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„Fractionation and characterization of an aqueous $NaHCO_3$-extract of Norway spruce balm“

verfasst von / submitted by

Tom Skibowski

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1 Introduction

The Norway spruce (*Picea abies* (L.) H. Karst; Pinaceae) is a coniferous tree, whose balm is used in traditional medicine for treating complicated wounds and skin infections (Sipponen A. et al., 2012). For these indications, spruce balm is used which differs from the resin of the tree in its phytochemical composition, color, and texture (ÖAB, 2019).

Several studies, which are mentioned in the course of this thesis, have shown evidence for antimicrobial as well as antifungal effects of the Norway spruce balm.

The following introduction gives a brief overview of the state of research as well as a description of the Norway spruce balm itself alongside its traditional and modern use.

Source: Saukel, J.

Source: Saukel, J.

Figure 1: Excretion of Norway spruce balm from the trunk.

Relevance of Norway spruce balm

There are many types of wounds which are easily treatable with commonly used substances like dexpanthenol, cortisol, or antibiotics. But there are also certain types of wounds, especially decubitus (pressure ulcers), castration wounds in animals, or large wounds in general which are difficult to close entirely and within an acceptable time.

Sipponen A. et al. stated in their study of 2007 that there is "no 'gold standard' for their treatment". There are a lot of products, hydrogels for example, which are frequently used but don't deliver a sufficient and satisfying result of wound healing. On top of that, these hydrogels neither have the preferred combination of wound healing properties, antimicrobial characteristics, nor protective abilities.

Because the Norway spruce balm is used in traditional therapy for treating topical wounds for a long time it gained the interest of research, especially for finding a phytotherapeutic alternative for complicated and infected wounds (Sipponen A. et al., 2012).

Traditional use of the Norway spruce balm

The preparation and processing of the Norway spruce balm has already been discussed sufficiently in former theses on this subject and is therefore left out in this thesis. It has been displayed in detail by Julia Langeder (2017) and Elisabeth Eichenauer (2018).
In this section the focus will lie on the traditional usage of the Norway spruce balm. The oldest known usage of Spruce balm goes back to ancient Egypt, and other forms of traditional use are reported from Finnish Lapland. With the boiled ointment they treated all kinds of wounds, for example burns, sores, or abscesses.

The table below shows the different types of usage in ancient Egypt and Finnish Lapland (Jokinen J. J. et al., 2016).

Table 1: Overview of the different forms of traditional usage of spruce balm in ancient Egypt and Finnish Lapland.

Country	**Indication**
Egypt (ancient)	Burns
Finnish Lapland	Suppurating burns
	Acute and chronic infections
	Sores
	Pressure ulcers
	Punctured abscesses
	Onychomycosis
	Paronychia

Antimicrobial and antifungal properties

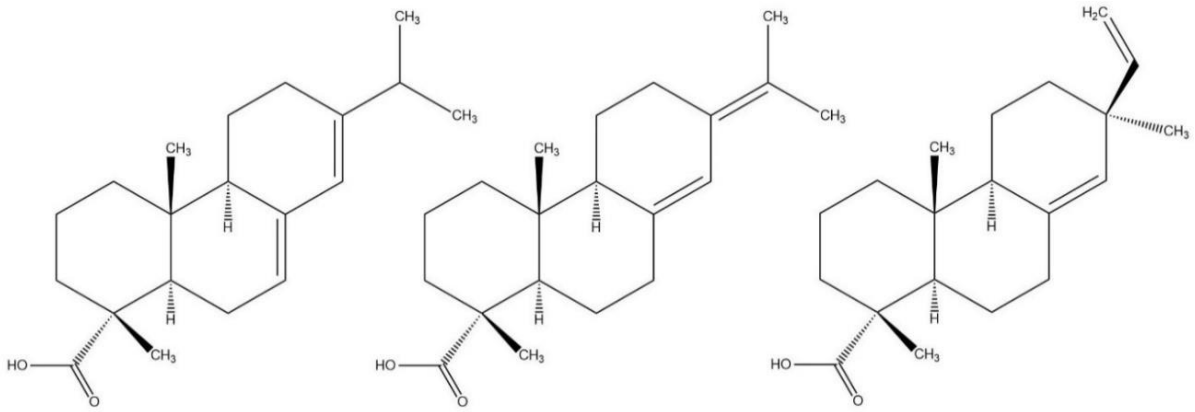
As mentioned above, the combination of protective, antimicrobial, and regenerative properties is estimated when using substances for treating complicated skin ulcers and infected wounds. Several studies have shown that Norway spruce balm contains substances that can deliver these three characteristics.

A study presented by Rautio M. et al. in 2012 showed antifungal effects of the resin salve using the agar plate diffusion test. The salve was effective against all types of dermatophytes, in particular species of *Trychophyton*. Exceptions were *Candida* yeasts, although there was a minor effect occurring on plates with *C. glabrata* and *C. krusei*.

Chemical composition of the Norway spruce balm

The Norway spruce balm consists of many different chemical compounds. The main components are resin acids, which belong to the category of terpenoids, precisely, diterpenoids. Other components are lignans as well as phenolic acids and various essential oils.

Many substances have already been isolated and characterized earlier, but the exact quantitative composition is still unknown (Goels et al., 2020). P-coumaric acid was detected and identified through gas-liquid chromatography. The same method allowed for the assessment of different types of lignans, like pinoresinol and matairesinol (Rautio M. et al., 2012).



Source: Eichenauer, E., diploma thesis, 2018

Figure 2: Chemical structures of resin acids of Norway spruce balm: abietic acid, neoabietic acid, pimaric acid (left to right).

Treatment of castration wounds in piglets

Not only for humans, but also for animals there is an increasing interest in phytotherapeutic products for treating impaired and infected wounds.

A recent study performed by Prokop D. et al. (2021) compared the wound healing and antimicrobial properties of Norway spruce balm and resin with pork lard, a control group, and an antimicrobial spray, which contained chlortetracycline HCl, on castrated male piglets. The data showed that those piglets which were treated with Norway spruce balm or resin had faster wound healing, more completely closed wounds and less bacterial and fungal growth.

Climate change and its effects on the Norway spruce

Like all living species on the planet the coniferous trees are affected by climate changes, for example the change in temperature, wind, and humidity. Norway spruce may be assigned as “threatened” regarding temperature increase and decrease in rainfall. They are extremely sensitive against draught because their roots don’t reach depth like other trees (Krisans O. et al., 2020). This study also implied a biomass measurement tool for gaining information about future damages in trees, especially in coniferous trees, which will be useful in estimating the amounts of spruce balm available for research and patients. Regarding future examination

and the development of Norway spruce balm related products for medicinal use it may become relevant to establish methods to secure enough output of the balm and resin to provide sufficient yields of ointments.

2 Aim of this work

Fractions and substances have been analyzed, characterized, and isolated earlier (Eichenauer E., 2018). Methods and solvents which are suitable for the substances contained in the Norway spruce balm have already been established. However, there are remaining fractions and substances of interest which aren't fully characterized and isolated yet.

The aim of this work is the fractionation and characterization of the $NaHCO_3$-extract of the Norway spruce balm, with the aim to isolate pure substances and elucidate their structure. These substances were presumed to belong to diterpenes, lignans, or phenolic acids, which are supposed to play a major role in the wound healing properties of Norway spruce balm (Sipponen A. et al., 2009).

3 Material and methods

In this chapter all materials and methods used in this work are described. It includes the preparation of the raw material, the separation to gather different extracts of the plant material, and the characterization of subfractions of the sodium hydrogen carbonate ($NaHCO_3$) extract to provide samples for further isolation and structure elucidation.

3.1 Plant material

The material used in this work was gathered in Lungau (Salzburg) and had already been processed in a way that allowed direct further handling (Eichenauer E., 2018). This pre-treatment provided the “boiled Norway spruce balm” which was the basic material for the present work.

3.2 Liquid-liquid extraction

For the liquid-liquid extraction an aqueous solvent and an immiscible organic solvent were used (see Figure 3, p. 8). To reach an optimal phase separation it was of great importance that the solvents used in this process had a sufficient difference in their density. Approximately 300 g of boiled spruce balm were dissolved in 400 ml of DCM. Alongside a saturated aqueous sodium hydrogen carbonate solution was prepared. Afterwards, this aqueous solution was used to extract the organic solution three times. Because of a poor phase separation, 100 ml of deionized water were added. Since Langeder (2017) and Eichenauer (2018) had used approximately half the amount of raw material they did not face those difficulties in this step. After the separation and combination of the aqueous phase, it was acidified to reach pH 3-4 using hydrochloric acid (HCl). The obtained solution contained free carboxylic acids. Now the process of re-extracting the aqueous solution with DCM started. The low pH value caused protonation of the present acids, which were consequently soluble in the organic phase. In the following step the organic phase was evaporated to yield **11.5 g** of the **$NaHCO_3$-extract.**

Since the first extraction process with $NaHCO_3$ led to incomplete separation of the two layers, it was decided to extract the DCM and "not separated" layer for another time using deionized water. The further procedure was as described above and shown in Figure 3 providing **10.9 g** of the **water-extract**. Likewise, an extraction with sodium hydroxide (NaOH) was performed to obtain the weak acids, for example phenols. Through NaOH they got deprotonated. The yield of the **NaOH-extract** was **124.3 g**.

The remaining DCM-phase was evaporated to obtain the **residual extract** containing all the nonpolar and alkaline substances. These substances were not deprotonated in former steps. The yield of this last extract was **69.5 g**.

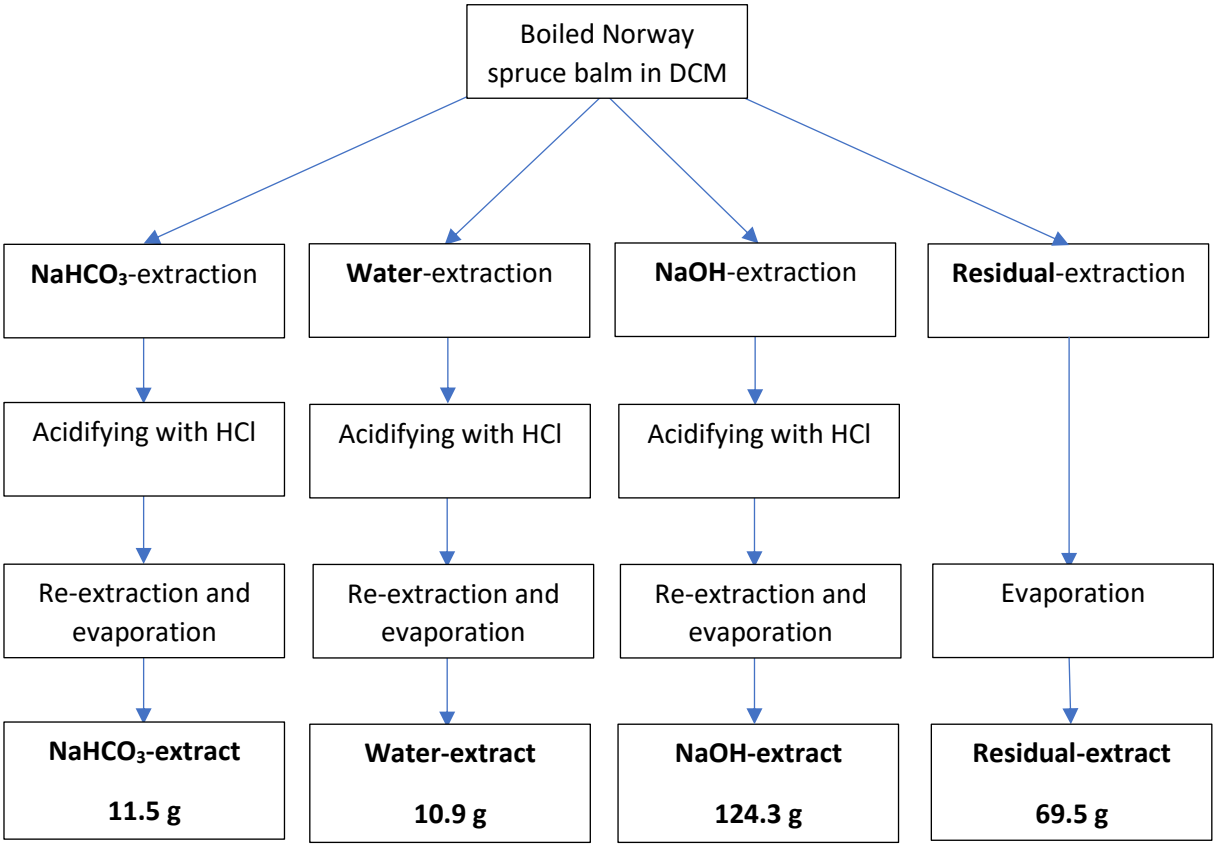


Figure 3: Resulting extracts and their yields after liquid-liquid extraction of the "boiled Norway spruce balm".

3.3 Chromatography

Chromatography can be used in an analytical or a preparative way. For each one, there are many different instrumentations used regularly in a laboratory scale. Both ways follow different goals: the analytical chromatography identifies, detects, and measures constituents in a certain mixture of analytes. The preparative chromatography aims at purification and isolation of specific substances (Muthuvelu K. S. & Arumugasamy S. K., 2019). In the beginning of this work, different types of analytical techniques were used to detect fractions, and later, substances of interest. With the supercritical fluid chromatography, a preparative technique was used as well and will be described in detail in this chapter.

3.3.1 Planar chromatography

Thin layer chromatography

Thin layer chromatography (TLC) is a type of planar chromatography. It can be used in an analytical and preparative manner and is therefore a flexible and valuable method. Due to its robust, inexpensive, and portable properties as well as the simple usage it is often used in laboratories for quick overviews regarding the purity of a solution or to perform an identification. The stationary phase is attached to an underlying, suitable plate consisting of glass or aluminum. The used stationary phase can vary between silica gel, cellulose, or aluminum. The size of the plate varies. Most used plates are between 20 cm x 20 cm and 5 cm x 10 cm, depending on the number of samples and their properties. It is recommended to use standardized TLC to ensure that the results are reproducible. The mobile phase is oftentimes a combination of organic solvents, like dichloromethane or acetone. Usually, an acidic substance is added in a small amount to increase the elution power which leads to an increased separation of the compounds. When the mobile phase has contact with the stationary phase it moves through the stationary phase because of capillary force while carrying the applied substances along (Flanagan R. J. et al., 2020). The table below shows the instrumentation as well as the software which were used during this project.

Table 2: Instrumentations and software that were used in the analytical TLC.

Instrumentation	CAMAG TLC Visualizer
	CAMAG ADC2 Automatic Developing Chamber
	CAMAG Chromatogram Immersion Device (III)
Software	CAMAG Vision Cats

Analytical TLC

TLC, as mentioned above, can be used in an analytical way. In this work analytical TLC was performed after each separation step to identify fractions potentially containing substances of interest and to perform purity checks.

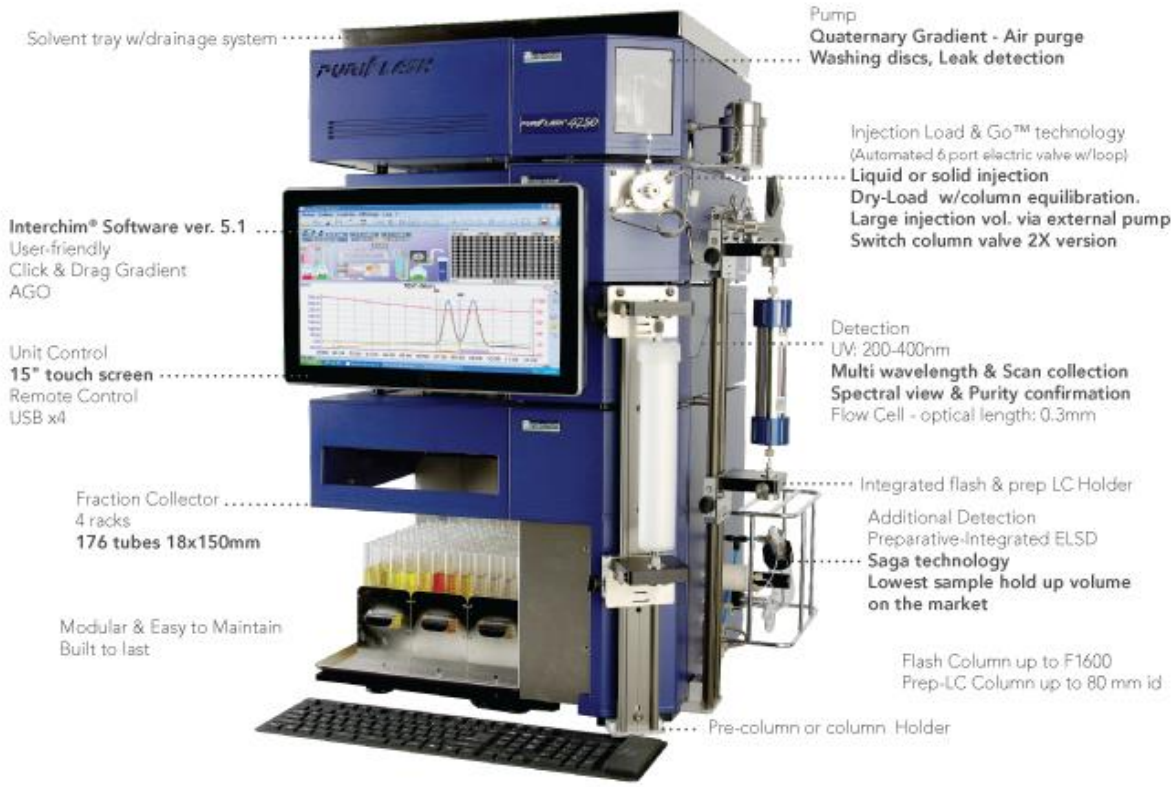
In this case Silica 60 F254 plates (MERCK) were used, which contained a particle diameter of 9.5 mm to 11.5 mm placed on an aluminum sheet. Zinc silicates were responsible as indicators for the fluorescence. The mobile phase contained dichloromethane, acetone, and formic acid in a proportion of 80+18+2. Before placing the samples on the TLC with a small glass capillary in lanes, the samples were dissolved in acetone. After drying the plates (room temperature), the CAMAG devices were used for development and documentation purposes. At first, the dried plates were photographed under white light as well as wavelengths 254 nm and 366 nm, using the CAMAG TLC visualizer. The undeveloped TLCs were then derivatized by using anisaldehyde/sulphuric acid in the CAMAG chromatogram immersion device. After heating the plates for approximately two minutes (105°C), again pictures were taken from the developed TLCs. Parameters for the analytical TLC are displayed in Table 3 (p. 10).

Table 3: TLC parameters for analytical TLC.

Mobile phase	Dichloromethane – acetone – formic acid
Stationary phase	Silica 60 F254 (MERCK)
Corresponding ratio	80 + 18 + 2

3.3.2 Flash chromatography

Flash chromatography is an easy and fast way to scale up thin layer chromatography. The day-by-day use in a laboratory is relatively inexpensive and the results are reliable. Samples can be gathered automatically. On top, flash chromatography offers cartridges, which are disposable what makes the whole process more time efficient (Miller L. & Peterson E. A., 2015). Flash chromatography also offers the use of columns with smaller particle sizes. This delivers higher resolution and shorter elution times (Swathi G. et al., 2015). In this work, the Interchim puriFlash 4250 was used which is displayed in Figure 4 below (p. 11).



Source: https://www.interchim.com/pp/793/puriflash-4250.html

Figure 4: Overview and instrumentations of the Interchim puriFlash® 4250.

For the preparation of the samples, 1 g of $NaHCO_3$-extract was mixed with 4 g of silica gel (MERCK), diluted in acetone. The solvent was evaporated, and 2.5 g loaded silica gel were filled in a flash cartridge. The other 2.5 g of the sample were saved for a separation run

performed later. For the mobile phase, water was used as solvent A, and methanol (MeOH) as solvent B. As stationary phase a reversed phase column was utilized (puriFlash® Column 15 C18 HP 35G-35,0 g (22bar)). Prior to the run, the whole instrumentation was purged using isopropanol for 5 minutes. The following step was an equilibration step for the column at a ratio of 80% water and 20% MeOH. Next on, the filled cartridge was inserted, and the separation started. The exact elution program is displayed in the table below (Table 4, p. 12), as well as the exact instrumentation and software (Table 5, p. 12).

For detection purposes, the Interchim puriFlash® was equipped with a photo diode array (PDA) detector and an evaporative light scattering detector (ELSD). The PDA measured at wavelength 254 nm and made a scan between 200 nm and 600 nm. The resulting fractions were collected by an automated fraction collector based on detection by ELSD.

Table 4: Elution program used in flash chromatography.

Minute	**Solvent B (MeOH) concentration in %**
0	20
40	100
50	100

Table 5: Used instrumentations and software for flash chromatography.

Instrumentation	Interchim puriFlash® 4250
	PDA Detector (254 nm and Scan
	Preparative-integrated ELSD
Software	Interchim Software

Source: Langeder, J. (2017)

3.3.3 Supercritical fluid chromatography

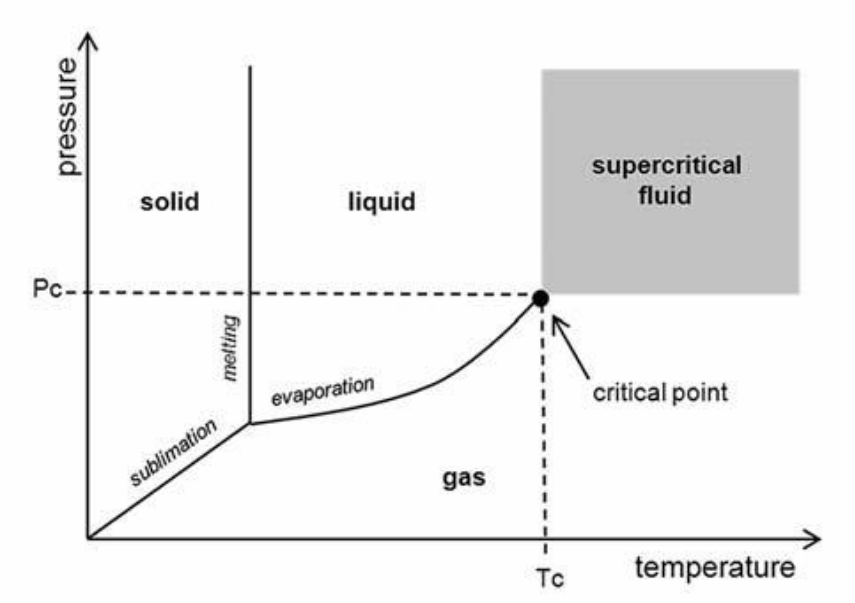
To formulate the aggregate of a supercritical fluid it is best described as a compressed gas which has a liquid-like density (Figure 6, p. 14). This special aggregate delivers distinct characteristics such as high diffusivity and low viscosity. This results in many advantages for its practical use: higher flow rates, increased solvating power and faster separations. Carbon-dioxide based fluids are seen as the ideal mobile phase because its critical temperature (31°C)

and pressure (74 bars) are easy to establish. In addition, carbon-dioxide is cheap, inert, and nonflammable. One of today's most important advantages is its neutral effect on the environment, and therefore it is considered as a "green" chemical. While using carbon-dioxide as a mobile phase, it is necessary to be able to vary the polarity. Therefore, solvents like MeOH, ethanol (EtOH), or acetonitrile can be added in different proportions – so called "modifiers". This allows the usage of normal and reversed phase columns (Hauptmann A. & Ganzera M., 2015). In this work, the 1-aminoanthracene column was selected due to its efficient performance in performed column screens. As a modifier, EtOH was used due to the best result in peak separation discovered in a mobile phase screen. The gradient for the modifier was set up with 10 – 20% over the course of 9 minutes.

The used modifiers, the gradient of modifier, the stationary phase as well as the duration per run and can be found in Table 6 (p. 14). The apparatus is displayed in Figure 5 (p. 13), the instrumentation and software in Table 7 (p. 14)

Source: https://www.waters.com

Figure 5: Instrumentation of WATERS SFC-apparatus.



Source: Hartmann A. & Ganzera M., 2015

Figure 6: Phase diagram displaying the underlying concept of supercritical fluid chromatography.

Table 6: Parameters for SFC.

Column	Torus 1-Aminoanthracene
Dimension in mm	4.6 x 250
Modifier	Ethanol
Gradient of modifier in %	10 - 20
Duration	9 min

Table 7: Instrumentation and software used for the supercritical fluid chromatography.

Instrumentation	WATERS 2767 Sample Manager
	WATERS Fluid delivery module
	WATERS Heat exchanger
	WATERS 515 HPLC pump
	WATERS Column Oven
	WATERS 2998 Photo diode array detector
	WATERS SFC Flow splitter
	WATERS Back pressure regulator
	WATERS 2424 ELSD
	WATERS Pump control module II
Software	WATERS MassLynx
	WATERS FractionLynx

3.3.4 High performance liquid chromatography

High performance liquid chromatography (HPLC) is a widely used tool for analytical and preparative purposes. It can be used for qualification, quantification, and separation of substances in a certain mixture. The stationary phase is packed in a stainless-steel column, which has a length of 25 cm, containing particles with a typical diameter of 5 µm. Flow rates up to several mL/min can be reached, and therefore high pressures are necessary. Through a special valve (Rheodyne®) the samples can be applied under normal pressure conditions. The smaller particle size of the stationary phase provides an increased surface, and therefore improves the interaction between the stationary phase and the analytes resulting in better separations. The significant parameters of HPLC are retention time and peak area (Muthuvelu K. S. & Arumugasamy S. K., 2019). For the detection of the analytes, a PDA is installed in the apparatus, which detects only those substances with a chromophore system. To detect substances without a chromophore system, an ELSD is installed, too (Gey M. H., 2008).
In this work, a SHIMADZU instrumentation was used (Table 8, p. 16). For the stationary phase a Licrosphere 100 RP 18e 5 µm (number: 098) was used. The mobile phase contained two solvents: solvent A, which was water plus 0,1 % formic acid, and solvent B, which was acetonitrile plus 0,1 % formic acid. The used gradient for the mobile phase (20 – 95 %) is described in the table below (Table 9, p. 16). Figure 7 (p. 16) displays the gradient. Before the application via the Rheodyne® valve, the samples were dissolved in MeOH.
The HPLC-setup for LC-MS contained a different instrumentation and will be described in chapter 3.4 and Table 10 (p. 18).

Table 8: Instrumentation of the high-performance liquid chromatography.

Instrumentation	SHIMADZU Degasser DGU-20A$_5$
	SHIMADZU Auto Sampler SIL-20AC HAT
	SHIMADZU Communications Bus Module CBM-20A
	SHIMADZU Liquid Chromatograph LC-20AD
	SHIMADZU Column Oven CTO-20AC
	SHIMADZU Diode Array Detector SPD-M20A
	SHIMADZU Low temperature evaporative light scattering detector (ELSD-LT)
Stationary phase	Licrosphere 100 RP 18e 5 5 µm (number: 098) Dimension: 250 x 4 mm
Mobile phase	Solvent A: water plus 0,1 % formic acid
	Solvent B: acetonitrile plus 0,1 % formic acid
Software	SHIMADZU Lab solutions

Table 9: Parameters for elution by HPLC.

Flow rate	1 ml/min
Solvent B concentration in %	20 – 95
Duration in min	37,5
Rate in % per min	2

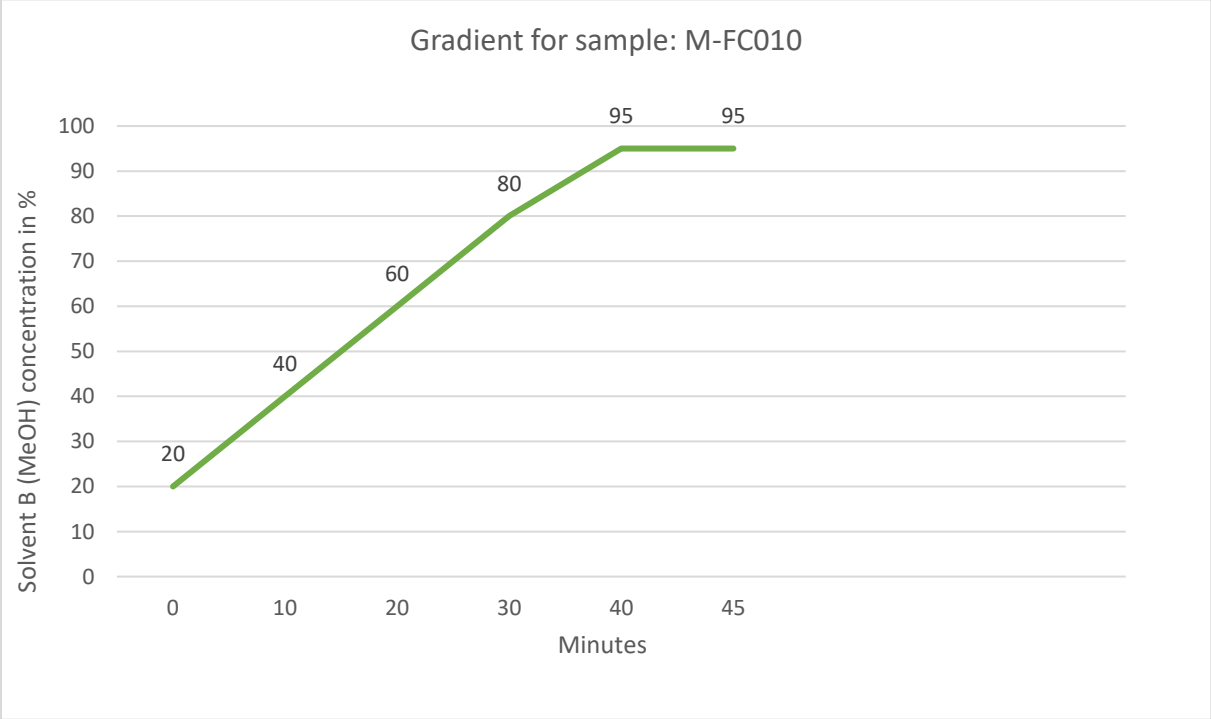


Figure 7: Gradient for sample M-FC010 for the purpose of a purity check via HPLC.

3.4 Mass spectrometry

Mass spectrometry is a technique widely used for the identification of a certain molecular structure and the molecular mass, which are both essential components for characterizations of yet unknown substances. For the analysis, the sample gets ionized. For this matter, there are various types of instrumentations (e. g. electron impact ionization (EI)). The ionized particles and fragments are accelerated and separated in a quadrupole, which leads to an ion trap, where the particles and fragments are gathered and analyzed. (Sticher O. et al., 2015). A detector records their mass-to-charge ratio (m/z), which is, together with the recorded and relative intensity, one of the two important parameters in mass spectrometry (Gey M. H., 2008).

In the course of this work, liquid chromatography was connected to a SCIEX X500R QTOF (see Figure 8, p. 17) mass spectrometer (LC-MS). The MS consisted of an electrospray ionization source (ESI) as well as a 3D quadrupole ion trap. The samples were dissolved in MeOH. The whole instrumentation is displayed in detail in the table below (Table 10, p.18).

Source: https://www.sciex.com

Figure 8: SCIEX X500R QTOF instrumentation used for MS.

Table 10: UHPLC-HRMS-Instrumentation.

UHPLC-Instrumentation	SCIEX EXION UHPLC
MS-Instrumentation	SCIEX X500R QTOF
	Turbo V ion source with Twin Sprayer ESI Probe
	Small molecules Top 5 IDA method
MS-Software	SCIEX OS ver. 2.02
Stationary phase	Kinetex 1.7 µm C18 100 A° 150 x 2.1 mm with security guard
Mobile phase	Solvent A: water plus 0,1 % formic acid
	Solvent B: acetonitrile plus 0,1 % formic acid
Gradient	0-6 minutes: 2 – 99% solvent B
	6-8 minutes: 99% solvent B
	8-10 minutes: 2% solvent B

For further analytical purposes, the exact molecular mass of substances is essential. To reach this goal, liquid chromatography was connected to a high-resolution mass spectrometry (LC-HRMS). The instrumentation for the MS was again an ESI followed by a quadrupole time of flight (Q-TOF) analyzer.

4 Results

4.1 Liquid-liquid extraction of the spruce balm

Liquid-liquid extraction was performed to gain the $NaHCO_3$-extract. The instrumentation and process, as well as the yields of the different extractions are displayed in detail in chapter 3.2 (p. 7-8).

Figure 9 displays an overview of the yielded extracts of the liquid-liquid extraction.

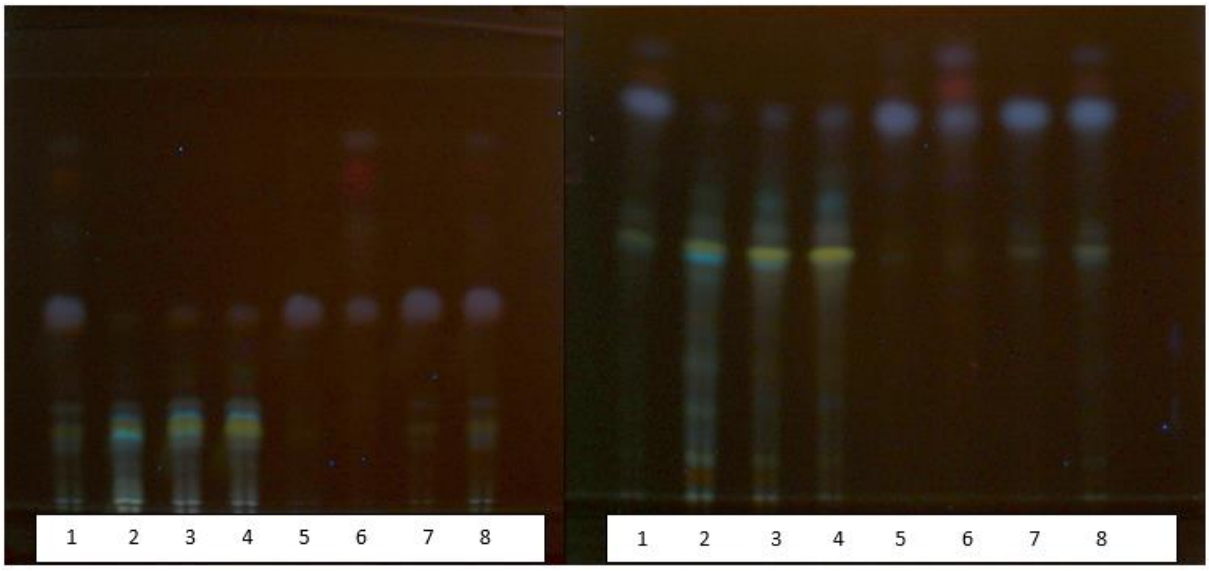


Figure 9: TLC-overview of the obtained extracts from the liquid-liquid separation (Legend: 1, 8: raw material in DCM; 2: $NaHCO_3$-extract; 3, 4: water-extract; 5: foam-phase; 6: residual-extract; 7: NaOH-extract).

4.2 Fractionation of the $NaHCO_3$-extract using flash chromatography

The $NaHCO_3$-extract (see Figure 3, p. 8) was fractionated by flash chromatography. The material and methods used for this separation are mentioned in chapter 3.3.2 (p. 11). The first run with die Interchim puriFlash provided 19 fractions according to detection with ELSD named FC01a/1-19. They were checked via analytical TLC (see A 11, p. 40). All together seven runs were performed, and the resulting fractions were combined according to TLC-analysis yielding 14 fractions named M-FC01 to M-FC14 (see appendix A 9 and A 10, p. 39). Their TLC-check and the yields are displayed below in Figure 10 and Figure 11 (p. 20).

Fraction M-FC10 was selected for further investigations, since its fingerprint showed promising bands which could not be assigned to any of the isolated compounds from Norway spruce so far (Figure 10, p. 20). With this fraction, analytical as well as preparative supercritical fluid chromatography were performed. For the corresponding spectra see Figure A 1 to A 7 (p. 31 – 37).

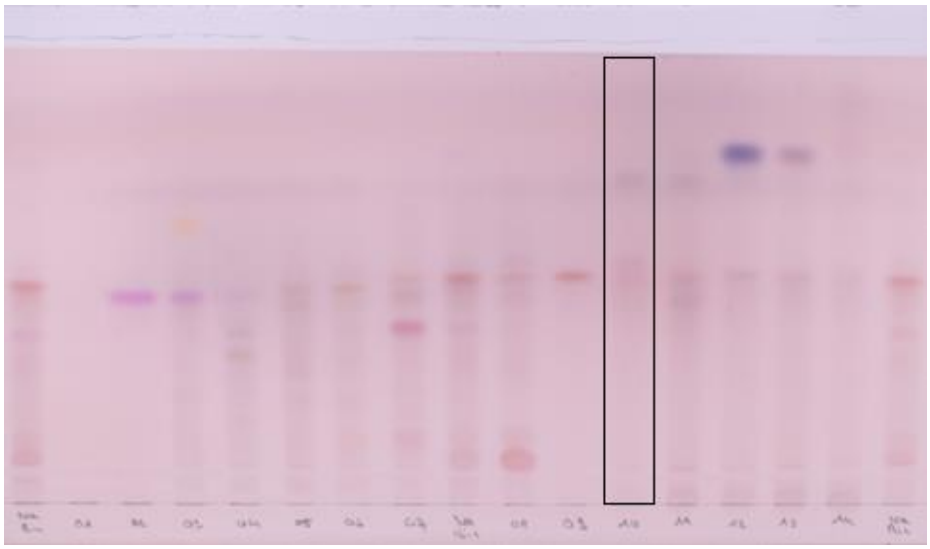


Figure 10: Analysis of fractions M-FC01-14 by TLC (system: see Table 2, p. 10). For "NaBic" the $NaHCO_3$-extract was used as a standard.

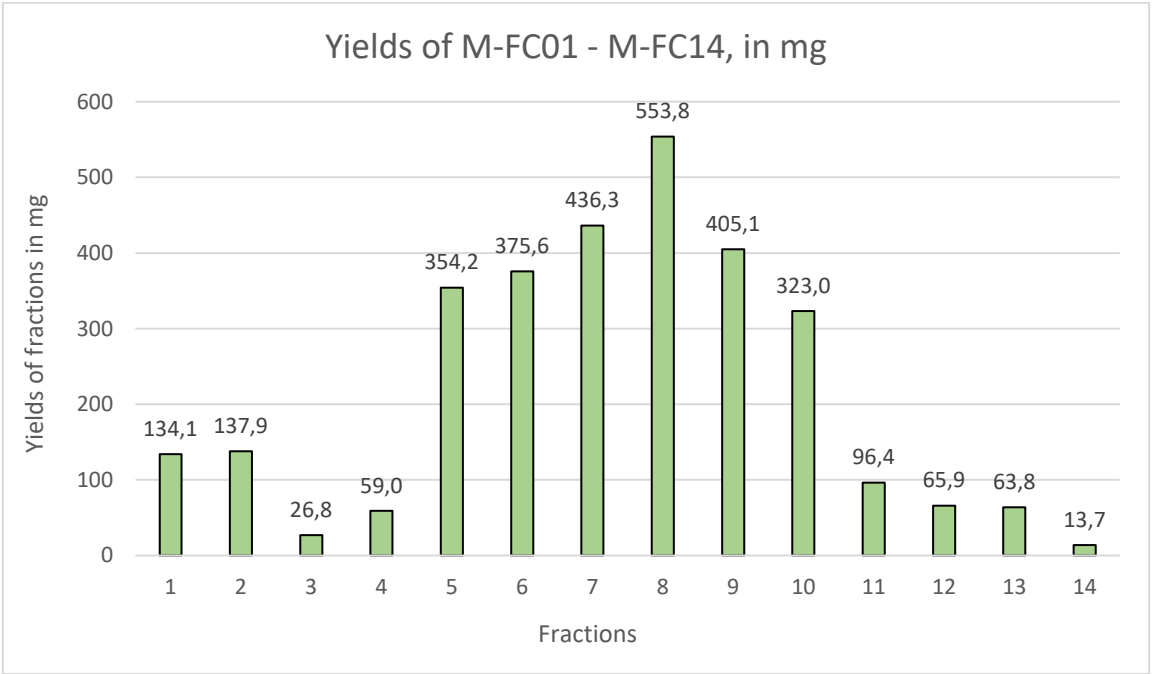


Figure 11: Yields of fractions M-FC01-14 gained after Flash-Chromatography.

4.3 Characterization of M-FC10 by supercritical fluid chromatography (SFC)

M-FC10 was further separated using SFC.

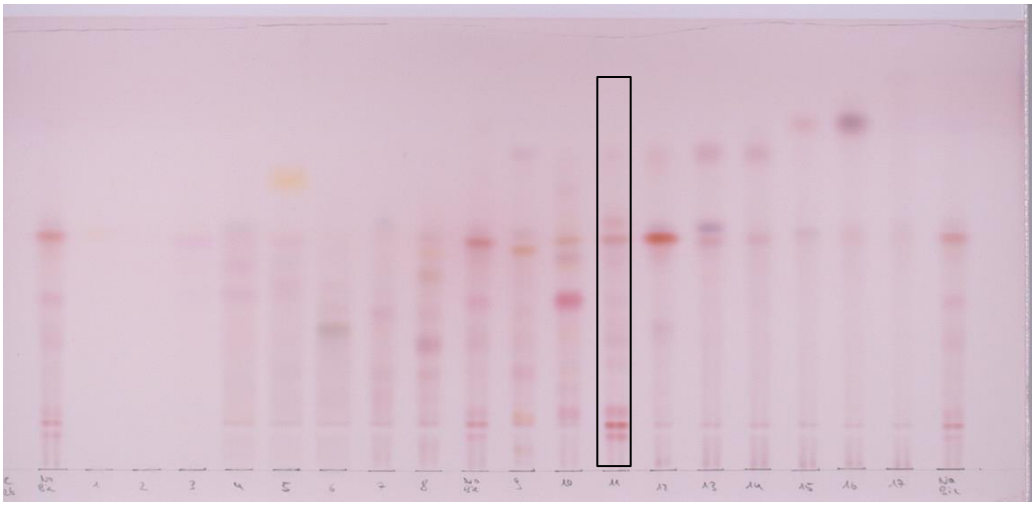


Figure 12: Fraction FC03a/1-17; fraction 13 yielded in M-FC10.

At first an analysis was performed to find an appropriate column as well as a suitable solvent and elution program. In this way the 1-aminoanthracen-column was chosen which unifies lipophilic and apolar properties (see appendix A 13, p. 41). Ethanol served as modifier (see appendix A 12, p. 40). Finally the separation of M-FC10 was performed by scale-up to a preparative column. The 323 mg were fractionated within eight single runs, all fractions were checked by TLC and combined to fractions B-01-08 (see Figure A 8, p. 38).

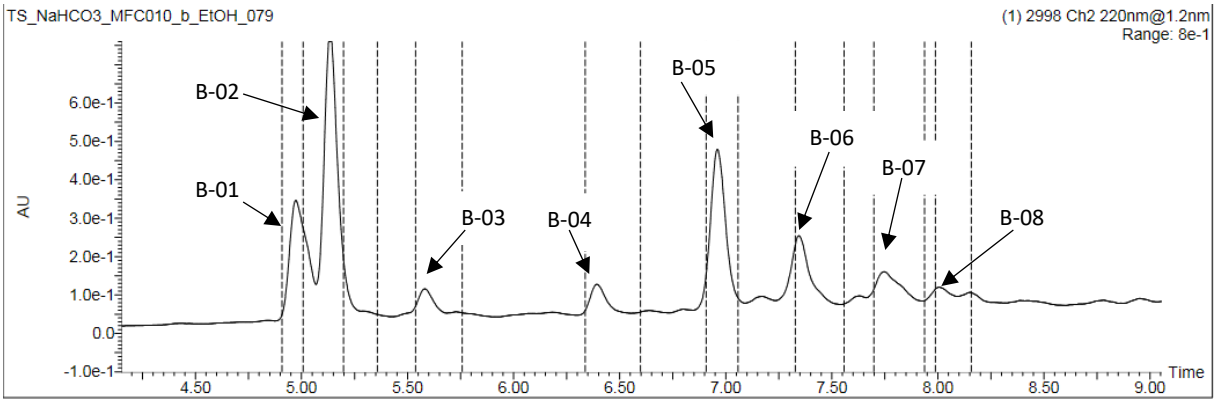


Figure 13: Analysis of M-FC10 by SFC (system: see Table 6 and 7, p. 14).

After combining the samples they were evaporated in a rotary evaporator, re-dissolved in MeOH and transferred in exactly weighed HPLC vials. After evaporating the solvent with compressed air the yield of each vial was recorded (Table 11, p. 22).

Table 11: Yields of the samples B-01 to B-08 (see Figure 13, p. 21), resulting from the separation of M-FC10 (see Figure 11, p. 20, and Figure 12, p. 21) by SFC (the samples used for mass spectrometry are marked bold).

Samples	Yield
B-01	0,48 mg
B-02	0,25 mg
B-03	**0,41 mg**
B-04	0,31 mg
B-05	**0,46 mg**
B-06	0,23 mg
B-07	0,15 mg
B-08	0,39 mg

4.4 Analysis of the fractions B-01 to B-08 by HPLC-ELSD/UV-PDA

In the next step the purity of the fractions B-01 to B-08, which were gained from the preparative SFC, was checked using HPLC-ELSD/UV-PDA (see Figure 14, p. 23). Beforehand, the samples were dissolved in acetone. The materials and methods used were described in chapter 3.3.4 (p. 15). The fractions B-03 and B-05 were selected for further characterizations because they stood out in purity compared to the other six fractions. With these two samples mass spectrometry was performed.

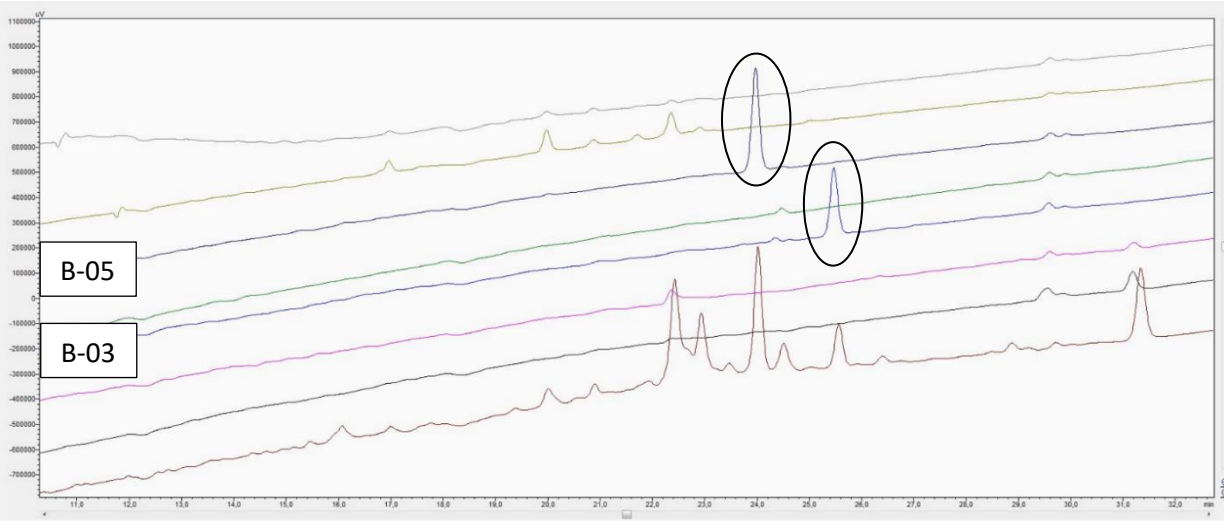


Figure 14: Analysis of fractions B-01 – B-08 (see Figure 13, p. 21) by HPLC-ELSD/UV-PDA (system see Table 8 and 9, p. 16; see appendix A 8, p. 38).

4.5 Mass spectrometry of fractions B-03 and B-05

Mass spectrometry, as described earlier in chapter 3.4, is an important tool for the characterization and structure elucidation of substances. During this thesis a liquid chromatography was used in combination with mass spectrometry to determine the molecular weight of the substances of interest. For MS, we used the fractions B-03 and B-05. The instrumentation for MS and HPLC used during these experiments are described in Table 10 (p. 18).

B-03

The interpretation of the mass spectrum of fraction B-03 recorded in the positive mode (appendix Figure A 14, p. 41) suggested a mixture of constituents with a molecular weight of 315,2 Da for the main compound. At 630,2 Da the related dimer appears. Table 12 (p. 24) explains the interpretation of the mass spectrum. The suggested sum formula is $C_{20}H_{27}O_3$.

Table 12: Two mass peaks and suggested interpretations after analysis of fraction B-03 (see Figure 14, p. 23) by LC-MS (system see Table 10, p. 18; mass spectrum: see Figure A 14, p. 41).

Positive mode [m/z]		
316,2 Da	$[M + H]^+$	[315,2 + 1]
631,4 Da	$[2*M + H]^+$	[2*315,2 +1]

B-05

The same process was repeated with the fraction B-05 (appendix Figure A 15, p. 41). Also, this fraction consisted of more than one substance. The main compound was suggested to have a molecular weight of 301,2 Da and a related dimer at 601,4 Da. For the interpretation see Table 13 (p. 24). The suggested sum formula is $C_{20}H_{28}O_2$.

Table 13: Two mass peaks and suggested interpretations after analysis of fraction B-05 (see Figure 14, p. 23) by LC-MS (system see Table 10, p. 18; mass spectrum: see Figure A 15, p. 41).

Positive mode [m/z]		
301,2 Da	$[M + H]^+$	[300,2 + 1]
601,4 Da	$[2*M + H]^+$	[2*300,2 +1]

5 Discussion and conclusion

In the course of this diploma thesis a characterization of fractions resulting from a $NaHCO_3$-extract was performed. Because of former works on this project there has already been plenty of knowledge regarding the solubility and suitable methods for the analysis of the Norway spruce balm. All the methods used were already established in former works on this project. Therefore, they were proven to be repeatable in a laboratory scale.

However, in this thesis a larger scale of raw material was used for liquid-liquid separation, during which problems regarding the separation of the polar and the non-polar phase appeared (chapter 3.2, p. 7). It seems, that because of the strong amphiphilic character of the compounds, especially of the diterpenes, high concentrations of these substances block the phase separation. This characteristic was addressed through addition of water and using larger glass appliances. In future works with Norway spruce balm, this should be considered before starting with liquid-liquid separation.

Through mass spectrometry, two substances were identified and characterized by molecular masses and suggested sum formulas. In future works, these findings can be used to perform structure elucidation with nuclear magnetic resonance spectroscopy.

6 Abstract/Zusammenfassung

Abstract

Norway spruce balm is an exudate gathered from the coniferous tree *Picea abies* (L.) H. Karst (Pinaceae). It has been used traditionally for centuries treating skin wounds and complicated skin infections. Due to its properties and established traditional use, the Norway spruce balm became a subject of interest for phytochemical research. Many of its constituents already have been isolated and characterized, but there are still more constituents to isolate and characterize.

This thesis focused on the fractionation and characterization of an aqueous $NaHCO_3$-extract. In the beginning, the raw, boiled, filtered Norway spruce balm was dissolved and extracted through liquid-liquid extraction yielding the $NaHCO_3$-extract. In the next step, this extract was further separated using flash chromatography. Through analytical TLC after each separation step of the flash chromatography, fractions for further separation and analysis were identified. This led to a combination of all gathered fractions and finally resulted in the main fraction (M-FC). With this main fraction, again TLC was used to identify promising fractions for further investigation. Due to this analysis, fraction M-FC10 was selected. M-FC10 was then used to perform supercritical fluid chromatography. After a solvent and column screen in the analytical scale, preparative SFC was used for the isolation of fractions B-01 to B-08. With these samples, a purity check was performed using HPLC-detection. The most promising samples, B-03 and B-05, were measured with LC-MS to determine the sum formulas and molecular weights of two compounds which represented the main constituents in a mixture of several compounds.

Further structure elucidation has to be performed by nuclear magnetic resonance spectroscopy in order to establish the chemical formulas.

Zusammenfassung

Fichtenfaulpech ist ein Exsudat, welcher vom Nadelbaum *Picea abies* (L.) H. Karst (Pinaceae) gesammelt wird. Es wurde traditionell über Jahrhunderte hinweg verwendet, um Hautwunden und komplizierte Hautinfektionen zu behandeln. Wegen seiner Eigenschaften und der etablierten, traditionellen Nutzung wurde das Fichtenfaulpech ein interessantes Thema für die phytochemische Forschung. Viele der enthaltenen Inhaltsstoffe wurden bereits isoliert und charakterisiert, aber es gibt noch immer Inhaltsstoffe, die zu isolieren und zu charakterisieren sind.

Diese Arbeit fokussierte sich auf die Fraktionierung und Charakterisierung eines wässrigen $NaHCO_3$-Extraktes. Zu Beginn wurde das rohe, gekochte und gefilterte Fichtenfaulpech gelöst und seine Inhaltsstoffe durch Flüssig-Flüssig-Extraktion getrennt, was den $NaHCO_3$-Extrakt ergab. Im nächsten Schritt wurde dieser Extrakt mittels Flash-Chromatographie weiter aufgetrennt. Durch analytische Dünnschichtchromatographie nach jedem Trennungsschritt mittels Flash-Chromatographie wurden Fraktionen für weitere Trennung und Analyse identifiziert. Dies führte zur Kombination aller gesammelten Proben und resultierte letztlich in der Hauptfraktion (M-FC). Mit dieser Hauptfraktion wurde abermals eine Dünnschichtchromatographie durchgeführt, um vielversprechende Fraktionen für weitere Untersuchungen zu identifizieren. Durch diese Analyse wurde M-FC10 ausgewählt. Mit M-FC10 wurde anschließend eine superkritische Fluid-Chromatographie durchgeführt. Nach einem Lösungsmittel- und Säulen-Screen im analytischen Maßstab wurde die präparative SFC benutzt, um die Fraktionen B-01 bis B-08 zu isolieren. Mit diesen Proben wurde eine Reinheitsprüfung mittels HPLC-Detektion durchgeführt. Die vielversprechendsten Proben, B-03 und B-05, wurden mit LC-MS vermessen, um Summenformel und Molekulargewicht von zwei Verbindungen zu bestimmen, welche die Hauptinhaltsstoffe in einer Mischung mehrerer Verbindungen repräsentierten.

Weitere Strukturaufklärungen müssen mittels Kernspinresonanzspektroskopie durchgeführt werden, um die chemischen Strukturen zu ermitteln.

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List of Abbreviations

DCM	dichloromethane
EI	electron impact
ELSD	evaporative light scattering detector
ESI	electron spray ionization
EtOH	ethanol
FA	formic acid
HPLC	high performance liquid chromatography
HRMS	high resolution mass spectrometry
LC	liquid chromatography
MeOH	methanol
MS	mass spectrometry
m/z	mass-to-charge ratio
$NaHCO_3$	sodium hydrogen carbonate
NaOH	sodium hydroxide
ÖAB	Österreichisches Arzneibuch, Austrian pharmacopoeia
PDA	photo diode array detector
RP	reversed phase
SFC	supercritical fluid chromatography
TLC	thin layer chromatography
UV	ultraviolet
Vis	visible light

Appendix

Figure A 1: Flash chromatography spectrum (01a) of the $NaHCO_3$-extract (see chapter 4.2, p. 19).

Title : FC01a		Author : Tom
Sample : NaHCO3 extract	Column : PURIFLASH COLUMN 15 C18 HQ 35G - 35.0 g (22 bar)	
Solvent A : H2O	Solvent C :	
Solvent B : MeOH	Solvent D :	
Channel 1 : UV600:SIG1 ==> 254 nm	Channel 2 : UV600:SCAN ==> 200-600	
Channel 3 : ELSD ==> 49°		
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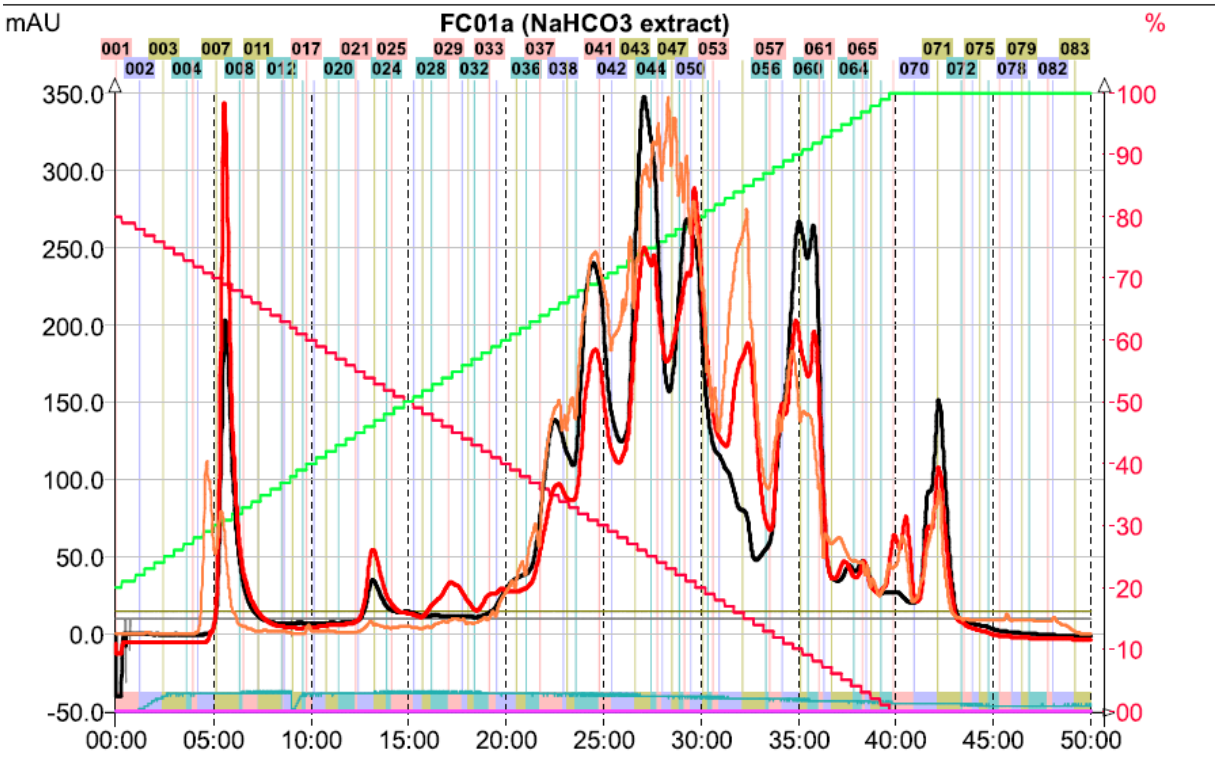

Figure A 2: Flash chromatography spectrum (01b) of the $NaHCO_3$-extract (see chapter 4.2, p. 19).

Title : FC01b		Author : Tom
Sample : NaHCO3 extract	Column : PURIFLASH COLUMN 15 C18 HQ 35G - 35.0 g (22 bar)	
Solvent A : H2O	Solvent C :	
Solvent B : MeOH	Solvent D :	
Channel 1 : UV600:SIG1 ==> 254 nm	Channel 2 : UV600:SCAN ==> 200-600	
Channel 3 : ELSD ==> 49°		
Equil+Inject Mode : Equil. (Simple) + Valve Inj. + Wait	Stop Mode : Pause	
RUN Time : 1/27/2020 2:33:31 PM		

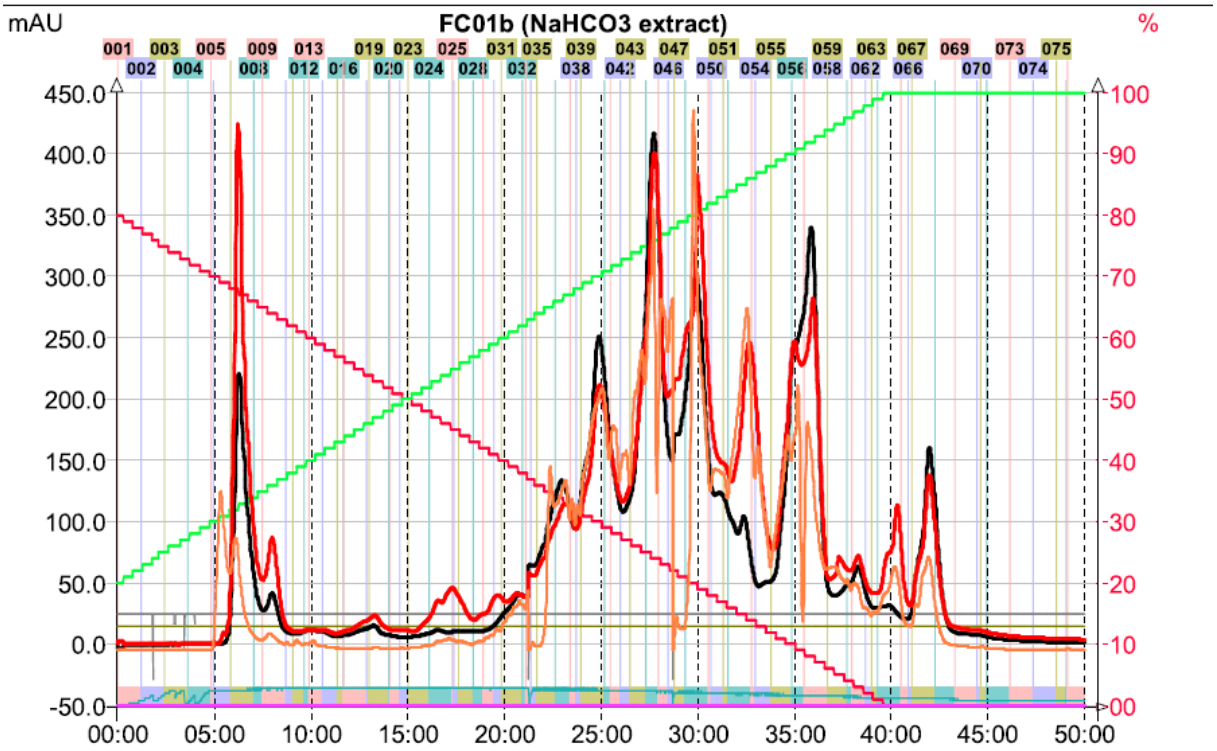

Figure A 3: Flash chromatography spectrum (02a) of the $NaHCO_3$-extract (see chapter 4.2, p. 19).

Title : FC02a	Author : Tom
Sample : NaHCO3 extract	Column : PURIFLASH COLUMN 15 C18 HQ 35G - 35.0 g (22 bar)
Solvent A : H2O	Solvent C :
Solvent B : MeOH	Solvent D :
Channel 1 : UV600:SIG1 ==> 254 nm	Channel 2 : UV600:SCAN ==> 200-600
Channel 3 : ELSD ==> 49°	
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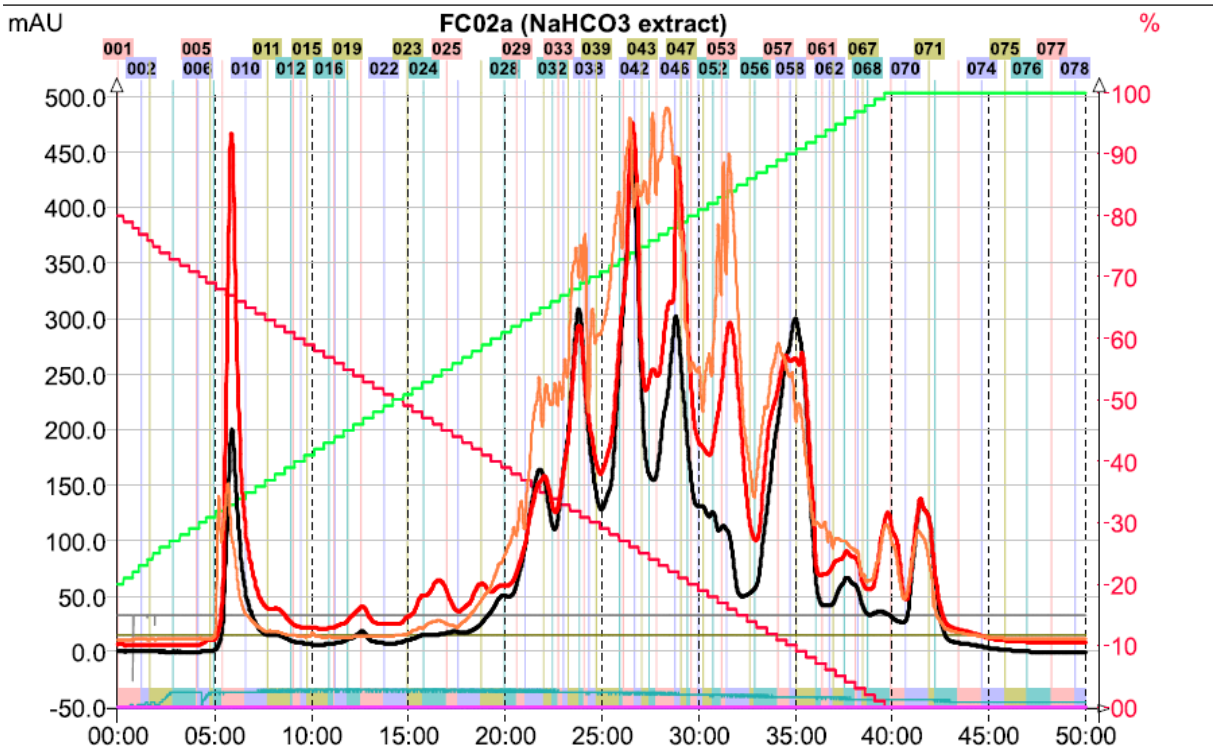

Figure A 4: Flash chromatography spectrum (02b) of the $NaHCO_3$-extract (see chapter 4.2, p. 19).

Title : FC02a		Author : Tom
Sample : NaHCO3 extract	Column : PURIFLASH COLUMN 15 C18 HQ 35G - 35.0 g (22 bar)	
Solvent A : H2O	Solvent C :	
Solvent B : MeOH	Solvent D :	
Channel 1 : UV600:SIG1 ==> 254 nm	Channel 2 : UV600:SCAN ==> 200-600	
Channel 3 : ELSD ==> 49°		
Equil+Inject Mode : Equil. (Simple) + Valve Inj. + Wait	Stop Mode : Pause	
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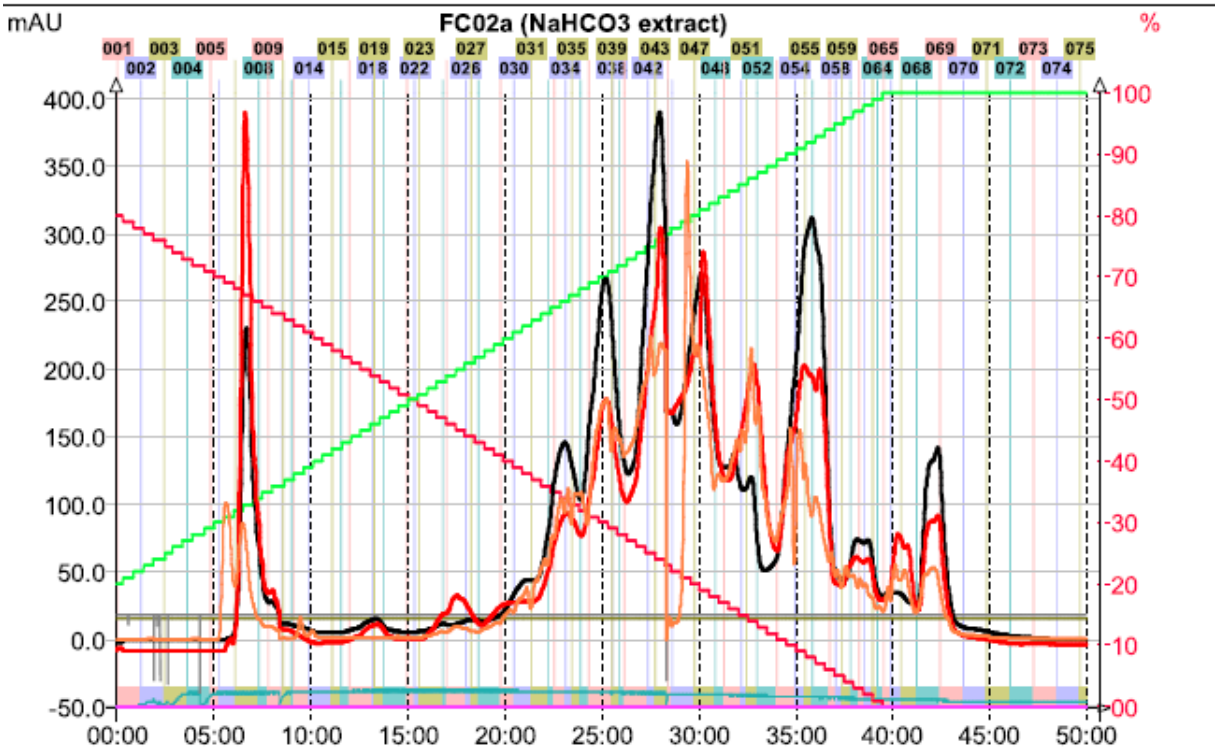

Figure A 5: Flash chromatography spectrum (03a) of the $NaHCO_3$-extract (see chapter 4.2, p. 19).

Title : FC03a		Author : Tom
Sample : NaHCO3 extract	Column : PURIFLASH COLUMN 15 C18 HQ 35G - 35.0 g (22 bar)	
Solvent A : H2O	Solvent C :	
Solvent B : MeOH	Solvent D :	
Channel 1 : UV600:SIG1 ==> 254 nm	Channel 2 : UV600:SCAN ==> 200-600	
Channel 3 : ELSD ==> 49°		
Equil+Inject Mode : Equil. (Simple) + Valve Inj. + Wait	Stop Mode : Pause	
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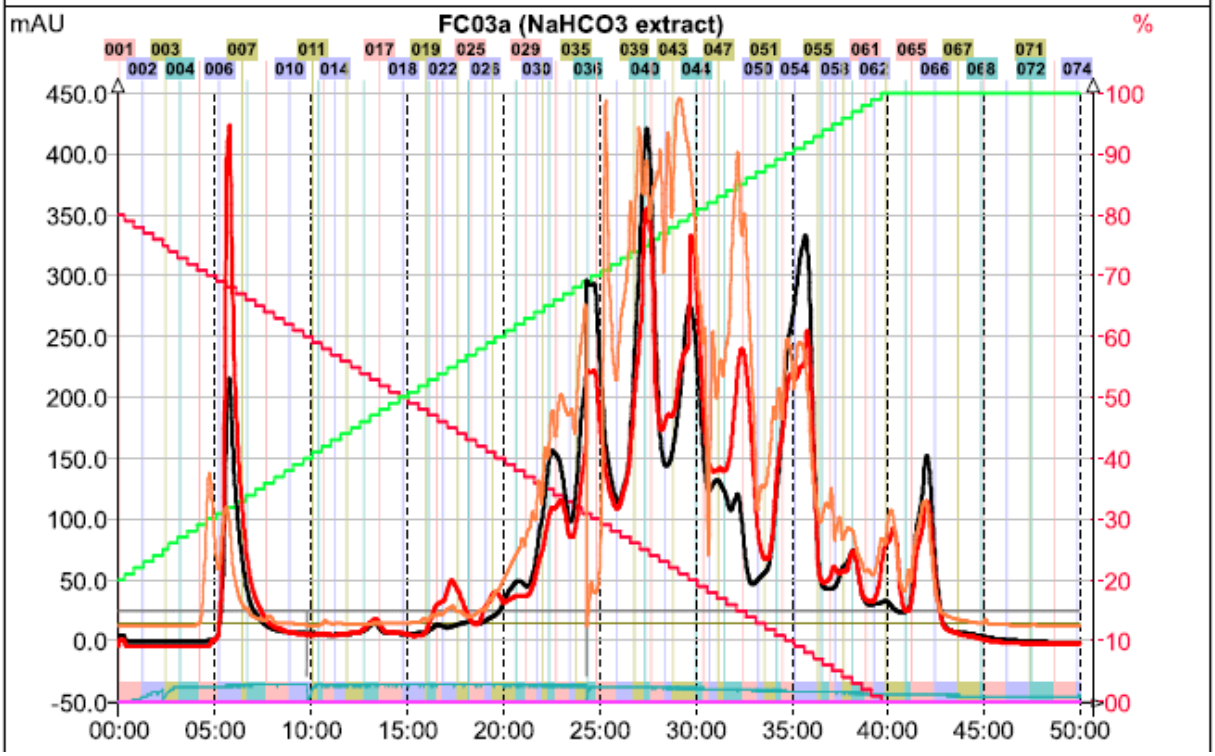

Figure A 6: Flash chromatography spectrum (03b) of the $NaHCO_3$-extract (see chapter 4.2, p. 19).

Title : FC03a		Author : Tom
Sample : NaHCO3 extract	Column : PURIFLASH COLUMN 15 C18 HQ 35G - 35.0 g (22 bar)	
Solvent A : H2O	Solvent C :	
Solvent B : MeOH	Solvent D :	
Channel 1 : UV600:SIG1 ==> 254 nm	Channel 2 : UV600:SCAN ==> 200-600	
Channel 3 : ELSD ==> 49°		
Equil+Inject Mode : Equil. (Simple) + Valve Inj. + Wait	Stop Mode : Pause	
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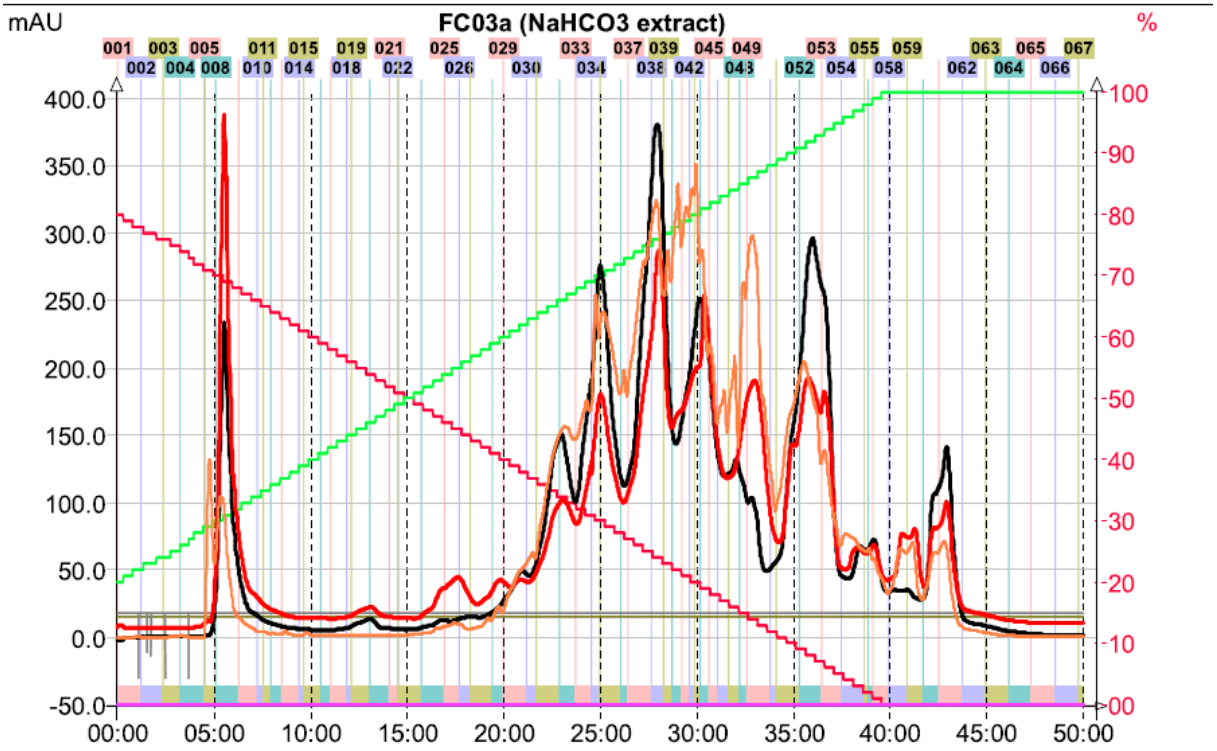

Figure A 7: Flash chromatography spectrum (04a) of the $NaHCO_3$-extract (see chapter 4.2, p. 19).

Title : FC04a		Author : Tom
Sample : NaHCO3 extract	Column : PURIFLASH COLUMN 15 C18 HQ 35G - 35.0 g (22 bar)	
Solvent A : H2O	Solvent C :	
Solvent B : MeOH	Solvent D :	
Channel 1 : UV600:SIG1 ==> 254 nm	Channel 2 : UV600:SCAN ==> 200-600	
Channel 3 : ELSD ==> 49°		
Equil+Inject Mode : Equil. (Simple) + Valve Inj. + Wait	Stop Mode : Pause	
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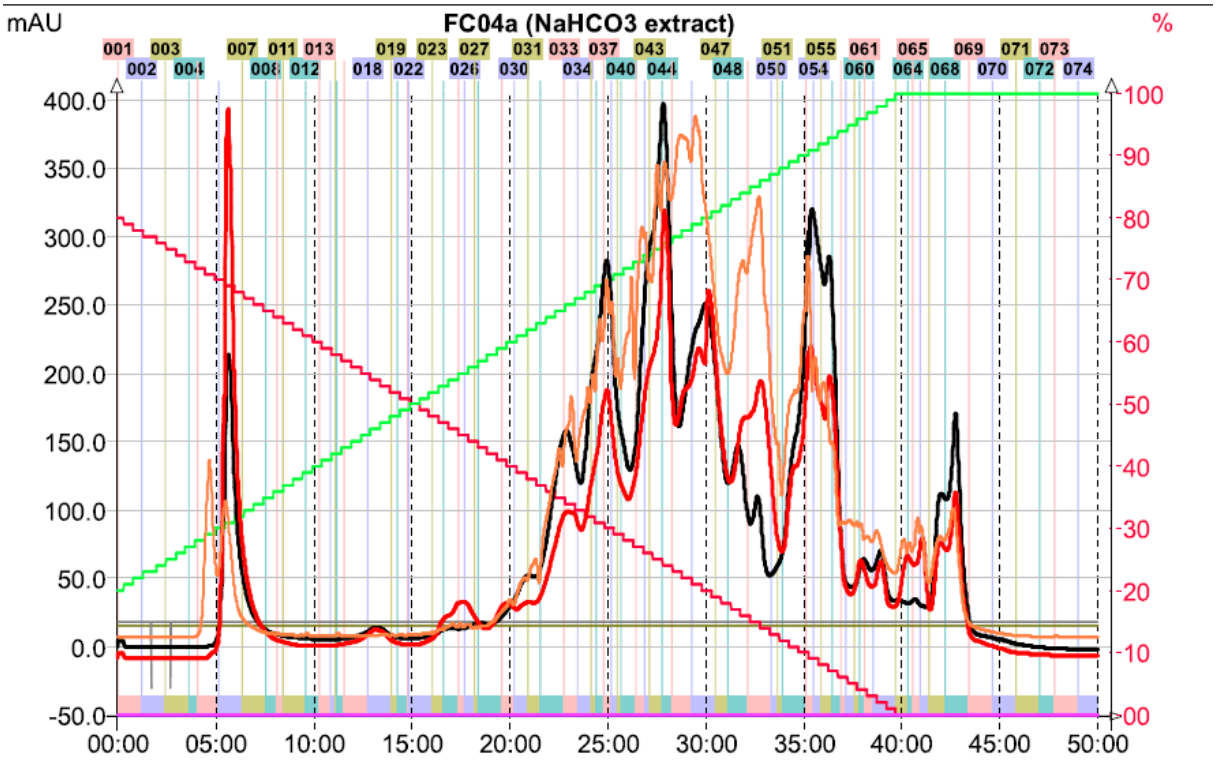

Figure A 8: Purity check of B-01 to B-08 via HPLC-ELSD (see chapter 4.4, p. 22).

Figure A 9: M-FC, postderivatized, Rem366 (see chapter 4.2, p. 19).

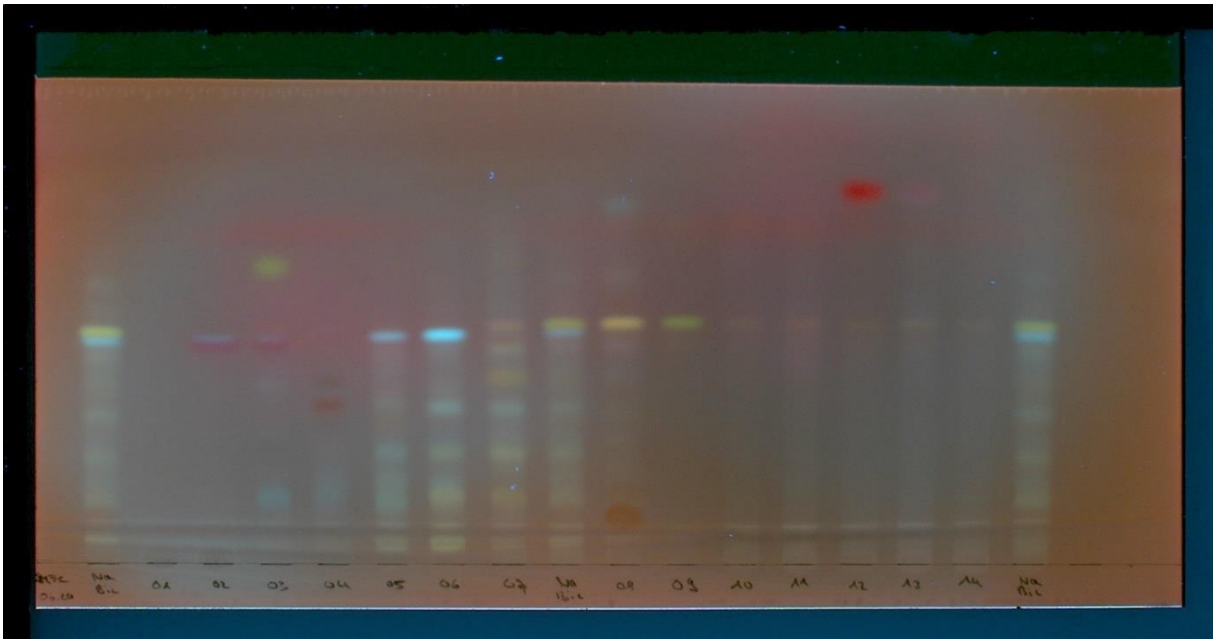

Figure A 10: M-FC, postderivatized, RemTransVis (see chapter 4.2, p. 19).

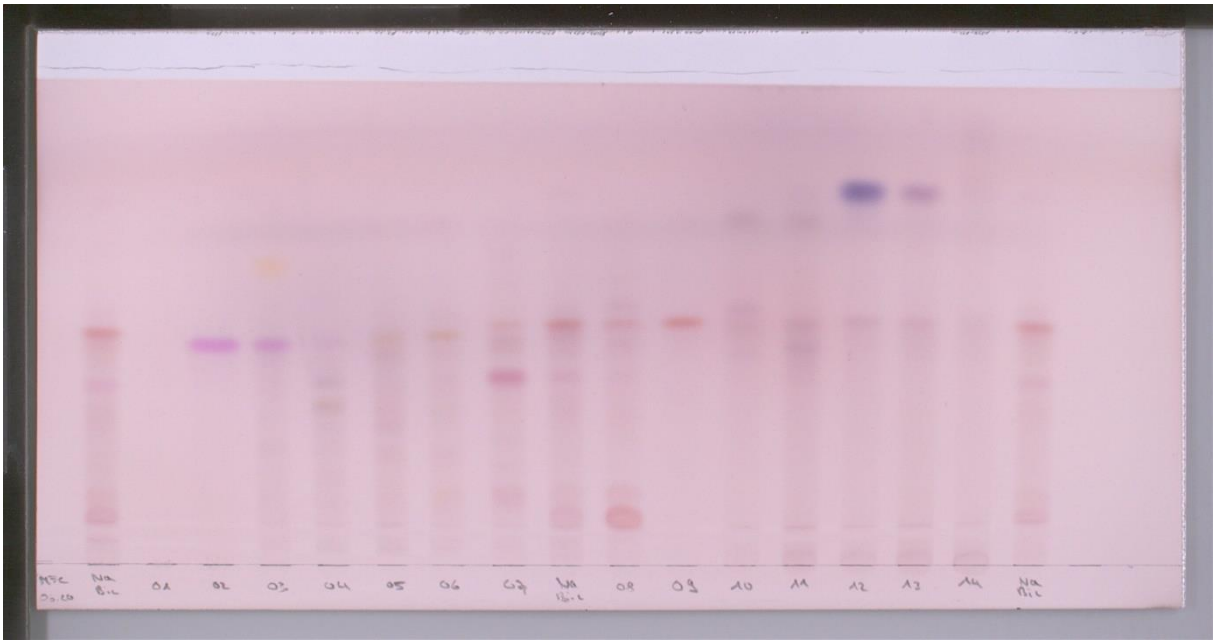

Figure A 11: FC01a (first Flash chromatography in this work); "NaBic" is the $NaHCO_3$-extract as a standard (see chapter 4.2, p. 19)

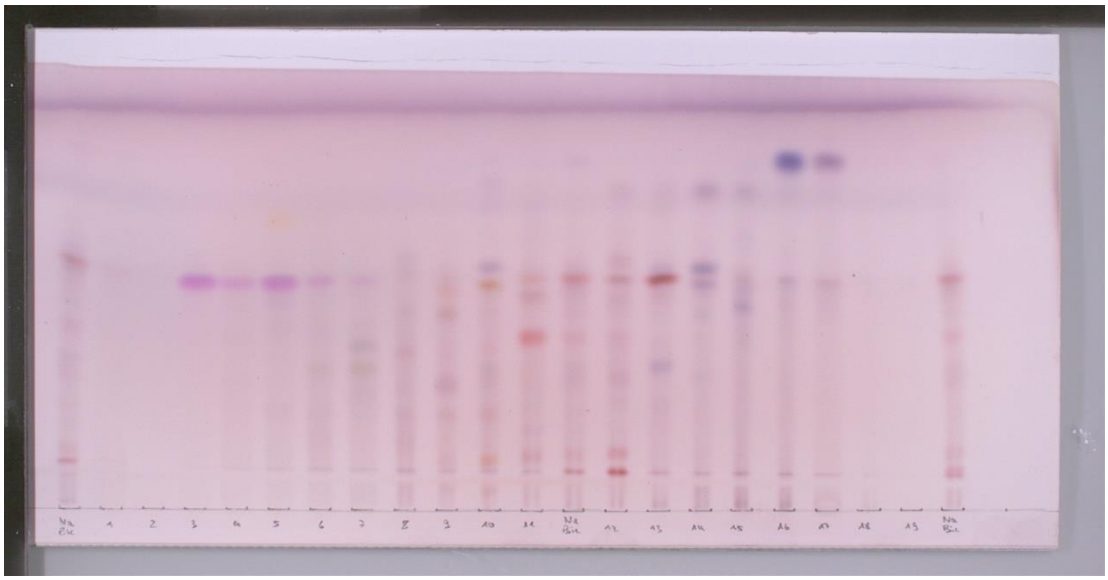

Figure A 12: Solvent screen, SFC (elected solvent: ethanol; chapter 4.3, p. 21).

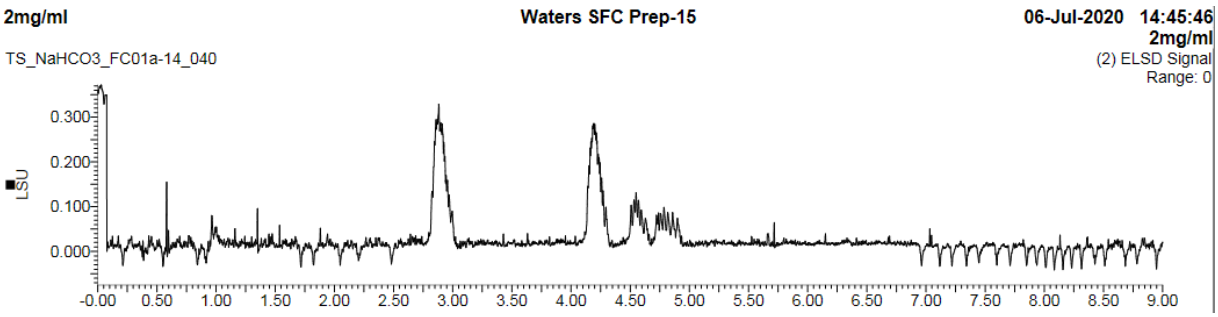

Figure A 13: Column screen, SFC (elected column: 1-AA; chapter 4.3, p. 21).

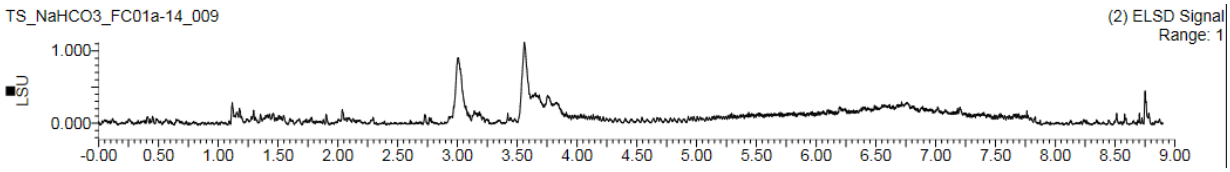

Figure A 14: B-03 mass spectrum (compounds: at 316,2 Da and its related dimer at 631,4 Da (chapter 4.5, p. 23 - 24)).

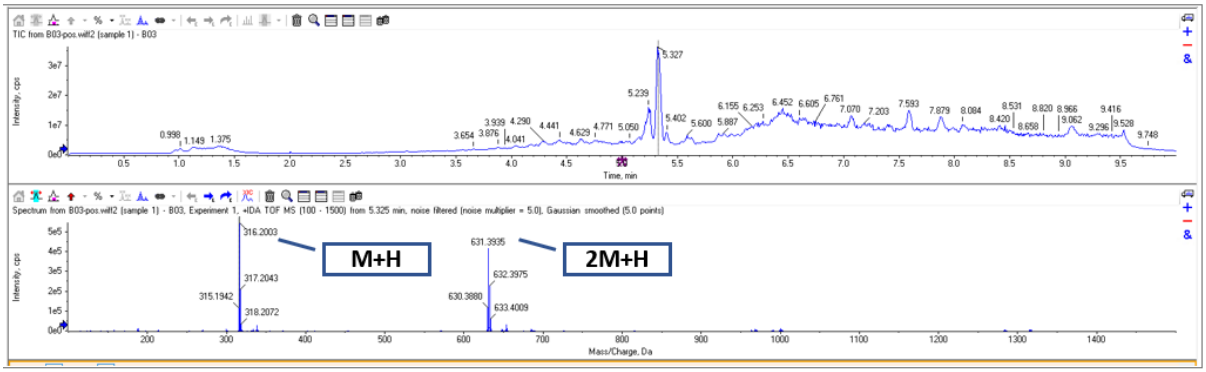

Figure A 15: B-05 mass spectrum (compounds: at 301,2 Da and its related dimer at 601,4 Da (chapter 4.5, p. 23 - 24)).