)

4.1.3. Role of bryozoans

Studies on coral reefs have shown that bryozoans also occur frequently on corals which is rarely mentioned in literature. They are often encrusting bryozoans but are not responsible for the building of reefs. But still they are supporting the formation of the coral reefs by encrusting or stabilising the lower parts of corals and, moreover, by filling the cavities of a coral colony partly (Cuffey, 1972).

Observations over more than two decades in the “Indian River Lagoon” have also pointed out that bryozoans are responsible for a part of the water purification. In fact, some colonies of these living water purifiers per m^2 are capable of clearing approximately 48 000 gallons of seawater daily (Winston, 1995).

4.1.4. The “animal” *Flustra foliacea* (hornwrack)

Bryozoans have very versatile phenotypes as mentioned before. One of the most washed ashore bryozoan is the species *Flustra foliacea* L. (Fig. 2). Indeed, this one has a foliaceous shape and looks like a small water plant at first sight (Gehrmann, 2011). But still, its exoskeleton is firm and is made of calcium carbonate (8,5%), scleroprotein (14%) and chitin (1%) (Hunt, 1972).

The moss animal itself is a tiny organism with a size of about 1 mm. Usually, it is organized in colonies, that can reach a diameter of 1 m and is found in chilly waters in the northern hemisphere (Lysek, 2002). The colonies of *Flustra foliacea* initially grow adhesive to the ground forming a low base before the growth of an erect colony begins (Stebbing, 1971).

Typical for this hornwrack are bushy clots with fronds that are in general lobed and may grow up to a size of 20 cm. In *Flustra foliacea* the zooids are organized bilaminar on the brown or grey fronds. Hornwracks are washed up on to the beaches sporadically and, if they are not completely dried, a typical lemon-like smell is apparent (MarLIN-The Marine Life Information Network, accessed on December 2017).

Figure 2: *Flustra foliacea*
(Flickr.com-©Alexander Semenov; accessed in December 2017)

4.1.5. Indole alkaloids of the physostigmine type

Indole alkaloids possess an indole- or indoline- (2,3-dihydro-indole) base frame and are derived from L-tryptophan. They are the largest group of alkaloids with more than 3000 different compounds. Pharmaceutical and toxicological important indole alkaloids are classified according to their structure. They are divided into physostigmine, β-carboline and ergoline types and monoterpenoid indole alkaloids.

Indole alkaloids of the physostigmine type form a small group which are common in the legumes species Physostigma and Dioclea. A major compound of this class is physostigmine from the beans of *Physostigma venenosum*. Physostigmine (Fig. 3) is a reversible acetyl choline esterase inhibitor and is today mainly used as an antidot for intoxications with atropine, benzodiazepines or tricyclic antidepressants. The toxic dose of physostigmine salts for humans is 6-10 mg (Teuscher, Melzig, & Lindequist, 2012).

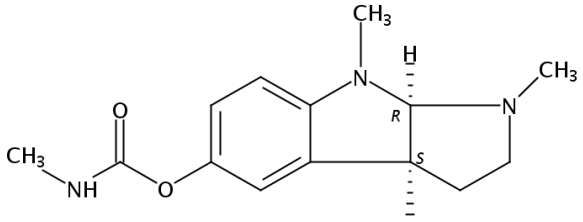


Figure 3: Physostigmine
(scifinder.cas.org; accessed in December 2017)

4.1.6. Chemical profile of *Flustra foliacea*

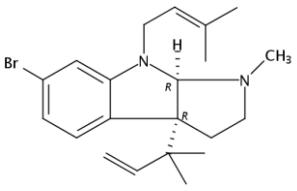
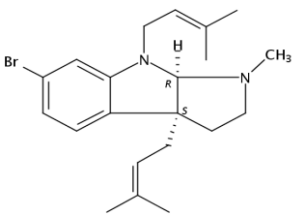
Organic compounds, especially from marine organisms, were intensively investigated in the past. Marine organisms did not go through the same biosynthetic pathway as their terrestrial counterparts, however, few isolated structures belong to the group of alkaloids. Only, a very low number of today's known alkaloids from marine organisms are bromo substituted. These are present in the bryozoan *Flustra foliacea*. Most of the alkaloids from this marine ectoprocta are characterized by a physostigmine skeleton with an unusual pattern of substitution (Carlé & Christophersen, 1979). The hornwrack´s alkaloids are "unique brominated pyrrolo[2,3-b]indoles" which show resamblance to the strong acetylcholine esterase inhibitor physostigmine which is used for the treatment of Alzheimer´s. So far, more than 15 of these secondary metabolites have been isolated and still the search for new compounds, also for

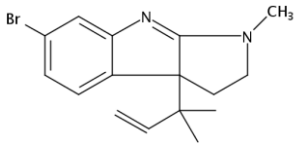
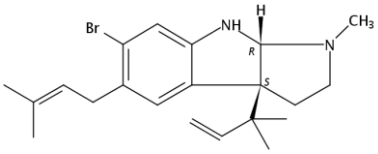
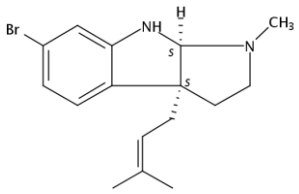
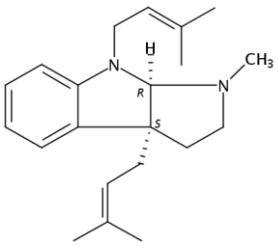
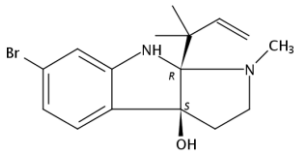
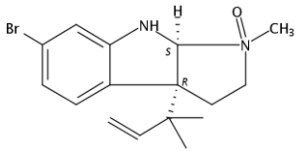
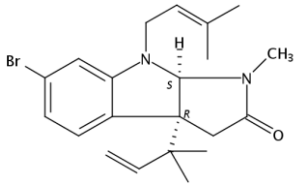
biosynthetic intermediates, keeps going on (Lysek et al, 2002).

Brominated alkaloids, so called flustramines present in the investigated bryozoan, are one of the first ones investigated from marine organisms. Moreover, flustramines are structurally highly related to “terrestrial” physostigmine alkaloids which are for example found in legumes (Rochfort et al, 2009).

As mentioned before, flustramines are pyrrolo[2,3-b]indoles which can be mono- or di-prenylated. All tricyclic flustramines are characterized by methylation at N-1 and, except for debromoflustramine B, they are brominated at C-6. In addition, at position C-3a there is an inverse prenyl group, except for fustramine B, flustramine E and debromoflustramine B. Others, like flustramine D and isoflustramine D, are showing prenyl substitution at C-5 and C-7. Some flustramines, such as flustraminol A and B are oxygenated derivatives possessing an OH-group at position C-3a instead of the prenyl group. Flustramines are structurally very versatile with N-oxides (dyhydroflustramine C N-oxide and flustramine D N-oxide) or lactams (flustramide A and B). There are also non-cyclic tryptamine derivatives which are synthesised after decarboxylation of tryptophan and built up to alkaloids with components that do not contain nitrogen. Besides bromination at C-6 a methyl group and a formyl group is added to the amino group of tryptamine (Lysek, 2002). An overview of the different substitution pattern is depicted in Table 1. An overview of the different substitution patterns is depicted in Table 1.

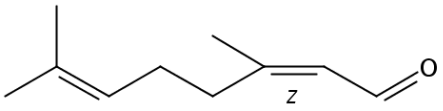
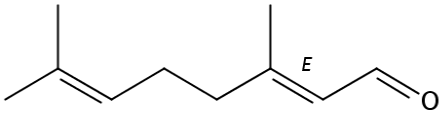
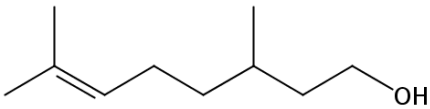
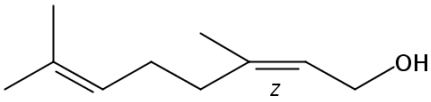
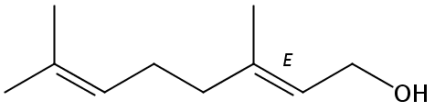
Table 1: Overview of the structural variety of flustramines (Scifinder)

Substance Name	CAS-Number	Molecular Weight	Structure
Flustramine A	71239-64-0	389.37	
Flustramine B	71239-65-1	389.37	

Flustramine C	78127-86-3	319.24	
Flustramine D	104387-12-4	389.37	
Flustramine E	158642-05-8	321.26	
Debromoflustramine B	158060-73-2	310.48	
Flustraminol A	78127-88-5	337.25	
Dihydroflustramine C N-oxide	104387-11-3	337.25	
Flustramid A	82534-44-9	403.36	

Besides flustramines, there are also other secondary metabolites in *Flustra foliacea*. Noticeable is the typical lemon-like smell of hornwracks due to the small amount of essential oil. Cis- and trans-citral, citronellol, nerol and geraniol have been identified as the main compounds of the essential oil (Table 2) and responsible for the remarkable odour, which could be associated with antimicrobial activity (Christophersen & Carlé, 1978).

Table 2: Compounds of the essential oil fraction of *Flustra foliacea* (Scifinder, accessed in December 2017)

Substance Name	CAS-Number	MolecularWeight	Structure
Cis-Citral	106-26-3	152.23	
Trans-Citral	141-27-5	152.23	
Citronellol	106-22-9	156.27	
Nerol	106-25-2	154.25	
Geraniol	106-24-1	154.25	

4.1.7. Biological activity

Antibacterial studies have shown that methylene chloride extracts of this organism have remarkable antimicrobial activity against *Bacillus subtilis* (Laycock et al, 1986). Furthermore, the raw extract and isolated flustramines A and B demonstrate muscle-relaxant activity *in vivo* and *in vitro* since they have an affect on smooth and skeletal muscles (Sjöblom et al, 1983). Additonally, flustramine A seems to have a non-specific potassium channel ($K_{v1.4}$) blocking activity (Peters et al, 2002). Apart from that, deformylflustrabromine and

deformylflustrabromine B block the subtypes (α4β2 and α7) of the nicotinic acetylcholine receptor (nAchR) (Peters et al, 2004). Another compound, deformylflustrabromine (dFBr), stimulates the cytotoxic response of biofilms on orthopaedic implants and low doses of dFBr can have a negative effect on osteoblast differentiation in cultures of MC3T3 cells (Rawson et al, 2014). Lysek et al (2002) have demonstrated that deformylflustrabromine has cytotoxic activity against the cancer cell line HCT-116 which appears in the human colon.

4.2. The fungus *Gloeophyllum odoratum*

4.2.1. Historical use of mushrooms

In human history fungi have always been of interest. They are used as food as well as for medicinal purposes to cure several diseases, particularly in Asia. Asian countries have early realised the benefit of mushrooms and their diverse therapeutic features (Fantasticfungi, accessed in December 2017). In Traditional Chinese Medicine (TCM) which goes back for thousands of years, mushrooms have been used as medicine. In the *Materia medica* dating back nearly 3000 BC, the fungus *Ganoderma lucidum* [Chinese: *lingzhi*, Japanese: *reishi*] is highlighted (Myko San, accessed in December 2017). Thus, especially the single compounds of fungi become more popular in the western world and interesting for the treatment of anti-cancer therapy (Sliva, 2006). The reason why interest among the eastern world has been raised is not only the longstanding ethnomedicinal use of mushrooms, but more the isolated constituents like fatty acids, steroids, terpenoids, flavonoids, polysaccarides and other substance classes, which are associated with immunomodulatory, blood lowering, antioxidant or anti-cancer activity (Zhu et al, 2017). All of these properties are the reasons why mycochemical and pharmacological investigations of bioactive compounds of fungi which can also serve as building blocks for new pharmaceutical drugs is of high interest (Elisashvili, 2012).

4.2.2. Description of fungi and their environmental role

Mushrooms are heterotroph organisms but in contrast to animals they do not eat their prey or substrate but excrete digestive enzymes for a so called “outer digestion”. Only if their food is available in liquid form they can absorb it. For providing a continuous growth the actual body of mushrooms consists of countless threads, so called hyphae which ensure an enormous total surface for food intake. The actual fungus is built up of the entirety of all hyphae and is called mycelium, the vegetative part of the organism. But what is often wrongly described as “mushroom” is the fruit body which is often visible with the naked eyes (Schmid & Helfer, 1995).

In general, the number of enzymes of different mushrooms is enormous. Hence, they are playing many distinct roles in the global ecosystem. On the one hand saprophytes are specialised on degradation of dead organic material and on the other hand there are parasites

stealing nutrients from their host organism. Apart from that, fungi and other organisms can live in symbiotic relationships with benefits for both sides (Campbell & Reece, 2009).

4.2.3. *Gloeophyllum odoratum*

Gloeophyllum odoratum (Wulfen) Imazeki (Fig. 4) mainly grows on stumps of spruces in Middle Europe but is rarely found on branches or trunks of spruces. It is responsible for brown rot, which leads to the destruction of the structure of the wood (Schmid & Helfer, 1995). Young fungi are bulbous with a mat velvety surface. They are orange brown in colour, whereas older mushrooms change their colour to red-brown and have got an uneven mat miserly surface. Characterized by perennail growth *G. odoratum* enlarges its surface from dark red-brown to a bright yellow colour.

Considering the anise- or fennel-like odour of the fruiting bodies or older parts, that are not too dry, this fungus is described as "*odoratum*". According to Laux it is not suitable for human consumption because of its bitter-sour taste (Pilzforum, accessed in December 2017).

Figure 4: *Gloeophyllum odoratum*
(Pilzforum.eu; accessed in December 2017)

4.2.4. Investigated compounds of *Gloeophyllum odoratum*

4.2.4.1. Volatile compounds

Besides a strong smell and an anise-like touch of *G. odoratum,* a fruity and rosy scent is also reported, which can be attributed to the main compounds citronellol, geraniol, linalool and methyl-p-methoxyphenyl acetate (Fig. 5). The latter one is the main reason for the anise-like smell and at the same time the main constituent. However, the production of volatiles in *G.odoratum* is not constant and can be influenced by chitosan, a natural part of the cell wall. It leads to the assumption that it plays a role as a growth modulator and inhibits the output of essential oil. Furthermore, studies on the brine shrimp larvae *Artemia salina* showed toxic activity after exposure to the volatiles (Kahlos et al, 1994).

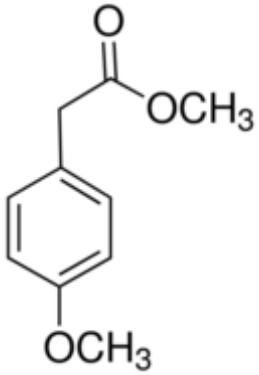


Figure 5: Methyl-p-methoxyphenyl acetate
(Sigma-Aldrich.com, accessed in January 2018)

4.2.4.2. Non-volatile compounds

Concerning non-volatile secondary metabolites, terpenoids, especially triterpenes of the lanostane type are very common. According to Grienke et al (2014), these compounds are characterized by many different functional groups such as acids, aldehydes and more and they are mentioned to exhibit various biological effects like anti-inflammatory or cytotoxic activities. One of the first isolated triterpenes have been trametenolic acid, trametenolic acid B and methyl-3β-hydroxylanost-8, 24-dien-21-oat (Prenner, 2016). In further studies of trametenolic acid B (Fig. 6) and ergosterol peroxide (Fig. 7) from *Inonotus obliquus* these compounds demonstrated an inhibition of NF-κB luciferase and (3β)-Lanosta-8,24-dien-3-ol inhibited the production of NO, targets which are vital mediators of inflammation and the development of tumours (Zwirchmayr, 2016). Additionally, other compounds of *G. odoratum*,

such as gloeophyllin B (Fig. 8) and (3β,4α,5α,12β)-12-(acetyloxy)-3hydroxy-4-methyl-ergosta-8,24-diene-4-carboxylic acid exhibited thrombin inhibition. This might be interesting regarding the fact that currently only one oral drug targeting thrombin directly, i.e. dabigatran, is on the market (Cateni et al, 2015). Further bioactivity investigations on STS and NA inhibition of former diploma students of the Department of Pharmacognosy of Vienna, Austria, are reported in the diploma theses of N. Lukovic (2015), N. Prenner (2016) and J. Zwirchmayr (2017).

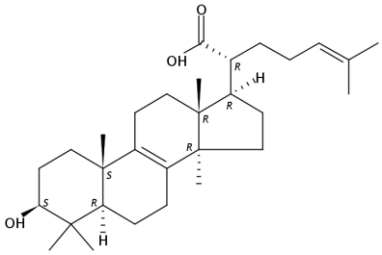


Figure 6: Trametenolic acid B

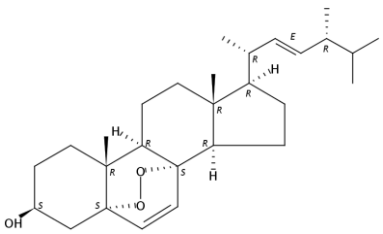


Figure 7: Ergosterol peroxide

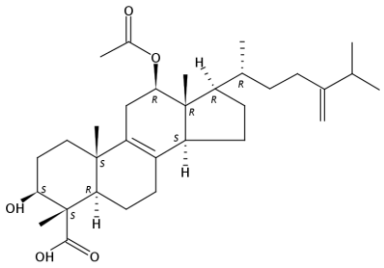


Figure 8: Gloeophyllin B

(Fig. 6-8 have been retrieved from Scifinder in January 2018)

4.2.5. Role of hormone dependent cancers

4.2.5.1. Tumour development

Under physiological conditions useless or damaged cells are eliminated. This highly automatic procedure is called apoptosis and appears ubiquitously in living organisms. In case of tumour diseases tumour cells are often resistant to tumour suppressant proteins, such as p53 which usually induce apoptosis. Consequently, it is plausible that there are numerous cell changes leading to the development of an actual tumour. Tumour diseases are also often associated with the absence of distinct genes or congenital disposition in combination with a dysfunctional immune system. Furthermore, hormones can promote cancer growth, like breast or prostate cancer. Hormones themselves are not responsible for the development of cancer but rather act as co-carcinogens by increasing the tissue of tumours due to the enhanced error rate during proliferation (Mutschler, 2013).

4.2.5.2. Hormone dependent cancers

Several studies investigated the role of hormones on the development of cancer and showed that mammary cancer and other gynaecological tumours are frequently hormone dependent

(Schüler-Toprak et al, 2017). Particularly, they are dependent on estrogen which is a key trigger for the formation of several gynaecological diseases (Rizner, 2016). Estrogen induces the secretion of TGFα, an important mediator of proliferation in estrogen dependent breast cancer cells (Leidenberg, 2013).

4.2.5.3. Therapy

Numerous types of cancers are dependent on sex hormones and therefore castration was a common procedure in the past. Today, pharmacological progress allows medicinal therapy with anti-hormones or hormone blockage. Due to the high concentration of GnRH in the body, synthetic analogues of GnRH suppress the secretion of LH and FSH which would usually induce the formation of estrogen and testosterone. Other vital substances are selective estrogen modulators, SERMs. As counterparts to estrogen, such as Tamoxifen, they are blocking its receptors and inhibit its effect on proliferation of tissue. Enzymes like aromatase which catalyse the production of estrogen are also very important targets in anticancer therapy, particularly fighting against breast cancer. Though, they are strictly advised only for postmenopausal women. Moreover, gestagens are also used for the treatment of mammary cancer by regulating the secretion of estrogen (Onkologie HEXAL, accessed in January 2018).

4.2.6. Steroid sulfatase

The enzyme steroid sulfatase, STS, catalyses the hydrolysis of the inactive and sulfated precursors of steroids to their unsulfated active form and presents an important target in the regulation of steroids (Palmieri et al, 2017).

STS is a small monomer and appears ubiquitously with high concentrations in placenta, breast or ovary and other tissues and converts the sulfated form of estrone (E1S), dehydroepiandrosterone (DHEAS), pregnenolone and cholesterol to their equivalent unconjugated active steroid. Due to the activation of sexual hormones like estrogen, which is associated with breast cancer and other gynaecological diseases, STS is of great interest for further investigation of STS inhibitors.

Steroid sulfotransferases, SULTs, represent the counterpart of STS by transferring sulfuryl groups to various substrates including hormones. SULTs are of significant importance for

inactivating and controlling the hormonal pool (Rizner, 2016). Fig. 9 gives an overview of the impact of STS and SULT on the regulation of steroids.

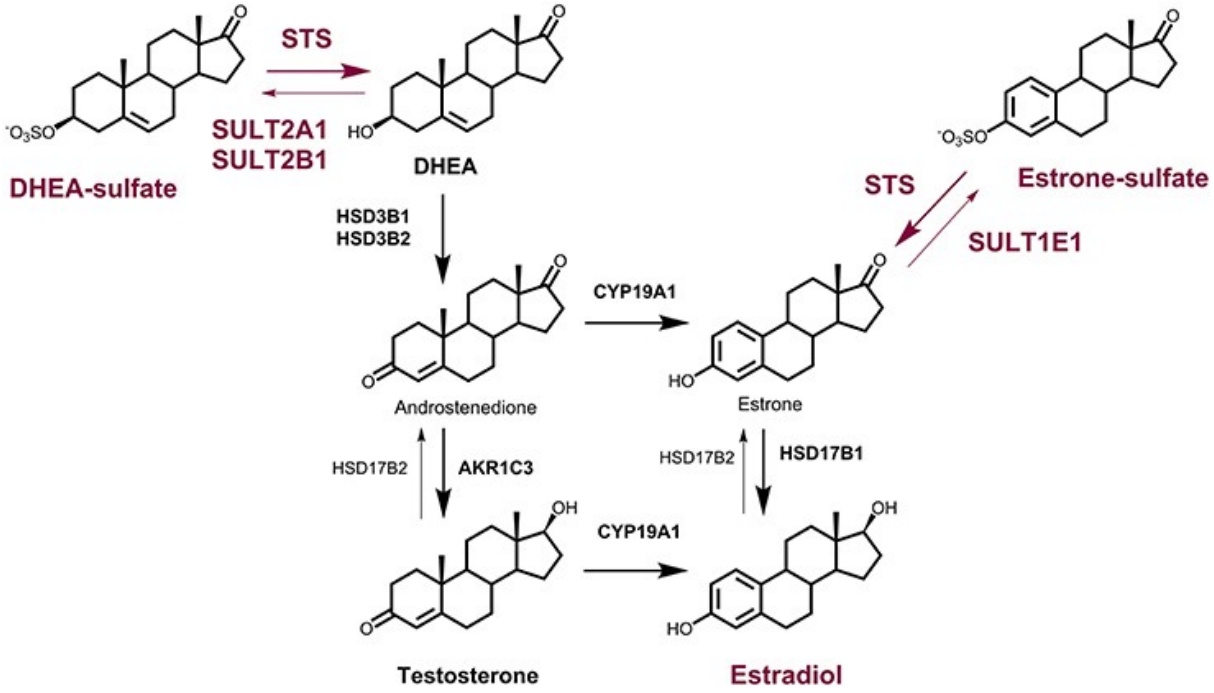


Figure 9: Steroid regulation by STS and SULT. Estradiol (E2) synthesised from DHEAS and otherwise from estrone sulfate (E1); HSD3B1, HSD3B2: 3β-hydroxysteroid dehydrogenase-Δ^4-isomerase; CYP19A1: aromatase; HSD17B1: 17-ketosteroid reductase; AKR1C3: aldo-keto reductase. SULT2A1 and SULT2B1 transfer sulfuryl groups to DHEA and SULT1E1 to E1. Oxidation of E2 to E1 by HSD17B2 (Rizner, 2016).

4.2.6.1. Structure properties

The crystallized structure of the enzyme steroid sulfatase (Fig. 10) is shaped like a fungus with two non-polar “anti-parallel α-helices” which are responsible for the connection to the membrane of the endoplasmic reticulum (ER). Deep in the “polar globular domain” of the protein there is the catalytic site hidden capable of converting inactive steroid precursors in active hormones (Nussbaumer & Billich, 2004).

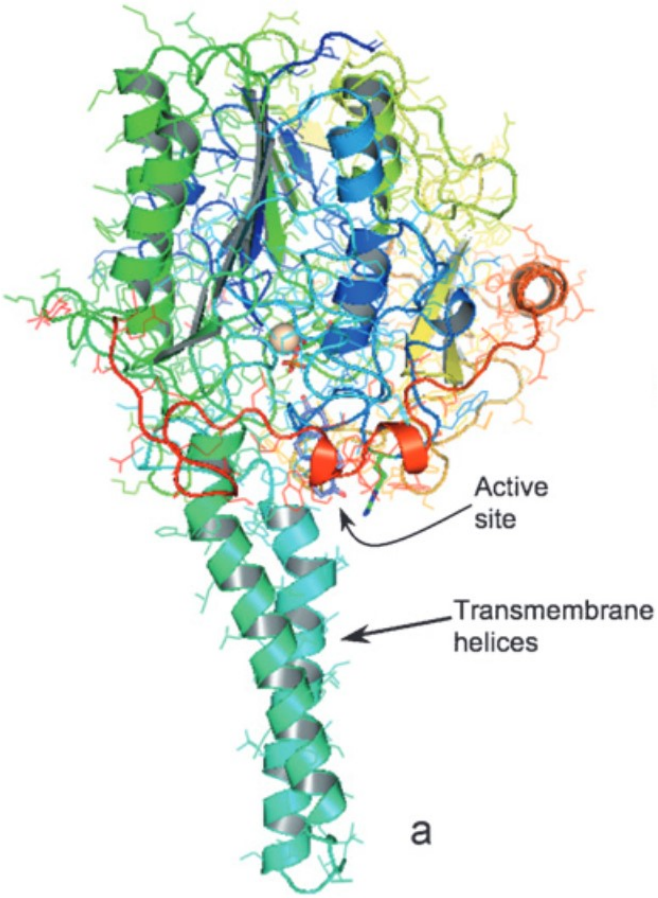


Figure 10:Crystal structure of the human placental STS (Ghosh, 2007)

5. Results and Discussion

5.1. Analysis and chemical investigation of the marine bryozoan *Flustra foliacea*

In the course of the examination of the bacterial flora occurring in the marine bryozoan *Flustra foliacea* performed by Univ.-Prof. Dr. Sergey B. Zotchev (Department of Pharmacognosy of Vienna, Austria) it was also of interest to analyse its chemical composition and to compare it with data from the literature. Due to the very small amount of starting material, it was only possible to give a rough overview about the chemical composition of the rarely described bryozoan. In the following chapters optimization of TLC and HPLC systems and interpretation of the MS data are discussed.

5.1.1. TLC analysis

After extraction of the sample FlufolADMUVIE_1 various TLC systems with different solvents and concentrations were tested to obtain a satisfying separation of the constituents.

According to literature, TLC investigations of *Fustra foliacea* were not easy to interpret due to the very complex mixture of flustramines which often have the same molecular weight and polarity. Lysek (2002) and Rochfort et al (2009) showed that the majority of flustramines are located at about R_f 0,5/0,6 ($CHCl_3$:MeOH; 9,5:0,5/9:1/8:2) or rather R_f 0,2/0,3 (100% EtOAc as mobile phase).

Finally, the mixture of dichloromethane (DCM) and methanol (MeOH) at a ratio of 9,5:0,5 turned out to be the best one for the sample FlufolADMUVIE_1 (Fig. 11 and Fig. 12). Detailed information about the extraction process is described in chapter 7.2.3 “Generation of FlufolADMUVIE_1”, p. 41.

Despite the rather good separation, there was a violet spot at about R_f 0,7 which is not described in the literature. This could be the result of using a highly hydrophobic extraction solvent or these might be constituents which do not belong the class of flustramines.

TLC parameters are depicted in Table 5, p. 42.

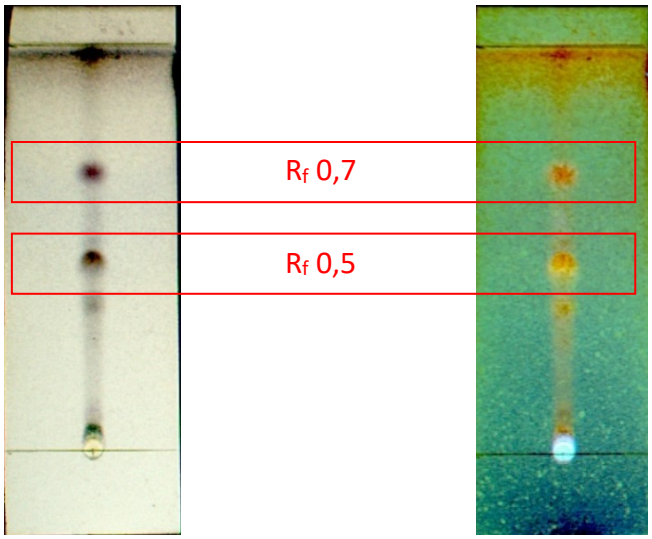


Figure 11:

TLC of FlufolADMUVIE_1 after derivatisation at daylight

Figure 12:

TLC of FlufolADMUVIE_1 after derivatisation at UV 366 nm

5.1.2. HPLC analysis of FlufolADMUVIE_1

Initial runs were first performed with MeOH and water (H_2O) as mobile phase. The main challenge for the separation was the complexity of the crude extract.

First optimization steps were performed with different concentrations of MeOH and H_2O, however, with insufficient separation. MeOH was replaced by acetonitrile (ACN) and the total run time was prolonged to 100 min starting from 10%. This resulted in a much better separation of the peaks in comparison to the previously used mobile phase MeOH/H_2O.

Figure 13 shows that there are some sharp peaks such as those at about RT = 38 min, 55 min, 73 min or 90 min. However, considering the complexity of the extract these peaks could also be a result of overlapping substances eluting at the same time.

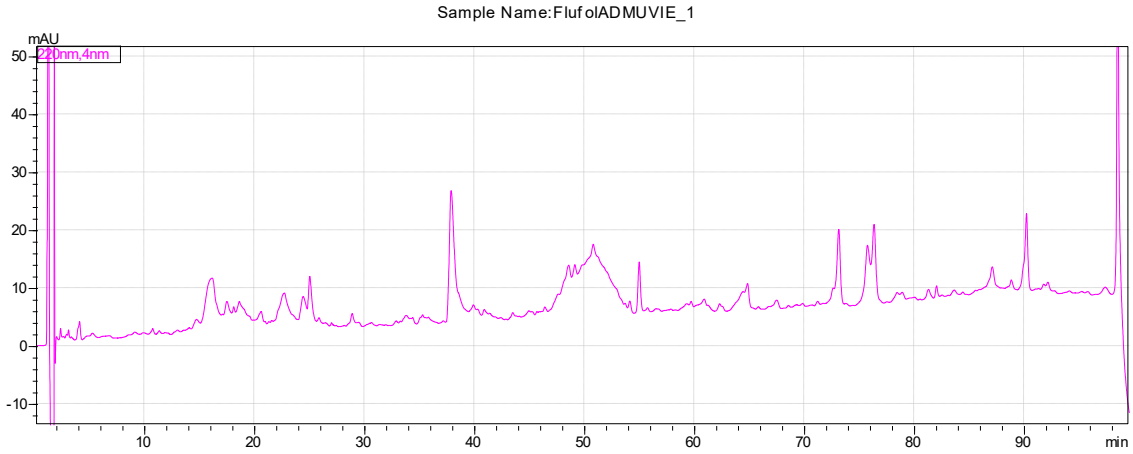


Figure 13: HPL-chromatogram of FlufolADMUVIE_1 at 220 nm
HPLC parameters and stationary phase are depicted in Table 6, p. 43
Gradient of the mobile phase is depicted in Table 7, p. 43

5.1.3. Dereplication of FlufolADMUVIE_1 using HPLC-MS

After optimization of the HPLC method, HPLC-MS was performed to determine the molecular mass of the compounds. Brominated alkaloids show a typical pattern of two bromine isotopes which occur at the ratio of nearly 1:1 (Fig. 14). This pattern was found over the entire range of the chromatogram.

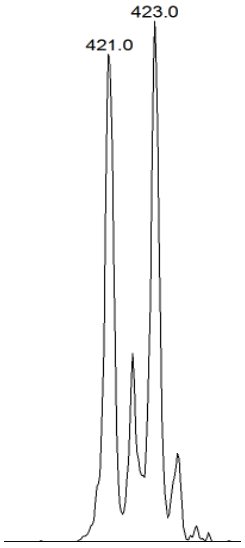


Figure 14: Bromine isotopes

Base peak chromatograms in positive and negative mode allowed for the differentiation between protonated compounds or non-protonated ones. Most of the analytes eluting at the

beginning (5-50 min) showed protonation, while those appearing in the negative mode eluted in the range of 60-80 min (Fig. 15).

Moreover, the molecular mass of the single peaks was obtained from their respective ion fragments. Univ.-Prof. Dr Sergey B. Zotchev provided data from the dictionary of natural products (DNP) dealing with already known compounds from *Flustra foliacea*. Thus, it was possible to assign the obtained masses to their assumed names. Otherwise, it is important to notice that the mass spectrometer was not calibrated properly when the masses were recorded which led to minimal deviations from literature. A compilation of the assigned (possible) compound names is given in Table 3.

Nevertheless, it is important to emphasize the mixture was very complex and therefore the multitude of peaks was enormous. Hence, at this point of investigation it was not possible to determine if some of the peaks were putatively new structures or derivatives of already known compounds.

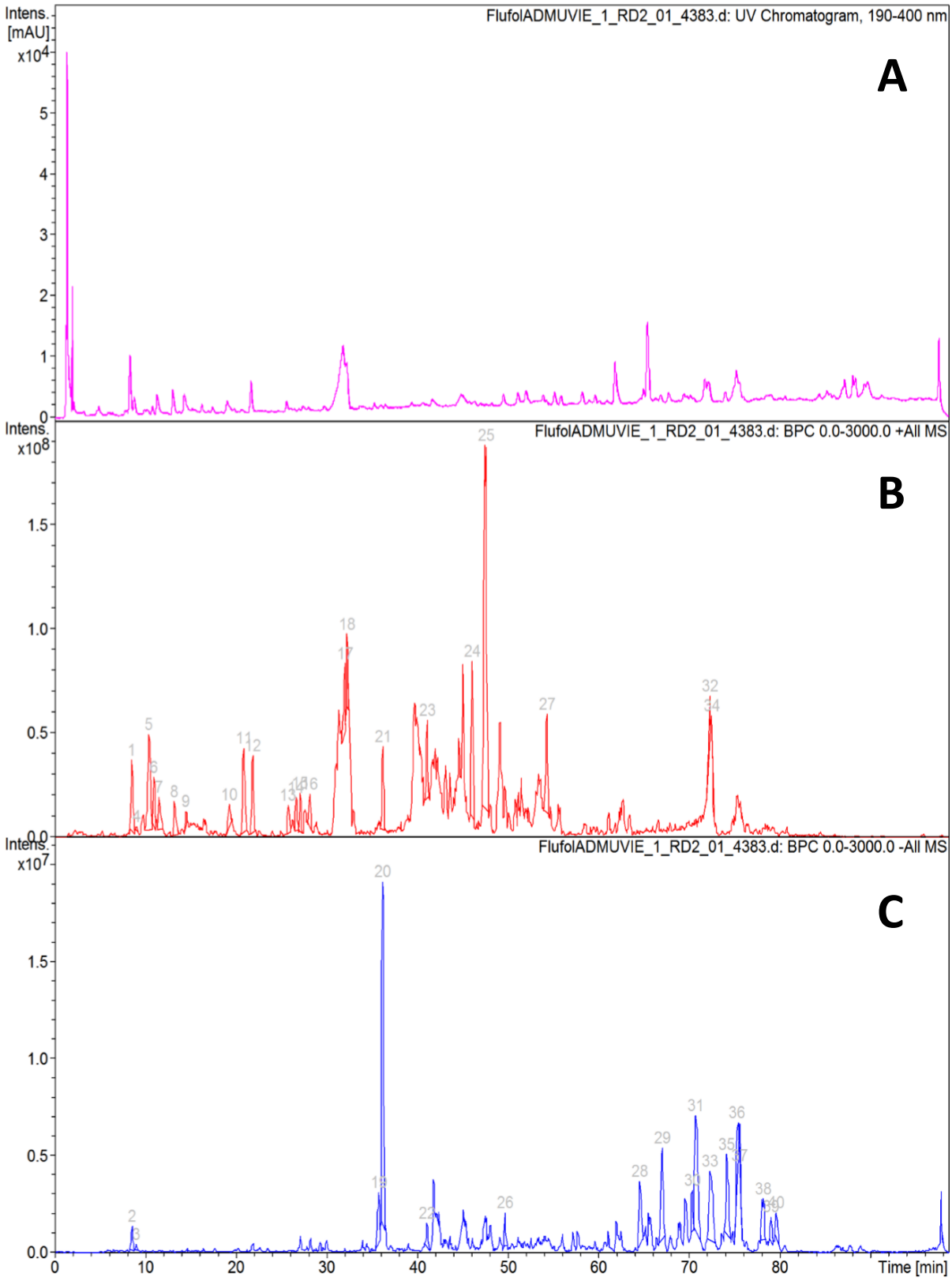


Figure 15: A: HPL-chromatogram of FlufolADMUVIE_1 detected at 190-400 nm; B: Base peak chromatogram in positive mode; C: Base peak chromatogram in negative mode. HPLC-parameters are depicted in Table 6 and 7, p. 43. MS-parameters are given in Table 8, p. 44

Table 3: Proposed structures, retention time, and HRMS data of the compounds tentatively identified in the extract of FlufolADMUVIE_1.

Peak	Retention time [min]	LC-MS mass number (m/z)		Molar mass esti-mated	Exact molar mass	Proposed structures
		Positve mode	Negative mode			
1+2	8,5	337/339 Br	335/336 Br	336	337	Flustramine C; Debromo, 7-bromo, 4-hydroxy, 8,8a-dihydro Flustramine C; 8,8a-Dihydro, N1-oxide Flustraminol A Flustraminol B
3+4	9	351/353 Br	349/350 Br	350	349	6-Bromo2-(1,1-dimethyl-2-propenyl)-tryptamine; Nb-Me,Nb-formyl
5	10,5	495/497 Br	n.c.	494		
6	10,9	421/423 Br	n.c.	420	419	Flustramine M
7	11,5	321/323 Br	n.c.	320	321	6-Bromo-2-(1,1-dimethyl-2-propenyl)-tryptamine; Nb-Me Flustramine C Flustramine E Dihydroflustramine C
8	13,2	321/323 Br	n.c.	320	321	6-Bromo-2-(1,1-dimethyl-2-propenyl)-tryptamine; Nb-Me Flustramine C Flustramine E Dihydroflustramine C
9	14,4	335/337 Br	n.c.	334		
10	19,2	337/339 Br		336	337	Flustramine C; Debromo, 7-bromo, 4-hydroxy, 8,8a-dihydro Flustramine C; 8,8a-Dihydro, N1-oxide Flustraminol A Flustraminol B
11	20,8	489/491 Br	n.c.	488		
12	21,8	290/292 Br	n.c.	289		
13	25,7	358/360 Br	n.c.	357		
14	26,6	472	n.c.	471		
15	27,1	482	n.c.	481		

16	28,1	472	n.c.	471		
17	32	405/407 Br	n.c.	404	405	6-Bromo-3a-(3,7-dimethyl-2,6-octadienyl)-1,2,3,3a,8,8a-hexahydro1-methylpyrrolo[2,3-b]-indol-7-ol Flustramine H Flustramine L Flustramine N Flustrarine B
18	32,2	389/391 Br	n.c.	388	389	Flustramine C; 8,8a-Dihydro, 8-(3-methyl-2-butenyl) Flustramine D Flustramine A Flustramine B
19	35,7	n.c.	643	644		
20	36,1	n.c.	641	642		
21	36,2	625	n.c.	624		
22	41	n.c.	438	439		
23	41	454	n.c.	453		
24	46	506	n.c.	505		
25	47,5	482	n.c.	481		
26	49,5	n.c.	412	413		
27	54,3	510	n.c.	509		
28	64,5	n.c.	253	254		
29	67	n.c.	339	340		
30	70,3	n.c.	305	306		
31	70,7	n.c.	255	254		
32	72,3	405/407 Br	n.c.	404	405	6-Bromo-3a-(3,7-dimethyl-2,6-octadienyl)-1,2,3,3a,8,8a-hexahydro1-methylpyrrolo[2,3-b]-indol-7-ol Flustramine H Flustramine L Flustramine N Flustrarine B
33	72,3	n.c.	281	282	281	6-Bromotryptamine; Nb-Me, Nb-formyl

34	72,5	404/407 Br	n.c.	404	405	6-Bromo-3a-(3,7-dimethyl-2,6-octadienyl)-1,2,3,3a,8,8a-hexahydro1-methylpyrrolo[2,3-b]-indol-7-ol Flustramine H Flustramine L Flustramine N Flustrarine B
35	74,1	n.c.	307	308		
36	75,4	n.c.	307	308		
37	75,5	n.c.	307	308		
38	78	n.c.	283	284		
39	79	n.c.	309	310	310	Flustramine E; Debromo, N8-(3-methyl-2-butenyl)
40	79,6	n.c.	309	310	310	Flustramine E; Debromo, N8-(3-methyl-2-butenyl)

Despite the complexity of the extract and the similarity of the compounds, it was still possible to assign the majority of the main constituents of FlufolADMUVIE_1 to their putative compound names. Unfortunately, it is only possible to speculate about the identity of some constituents overlapping in the chromatogram. A fractionation of the sample which would have resulted in a better resolution of the peaks was impossible due to the small amount of starting material.

The flustramines are structurally very similar. Accordingly, some of them often possess the same retention time in HPL-chromatograms. After using LC-MS with the optimized HPLC-parameters it was possible to assign some of the recorded molecular masses to already known constituents from the genus *Flustra*. There were also masses that were not reported in literature so far. They hint towards either new structures of undisclosed natural products or derivatives of already known compounds. Limited starting material, however, does not allow for the isolation and unambiguous identification of these putatively new constituents.

5.2. Generation of a separation protocol for a selected fraction for polar and non-polar triterpenes in *Gloeophyllum odoratum*

The aim of this part of the diploma thesis was to determine an appropriate separation protocol for polar and non-polar triterpenes in *G. odoratum* which was already worked on by former diploma students. They already investigated the fractions GOEW (Fig. 34, p. 65) (aqueous fraction of the ethanolic extract, GOE), GOEP (Fig. 33, p. 64) (petrol ether fraction of the extract) and GOED (Fig. 31, p. 63) (dichloromethane fraction of the ethanolic extract of GOE). Another fraction, which was initially neglected was the non-soluble fraction (GOEF, Fig. 32, p. 64), a residue after liquid-liquid distribution of GOE (Fig. 30, p. 63) between dichloromethane and H_2O. Therefore, it was of great interest which triterpenes are in this fraction and if they had an influence on STS. Figure 16 gives an overview of the already processed fractions.

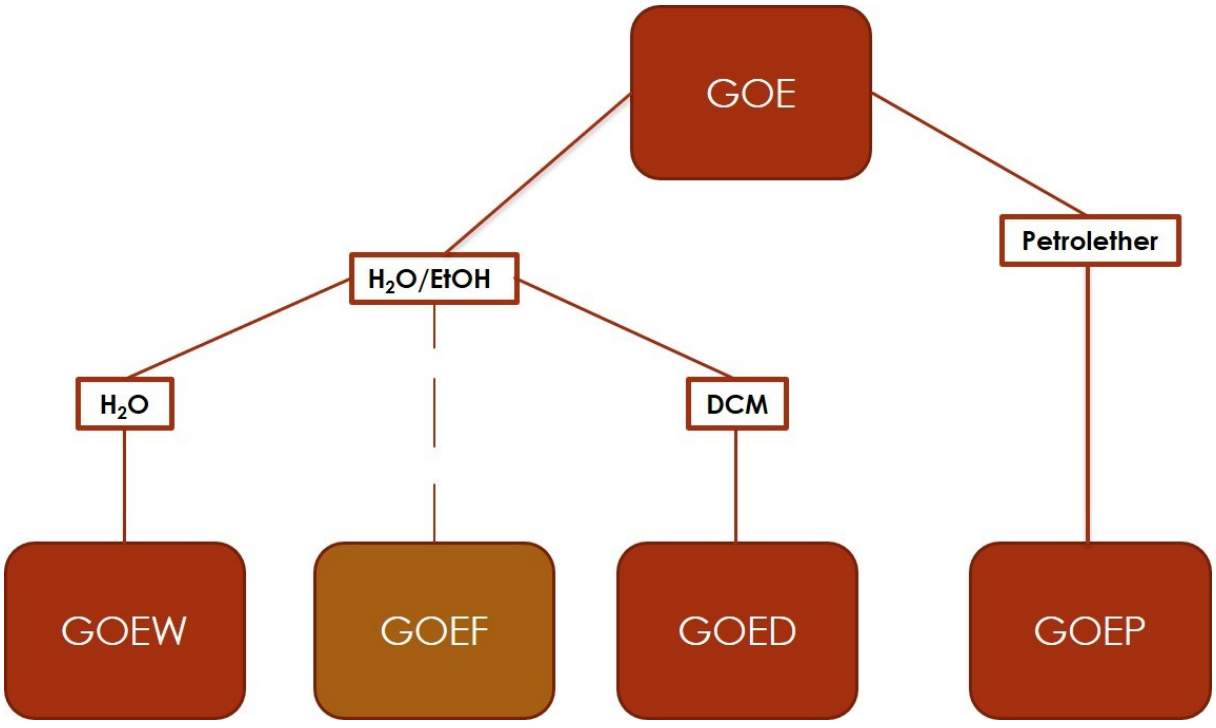


Figure 16: Already obtained fractions GOEW, GOEF, GOED and GOEP from the ethanolic extract of *G. odoratum*

5.2.1. Preliminary tests with GOEF

For further separations with flash chromatography it was important to find an appropriate solvent mixture. This was done by TLC trials, where a small amount of GOEF was applied on the TLC plate's starting zone. Different solvents (pure or as mixtures) were tried out as mobile phases to find the one that is able to slightly move the spot from the starting zone. Since the fraction GOEF was previously obtained as insoluble part from the liquid-liquid partition between dichloromethane and water, it was obvious that solvents with low to medium polarity were required. Mixtures of n-hexane/acetone in different concentrations were not able to move all compounds from the spot at the starting zone. This was only achieved with pure acetone.

5.2.2. GOEF01

Nevertheless, it was worth trying to see if there was a separation using a gradient of acetone and n-hexane as a mobile phase for flash column chromatography with normal phase silica columns. After applying 91,98 mg of GOEF the concentration of 90% n-hexane was held for 5 min and then the concentration of acetone slowly increased till it reached 100% after 90 min. Since this was not optimal but meaningful enough, the obtained fractions were finally re-combined and re-used for further separation trials.

5.2.3. GOEF02

Another option was to try out reversed phase flash column chromatography on an RP-18 column using a gradient of H_2O and acetonitrile as a mobile phase. For this trial 89,24 mg were used. However, this was not successful. Thus, we decided to optimize the method on silica columns in normal phase mode.

5.2.4. GOEF03

Finally, an optimised gradient starting from n-hexane going to acetone was used for the following fractionation of one portion of GOEF (9,909 g). For receiving adequate fractions, it was reasonable to perform a very long run with a slowly increasing gradient till the concentration of acetone reached 100%. Considering such a slow gradient, first n-hexane could dissolve the very non-polar compounds and the subsequently increasing acetone

concentration slowly eluted the undisclosed more hydrophilic ones.

After flash column chromatography, TLC monitoring allowed to combine the 832 single fractions into 17 collective fractions (TLCs of all fractions are given in Fig.24-29, p. 61-62). At VIS (Fig. 17) as well as at 366 nm (Fig. 18), the TLCs showed an appropriate separation of the compounds in GOEF. However, one substance nearly occurs in each fraction in a high concentration (blue violet spot at R_f 0,5).

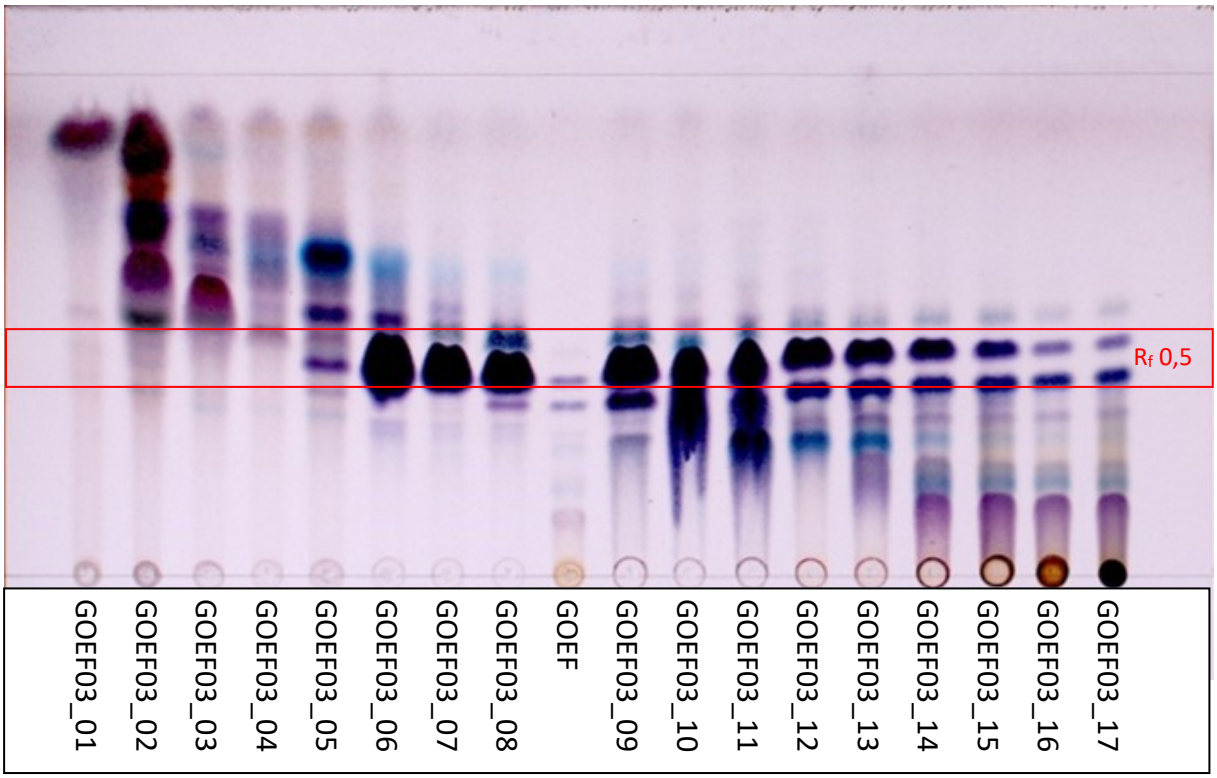


Figure 17: TLC in VIS of GOEF03_01 to 17 including the reference GOEF after derivatisation with H_2SO_4/Vanillin. TLC parameters are depicted in Table 9, p. 44

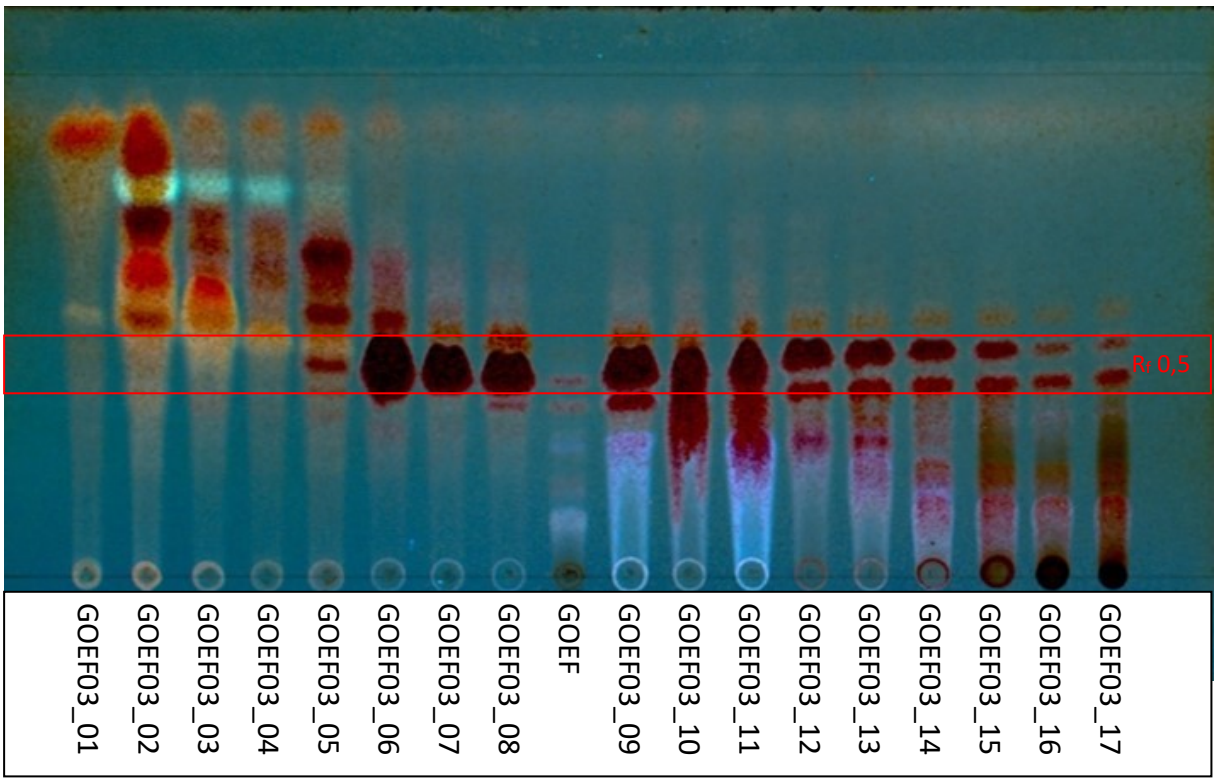


Figure 18: TLC of GOEF03_01 to 17 including the reference GOEF after derivatisation with H_2SO_4Vanillin. Detection at 366 nm wavelength. TLC parameters are depicted in Table 9, p. 44

Another point which was observed was the strong change in colour of the silica column. Instead of pure white columns they were completely dyed red-dark brown after each run, which could putatively be traced to the many pigments occurring in *G. odoratum* and especially in the fraction GOEF. These compounds could not be washed out even after a long post-run.

The 17 fractions obtained were afterwards investigated by HPLC-CAD (= charged aerosol detector) to better monitor the fractionation. HPLC-CAD was performed with acetonitrile/H_2O using a reversed phase column. The gradient of the mobile phase and HPLC-CAD parameters are given in Table 15, p. 49, and Table 16, p.50. A tall peak at RT= 27 min (Fig. 19), which decreased till fraction GOEF03_17, going through all fractions (Fig.35-51, p.66-74), could probably be assigned to the spot at R_f 0,5 in the TLCs.

All in all, a rough separation of polar and non-polar compounds in the fraction GOEF was achieved and depicted in Figure 19 and 20. The chromatogram of GOEF is depicted in Fig 32, p. 64.

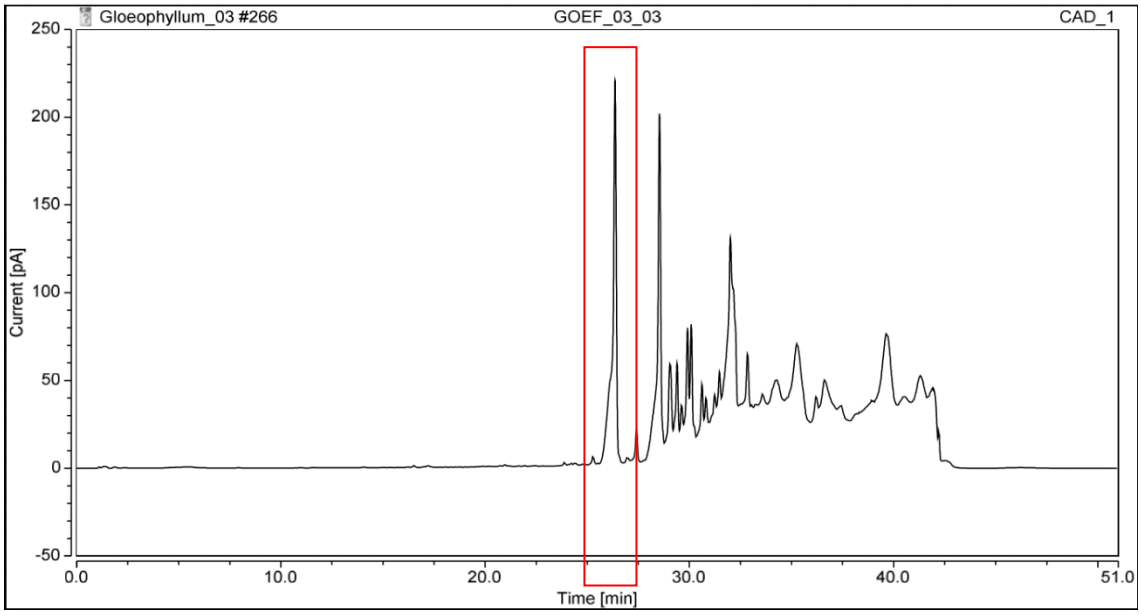


Figure 19: Non-polar constituents of GOEF eluting after 27 min. The peak at RT= 27 min appeared in all fractions of GOEF.

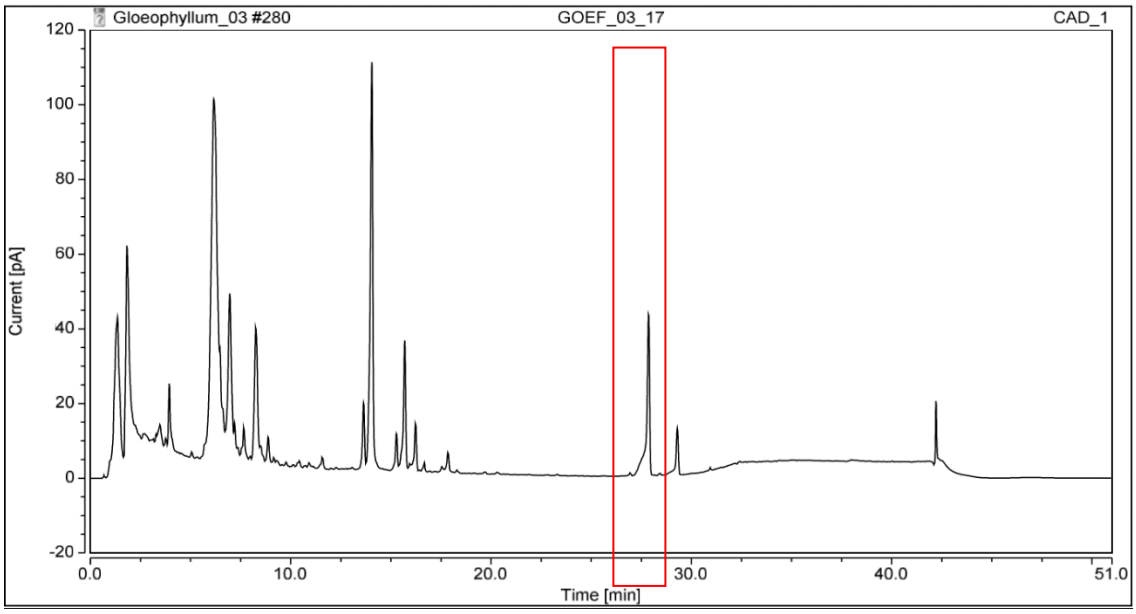


Figure 20: Polar compounds of GOEF eluting till RT= 15 min were separated from non-polar ones. The peak at RT= 27 min appeared in all fractions of GOEF.

Further two portions of GOEF were fractionated in GOEF04 (9,949 g) and GOEF05 (6,945 g) using the same parameters as in GOEF03. In this way, almost everything of the huge quantity of GOEF starting material, except for a small part needed as reference was used for fractionation.

5.2.5. STS inhibition by GOEF fractions

Regarding the importance of innovative approaches for the treatment of hormone dependent cancers, it was worth knowing if some fractions of GOEF are capable of STS inhibition. Hence, small amounts of the 17 fractions of GOEF03 as well as the fractions GOEF and GOEP were sent for bioactivity testing to a cooperation partner at University of Birmingham, United Kingdom. Dr. Paul A. Foster and his team performed STS inhibition assays using the positive control STX64. This is a coumarin sulfamate also known as Irosustat/667 Coumate, i.e. the most potent STS inhibitor for the treatment of hormone dependent mammary cancer or prostate cancer in clinical studies (El-Gamal et al, 2016). At the applied concentration of 100 µg/ml the fraction GOEF03_17 inhibits STS activity by 55% in comparison to GOEF with 50% inhibition. Due to these results further woking steps were performed with GOEF03_17 to isolate possible bioactive compounds of *G. odoratum*.

In Figure 21 the results of the STS assay are shown. STS activity of the fractions GOEF03_01-17 are given as a percent value (% STS activity). Besides some less active fractions there are some relevant bioactive fractions, such as GOEF03_05, GOEF03_11-13 and the most active one GOEF03_17. Compared to the ethanol extract GOE the fraction GOEF03_17 showed almost the same inhibition of the enzyme. Thus, this fraction was assumed to contain interesting bioactive natural products.

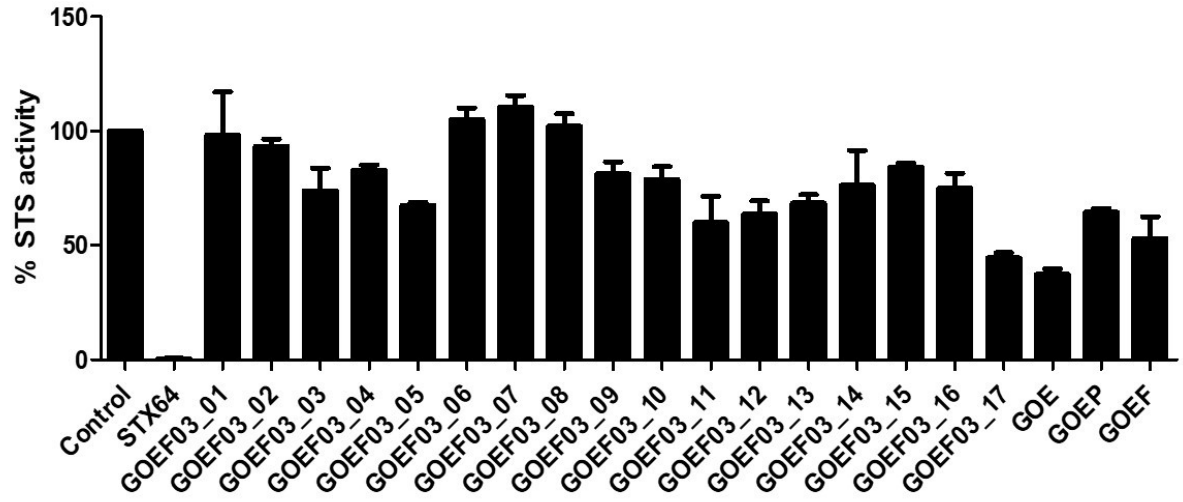


Figure 21: STS assay performed with a vehicle control, a positive control (STX64), GOEF03 fractions 01 to 17, GOE, GOEP and GOEF

5.2.6. Purification of GOEF03_17

Based on the bioactivity results for GOEF03_17 we considered to take a closer look at this fraction and tried to reduce or, if possible, to remove the distracting brown pigments of the extract for further chromatographic methods.

Sephadex LH-20 chromatography was tried out but did not lead to satisfying results. A separation via SFC was also not possible due to technical problems. Therefore, a liquid-liquid partition using different solvents was performed. After various separation steps (see chapter 7.3.6. “Preliminary tests for further purification steps of GOEF03_17”, p. 51) a DCM fraction was obtained which contained less brown pigments but most of the secondary metabolites present in GOEF03_17, except the very hydrophilic compounds. Moreover, after a first liquid-liquid distribution with ethyl acetate (EtOAc) and H_2O, the EtOAc phase was further processed with DCM/H_2O where a so called “interphase” was formed due to compounds

which were not dissolved neither in polar nor non-polar solvents. Figure 22 presents the HPL-chromatograms of the H_2O phase, the “interphase”, the DCM phase and GOEF03_17 as a reference.

The obtained fractions were monitored by TLC and HPLC-CAD and could be vital and promising for further separation of compounds present in *G. odoratum*.

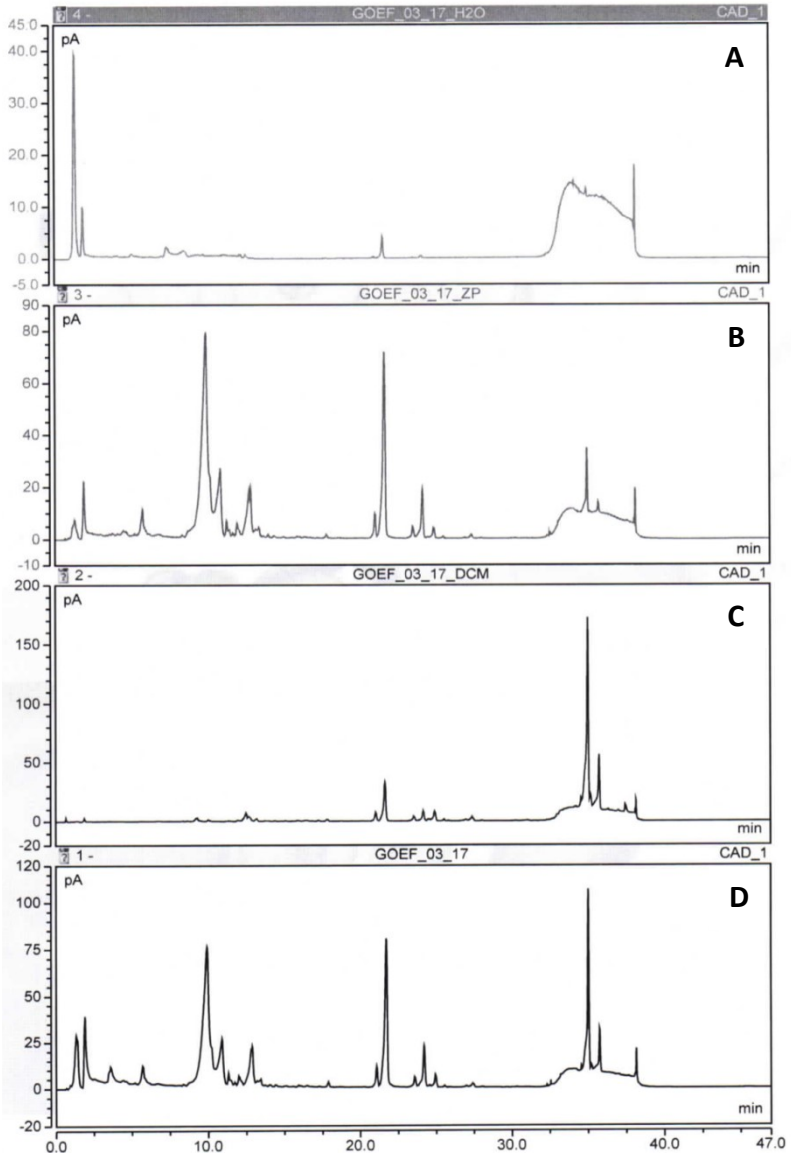


Figure 22: A: GOEF03_17_H_2O, B: GOEF03_17_ZP (= “interphase”), C: GOEF03_17_DCM, D: GOEF03_17

6. Conclusion

6.1. Analysis and chemical investigation of the bryozoan *Flustra foliacea*

As part of this thesis, it was of interest to investigate a marine organism. Often wrongly described as an ordinary seaweed waving in the ocean current, *Flustra foliacea* has to offer various qualities, such as many biological activities. Concerning these properties of the constituents, especially brominated indole alkaloids of the physostigmine type or more suitably called flustramines could open new perspectives for the treatment of several diseases. Therefore, they could feature new interesting natural building blocks for design and synthesis of potent drugs if once the whole chemical profile and the biological properties of each compound is described.

Still, investigations of *Flustra foliacea* are not frequently performed because of the low amounts of available starting material, difficulties in the collection of this marine animal and very time-consuming isolation and structure elucidation procedures. Particularly in this thesis, it was not possible to properly classify structures based only on MS-data. The complexity of the extract and similarity of the contained compounds which only differ from each other in the substitution pattern of their prenyl residues, made an interpretation of the chromatograms a very challenging task. Additionally, the flustramines´ structural likeness also resulted in the same retention time and in many cases in the same molecular weight. At this point, it was not possible to distinguish the investigated structures from each other without any isolation and subsequent 1D or 2D NMR experiments. But the fact that there was only a very small amount of sample available, allowed neither further examination of the specific chemical constituents of *Flustra foliacea* nor the isolation and identification of single flustramines.

Despite of these limitations it was still possible to assign some of the detected masses to already known constituents. On the other hand, some single peaks which could not be assigned to compounds reported in the literature so far hint towards either new unfamiliar natural compounds or derivatives of known structures from the genus *Flustra*. Overall, the investigation of marine bryozoans and their biologically active compounds seem to be a thrilling mission provided that there is enough starting material to gain fractions for a better

and easier isolation and structure elucidation.

6.2. Generation of a separation protocol for a selected fraction of polar and non-polar triterpenes in *Gloeophyllum odoratum*

Within the scope of this diploma thesis and considering the limited investigation of the marine bryozoan *Flustra foliacea* an already prepared fraction of the mushroom *Gloeophyllum odoratum* represented the longer and complex part of this work. Considering the previous analysis of other fractions, such as GOEP, GOED, and GOEW, it was of interest to investigate which mycochemical composition the non-soluble fraction GOEF had. Since the previous isolation approaches and structure elucidation of new compounds in other fractions, the assumption of finding other unknown structures in this fraction was promising. Therefore, a separation protocol was generated with the aim of achieving a separation of polar and non-polar compounds.

Since there was a huge amount of starting material of GOEF available, a proper large-scale separation resulting in numerous fractions could be performed. The flash chromatography separations GOEF03, GOEF04 and GOEF05 yielded 832 single fractions each which were then combined into 17 fractions each for further working steps. TLC analysis of these fractions showed a promising result of the fractionation by flash chromatography. Following HPLC-CAD examinations also pointed out that there was a successful separation of polar and non-polar compounds. The comparison of the chromatograms obtained with a normal phase gradient from flash chromatography and the obtained HPLC-CAD chromatograms showed a relatively high concentration of very hydrophobic compounds. In the normal phase separation, a slowly increasing concentration of acetone turned out to be a key point for dissolving polar substances. Moreover, a noteworthy fact was also the appearance of a distinct peak in the HPLC-CAD chromatograms at RT= 27 min which was present in all fractions. It can be assumed that the compound related to this peak occurs in a very high concentration in this fungus.

Since previous biological tests of GOEP, GOED and GOEW showed noticeable results on STS inhibition, small amounts of the fractions of GOEF03_01-17 were sent to Dr. Paul Foster, University of Birmingham, UK. Indeed, some of these fractions showed very promising STS inhibition compared with the ethanol crude extract of *G. odoratum*. Especially GOEF03_17

turned out to be the most potent fraction inhibiting STS activity by 55% (at c = 100µm/ml) which is nearly the same as GOE did (60%). As a result, it was obvious to take a closer look at this fraction and start a further study focussing on its purification.

Red-brown pigments contained in this fraction might cause issues considering further purification steps with sensitive instruments. Therefore, a protocol was developed to remove most of the pigments. Concerning my diploma thesis, the work on *G. odoratum* was finished at this step but the established protocols will be useful for further investigations.

Altogether, there remains a high request for potent compounds inhibiting the enzyme STS as a treatment for hormone dependent cancers. The STS inhibiting activity of the fractions obtained from *G. odoratum* are quite positive and promises a great outlook for further work on STS inhibition by compounds derived from this mushroom.

7. Materials and Methods

The following chapter lists detailed information about all used materials and procedures which were mentioned in chapter 5 “Results and Discussion”.

7.1. Chromatographic techniques

7.1.1. Thin Layer Chromatography

Thin layer chromatography (TLC) represents a very simple and fast chromatographic technique giving an overview of the constituents in a sample. Typically, on a plate coated with an adsorbent (stationary phase) a sample is applied on a marked starting point on the lower part of the plate and then put in a vessel filled with solvents (mobile phase) that nearly reach till the starting point. Over the time the solvents are slowly running upwards the TLC plate by capillary effect and lead to the separation of the mixture due to the different solubility in the solvents and adsorption to the stationary phase. After chromatography, the separated spots are visible at daylight or under UV light (Organic Chemistry at CU Boulder, accessed in January 2018).

7.1.2. High Performance Liquid Chromatography

High performance liquid chromatography (HPLC) is a commonly used chromatographic method for analytical investigations and the standard equipment of each modern laboratory nowadays. Under high pressure, solvents (mobile phase) pass through a column (stationary phase) filled with adsorbent of very small particle size. Thus, it makes it a very fast and efficient method for chromatography combined with detection methods such as UV or MS detection (Chemguide, accessed in January 2018). The separation of an apllied sample into its single compounds depends on their interactions with the mobile and stationary phase used (LaboratoryInfo.com: All About Medical Laboratories, accessed in January 2018).

7.1.3. Flash Column Chromatography

As a preparative method, flash column chromatography is used for fast separation steps performed with air pressure. The chosen solvents are pumped through a dry load cartridge containing the sample and transport the dissolved compounds through the analytical column.

The columns are normally filled with silica or RP-18 materials of a defined particle size (Dinesh-Kumar, 2016). The separated compounds are eluting one by one depending on their affinity to the solvents and the columns and are collected in numerous fractions during the entire chromatography. Nowadays this process often runs automatically by a sample collector and is connected to analytical instruments such as ELSD or UV spectrometers (Wikipedia, accesed in January 2018).

7.2. Analysis and chemical investigation of the marine bryozoan *Flustra foliacea*

7.2.1. Animal material

The animal material for this diploma thesis has been provided by Univ.-Prof. Dr. Sergey Zotchev. The frozen sample originating from the White Sea, Russia, was freeze dried to remove the residual water resulting in 1063 mg dried sample.

7.2.2. Labelling *Flustra foliacea´s* extract

The extract of *Flustra foliacea* was named FlufolADMUVIE_1 due to a distinct guideline. In Table 4 labelling is explained.

Table 4: Labelling of the extract of *Flustra foliacea*

Signs	Description
Flufol	First three letters of genus and species
A	Contained compound class (A = alkaloids)
DM	Used extracting solvents DCM and MeOH
UVIE	University of Vienna
_1	Extract number 1

7.2.3. Generation of FlufolADMUVIE_1

The lyophilized animal (1,0634 g) was frozen to -80°C to make it easier grounding it to a pulp. Especially at this step the lemon-like smell of the compounds was recognizable. Afterwards the material was placed into glass tubes, defatted with 18 ml n-hexane and shaken for about one minute. The hexane extract was discarded after centrifugation (10 min, 3500 rpm). The residue was first extracted with 25 ml dichloromethane (DCM) for 15 min in the ultrasonic

bath at room temperature to extract non-polar compounds and then centrifuged (10 min, 3500 rpm). Then the extract was filtered through a pipette stuffed with cotton wool and transferred into a round-bottom flask. The second extraction of the remaining material was performed with 46 ml methanol (MeOH) for 15 min in the ultrasonic bath at room temperature to extract polar substances and centrifuged (10 min, 3500 rpm) afterwards. The extracts of MeOH and DCM were combined and dried under reduced pressure. The concentrate was dried in the desiccator to finally obtain 338,06 mg of the extract, which was named FlufolADMUVIE_1.

7.2.4. TLC system for FlufolADMUVIE_1

Table 5: Stationary phase, mobile phase, spraying reagents and detection for TLC investigation of FlufolADMUVIE_1

Stationary phase	TLC Silica gel 60 F_{254}
Mobile phase	DCM:MeOH (9,5:0,5)
Spraying reagents	1) 5% sulfuric acid in MeOH 2) 1% vanillin in MeOH
Detection	VIS, UV_{254}, UV_{366}

7.2.5. HPLC optimization

HPLC was performed with a Shimadzu UFLC XR instrument and a Phenomenex® Gemini-NX column (5 µm C18 110A; 150 x 3,00 mm) as stationary phase. The parameters for the optimized system are depicted in Table 6 and the gradient of the mobile phase acetonitrile/H_2O is given in Table 7.

Table 6: HPLC device, column (stationary phase) and configured parameters

HPLC Device	Column	Temp. [C°]	Flow rate [ml/min]	Inject. Vol. [µl]	Det. Wavel. [nm]
Shimadzu UFLC XR	Phenomenex® Gemini-NX 5 µm C18 110A (150x3,00 mm)	35	0,5	10	220 250 280

Table 7: Gradient of H_2O and acetonitrile

Time [min]	H_2O [%]	Acetonitrile [%]
0	90	10
70	20	80
85	2	98
95	2	98
96	90	10
100	90	10

7.2.6. Mass spectrometer parameters

HPLC was coupled to MS and led to fragmentation patterns gained with electrospray ionization (ESI) with following parameters (Table 8).

Table 8: ESI ion source parameters

Capillary Voltage [kV]	Nebulizer [N_2] [psi]	Dry Gas Flow [N_2] [L/min]	Dry Temp. [°C]	Target Mass [m/z]
+3,5/-3,7	26	9	340	500

7.3. Generation of a separation protocol for a selected fraction of polar and non-polar triterpenes in *Gloeophyllum odoratum*

7.3.1. Origin of GOEF

The non-soluble fraction GOEF was already obtained by former diploma students performing a liquid-liquid partition of the ethanol crude extract GOE with DCM/H_2O. Thereupon, an insoluble residue (GOEF) was left between the DCM and water phase. In comparison to the other fractions GOEW, GOEP and GOED the non-soluble fraction GOEF had the highest yield with 27,12 g. This huge amount was divided for three identical separations, i.e. GOEF03, GOEF04 and GOEF05. 102,98 mg of GOEF were kept as a reference.

7.3.2. TLC system for GOEF

The parameters for the optimised TLC systems are given in Table 9. After derivatisation with spraying reagents the TLC plates were dried in the oven for 5 min at 80°C.

Table 9: Stationary phase, mobile phase, spraying reagents and detection for all fractions of GOEF

Stationary phase	TLC Silica Gel 60 F_{254}
Mobile phase	DCM:MeOH:H_2O (10:1:0,25)
Spraying reagents	1) 5% sulphuric acid in MeOH 2) 1% vanillin in MeOH
Detection	VIS, UV_{366}

7.3.3. Flash chromatography of GOEF03, GOEF04 and GOEF05

Flash chromatography was performed with acetone and n-hexane with a distinct gradient (Table 10) operated at normal phase mode using silica columns. The dry load cartridge contained about 10 g of GOEF which was carefully mixed with silica gel. The liquid application of the sample was not possible since the fraction was too sticky and clumpy and would have led to an over-pressure of the column.

The concentration of n-hexane was held for 10 min at 90% to obtain a separation of the non-polar compounds from the polar ones which then eluted with increasing acetone concentration. For detection the evaporative light scattering detector (ELSD) was used. Detailed parameters about column, detection, temperature, etc. are depicted in Table 11.

Table 10: Gradient of the solvents n-hexane and acetone

Time [min]	Flow Rate [ml/min]	n-Hexane [%]	Acetone [%]
0	46	90	10
10	46	90	10
180	46	0	100

Table 11: Column, detection, temperature, collected fractions and volume per test tubes for fractionation of GOEF03, GOEF04 and GOEF05 by Flash Chromatography

Column	PURIFLASH Column 25 Silica HC-120,0 g (20 bar)
Detection by ELSD [nm]	250-600
Temp. [°C]	30
Fractions collected	831
Vol. per test tube [ml]	10

7.3.3.1. Applied amount of the samples GOEF01, GOEF02, GOEF03, GOEF04 and GOEF05

- GOEF01: 91,98 mg
- GOEF02: 89,24 mg
- GOEF03: 9,909 g
- GOEF04: 9,949 g
- GOEF05: 6,945 g

7.3.3.2. Fraction yields of GOEF03, GOEF04 and GOEF05

Fraction yields of mentioned fractions are listed in Table 12-14.

Table 12: Fraction yields of GOEF03

Sample	Collected Fractions	Vial Weight [g]	Vial + Substance [g]	Yield [mg]	Amount [%]
GOEF03_01	1-42	9,81536	9,83284	17,48	0,18
GOEF03_02	43-50	9,73003	9,74285	12,82	0,13
GOEF03_03	51-65	9,51620	9,53492	18,72	0,19
GOEF03_04	66-95	9,80945	9,82002	10,57	0,11
GOEF03_05	96-125	9,92673	9,94139	14,66	0,15
GOEF03_06	126-150	9,78054	9,84574	65,2	0,66
GOEF03_07	151-165	9,68802	9,84003	152,01	1,53
GOEF03_08	166-195	9,71723	9,97334	256,11	2,58
GOEF03_09	196-215	9,60954	9,74931	139,77	1,41
GOEF03_10	216-235	9,84002	9,98462	144,6	1,46
GOEF03_11	236-270	10,10457	10,34564	241,07	2,43
GOEF03_12	271-310	9,74608	9,97202	225,94	2,28
GOEF03_13	311-350	10,22132	10,41851	197,19	1,99
GOEF03_14	351-450	10,22593	10,95784	731,91	7,39
GOEF03_15a	451-560	10,29427	10,73615	441,88	8,43
GOEF03_15b	451-560	9,71569	10,10903	393,34	
GOEF03_16a	561-690	9,79660	10,41548	618,82	10,31
GOEF03_16b	561-690	10,23813	10,64068	402,55	
GOEF03_17a	691-832	10,12934	10,68048	551,14	10,53
GOEF03_17b	691-832	9,84294	10,33497	492,03	
				Σ: 5127,81	**51,75**

Table 13: Fraction yields of GOEF04

Sample	Collected Fractions	Vial Weight [g]	Vial + Substance [g]	Yield [mg]	Amount [%]
GOEF04_01	1-42	10,13922	10,15203	12,81	0,13
GOEF04_02	43-50	10,11803	10,12025	2,22	0,02
GOEF04_03	51-65	10,00036	10,00369	3,33	0,03
GOEF04_04	66_100	10,06382	10,08096	17,14	0,17
GOEF04_05	101-125	10,45605	10,46423	8,18	0,08
GOEF04_06	126-149	9,97926	10,00331	24,05	0,24
GOEF04_07	150-165	10,100205	10,26804	167,84	1,69
GOEF04_08	166-185	10,00552	10,16656	161,04	1,62
GOEF04_09	186-200	10,00596	10,10059	94,63	0,95
GOEF04_10	201-240	10,19075	10,62533	434,58	4,37
GOEF04_10 KR	201-240	10,22117	10,29001	68,84	0,69
GOEF04_11	241-270	10,15119	10,46591	314,72	3,16
GOEF04_12	271-310	10,23092	10,40025	169,33	1,70
GOEF04_13	311-350	10,24831	10,29422	45,91	0,46
GOEF04_14	351-365	10,05460	10,15015	95,55	0,96
GOEF04_15	366-410	9,98889	10,49740	508,51	5,11
GOEF04_16a	411-610	10,55208	11,98424	1432,16	23,78
GOEF04_16b	411-610	10,25519	11,18952	933,89	
GOEF04_17a	611-831	10,10144	10,93952	838,08	15,36
GOEF04_17b	611-831	10,50675	11,19683	690,08	
				Σ: 6022,89	**60,53**

Table 14: Fraction yields of GOEF05

Sample	Collected Fractions	Vial Weight [g]	Vial + Substance [g]	Yield [mg]	Amount [%]
GOEF05_01	1-42	10,12243	10,13467	12,24	0,18
GOEF05_02	43-50	10,24693	10,24905	2,12	0,03
GOEF05_03	51-65	10,04814	10,05275	4,61	0,66
GOEF05_04	66-95	10,16560	10,18186	16,26	0,23
GOEF05_05	96-110	10,28984	10,29430	4,46	0,06
GOEF05_06	111-150	10,17854	10,27280	94,26	1,36
GOEF05_07	151-175	10,44327	10,65911	215,84	3,11
GOEF05_08	176-225	10,52543	10,95415	428,72	6,17
GOEF05_09	226-300	10,17996	10,57590	395,94	5,70
GOEF05_010	301-350	10,48808	10,77434	286,26	4,12
GOEF05_011	351-420	10,16802	10,73253	564,51	8,13
GOEF05_012	421-460	10,29711	10,59681	299,70	4,32
GOEF05_013	461-540	10,51493	10,93775	422,82	6,09
GOEF05_014	541-590	10,19380	10,44978	255,98	3,69
GOEF05_015	591-670	10,12988	10,42309	293,21	4,22
GOEF05_016	671-750	10,07075	10,38457	131,82	1,90
GOEF05_017	751-829	10,29118	10,66335	372,17	5,36
GOEF05_NL		10,16622	10,29863	132,41	1,91
				Σ: 3933,33	**57,24**

7.3.4. HPLC-CAD of GOEF03, GOEF04 and GOEF05

For investigation of the obtained fractions, HPLC-CAD was performed with following parameters (Table 15).

Table 15: HPLC-CAD parameters of GOEF03, GOEF04 and GOEF05

Column	Inj. Vol [µl]	Flow Rate [ml/min]	Temp. [°C]	Det. Wavel. [nm]
Agilent Zorbax SB-C18	10	1	35	190 254

The gradient of the mobile phase acetonitrile/H_2O is shown in Table 16.

Table 16: Gradient of the HPLC-CAD method

Time [min]	Acetonitrile [%]	H_2O [%]
0	35	65
30	98	2
40	98	2
41	35	65
51	35	65

7.3.5. Prepared fractions sent to Dr. Paul Foster for testing on STS inhibition

For STS assays 3-4 mg of GOEP, GOEF and GOEF03_01-17 were prepared (Table 17).

Table 17: Sent fractions for testing on STS inhibition

Sample	Amount [mg]
GOEF	4,12
GOEP	4,22
GOEF03_01	3,21
GOEF03_02	3,37
GOEF03_03	3,24
GOEF03_04	3,19
GOEF03_05	3,14
GOEF03_06	3,32
GOEF03_07	3,52
GOEF03_08	3,27
GOEF03_09	3,25
GOEF03_10	3,50
GOEF03_11	3,16
GOEF03_12	3,38
GOEF03_13	3,22
GOEF03_14	3,29
GOEF03_15	3,43
GOEF03_16	3,45
GOEF03_17	3,18

7.3.6. Preliminary tests for further purification steps of GOEF03_17

In a separatory funnel GOEF03_17 was distributed between several solvents with the aim to reduce or remove the brown pigments of the extract. Ideally, as a second goal, this procedure should also lead to a rough separation of polar and non-polar compounds.

First 100 mg of GOEF03_17 were distributed between EtOAc/H_2O (1:1; 20 ml each), which resulted in three phases – H_2O phase, "interphase" and EtOAc phase. Then the EtOAc phase was placed into glass tubes and after centrifugation the supernatant was mixed with DCM/H_2O (1:1; 20 ml each) and again centrifuged. The H_2O phase was set aside, and the DCM phase was washed with H_2O twice. After each washing step the so called "interphase" was removed. After combining all "interphases" of the EtOAc/H_2O and DCM/H_2O steps it was distributed between butanol/H_2O (1:1; 20 ml each) after centrifugation. Finally, the butanol phase was washed with H_2O twice. H_2O phase, DCM phase and "interphase" of GOEF03_17 were examined with HPLC-CAD starting with 30% acetonitrile instead of 35% with a total running time of 47 min (Table 18). The workflow for the liquid-liquid separation of the most active fraction GOEF03_17 is depicted in Figure 23.

Table 18: Modified gradient for HPLC-CAD

Time [min]	Acetonitrile [%]	H_2O [%]
0	30	70
30	70	30
31	98	2
36	98	2
37	30	70
47	30	70

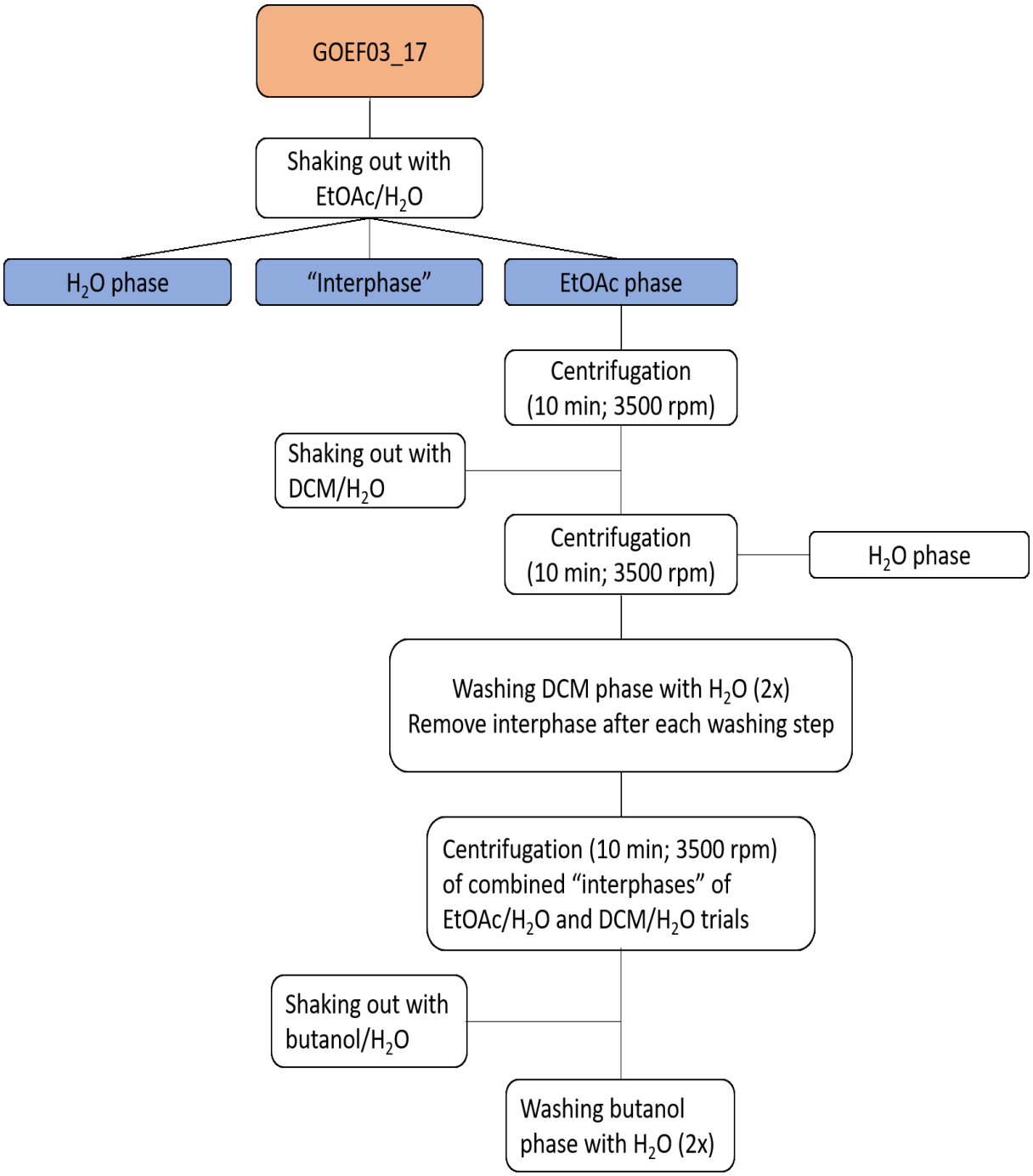


Figure 23: Workflow for the liquid-liquid separation of GOEF03_17

7.4. Instruments, solvents and reagents

7.4.1. Instruments

Device	Description
CAD	Charged Aerosol Detector, Corona Ultra RS
Concentrator	Sample concentrator FSC400D, Techne
ELSD	Evaporative Light Scattering Detector
Flash Chromatography	Interchim Puriflash, Software: Interchim soft
Hot Air Dryer	HG 2000 E, Steinel
HPLC for *F. foliacea*	Shimadzu UFLC XR
HPLC for *G. odoratum*	Dionex Ultimate 3000
Mass Spectrometer	Bruker Esquire Ion Trap
Rotary Evaporator	Rotavapor R II, Büchi
TLC Spray Cabinet	CAMAG TLC Spray Cabinet II
TLC Visualizer	CAMAG
Ultrasonic Bath	Transsonic T 460, Elma
UV Lamp	CAMAG
Vacuum Pump	V-710, Büchi
Vortex	Genius 3, IKA

7.4.2. Solvents and reagents

Solvent/Reagent	Description
Acetone	Rectapur; ÖAB, distilled
Acetonitrile for HPLC	Hypersolv Chromanorm VWR BDH 83639.320
Butanol	AnalR Normapur, VWR,99,9%, Lot: 16G084003
DCM	Rectapur; ÖAB, distilled VWR BDH 25631.362
Ethanol 96%	ÖAB, distilled Brenntag CEE
Ethyl acetate	Rectapur; ÖAB, distilled
H_2O for HPLC	purified by ion exchanger
Methanol	ÖAB, distilled
Methanol for HPLC	HiPerSolv Chromanorm VWR BDH 83638.320, 16Z0432
n-Hexane	Normapur; VWR BDH 24577.460
Petroleum ether	Rectapur; VWR BDH 23826.464
Sulphuric acid	Sigma-Aldrich, Lot: SZBF0140V
Vanillin	ReagentPlus, 99%, Sigma-Aldrich, S/No: V1104-500G

7.4.3. HPLC column for investigation of *F. foliacea*

- Phenomenex® Gemini-NX 5 µm C18 110A (150x3,00 mm)

7.4.4. HPLC column for HPLC-CAD

- Agilent Zorbax SB-C18

7.4.5. Flash Chromatography columns

- Precolumn: Cartridge for 120 g (silica gel and applied sample)
- Analytical column: PURIFLASH Column 25 Silica HC-120,0 g (20 bar)

7.4.6. Spraying reagents for TLC

- Solution 1: 5% H_2SO_4 in MeOH

  15 ml concentrated sulphuric acid diluted with 285 ml MeOH

- Solution 2: 1% vanillin in MeOH

  4 g vanillin dissolved in 400 ml MeOH

7.4.7. TLC plates

TLC silica gel 60 F_{254}

Aluminum sheets 20 x 20 cm, 0.2 mm

Merck, 1.0554.0001

8. List of Figures

Crystal Structure of the human placental STS **(Fig. 10)**

Ghosh D. (2007) Human sulfatases: A structural prospective to catalysis, *Cell. Mol. Life Sci. 64*, 2013-2022

Flustra foliacea - ©Alexander Semenov **(Fig. 2)**

https://www.flickr.com/photos/a_semenov/5439427201/

Methyl-p-methoxyphenyl acetate **(Fig. 5)**

https://www.sigmaaldrich.com/catalog/product/aldrich/199206?lang=de&region=AT

Gloeophyllum odoratum **(Fig. 4)**

https://www.pilzforum.eu/board/attachment.php?aid=126271

Scifinder **(Fig.3, 6-8 and Tables 1 and 2)**

https://scifinder.cas.org/scifinder/view/scifinder/scifinderExplore.jsf

Animation of a single zooid **(Fig.1)**

http://www.sleloinvasives.org/about-invasives/native-species/magnificent-bryozoan/bryozoan-individual/

Steroid regulation by STS and SULT **(Fig.9)**

Rizner T. L. (2016) The important roles of steroid sulfatase and sulfotransferase in gynecological diseases, *Front. Pharmacol. 7*, 30

Ich habe mich bemüht, sämtliche Inhaber der Bildrechte ausfindig zu machen und ihre Zustimmung zur Verwendung der Bilder in dieser Arbeit eingeholt. Sollte dennoch eine Urheberrechtsverletzung bekannt werden, ersuche ich um Meldung bei mir.

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10. Appendix

10.1. TLC fractions of GOEF03 after Flash Chromatography

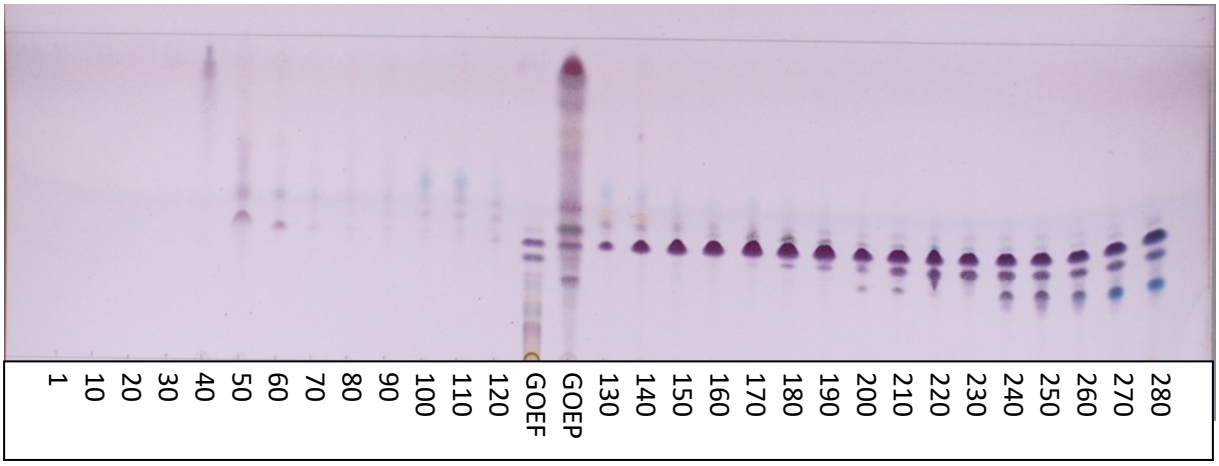

Figure 24: Fractions 1-280 of GOEF03. Detection VIS after using spraying reagents H_2SO_4/Vanillin

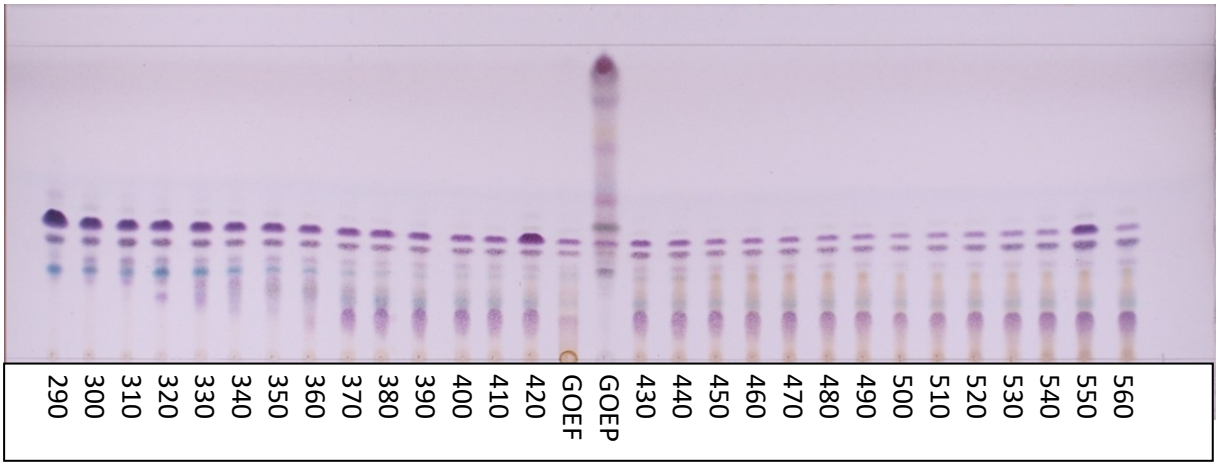

Figure 25: Fractions 290-560 of GOEF03. Detection VIS after using spraying reagents H_2SO_4/Vanillin

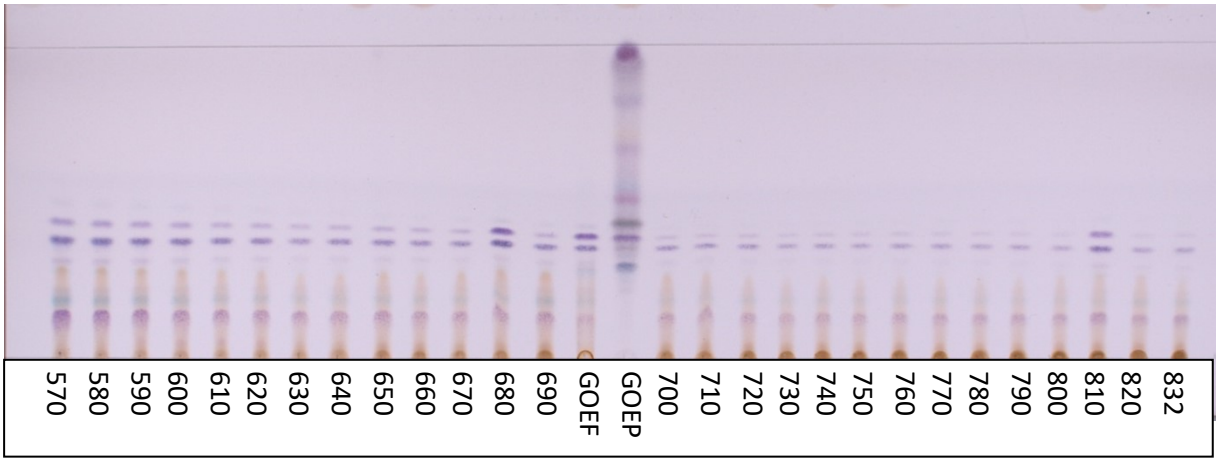

Figure 26: Fractions 570-832 of GOEF03. Detection VIS after using spraying reagents H_2SO_4/Vanillin

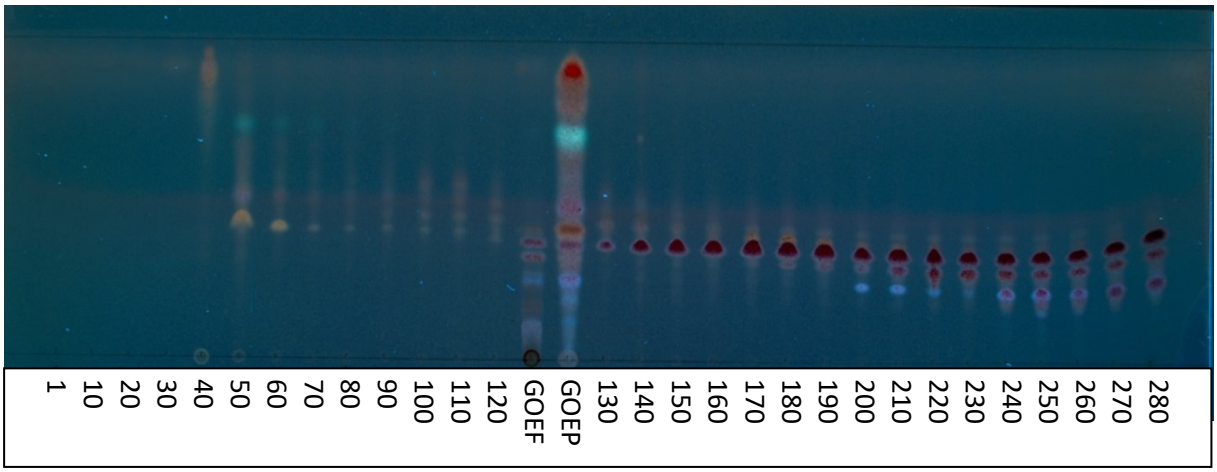

Figure 27: Fractions 1-280 of GOEF03. Detection at 366 nm after using spraying reagents H_2SO_4/Vanillin

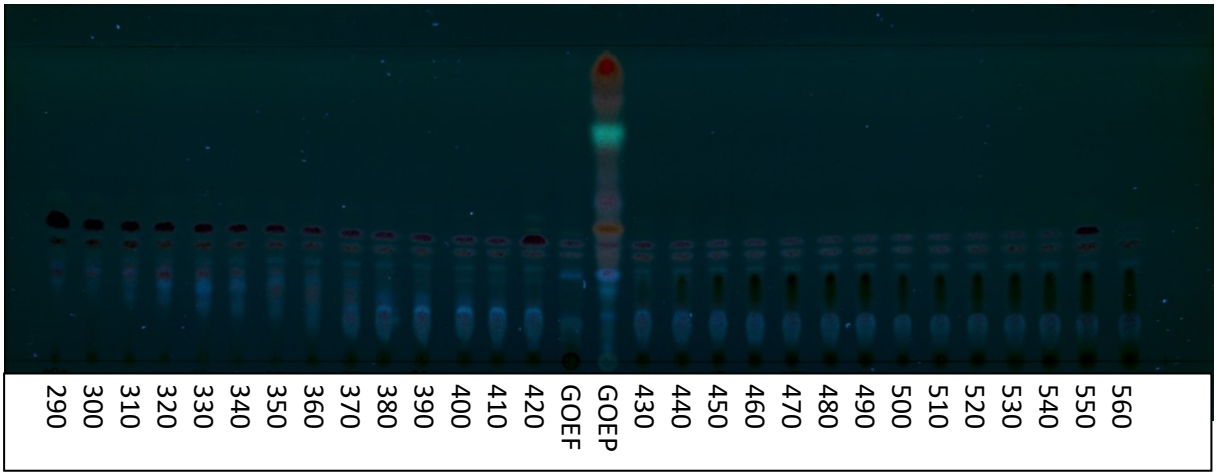

Figure 28: Fractions 290-560 of GOEF03. Detection at 366 nm after using spraying reagents H_2SO_4/Vanillin

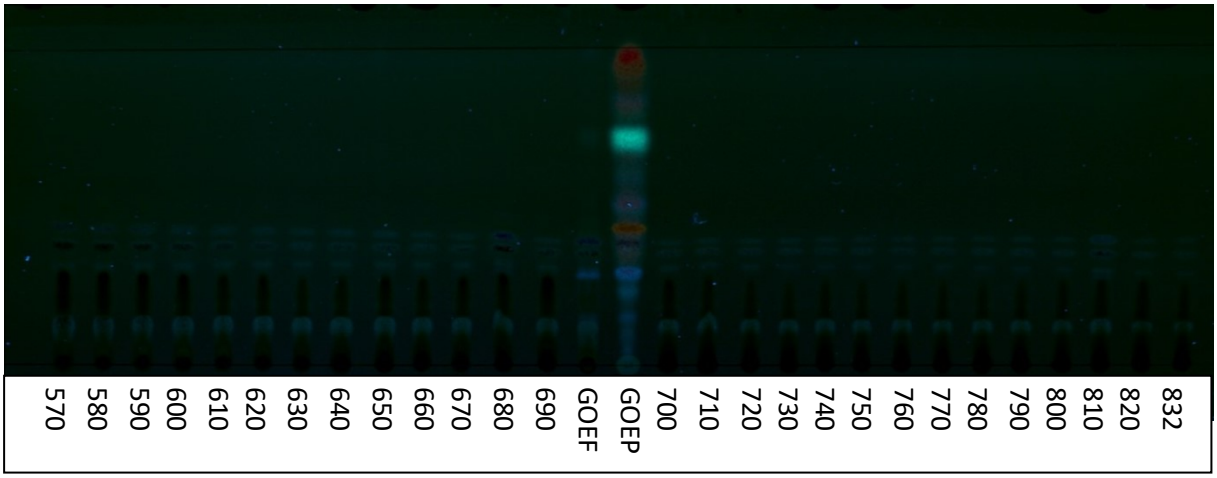

Figure 29: Fractions 570-832 of GOEF03. Detection at 366 nm after using spraying reagents H_2SO_4/Vanillin

10.2. Chromatograms of GOE, GOED, GOEF, GOEP and GOEW

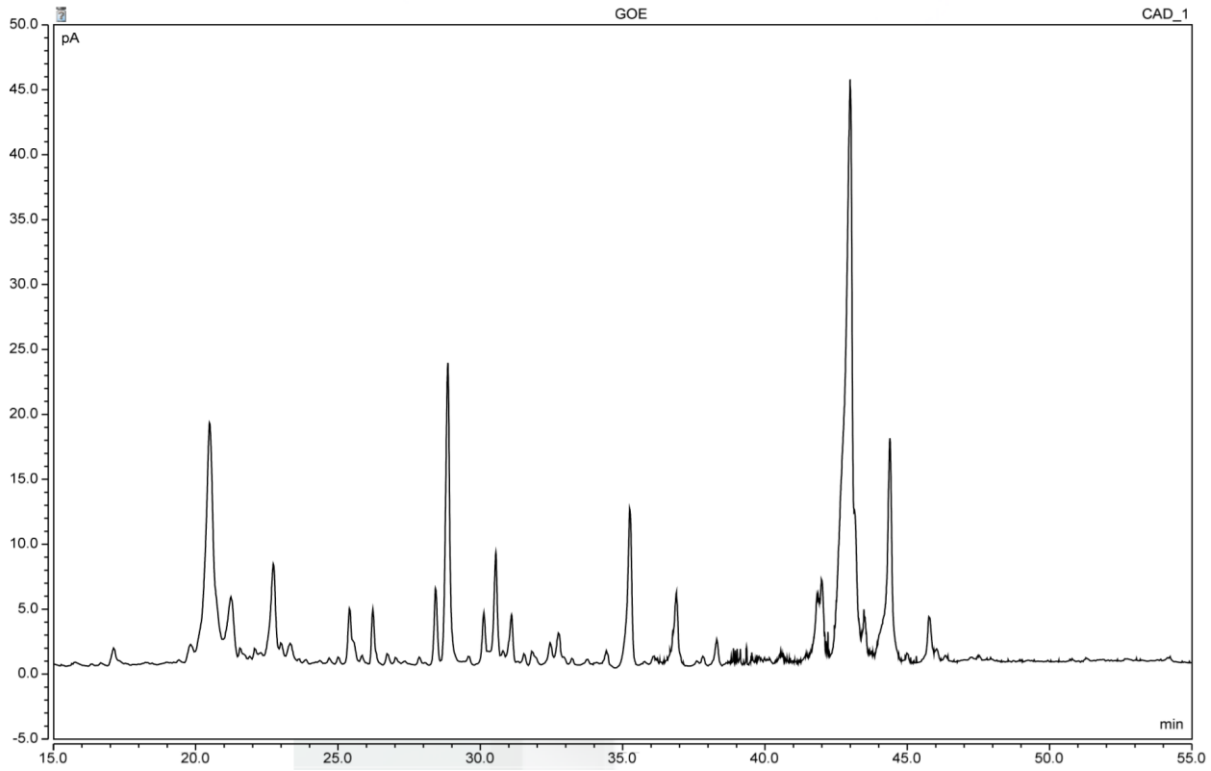

Figure 30: GOE

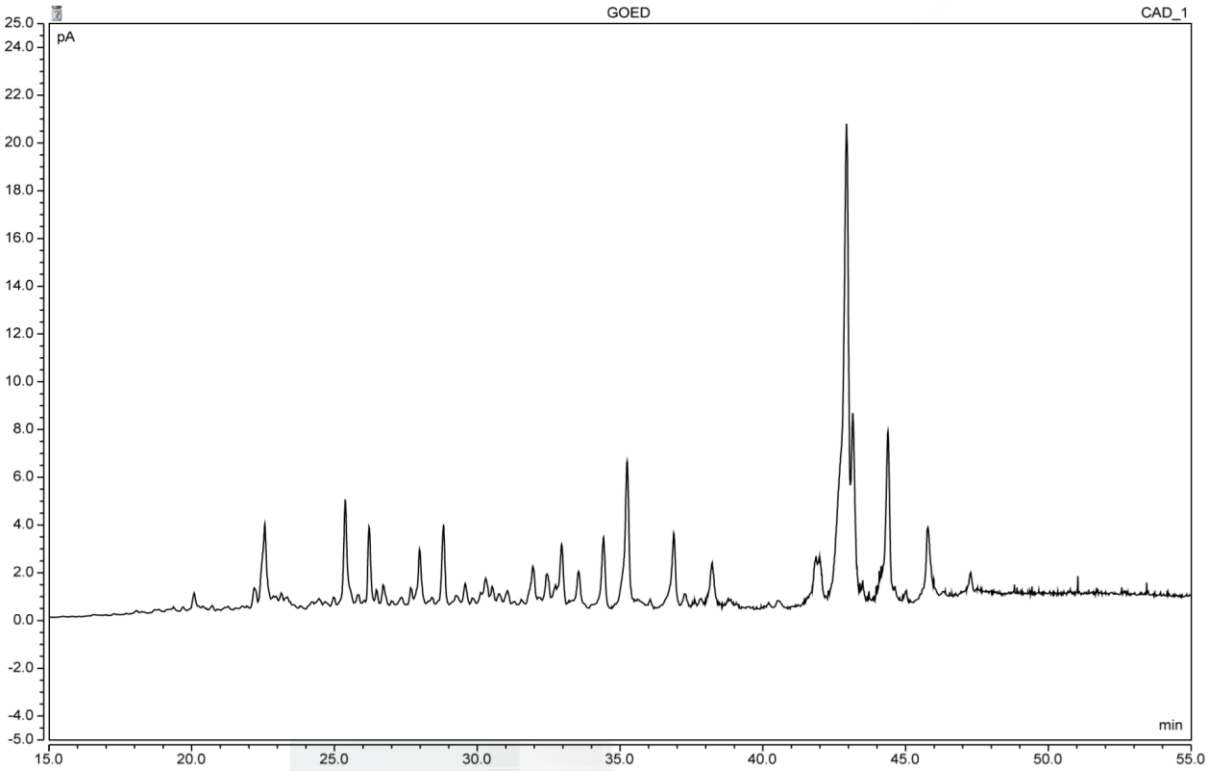

Figure 31: GOED

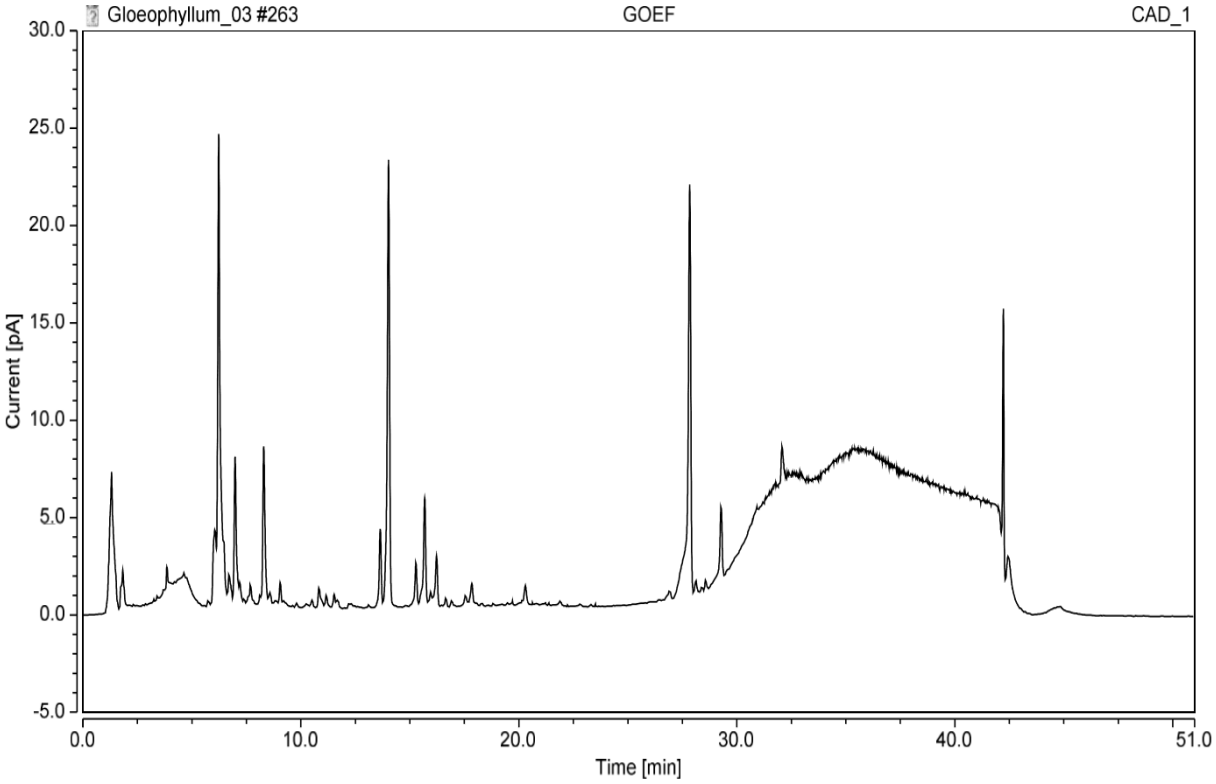

Figure 32: GOEF

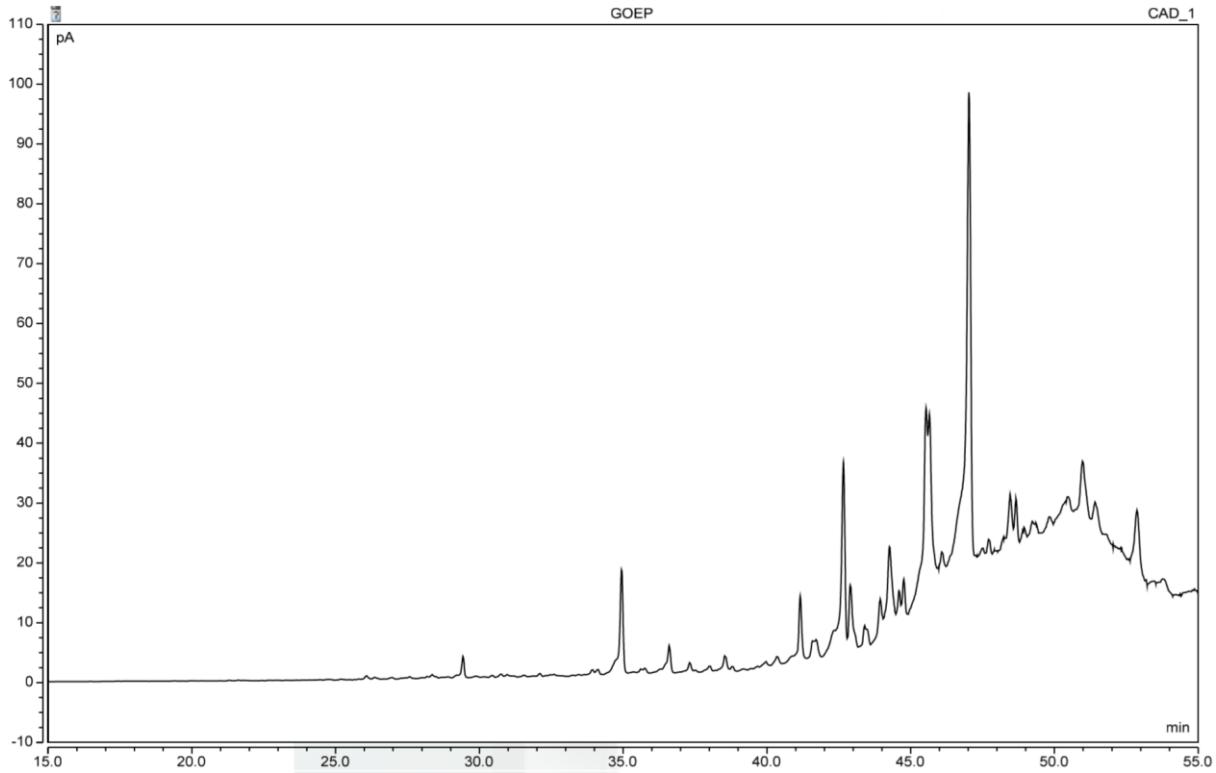

Figure 33: GOEP

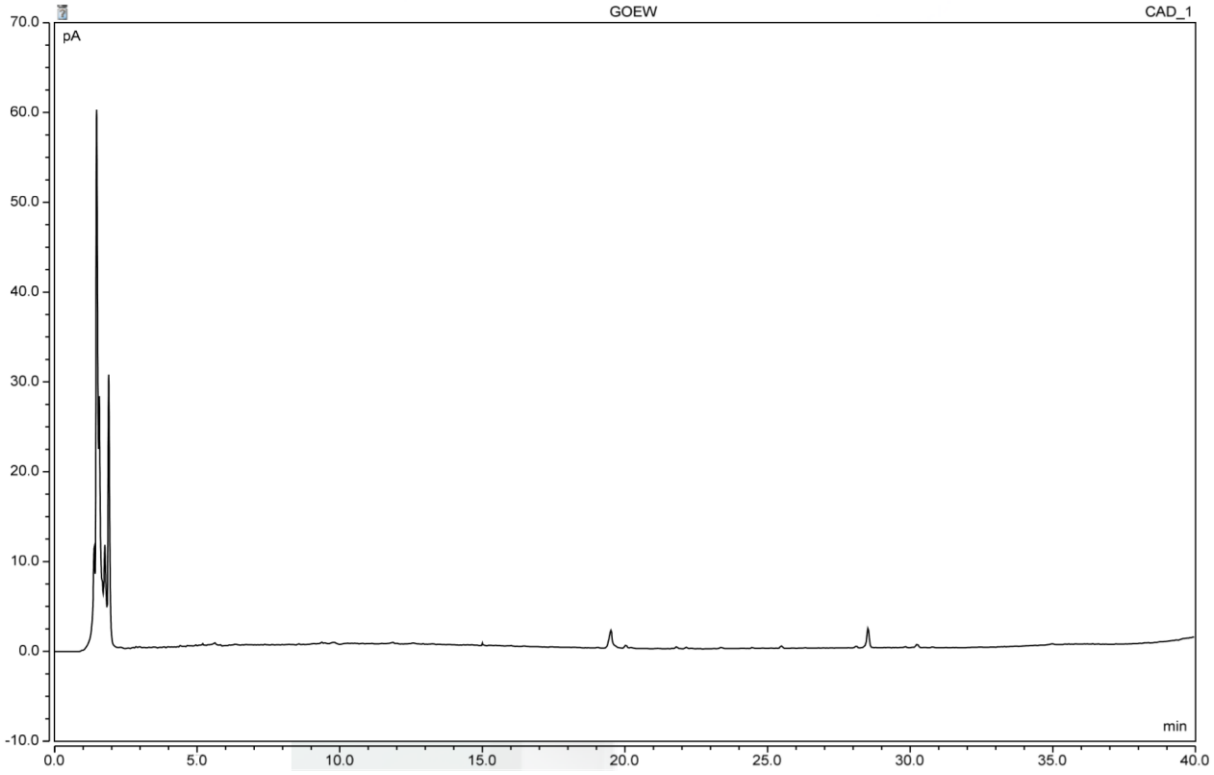

Figure 34: GOEW

10.3. Chromatograms of GOEF03_01-17 by HPLC-CAD

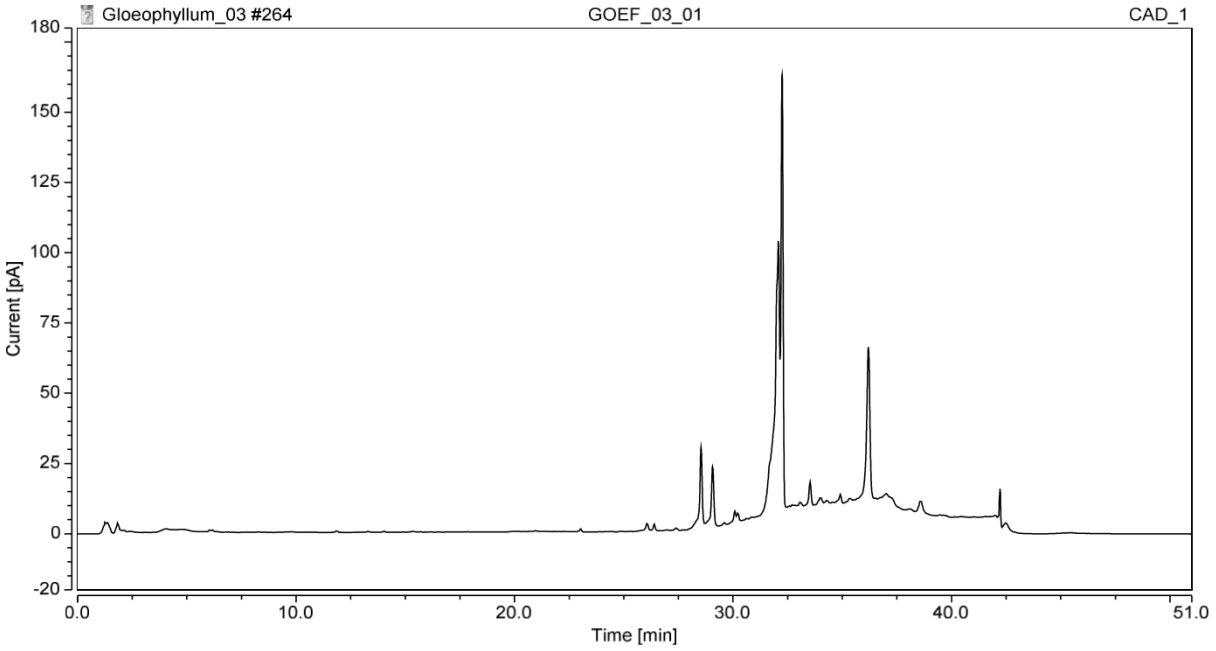

Figure 35: GOEF03_01

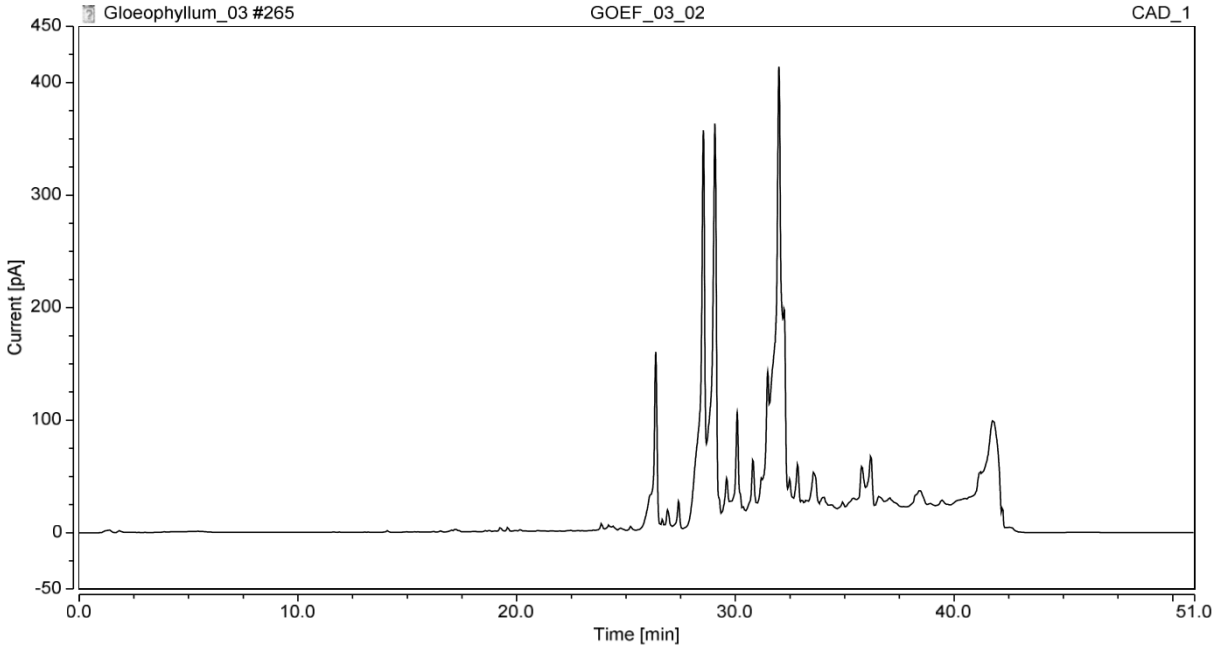

Figure 36: GOEF03_02

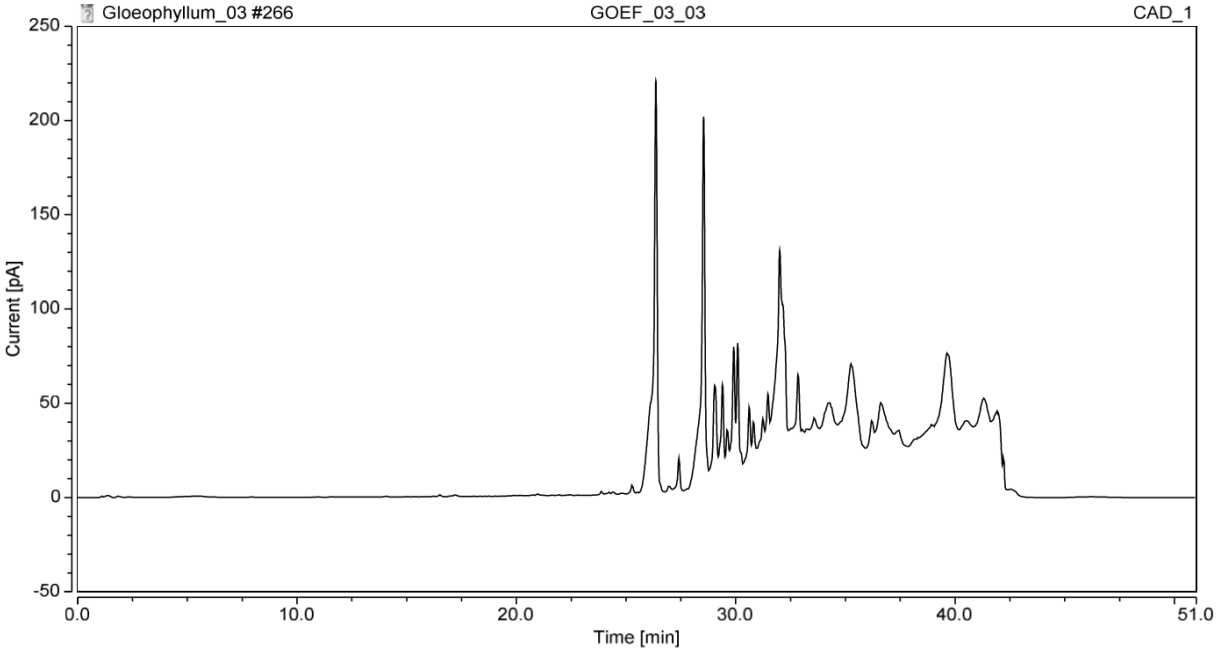

Figure 37: GOEF03_03

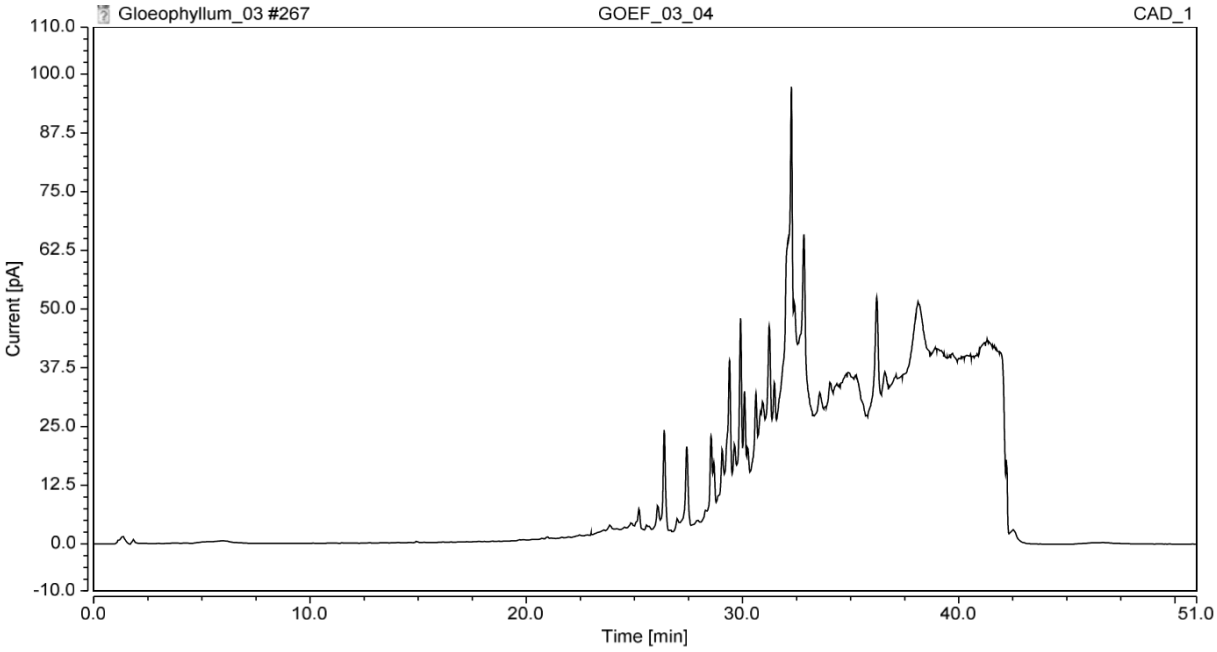

Figure 38: GOEF03_04

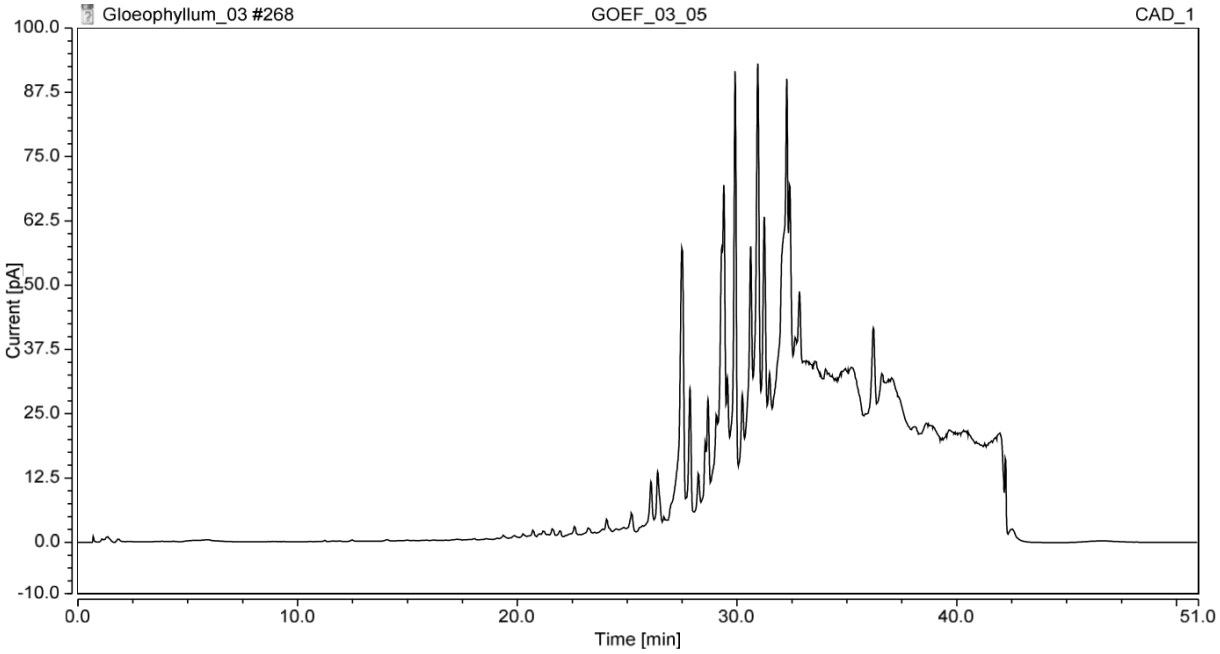

Figure 39: GOEF03_05

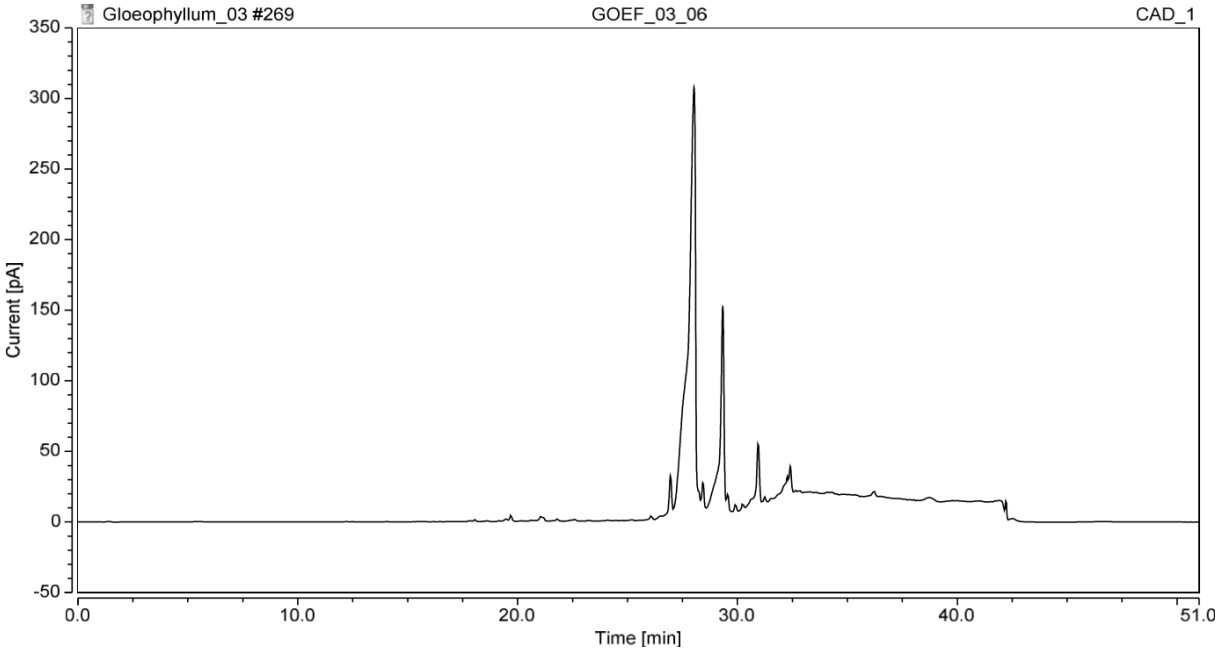

Figure 40: GOEF03_06

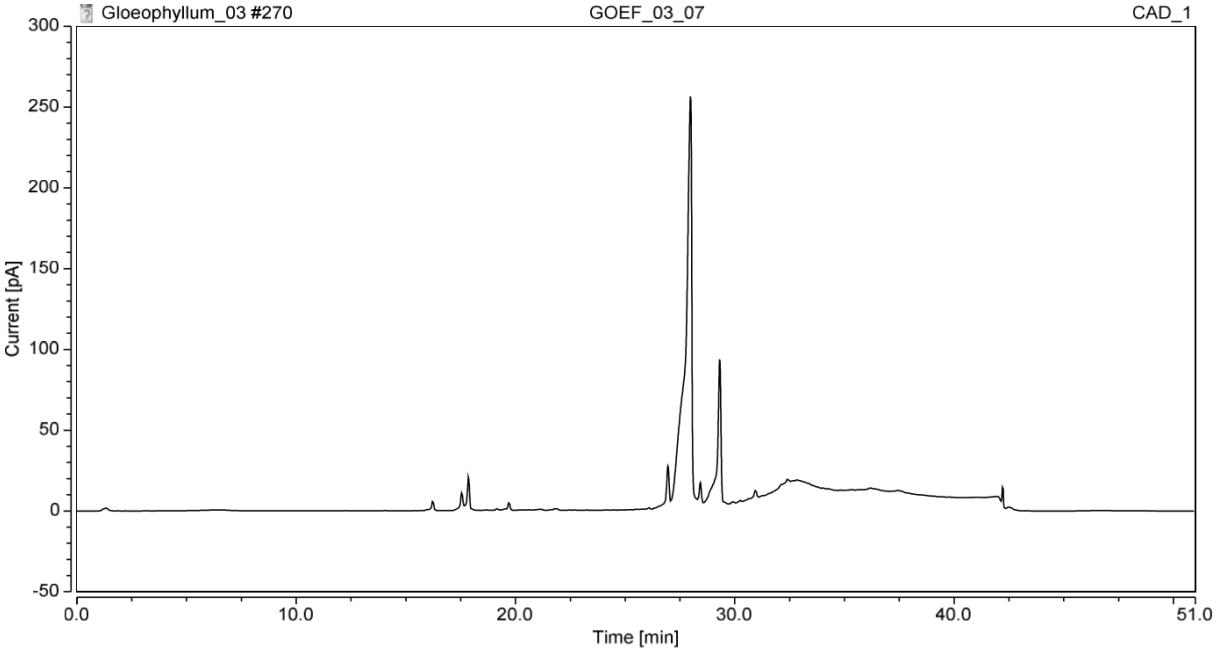

Figure 41: GOEF03_07

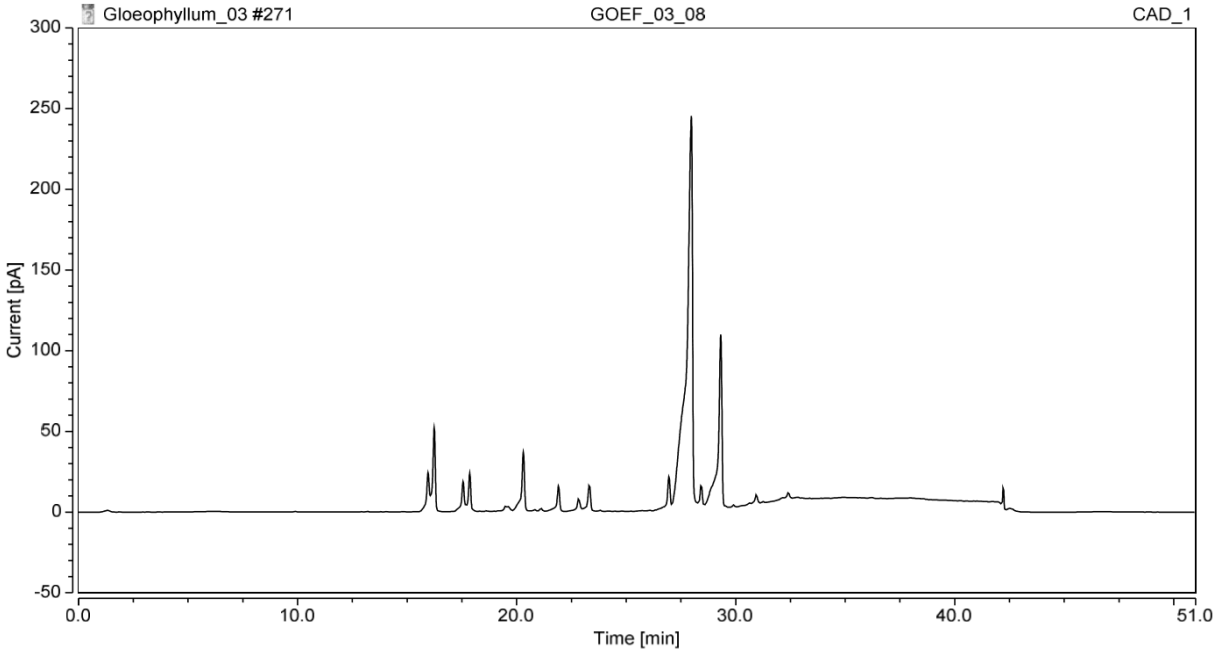

Figure 42: GOEF03_08

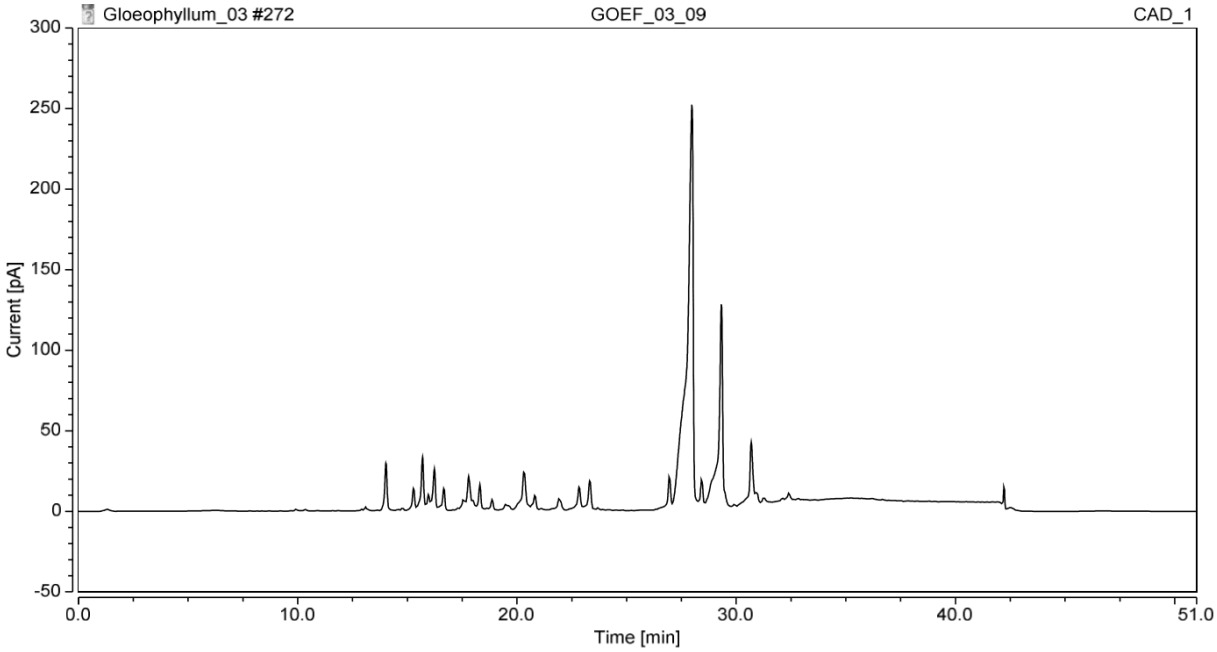

Figure 43: GOEF03_09

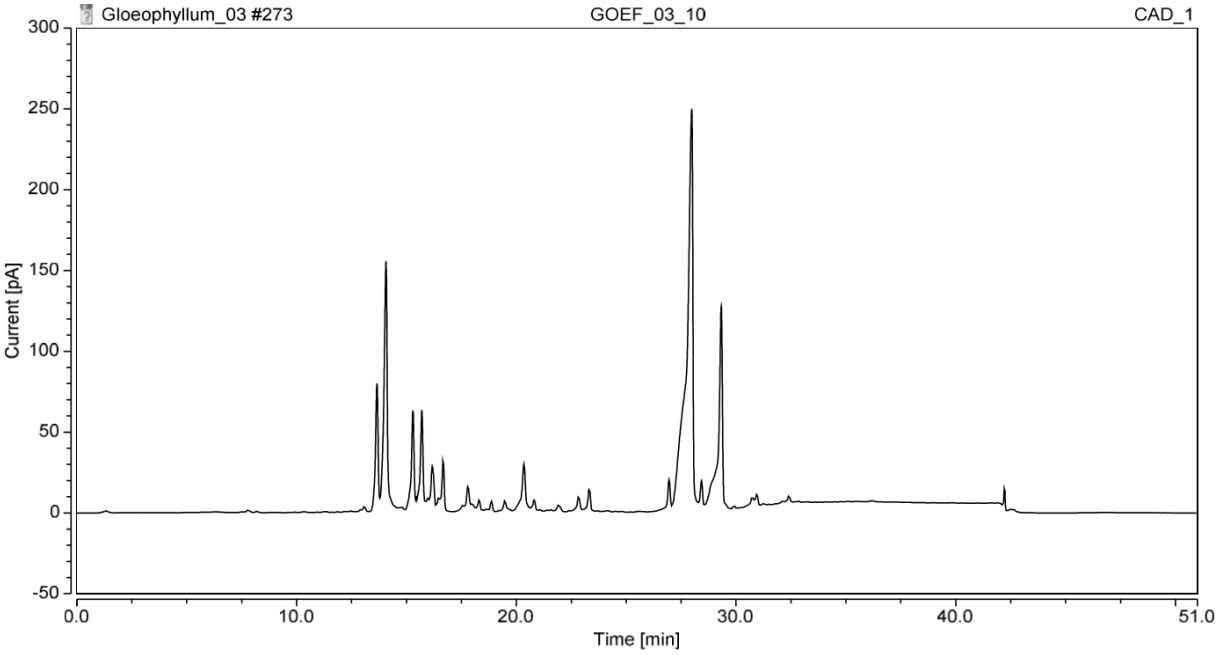

Figure 44: GOEF03_10

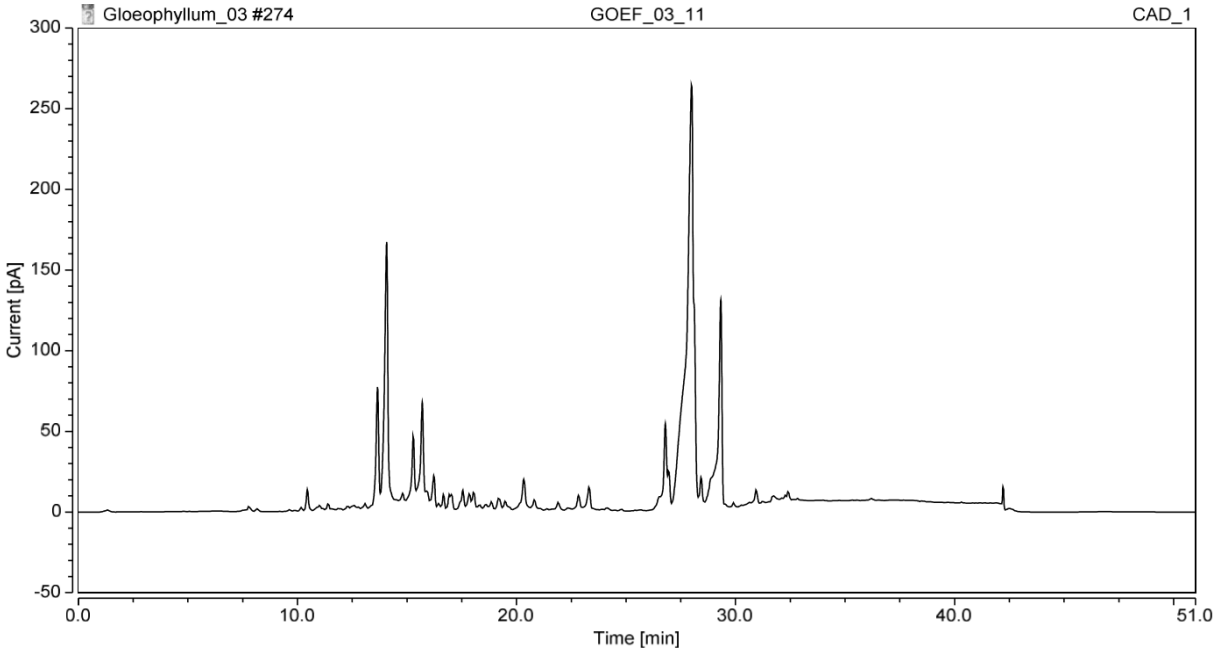

Figure 45: GOEF03_11

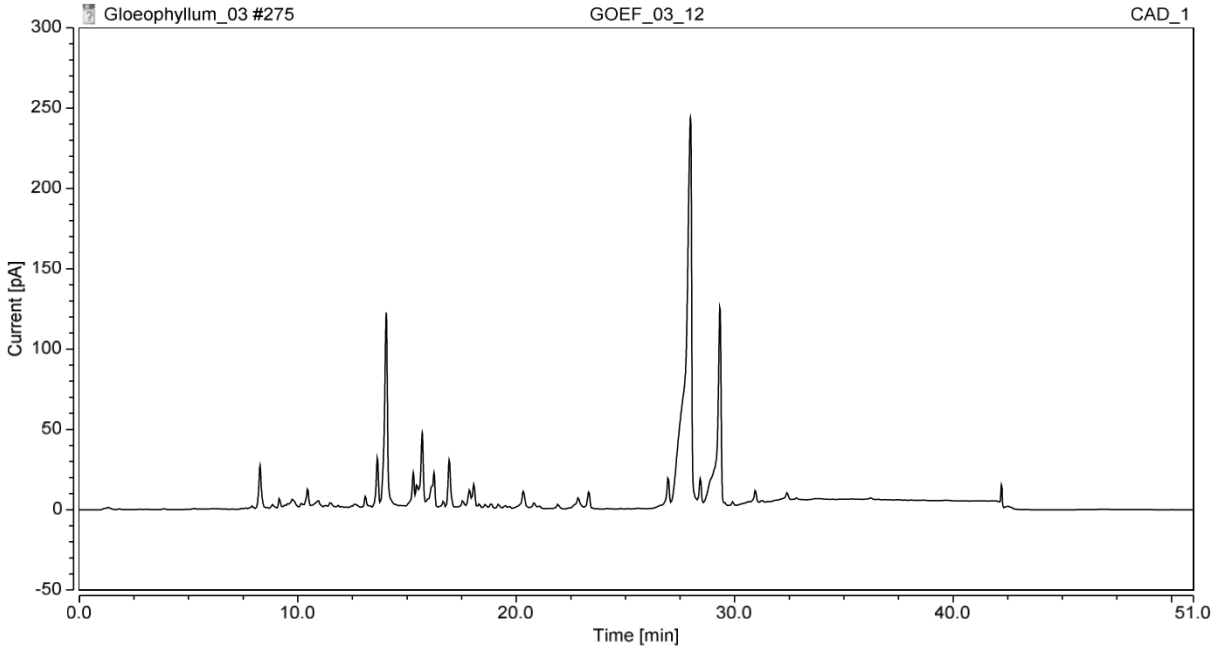

Figure 46: GOEF03_12

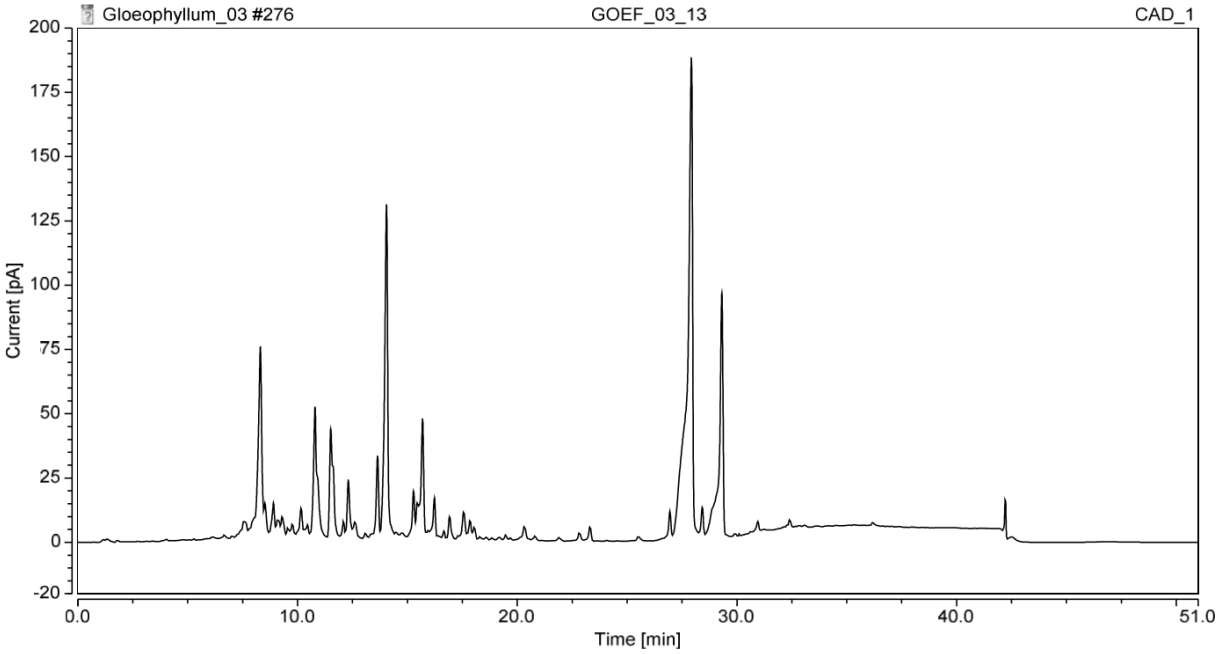

Figure 47: GOEF03_13

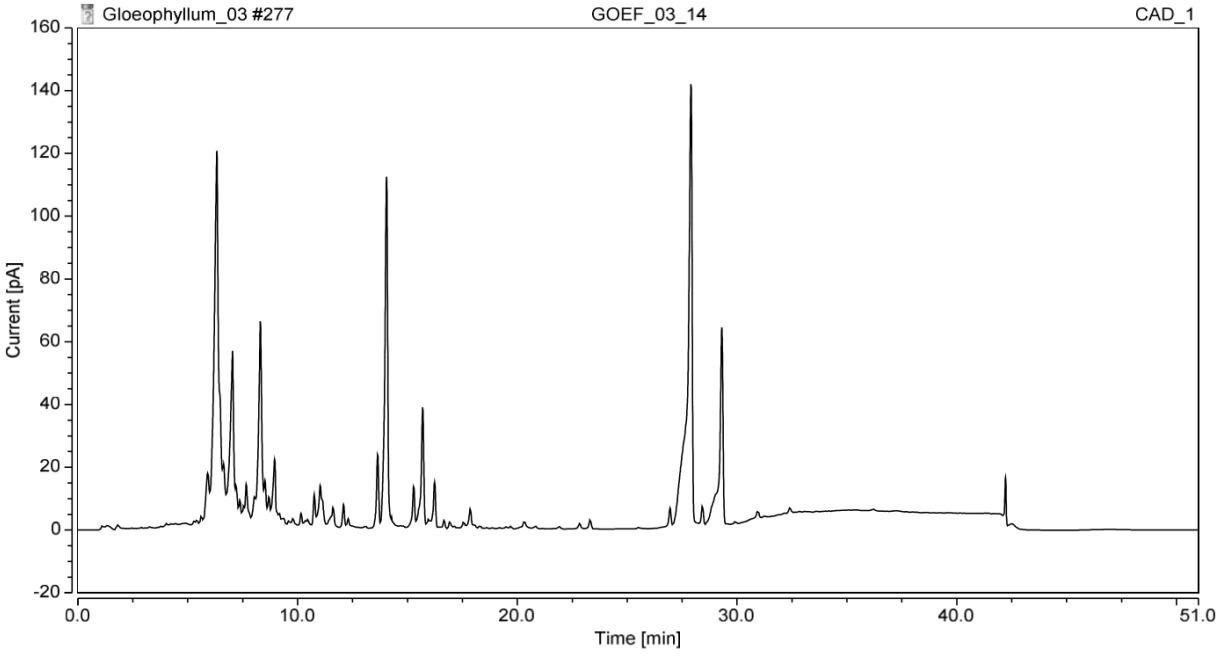

Figure 48: GOEF03_14

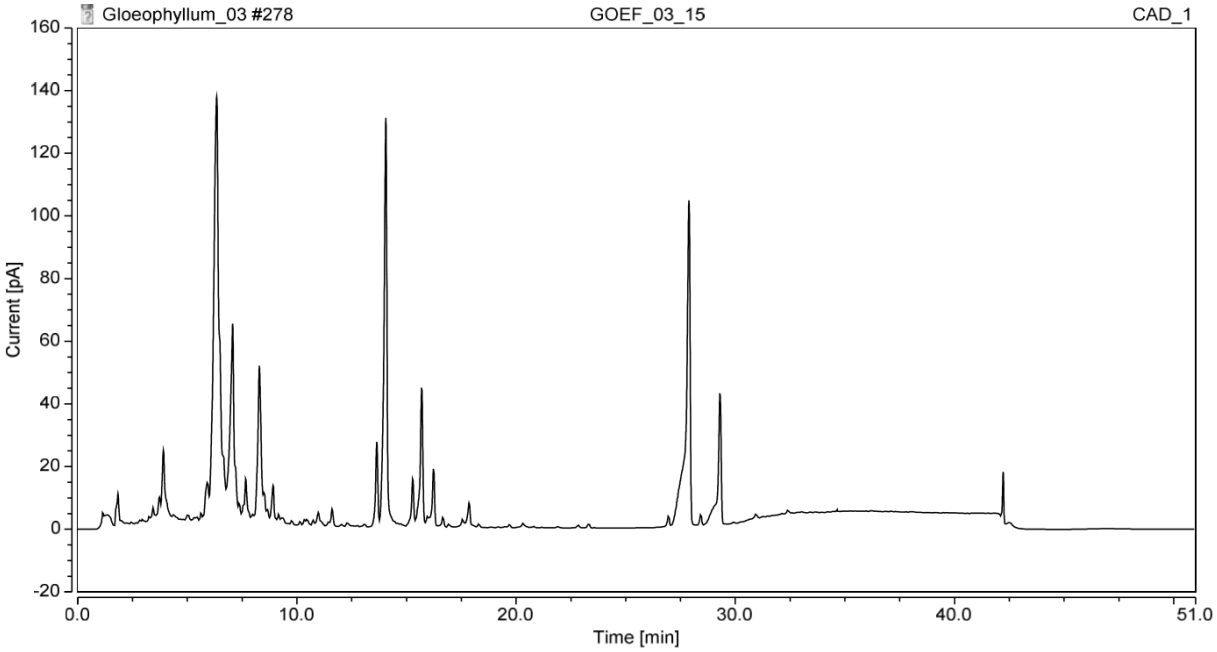

Figure 49: GOEF03_15

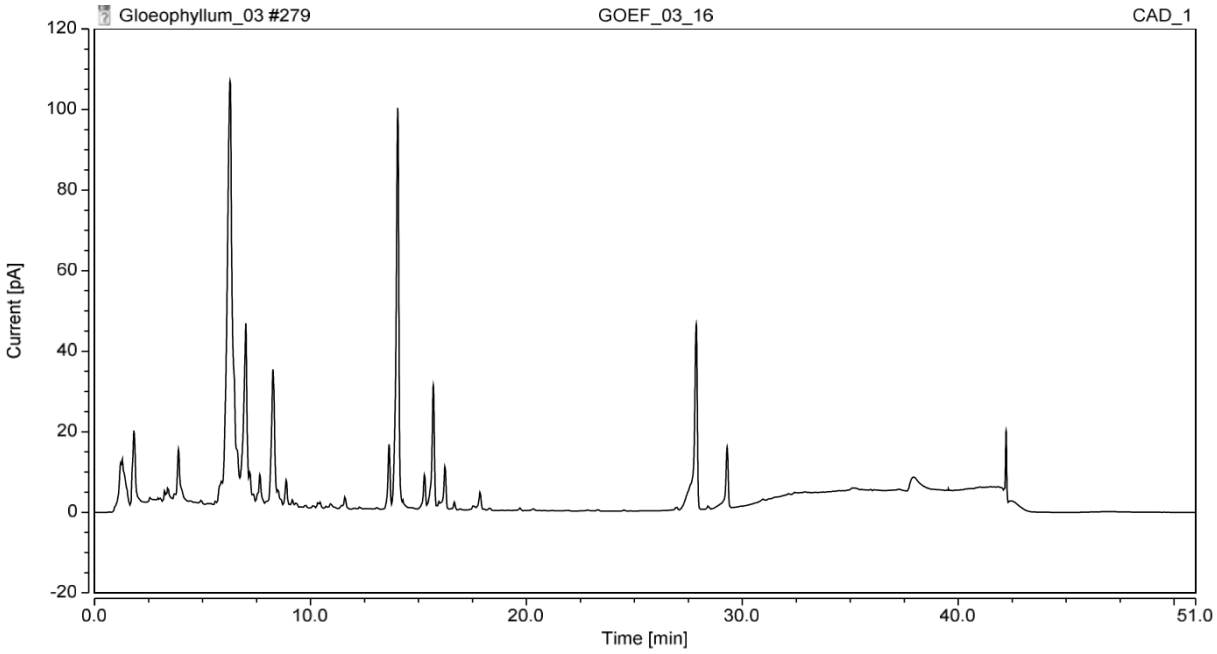

Figure 50: GOEF03_16

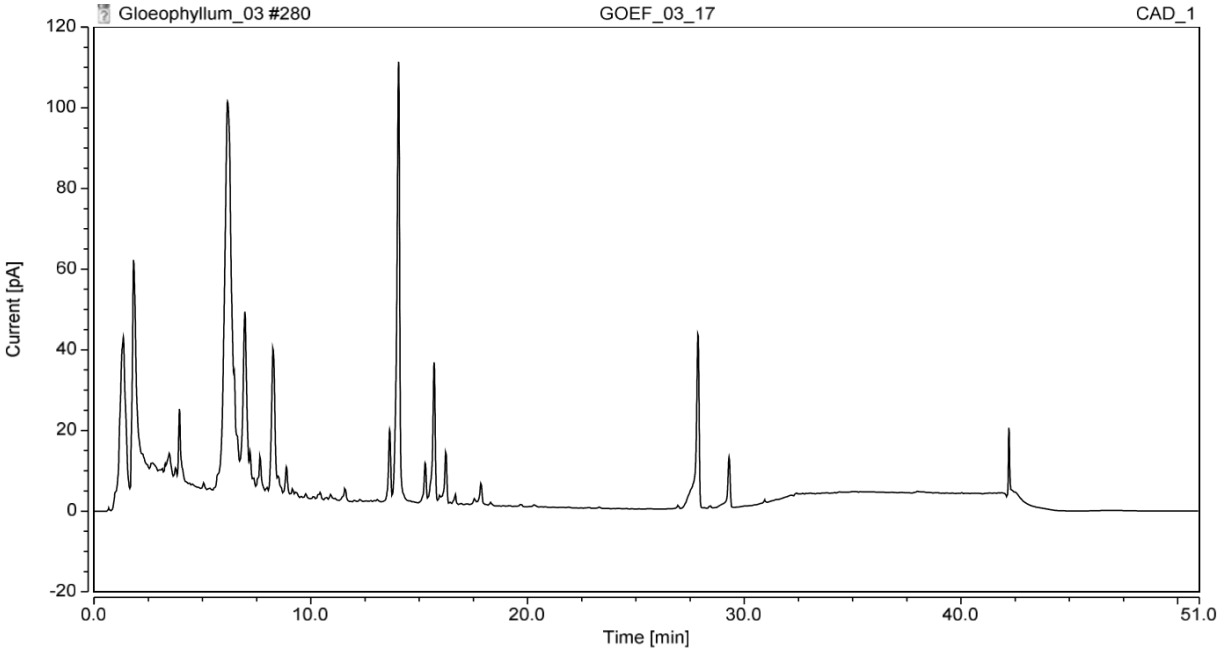

Figure 51: GOEF03_17