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„Evidence from traditional use of Norway spruce balm and ion exchange chromatography for the depletion of resin acids“

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List of Abbreviations

CA	carboxylic acid
$CHCl_3$	chloroform
CO_2	carbon dioxide
COO^-	carboxylate group
DCM	dichloromethane
ELSD	evaporative light scattering detector
et al.	et alii (lat.)
FA	formic acid
FC	flash chromatography
HCl	hydrochloric acid
HPLC	high performance liquid chromatography
H_2O	water
IEC	ion exchange chromatography
LC	liquid chromatography
LC-MS	liquid chromatography - mass spectrometry
Ma	Dowex marathon wba
MeOH	methanol
Mo 66	Dowex monosphere 66
MS	mass spectrometry
m/z	mass-to-charge ratio
NaCl	sodium chloride
$NaHCO_3$	sodium hydrogen carbonate
NaOH	sodium hydroxide
NMR	nuclear magnetic resonance
OH	hydroxyl group
ÖAB	Austrian pharmacopoeia
PDA	photo diode array
Phe	phenols/phenolic compounds
R_f	retention factor
RP	reversed phase
SFC	supercritical fluid chromatography
TFA	trifluoroacetic acid
TLC	thin layer chromatography
UV	ultra violet
Vis	visible light

1 Norway spruce balm ("Fichtenfaulpech") – a general Introduction

"Fichtenfaulpech", as Norway spruce balm is referred to in rural Austria, was first described 2016 in the Austrian pharmacopoeia (ÖAB) as *Picea abies pix putorius.*

The balm is described as a resinous excretion of the indeciduous tree Norway Spruce (*Picea abies* (L.) H.Karst.)[1], which is obtained by scraping it off from a cavity (see Figure 1, page 1). Due to the fact, that *Picea abies* has the ability to produce different types of excretions, the inhabitants of the alpine regions distinguish between two types. The first one represents the already mentioned balm with a soft and kneadable texture with strong fragrance and white-yellowish color. In contrast, the common sticky resin depicts the second type, which runs down the tree trunk and is typically transparent and amber colored (ÖAB - Österreichisches Arzneibuch, 2016). Both types are shown in Figure 2 on page 1.

Source: F. Fröstl

Figure 1: Cavity, where balm was harvested from with blunt instruments in order to prevent damages.

Source: J. Saukel

Figure 2: The encircled area represents the excreted Norway spruce balm (located above). Below, in thin lanes, the unusable excreted common resin is located.

[1] The generic name Picea can be derived from the excreted resin of the tree – Pix = Pech (Laudert, 2003, 127)

The term “Faulpech” is also used in the region Pinzgauer Saaltach, where people define it as a kneadable, not solidified texture (TEH® Verein, 2018).[2] Apart from these exceptions, the name “Fichtenfaulpech” or “Faulpech” does not occur in German literature. Other terms, such as “Fichtenpech/Fichtenharz” (spruce resin/pitch) or “Harz der Fichte/Pech der Fichte” (resin/pitch of spruces) are used more frequently. Due to this vague descriptive nomenclature, it remains uncertain which specific type of excretion they designate. That is due to the fact, that spruce resin is also a collective term, defining not only one type of excretion (see chapter 3, page 4).

Excretions by conifers serve as defense against insects and microbial pathogens (Rautio et al., 2012). The main components of Norway spruce balm are resin acids, which belong to the group of diterpenes, including dehydroabietic, abietic, neoabietic, levopimaric, pimaric, palustric and isopimaric acid, among others. Furthermore, they contain lignans such as lariciresinol, pinoresinol and matairesinol (Jokinen and Sipponen, 2016).

Norway spruce balm is primarily used on the skin to treat wounds, burns and ulcers due to the wound healing features traditionally attributed to it. It is applied topically as crude resin or processed in a salve or plaster (Gerlach, 2007). A plaster is manufactured by putting resin salve on linen and applying it on the injured area (Grünn, 1960).

In Europe, especially in the Nordic countries like Lapland and Northern Finland, salves made by the excretion from Norway spruce play an important role in traditional folk medicine. This home-made therapeutic salve, which is prepared by heating resin together with butter or animal fat, has been used for centuries to heal skin ulcers and infected wounds (Rautio et al., 2007). Despite its extensive use and the fact that the treatment with resins has shown its positive effect over a long time, first systematic studies regarding its effectiveness and mechanisms of action were carried out only in the 21st century (Jokinen and Sipponen, 2016).

For instance, Sipponen (2013) and others initiated the so-called “resin-project” to investigate the antimicrobial features of Norway spruce balm and of the processed salve as well as its efficacy, feasibility and safety for wound care. The doctoral thesis of Sipponen A. provides an overview of these results. Four microbiological studies show antimicrobial effects against gram-positive and negative bacteria including typical resistant bacterial strains like *Staphylococcus aureus* (MRSA)

[2] The so-called “Pinzgauerinnen” – people who live in Saaltach – shared their knowledge of healing in the course of qualitative interviews which are conducted in 2005. They provided an insight into the folk medicine of their region. The most frequently mentioned remedy is resin/pitch from conifers due to their astringent and disinfectant effects. Most of all, “Faulpech” is used preferentially in this region.

and *Vancomycin-resistant enterococcus* (VRE). Antifungal properties against common yeasts like *Candida albicans* and other dermatophytes have also been demonstrated *in vitro* and *in vivo* (Rautio et al., 2012; Repolar, 2018). Moreover, it has been shown that the natural coniferous resin salve has positive healing effects in the treatment of pressure ulcers and chronic surgical wounds, demonstrated in two clinical trials (Sipponen, 2013).

Abilar, a 10 % resin salve, is used not only for small injuries like cuts, abrasions or burns but also for treating more difficult injuries including ulcers and decubitus (Pelpharma, 2015). Regarding this product's wound healing properties, several clinical and mycological trials, as well as electrophysiological and morphological investigations were conducted (Sipponen, 2013).

2 Aim of the Work

Despite previous studies and investigations of Norway spruce balm, the chemical composition has not yet been entirely elucidated and the molecular mechanism of the wound healing properties are still unknown. Up to date, research dealt with either clinical effects or phytochemical investigations, but so far, they have not been closely linked. A forthcoming doctoral thesis on the Department of Pharmacognosy at the University of Vienna aims at closing this gap. The research of my diploma thesis takes places in the context of this doctoral thesis and comprises two parts. The first one consists of an extensive literature review on Norway spruce balm and its importance in traditional medicine. The second – experimental – part focuses on isolating and identifying the constituents of the balm.

3 Terms and Definitions

Plant resins are secondary metabolites, containing different physical properties and chemical compositions. Due to their wide variation, a uniform definition and nomenclature is not determined (Howes, 1949). Concerning their physicochemical qualities and main components, resins differ from other herbal products, such as oils, gums, waxes and fats (Bergmann, 2004).

According to Moeller (1906), Bergmann (2004) and Schnabl (2011) a plant's **excretion** can be classified as **balm** ("Weichharz/Balsam"), **hard resin** ("Hartharz/gewöhnliches Harz") or **gum resin** ("Gummen/Schleimharz"), depending, among other things, on the respective plant's age and the collection period. As pointed out by Schnabl (2011), the composition of resin species varies due to climatic conditions and their excretory organs. These compositions comprise different mixtures of volatile, liquid and solid groups of substances. The term **balm** describes freshly secreted excretions, comprising a viscous mix of solid and volatile compounds. Turpentine, which is a subtype of balms, is limited to excretions of conifers. **Resin** is a collective term for herbal exudates, but in our context, it can also be considered a solidified daughter product of balms after volatile substances have partly or completely evaporated. Resins can be obtained artificially by removing essential oil from the respective balms. As a result, balms differ from resins only due to their higher content of essential oils. Hence they are more liquid and have a more intense taste and smell (v. Jacquin, 1798).

In German-speaking regions, especially in Austria, people also use the term "Pech" (**pitch**) instead of resin, in regards to the excretions of plants. Additionally, pitch (lat. pix) describes the resulting residue during distillation of petroleum and coal tar.

Hard resins, such as colophony and dragon's blood ("Drachenblut") are of a hard and brittle consistency at normal temperature (Schnabl, 2011). **Gum resins** such as Galbanum and Olibanum differ from hard resins with respect to the concentration of gums. **Balms** or so-called soft resins, which include Canada balsam and turpentine, are rich in essential oils.

Resin products originate from turpentine producing trees and are classified as **ordinary resins** ("gemeine Harze"), according to Wiesner (1900). On the one hand, they can arise naturally, after essential oil has evaporated from the balm. They are then referred to as **natural spruce resin** ("natürliches Fichtenharz"), **root pitch** ("Wurzelpech"), **wood frankincense** ("Waldweihrauch"), **scrape resin** ("Scharrharz"), ***Galipot, Sarape or*** **overgrowth resin** ("Überwallungsharz"). When the essential oil is removed artificially by distillation on the other hand the product is termed **boiled turpentine** (“gekochtes Terpentin”), **white pitch** (“Weisspech/Wasserharz”) and **colophony** (“Kolophonium”).

While **natural spruce resin** has a semi-soft to solid texture and is yellowish/brownish with a turpentine-like smell, the consistence of **root pitch** is brittle and hard and its color is sulphur yellow. With regard to the latter, resin plates accumulate in branches of root, between bark and wood and are collected in the region Bohemia (Czech Republic). A special form of white, yellow or red small grains is called **wood frankincense**, which can be picked up from the ground after having dropped down from young spruces. When burning it, a pleasant scent unfolds. Resin that consists of a stalagtite-shaped structure, gathering on wound surfaces of turpentine producing trees is referred to as ***Sarape*** in America, ***Galipot*** and ***Barras*** in France and **“Scharrharz”** in Lower Austria. The **overgrowth resin**, which is a pathological product, arises on overgrowths of stem injuries of conifers. The **boiled turpentine** is of matt yellow color and has a subtle smell and taste. The **white pitch** differs from the boiled turpentine in respect to the concentration of water. This is caused by the fact that white pitch is gained through melting crude balm while being stirred in water. **Colophony** results when turpentine is heated until it becomes a clear substance. The brittle residue can vary from pale yellow to brownish-black color (Wiesner, 1900).

4 Literature Part

The first part of my diploma thesis constitutes a literature review on the use of Norway spruce balm in different medical indications and its healing effects. With regard to the longstanding positive experiences of people treating themselves with this natural product, the objective of this part is to provide evidence of traditional use. Based on these findings a potential submission as traditional herbal medicinal product is strived for.

4.1 Methodology of Research

Given this part is a literature review, extensive efforts have been made to identify the most important sources with respect to the topic at hand. Regarding the research methodology, neither the time frame, nor the geographic location were limited. However, considering that this research aims at providing evidence of traditional use, Europe, and especially German-speaking countries were analyzed in more detail. Databases, like Scopus, PubMed and Google Scholar and libraries, including the Vienna University Library and Pharmacy and Nutritional Sciences Library have been used primarily. The following German and English keywords were utilized during research: “Fichtenfaulpech”, “Faulpech”, “Fichtenpech”, “Fichtenharz”, “Pech”, “Harz”, “Balsam”, “Fichte”, “Norway spruce balm”, “spruce resin/balm/pitch”, “resin”, “balm” and “spruce”.

The references of books, given in the text include the author, the year and the corresponding page numbers in brackets.

4.2 Historical usage

This chapter provides an introduction to the historical use of resins as a remedy. It describes various areas of application, by ancient Romans and Greeks like Pedanius Dioskurides, one of the most famous pharmacologists of the antiquity, and Hildegard von Bingen, a German healer of the Middle Ages, who was famous for her work on medical science and natural history (Eckart, 1998, 68,69; Hildegardis, 2011, 7).

4.2.1 Resins

Resins have a long-standing history in the fields of technology, medicine and pharmacy. Particularly in the pharmaceutical and medicinal industry they play important roles, mainly in the form of plasters and ointments (Dietrich, 1900, 21). In medicine, resins of different trees belong to the oldest and most common remedies (Schnabl, 2011).

The medical use of resins reaches back into antiquity (Sipponen, 2013). Even the ancient Romans and Greeks were aware of their healing effects. It was recommended by Dioskurides and Celsus to treat specific diseases, including asthma, toothache and earache by using these natural products. They have been utilized topically on moles, furuncles, ulcerations and open wounds. Applying crude resin in case of cuts or injuries was, according to Dioskurides, a feasible strategy to pull out dirt and accelerate the healing process. In India, people took advantage of its disinfectant and absorbing effects by using it on snakebites (Grünn, 1960, 24). They also utilized it in medicinal therapy in form of incense. Phoenicians are known to have prepared salves or incense out of resins and Egyptians used it for medical purposes as well as the embalming of corpses (Schnabl, 2011). Ancient Egyptian sources describe therapeutic effects of a salve manufactured of resins (Jokinen and Sipponen, 2016). Aristoteles and Teophrast of Eresos also paid close attention to different types of resins in their studies (Schmitz, 1998, 151,144). According to Schnabl (2001) prominent dosage forms were salves, plasters, incense, baths or the direct use of resins. In „die Medicin der Naturvölker“, Bartels (1893, 120) illustrates the utilization of tree-resins by indigenous people to produce plasters, using them for external and internal treatments. Lémery (1721, 887,888) mentions the healing properties of different types of “Pech”, for example *Galipot* and *Encens marbre*. Application forms, comprising salves and plasters, were described, but only for external use.

4.2.2 Resins of Conifers

Resins from coniferous trees have for a long time been referred to as the “gold of the wood”. Forest workers used the pure resin or a salve thereof (pitch salve “Pechsalbe”) in case of serious injuries to prevent infections and accelerate the process of wound closure (Stumpf et al., 2017, 133,134). In “die Pecher”, Grünn (1960, 26) shows that the so-called “Pecher”[3], who worked a lot with sharp tools, had, however, a smooth skin and no scars due to the direct treatment of injuries with crude resins. Wiesner (1900, 229) mentions the medical use of the resin (“gemeines Harz”) *Resina pini* by ancient Romans and Greeks. Dioskurides already was convinced, that the essential oil, rich in terpenoids, which is contained in the resin of conifers, has hyperemic and antiseptic effects (Kostenzer, n.d.). Plinius and Dioskurides pointed out several kinds of resins with regard to their therapeutic effects, with resins of conifers reported as being especially suitable (Schnabl, 2011; Schmitz, 1998, 24,25,176). According to Dioskurides, salves made from resins, especially from those of conifers, are effective against the swelling of tonsils, purulent ears, snake bites and tears on feet and fingers (Grünn, 1960, 25).

4.2.3 Spruce and its Healing Balm

Bingen, Paracelsus and Matthiolus knew about the healing effects of spruces. Their resins, needles and sprouts were applied as remedies against various diseases like gout, rheumatism and colds (Strassmann, 2003, 131).

Since the Middle Ages, people have taken advantage of the excretions by spruces due to their curative effects. Resin was used as incense material against diseases because of its antiseptic effects (Heindl, 2008). The secreted resins formed an important remedy in folk medicine because of the large occurrence of spruces. In case of injury, lumberjacks, among others, took advantage of this excretion by applying it directly on wounds (Pohl-Sennhauser, 1996, 175,176). Hippokratics[4] used the resin of spruces for wound dressings and hemostatis (Schnabl, 2011; Schmitz, 1998, 135,136).

V. Burgsdorf (1791, 213) and Jacobsson (1793, 102) describe salves and plasters made from the excreted resin and white pitch from spruces *Pinus picea*. A special pitch, so-called “Tropfenpech”,

[3] “Pecher” are people, who collect resin.
[4] Disciples of Hippocrates

has been used as salve and plaster due to its healing power. Furthermore, “Wurzelpech”, which has specific healing effects in cases of fractures, has been mentioned in the “Knaffl-Handschrift” – an upper Styrian manuscript from 1813 (Knaffl, 1928, 33).

In Nordic countries ointments prepared from pure resin of Norway spruce have been among the most popular remedies for hundreds of years. The described application areas include “infected wounds, sores, pressure ulcers, punctured abscesses, suppurating burns, onychomycosis and paronychia” (Jokinen and Sipponen, 2016).

4.3 Indications

This chapter presents an overview of the different applications of Norway spruce balm. For this purpose, the section is structured according to specific indication areas which, together with the kind of excretion, were mentioned frequently in literature.

As already mentioned, the Austrian expression “Fichtenfaulpech” was first published in 2016, hence, this term did not exist in earlier literature. Instead, other terms for excretions by spruces have been included. Therefore, it remains somewhat unclear whether the desired soft and kneadable Norway spruce balm is referred to.

A common source is “Volksmedizin in Tirol” – an EU-Interreg-II-Project – by Ausserer (2001, 228,229,237), where people from North and South Tyrol are reporting their experiences with Norway spruce balm in cases of injuries. Some of them have revealed the composition of their preparations. Around 200 people, between the ages of 44 and 97 years participated in this project. However, the cited recipes and applications are not recommended for imitation. The aim of this historico-cultural work was to collect ancient knowledge about folk medicine. The knowledge about traditional folk remedies, an invaluable cultural asset, are in constant danger of falling into oblivion due to the ever-increasing number of scientific innovations. This was the motivation for the collection of traditional knowledge and recipes in the above-mentioned project.

4.3.1 Wounds, Inflammations, Skin swellings, *Rhagades*, Cuts and Abrasions

Spruce resin is often applied in the healing of **wounds** due to its disinfectant and coagulating effect, hence, it is a commonly used component of salves or plasters (Machatschek, 2011).

Grünn (1960, 26) and Kostenzer (n.d.) explain that pitch or crude resin, especially from conifers like spruces, are a well-known cure of **ulcerative wounds**. A special preparation process before application is handed down in the region Brixlegg: so-called wash-pitch “Waschpech” is being knocked and kneaded in water until it becomes pliant. In the region Wildschönau, people are preparing salves from of pitch, St John’s wort oil and lard, to treat **weeping wounds**. In contrast, Lumberjacks in the region Brandenberg, took advantage of a salve made from pitch and lard to treat injuries (Kostenzer, n.d.). A 97 years old woman living in the last-mentioned region, uses a spruce resin, which had to be beaten until it gets soft under water, to cure **inflammations** (Ausserer, 2001, 588).

Lettner (2000) mentions a pitch salve against **inflammations**, sprains, ulcers and gangrenes, in the course of a field-research in the region Rechberg. The ingredients for the mixture should be slowly cooked and are as follows: 1 l lard, 7-8 handful of pitch, one piece beeswax, 1 small onion, 12-15 pieces of camphora. A wound healing salve, consisting of spruce/larch pitch, lard, beeswax or olive oil, is, according to the "Pinzgauerinnen", a traditional remedy for the treatment of **inflammations** and ulcus cruris (TEH® Verein, 2018).

As pointed out by Pohl-Sennhauser (1996, 111,112,174,175), a black plaster, including spruce resin, beeswax, hematite, camphor, turpentine pitch and minium (a rare mineral) is utilized on **inflammations and skin swellings**. In case of **rhagades on female nipples**, a warmed-up mixture of resin from *picea excelsa/picea abies* and butter can be applied. A salve, comprising beeswax, salad oil and spruce resin is said to have wound healing features.

With regard to **wounds**, in particular **open/fresh wounds and cuts**, Ausserer (2001, 92,250-270,310,410,411,507,588-592,608) discusses different types of healing methods. Preparations of pitch salves (Antholz, Deutschnonsberg), salves made of lard, honey and spruce pitch (Zillertal) or warmed up spruce pitch, in crude form (Taufers/Ahrntal, Etschland) or mixed with camphora (Deutschnonsberg) were used in different regions for the treatment of **wounds**. Spruce pitch in crude form (Wildschönau) or in a mixture with beeswax and lard (Innsbruck), can be used in case of **open** or **fresh wounds**. In the region Pitztal, people are applying a mix of arnica flowers, chamomile buds, butter and pitch on injuries like **cuts**.

Another wound healing salve, containing spruce pitch and lard, can be applied in case of **cuts** due to its antibacterial, anti-inflammatory, antiviral and astringent effects (Fauma, 2016, 125).

A 300 years old recipe of a wound healing salve which includes 60g spruce resin, 40g beeswax and 200g olive oil, was found in the "Loferer Rezeptbüchl". The salve is used to help the healing process of **cuts, abrasions** and against cold feet due to the astringent, disinfectant and anti-inflammatory effects of the resin (Buchart et al., 2016, 130). An illegible and incomplete copy of the original prescription can be found in the appendix (Figure A 1, page 60).

Margot Adler [5] has tried to transcribe the “Loferer Rezepetbüchl”. An excerpt of the book is given below.

„Ein auch dermaßen gar guette Salben
Nimb ein feichteners Bech (Fichtenpech), das lind seye und nimb also vill lerchenen Lerget (Lärchenpech) und zerlaßes gemächlich ergehen auf einen Kolfeuer und mach das Bech zu kleinen Bröcklein, so zerget es desto ehr. Darnach wasch das aus 9 kalten Wasser wohl und alleweg wider herabgießen. Darnach nimb halbes so vill neies Wax und ein wenig Bämöll, auf ein viertel Salben ein drinckl? Bämöll, Butterschmalz yst fast noch bösser, das in kein Wasser kommen yst, oder yndes halb so vill genomen.“

„Ein Salbe von Stöckl gemacht
Item nimb ein hört Feichtespech und lauteres scheenes Wax und Bämöll, und Hennig gleich vill und ein altes schmer, ye älter ye besser und ein Hirschtalg yst auch guet und eshailt dermaßen alle Wunden und Schaden.“

A blistering ointment (“Zugsalbe“) comprising pure pitch, which can be used alone or in addition to lard and/or beeswax, is, according to Vogl-Lukasser et al. (2006), an effective cure. Pitch from spruces is a remedy for the treatment of **wounds**, **injuries**, ulcers and abscesses**.**

Prevedel (2011) pointed out a salve made of spruce pitch “feichtes Pech”, larch resin “lergat”, “Raglwachs”, “Schmer”, beef marrow and “Hirschinselt” to be helpful against **open wounds.** In case of **injuries** and **fresh wounds**, a salve recipe from the 16th century, including arnica juice, houseleek, lard and spruce resin, is described as well by Preveder (2011).

The detailed preparation of a resin/pitch salve, including 60g spruce resin, 200ml olive oil and 30g beeswax is indicated in “Mythische Bäume”. The salve is used in the following cases: **badly healing wounds**, cold feet, joint pain, colds, muscle cramps, neuralgia, infections and splinters (Stumpf et al., 2017, 133,134).

In case of **injuries,** a balm containing 6g beeswax, 60g spruce resin and 50ml St John`s wort oil, is suitable (Strassmann, 2003, 293,294**)**.

[5] Margot Adler, a woman who lived in the Region Lofer, has studied old manuscripts.

4.3.2 Dislocations, Rashes, Strains, Sprains and Gangrenes

With respect to these indications, pitch plasters have often come into use, according to literature.

Swellings, caused by **dislocations** and bone fractures can be cured, according to Paßler Ursche[6] by applying a pitch plaster which includes a mixture of rye flour, pitch and "Pollestein" (Müller, 1985, 189-191). An 80 years old man from the region East Tyrol (Defreggen, St. Jakob) also suggested a pitch plaster to cure **dislocations** (Ausserer, 2001, 257).

As mentioned by "Österreichische Akademie der Wissenschaften, Bayerische Akademie der Wissenschaften", (1976, 751), pitch can be used in the form of plasters to treat **dislocations** and **rashes** (see chapter 4.3.9, page 19). Gerlach (2007) reports also a spruce pitch plaster which relieves **dislocations and strains.**

Pohl-Sennhauser (1996, 111,112) has pointed out a "red plaster" consisting of *Dentaria enneaphyllos* („Saunigelwurzel") and turpentine or spruce pitch, which is used for **strains of muscles.**

Another plaster, called "Dörrband" is described as a very helpful remedy in the case of **dislocations and sprains**. For this, a mixture of powdered salsify, wheat flour, marshmallow root and herb, "Bole Ameni", oak bar, spruce pitch ("Feichtenpech") and larch pitch should be heated up (Koren et al.,1975, 267,268) (see appendix; Figure A 2, page 61). The ingredients of a pitch salve, according to Lettner (2000) helpful against **sprains** and **gangrenes**, were mentioned in chapter 4.3.1 on page 10.

[6] The landlady Ursula Steinkasserer – from the Antholzer Valley ("Antholzer-Tal") – was known as an alternative practitioner, who said to cure wounds of all kinds.

4.3.3 *Ulcus cruris*, Ulcer, Burns, Boils, Abscesses, Sciatic, Cracks, Furuncles and Ulcerations

Stöger (2010, 362) describes a recipe of a salve against circulation disturbances like ***ulcus cruris***, passed down from her mother and grandmother. The so-called "Steffisalbe" contains spruce pitch, larch pitch, beeswax and lard, amongst others. Its detailed composition is given in the appendix (Figure A 3, page 62). According to Strigl (1995), ***ulcus cruris*** can be treated with another special house salve. The ingredients of this salve include spruce resin (white pitch), butter or beef fat, beeswax and possibly the addition of larch resin. The original detailed preparation of this house salve is attached in the appendix (Figure A 4, page 62). An example is a 71 years old farmer from Zell am See (Austria) known for her special pitch salve which is said to be universally usable due to its healing power. Even her mother-in-law and their grandfather have used the same recipe of the so-called "Mitteregg-Salve". One time, as her mother in law suffered from "open feet" (***ulcus cruris***), the pitch salve showed positive effects in the healing process (Friedl, 2009, 51). As discussed in chapter 4.3.1 on page 10, a salve with wound healing features, comprising amongst others spruce pitch, can also be used in this clinical picture (TEH® Verein, 2018)**.** Machatschek (2011), in turn, explains, that pitch plasters have a long history in the treatment of ***ulcus cruris,*** due to their wound healing features.

Spruce pitch is, according to Vogl-Lukasser et al. (2006), a remedy for **ulcers** and **abscesses** (see chapter 4.3.1, page 10). **Abscesses**, among other indications, can be treated in accordance with Pohl-Sennhauser (1996) with a preparation of a salve, including pitch, which will be discussed in chapter 4.3.5, page 17. **Ulcers** can be treated with pitch plasters, as described by „Österreichische Akademie der Wissenschaften, Bayerische Akademie der Wissenschaften" (1976, 751), or pitch salves (Lettner, 2000) (see chapters 4.3.1, page 10 and 4.3.9, page 19).

In "Baumheilkunde", Strassmann (2003, 293,294) mentions an old house salve of a smooth texture, effective against **ulcers, abscesses and boils.** For this, 50g spruce resin had to be mixed with 40ml linseed oil. A balm containing 6g beeswax, 60g spruce resin and 50ml St John's wort oil is suitable for ailments like **abscesses, boils and sciatic** but also against colds, sinusitis, rheumatism and gout. By applying spruce pitch, turpentine and a plaster made of sugar, yolk and flour on **ulcers**, a positive result should be achieved (Renner and Renner, 2011, 923).

To treat **ulcers** and **burns** it is recommended to use a plaster made from cooked spruce pitch, which is spread on linen before application. These plasters have quite similar effects as essential oil drugs and are particularly effective, because for one thing they do not stick together with wounds and additionally, the wounds are always kept moist (Gerlach, 2007).

Hermann Jud, from East Styria reveals his family recipe of a pitch salve in the documentation „Tradition des Heilens- Auf den Spuren der Volksmedizin"– by Niedermair W. (2018). The composition of lard, beeswax, olive oil and resin, which has to be solved under heat, is presented to be helpful in the treatment of **ulcers**.
In Russia, a salve, containing protein, spruce resin, wax and olive oil, is used for the treatment of **ulcers**. A woman from the Netting region, recommends a salve made from resin to treat **ulcers** and throat diseases. The healing powers of such a salve were already known to Dioskurides, who had applied it on feet and finger **cracks**. In addition, the crude balm is also very helpful in the therapy of **ulcers** (Grünn ,1960, 25,26).

Ausserer (2001, 92,250,261,296,309,327,341,359,379,382,410,458,472,520-535,596) also mentions that **ulcers** can be treated by applying crude spruce pitch. In Schmirnertal people took advantage of the so-called "Tripflapech", which describes the small sticky drops of pitch on the trunk of spruces.

In this indication, salves containing spruce pitch, are frequently mentioned in "Volksmedizin in Tirol". A pitch salve made of beef fat, butter, linseed oil, beeswax and spruce pitch is referenced. Another preparation includes the following components: lard, beeswax, beef fat, lysoform, salt and spruce pitch. A blistering ointment, containing lard, pitch, camphora and onion, is also described. A woman from Lienz swears by a salve, comprising butter, beeswax, spruce pitch and "rote Polis". In Ötztal, a salve consisting of honey and spruce pitch is described by a woman. A composition containing spruce pitch, lard, beeswax and camphor had to be put on linen before applying it.

Additionally, in Wildschönau, spruce pitch plasters and blistering plasters from spruce pitch, larch pitch ("Lörget") and "santel pulver" are common as well.

According to a man from Ötztal, **ulcus cruris** can be treated with a salve containing spruce pitch or white pitch, butter or beef fat, beeswax, larch pitch ("lörget"), arnica liquor, cooking oil, honey and cod liver oil. A plaster made of tree resin and honey is reported to be helpful in the treatment of **ulcers, ulcerations** and **abscesses**. A woman from Antholz mentions a pitch salve, in connection with **abscesses**. In the regions Martell and Navis people have suggested a pitch plaster in case of **furuncle**.

4.3.4 Rheumatism and Gout

The healing power of spruce resin in the treatment of **rheumatism, gout** and colds has already been known for centuries (Strassmann, 2003, 131).

People from Lower Austria recounted their positive experiences with a plaster which they called “Dürrband” in “die Pecher”. The mix of spruce pitch (“Fichtenpech”), “Rottenbullos”, dry blood (“Trockenblut”), Sulphur flower (“Schwefelblüte”), salt, lard, “Hirschinslet, “Granabeer” and camphor gives a grease. In case of diseases like **rheumatism** this mixture can be applied on linen to the affected area. A “Pecher” from St. Veit raves about a preparation of pitch and lard, which he uses in the treatment of **rheumatism**. Regarding this condition, Swiss, Slavs and people from other regions like Vogtland, took advantage of resins in the form of plasters (Grünn, 1960, 24,25).

Applying a mixture of pitch and lard is according to Österreichische Akademie der Wissenschaften, Bayerische Akademie der Wissenschaften (1976, 751) a good method to treat **rheumatism**. Moreover, in case of **rheumatism and gout** a certain balm is suitable. The ingredients are discussed in chapter 4.3.3 on page 14 (Strassmann, 2003, 293,294).

In „Volksmedizin in Tirol“, different treatment methods for diseases like **rheumatism** and **gout** were discussed by people of different regions (Ausserer, 2001, 93-114,183-190,248,388,410-488). **Rheumatism** is often mentioned in connection with pitch plaster treatments. In the regions Antholz and Zillertal for example, people took advantage of this plaster made from pitch and in Deutschnonsberg and Sarntal spruce pitch was specifically mentioned as an ingredient of such a plaster. A salve to cure **rheumatism** made of pitch and ants is alluded to by a woman from the region Passeiertal. In Pitztal and Ridnaun, though, people describe good experiences with a different type of composition that includes 50g yellow wax, 40g olive oil, mutton fat, spruce resin solved in turpentine and honey. A woman from Stubai uses spruce pitch as a plaster for **gout**.

4.3.5 Inflamed gums, Toothache, Sore throat and Oral thrush

According to Pohl-Sennhauser (1996, 113) a preparation of a salve, which includes pitch, butter, deer suet, roots from blue gentian and sanicle, and, if required, arnica and calendula blossoms, camphora, thyme, white beeswax and yarrow leaves, is used in the treatment of **sore throat, toothache,** abscesses and bruises.

A special salve based on resin is also recommended in case of **throat diseases** by Grünn (1960, 25) in chapter 4.3.3 on page 14.

It is useful to chew spruce pitch or put some of it on the **inflamed** area in case of **toothache** and particularly **oral thrush**. In the regions Wildschönau and Unterland, people took advantage of this treatment option (Fauma, 2016, 125; Ausserer, 2001, 542,588).

4.3.6 Fractures and Bruises

Spruce pitch from the tree *Picea abies* is described in "Veränderung der Almwirtschaft in Salzburg und altes Erfahrungswissen" as an ingredient which should not be left out of any salve. It was used to accelerate the healing process of **bone fractures** (Hönegger, 2008). Machatschek (2011) also mentioned that salves consisting of spruce resin are used in case of these physical conditions, due to their coagulating effects. As discussed in chapter 4.3.2 on page 13, swellings caused by **bone fractures** can be cured by applying a pitch plaster (Müller, 1985, 189-191).

According to the doctorial thesis „Die Hausmittel- und Heilrezeptsammlung des Tiroler Bauerndoktors Anton Auer im Vergleich mit der Encyklopädie der Volksmedicin des Arztes Georg Friedrich Most", the preparation of a pitch plaster, including "Polus", spruce pitch and an egg, can be applied on **small fractures** and is described as follows: *„Für einen kleine Bruch nimmt man Polus, feichtes Pech (=Pech der Fichte), alles mitsammen sieden abrühren und ein Ei; früher muß man sich mit Terpentinöl fleißig einreiben, nachher legt man das Pflaster auf …*(Prevedel, 2011).*"*

The experiences and treatments of **bone fractures** by different people are listed below.

People from Antholz, Matrei am Brenner, Pitztal, Schmirnertal-Valsertal and Villgraten reported plasters with different ingredients. In addition to spruce pitch, plasters can contain camphor, houseleek, turpentine or arnica liquor. In Gschnitztal, a 70 years old man uses a salve made of spruce resin, marshmallow root, linseed oil, clarified butter, kidney fat, sanicle root, lovage and bacon. Laying spruce pitch on linen and putting it on the fractured area can be an alternative, if the salve is out of stock. The linen serves as a support for the diseased area after having gotten hard. Additionally, another pitch plaster with healing properties on **broken limbs** was mentioned (Ausserer, 2001, 93,248,293,295,345,423,467,468,553).

Gerlach (2007) pointed out a plaster advantageous in the treatment of **broken bones.**

In case of **bruises**, chapter 4.3.5 (page 17) describes the preparation of a helpful salve (Pohl-Sennhauser, 1996, 113).

4.3.7 Joint/Back/Hip-pains, *Lumbago*, Neuralgia, Muscle cramps, Stone formation in organs, Frostbites, Colds, Cold feet, Sinusitis

The special treatment of **colds** with spruce resin has been known for centuries (see chapter 4.3.4, page 16). The balm, already described in chapter 4.3.3 on page 14 is – among others – useful against this condition and **sinusitis** (Strassmann, 2003, 131,293,294). Furthermore, **colds** and other physical ailments such as **muscle cramps, neuralgia, cold feet** and **joint pains** can be treated with a resin/pitch salve, according to Stumpf et al. (2017, 133,134). The corresponding ingredients are listed in chapter 4.3.1 (page 10). In case of **cold feet**, a 300 years old preparation of a salve using spruce resin is also mentioned above (chapter 4.3.1 on page 10).

As Machatschek (2011) discussed, spruce resin or spruce pitch, respectively, is a remedy, which can be used in the treatment of **stone formation in organs** and **pain in the hips** due to its disinfectant effect.

According to Ausserer (2001, 91,248,318,429,436,444) a plaster is applied in three different indications of sicknesses. A plaster containing beeswax, butter, salt, pitch and pitch oil can be utilized in case of **frostbites**. In the treatment of ***lumbago***, people from Antholz and Ridnaun took advantage of a pitch plaster. The precise description of spruce pitch as an ingredient for this plaster is mentioned by a woman from Ridnaun. In Innsbruck and Sarntal, people report that **back pain** can also be treated with a plaster made from of pitch.

4.3.8 Corns and Warts

Corns can be treated by applying hot spruce pitch within 24 hours, which is attached in the form of a plaster. After a saltwater-footbath the corn can be carefully removed (Weidinger, 1988, 185). According to Paßler Ursche's recipes from "Dorfbuch Antholz", persistent **corns** can be melted with plasters and salves comprising compositions such as a pitch plaster of rye flour, pitch and "Pollestein" (Müller, 1985, 189-191).

In "Volksmedizin in Tirol", a 78 years-old woman from Deutschnonsberg (St. Felix) mentioned "pitch" in relation to **warts** (Ausserer, 2001, 267).

4.3.9 Hemostasis and Amputations

In earlier times people faced problems in the treatment of wounds. Amongst others, amputations and the stopping of bleedings were often problematic. Nevertheless, to accelerate the wound healing process, Teutons and people in Morocco, employed hot pitch (Bartels, 1893, 285,286; Engl, n.d.).

In „Volksmedizin in Tirol", a woman gives an example regarding the use of pitch. The great-grandfather of her husband had an accident with an iron piece. As a result, his toe was jammed and he had no other choice than to **amputate** it. To close his wound, he took advantage of pitch (Ausserer, 2001, 597).

As illustrated by this example, pitch has been known as an antiseptic remedy for a long time. In 1976 it was primarily used as a plaster for **hemostasis** and against ulcers, dislocations and rashes (Österreichische Akademie der Wissenschaften, Bayerische Akademie der Wissenschaften, 1976, 751).

4.3.10 Infections and injuries with Splinters

The healing power of pitch/resin salves helps to disinfect wounds and prevent **infections**. That is one of the reasons why these salves are applied on **splinters** to pull them out (Pechsalbe, 2012). Stumpf et al. (2017, 133,134) also mentions a pitch/resin salve, which can be applied in case of

infections and injuries with **splinters**. The ingredients are given in chapter 4.3.1 on page 10. According to Gerlach (2007), a foreign object can be pulled out of the skin by using resin or pitch externally as crude balm or plaster.

The treatment of injuries with **splinters** was discussed by 9 individuals in „Volksmedizin in Tirol“, who applied pure spruce pitch or salves thereof, like pitch salves (“Pechsalbe”), blistering ointments (“Zugsalben”) or larch salves (“Lörgetsalben”) (Ausserer, 2001, 338,427-468,513,553,588,592).

4.3.11 Ingrown Nails, Umbilical hernia, Insect bites and Chilblains

As reported by Ausserer (2001, 252,306,308,419), a 95 year old woman from Pitztal applied a plaster, the so-called spruce pitch plaster, which was useful for **softening nails**. In cases of **insect bites**, a woman from the region Niedertal spreads a liquid pitch on the bite area. In the treatment of **Chilblains** two persons from Innsbruck proposed salves made from different mixtures of ingredients. The first mix includes fat, wax and pitch, while the second one consists of beeswax, butter, salt, pitch oil and pitch.

A Bavarian treatment of **umbilical hernia** among children through applying pitch is described by Grünn (1960, 26).

4.3.12 Worms, *Periostitis/Periostalgia, Paronchya*

A salve consisting of pitch, camphor and lard is, according to Ausserer (2001, 431,467,468,588,589), mentioned by a woman (Ridnaun) in case of “**worm**” and ***Periostitis***. Furthermore, a pitch plaster is noted in connection with “**worm on finger**” and ***Paronchya*** by another woman from the region Schmirnertal-Valsertal. The explicitly mentioned spruce pitch, which can be applied in case of “**worm on fingers**”, is used by a woman, who lives in the region Wildschönau.

4.4 Customs

The first exsudate of the trees at the beginning of the year, the so-called “Maipech“, is supposed to have very special healing powers (Grünn, 1960, 27). In Zillertal, people are collecting the healing pitch on the third day of May. Before it can be used in the handling of sprains, open wounds, fractures, inflammations and ulcers, the pitch has to be washed and kneaded in nine different waters until it gets hot (Ausserer, 2001, 619-622).

5 Experimental Part

While the first part presents a literature review on the range of applications regarding the treatment with Norway spruce balm, the second, experimental part focuses on the isolation and identification of its constituents. The objective is the depletion of diterpene acids from Norway Spruce balm and the phytochemical investigation of the resulting fractions.

5.1 Current state of Science and Aim of the Experimental Part

Herbal medicinal products have become increasingly important in western countries for basic medical care. 80 % of people worldwide are using these products and relying on them (Ekor, 2014). Considering the increasing use of natural products, it is pivotal to identify the yet unknown substances, which could be responsible for their healing effects. Norway spruce balm plays an essential part in traditional medicine due to its wound healing and antimicrobial effects – as documented in the literature part. However, several ingredients, which could be responsible for these effects, remain unknown. Consequently, the direct correlation between phytochemistry and these effects is still missing. This thesis aims at contributing to the closure of this gap.

Prior research, performed by diploma students of the Department of Pharmacognosy at the University of Vienna, focused on the aqueous $NaHCO_3$-fraction of Norway spruce balm. As a result of these studies, several substances were isolated and identified. In the course of my work the focus lies on the NaOH-fraction, which accounts for the biggest amount in the results of the respective liquid-liquid-extraction (see Figure 4, page 24).

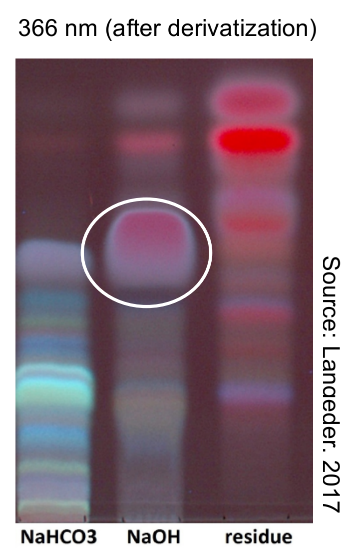


Figure 3: TLC-fingerprint comparison of $NaHCO_3$-fraction, Residue-fraction and NaOH-fractions (high accumulation of diterpenes is market)

Stationary phase: Silica gel 60G F_{254}
Mobile phase: $CHCl_3$ + MeOH + TFA (97 + 3 + 0.1)
Detection: Anisaldehyde/sulphuric acid solution: UV_{366}

Research has shown that resin acids are the main components of Norway spruce balm, which belong to the diterpene group. However, to understand the wound healing features, it is necessary to identify other substances, like phenolic compounds, which account for a considerably smaller fraction compared to resin acids. In Figure 3 on page 22, a TLC fingerprint comparison was conducted by Langeder (2017) showing a conspicuous big violet spot at R_f 0.6, representing the high proportion of resin acids. Thus, the thesis at hand concentrates on the depletion of these acids in order to investigate the resulting fractions phytochemically. As the purification method of choice ion exchange chromatography was used.

5.2 Material and Methods

This chapter lists the sources of used material and describes all methods which have contributed to isolate and identify the constituents.

5.2.1 Plant Material

In the course of a cooperation with Österreichische Bundesforste, crude Norway spruce balm (internal batch number LU01) was harvested and collected by scraping it off from trunks of Norway spruce *Picea abies* (L.) H. Karst in the Region Lungau. Subsequently, it was stored in a metal bucket at -20°C.

5.2.2 Liquid-Liquid Separation

As mentioned above, the experimental part of this diploma thesis focused on the NaOH-fraction. To obtain this fraction, the following liquid-liquid separation of boiled Norway spruce balm was conducted at the Department of Pharmacognosy:

Boiled Norway spruce balm was first dissolved in dichloromethane (DCM), then extracted with an aqueous sodium bicarbonate solution ($NaHCO_3$) and subsequently separated in an aqueous and an organic phase. The aqueous phase comprised strong acids such as carboxylic acids. This phase was acidified with hydrochloric acid (HCl) to release the acids into their free form and afterwards extracted with DCM. The remaining organic phase (DCM phase) was once more extracted with aqueous sodium hydroxide solution (NaOH), resulting in a separation between weak acids, such as phenols, and apolar compounds (residual fraction). The aqueous phase had to be acidified and extracted again with DCM.

As a result, three fractions arose from the liquid-liquid separation, as shown in Figure 4 (page 24). It is conspicuous that the fraction this thesis focused on yielded the highest amount.

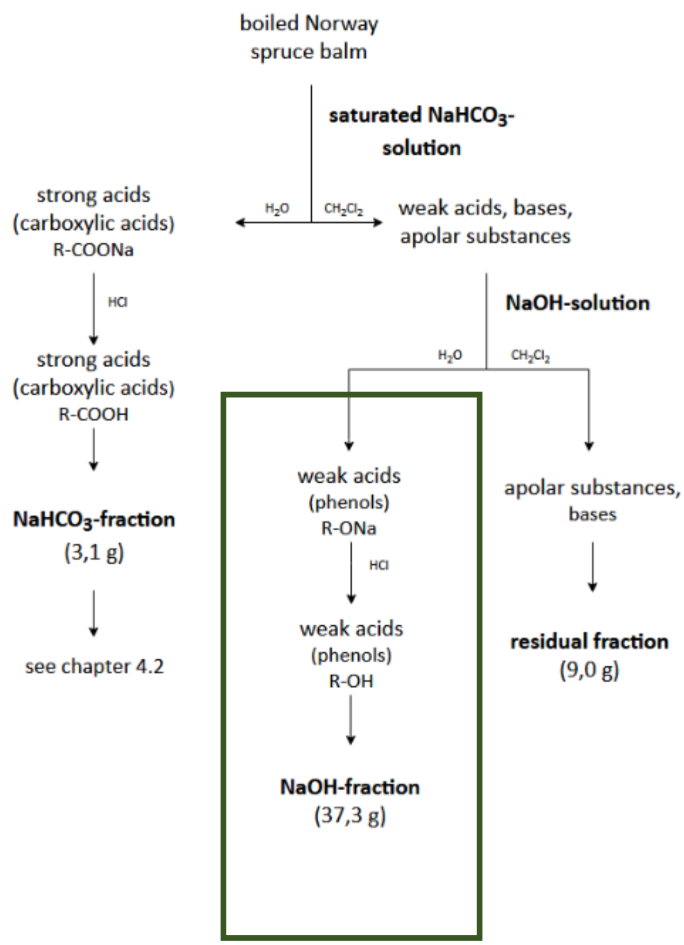


Figure 4: Liquid-liquid extraction steps made by Göls T. (Langeder, 2017)

5.2.3 Chromatography

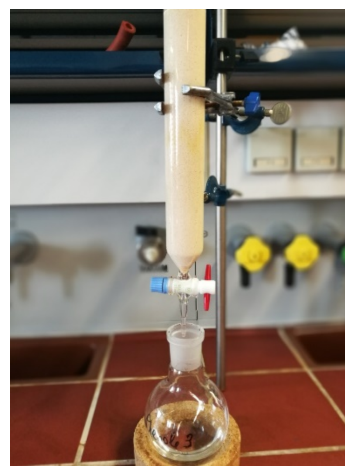
Chromatography allows the separation, identification and purification of mixtures of different molecules. The principle is based on a distribution of components in complex samples between two distinct phases, the stationary phase (solid phase) and the mobile phase (gaseous or liquid phase). Whether substances interact with one or the other phase depends on their differences in the distribution constants (Poole, 2003).

In the course of this diploma thesis, Ion Exchange Chromatography (IEC), Thin Layer Chromatography (TLC), Flash Chromatography (FC), High Performance Liquid Chromatography (HPLC) and Supercritical Fluid Chromatography (SFC) were used and will be discussed in this chapter.

5.2.3.1 IEC - Ion Exchange Chromatography

According to Durham (2006) and Dragull and Beck (2012), Ion Exchange Chromatography (IEC) is a technique for the isolation of ionizable and charged natural products by the exchange of ions (Figure 5, page 25).

The basis of the process is the interaction of charged molecules (M) with oppositely charged groups (G) attached to a supporting resin matrix (R). For this purpose, counter ions (C) of charged groups (G) are getting displaced with an equally charged molecule (M).



Source: F. Fröstl

Figure 5: Dowex Marathon WBA, during the exchange of ions

The stationary (or solid) phase represents an ion exchange resin, packed in a column, which includes an insoluble support matrix (R) associated with a functional group (G) and its reversible binding counter ion (C). The mobile phase comprises a charged molecule (M), solved in a solvent, which can replace the counter ion (C) by passing through the stationary phase. This change is possible because M is attracted more strongly to the exchange resin than C. After the so-called adsorption cycle the bound charged molecule can be eluted again considering that the process is reversible.

Depending on retaining either cation- or anion analytes, two types of ion exchangers are available. An anion exchanger, including a cationic resin as solid phase is necessary to retain anion analytes. Vice versa a cation exchanger, comprising an anionic resin as solid phase is needed to retain cation analytes. The respective equations are:

$$\text{Cation exchanger: } R - G^{-}C^{+} + M^{+} \rightleftharpoons R - G^{-}M^{+} + C^{+}$$

$$\text{Anion exchanger: } R - G^{+}C^{-} + M^{-} \rightleftharpoons R - G^{+}M^{-} + C^{-}$$

The pKa of a charged group (functional group) defines the effectiveness of an exchanger and depends on the group's ionized state. Weak basic anion exchangers, including primary, secondary or tertiary amines as functional groups and a free base as a counter ion had to be protonated at a pH below their pKa values.

Two weak basic anion exchange resins, Dowex Monosphere 66 and Dowex Marathon WBA, where tested. The latter proved to be better suited for the task. The product details are given in Table 1 on page 26. The support matrix (R) of the Dowex Marathon WBA is a Polystyrene-DVB macropore, which is macroporous to give a high internal surface area while the functional group (G) on the matrix is a tertiary amine representing a neutrally charged molecule ($-NR_2$). A free base represents the counter-ion (C), necessary for a neutral charge of the stationary phase. The stationary phase can be protonated and is able to carry a positive charge at a pH under 7 ($-NR_2H^+$).

Table 1: Product Information of Dowex Marathon WBA

Product	Type	Matrix (R)	Functional Group (G)	Counter Ion (C)
Dowex Marathon WBA	Weak Base Anion	Polystyrene-DVB macropore	Tertiary Amine ($-NR_2$)	Free Base

(Dow, 2018)

5.2.3.2 TLC - Thin Layer Chromatography

Thin Layer Chromatography (TLC) requires a stationary phase in a thin layer on a supporting material, such as an aluminum, glass or plastic sheet. Sorbents like aluminum oxide, polyamide, cellulose and silica gel are commonly used.

The sample is dissolved in the mobile phase, which normally consists of volatile, organic solvents, and applied in a concentration of 5-10 % (w/v). For the application, a glass capillary is used. A glass rectangular tank, lined with filter paper and filled with sufficient mobile phase, is required for the next steps. Before the thin layer plate can be inserted into the chamber, an equilibration step should be conducted to prevent unsteady solvent fronts. This vapor phase in the closed vessel is enhanced by the solvent-soaked filter paper. Regarding the development step, the plate has to be carefully placed into the saturated tank, which subsequently will be closed with a lid. The components of the sample start to migrate with the mobile phase, travelling upwards through the stationary phase by means of capillary force. The resulting separation of the components depends on their different interaction with the stationary phase. Shortly before the mobile phase reaches the top of the plate, it is removed. After drying, the results of the developed plate can be visualized first under UV light and second by derivatization (Wall, 2005; Poole, 2003; Coskun, 2016).

As part of the experimental work, TLC plates made from aluminum foil (support matrix) and silica gel (stationary phase) – produced by MERCK – were used. Either acetone or methanol were utilized as solvents for the samples, which were applied manually in bands of 2 microliter portions. The exact composition of the mobile phase is a mixture of chloroform, methanol and TFA (97+3+0.1). To achieve a refined result, a derivatization step, where the plate is dipped into an anisaldehyd/sulphuric acid solution, was conducted. Thereafter the plate was heated up for approximately 2-3 minutes to a temperature range between 100-105°C. The detection of the developed TLC plates was implemented using a CAMAG TLC visualizer allowing for the plates to be photographed before and after derivatization with white light illumination and with a UV-lamp at wavelengths of 254nm and 366nm.

The fractions resulting from the adsorption and elution step of the ion exchange chromatography were analyzed by TLC to validate the depletion of acids.

5.2.3.3 FC - Flash Chromatography

In Flash Chromatography (FC) short separation columns, which are packed with intermediate sized particles (40-63 micrometers), are the key component. The mobile phase can flow through these columns by means of moderately elevated pressure. The advantages, compared to conventional column chromatography, are not only shorter elution times and reduces solvent consumption, but also higher purity of the isolated compounds, resulting from higher resolution between bands (Poole, 2003).

For the thesis at hand Interchim's puriFlash® 4250 system was used. The Modules and Software are given in Table 2 on page 28.

Table 2: FC Instrumentation

FC-Instrumentation	**Interchim puriFlash® 4250**
	PDA Detector (254 nm and SCAN 200-600 nm)
	Preparative-integrated ELSD
Software	Interchim Software

For fraction FC01-04, a Puriflash reversed phase Column 15 C18 HP 6G (22bar) was utilized as stationary phase. A mixture of water and methanol served as mobile phase (see Table 3, page 28). The gradient of methanol is depicted in Table 4 on page 28.

Table 3: Stationary and mobile phase of FC

Fraction	**Column**	**Solvent A**	**Solvent B**
FC01-04	PURIFLASH COLUMN 15 C18 HP 6G (22bar)	H_2O	methanol

Table 4: Solvent gradient of FC

FC01-04		
Time (min)	**Solvent B concentration in %**	**Flow Rate in ml/min**
0	60	5.0
40:00:00	100	5.0
50:00:00	100	5.0

The sample was loaded in dry form, adsorbed on silica gel. Hence the sample, which was diluted with acetone or methanol (1:10), had to be mixed subsequently with the fourfold amount of silica gel. The solvent was removed from the mixture by evaporation under reduced pressure by means of a rotary evaporator. Suitable cartridges were first loaded with the dry sample-silica gel mixture, and then filled with pure silica gel. The FC system was purged and equilibrated before the cartridges were employed.

Concerning this thesis, FC served primarily as a fractionation and purification method of phenolic compounds. The aim of isolating pure compounds from the phenol fraction can be facilitated by using FC.

5.2.3.4 HPLC - High Performance Liquid Chromatography

High Performance Liquid Chromatography (HPLC), a rather recent form of LC, is one of the most widely used analytical techniques. By this method excellent separation of components in mixtures can take place within short time. Very small particles packed into columns, make up the stationary phase. The liquid mobile phase, together with the dissolved sample substances, is forced to flow through the column at high pressure. Depending on the choice of the mobile and stationary phase, different interactions occur with the dissolved sample. If the stationary phase is polar, the process is called normal phase. Most of the time silica gel is utilized for retaining polar analytes. Reversed phase separations, where the stationary phase apolar, count to the most common methods. In such cases interactions between non-polar sample compounds and the functional groups of the modified silica gel of the stationary phase take place. In both categories the mobile phases have the opposite polarity to the stationary phases (Ho et al., 2003; Meyer, 2004; Dong, 2006).

The study at hand worked with a Shimadzu instrumentation and reversed phase columns (see Table 5, page 30). Thus, the polar mobile phase consisted of a mixture of water with 0.1 % concentrated formic acid (solvent A) and acetonitrile with 0.1 % concentrated formic acid (solvent B). As an apolar stationary phase an RP 18 column was chosen. The samples were dissolved in acetone or methanol in a 1+1 dilution. A gradient from 35-95 % in 30 minutes was applied (see Table 6, page 30). For detection an ELSD and PDA (190nm and 254nm wavelengths) were utilized and the software program “Shimadzu Lab solutions” was used for recording and data evaluation.

Table 5: HPLC Instrumentation

HPLC- Instrumentation	SHIMADZU Degasser DGU-20A5
	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
Software	SHIMADZU Lab solutions
Stationary phase	Licrosphere 100 RP 18e 5µm (Number: 098)
	Dimension: 250 x 4 mm
Mobile phase	Solvent A: H2O + 0,1 % FA
	Solvent B: Acetonitrile + 0,1 % FA

Table 6: Solvent gradients used for NaOH-fraction

Fraction	Gradient (%solvent B)	Duration (min)	Rate (% per min)
NaOH-fraction	35-95 %	30	2

5.2.3.5 SFC - Supercritical Fluid Chromatography

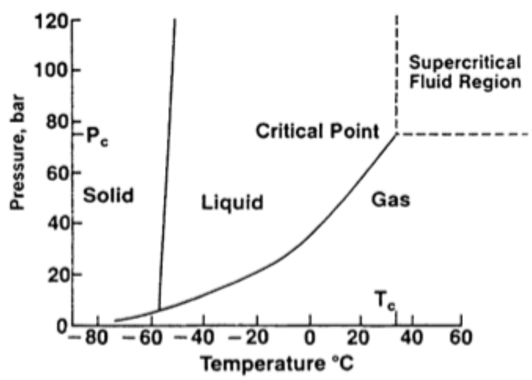


Figure 6: Phase diagram for carbon dioxide

As shown in Figure 6 (page 30), the supercritical state arises when a gas or liquid is brought to a temperature and pressure above its specific critical point (Poole and Poole, 1991).

Their properties like solubility, density and viscosity are between two states: gaseous and liquid. Due to that fact SFC fills the gap between gas chromatography and liquid chromatography. Due to its low critical temperature and non-toxicity, the most suitable mobile phase for supercritical fluid chromatography is carbon-dioxide (CO_2) (Harris, 1998).

An instrumentation from WATERS was used for the analyses (see Table 7, page 31). The utilized columns and corresponding solvent gradients are given in Table 8, page 31.

Table 7: SFC Instrumentation

SFC-Instrumentation	WATERS 2767 Sample Manager
	WATERS Fluid delivery module
	WATERS Heat exchanger
	WATERS Column Oven
	WATERS 515 HPLC pump
	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

Table 8: Stationary phases and corresponding solvent gradients for SFC

Column	**Dimension (mm)**	**Modifier**	**Gradient (% modifier)**	**Duration**
Bridged-Ethylene-Hybrid (analytical)	4,6 x 250	Methanol	5 - 50 %	9 min
Charged-Surface-Hybrid Fluoro-Phenyl (analytical)	4,6 x 250	Methanol	5 - 50 %	9 min
Bridged-Ethylene-Hybrid 2-Ethylpyridine (analytical)	4,6 x 250	Methanol	5 - 50 %	9 min
Silica 2-Ethylpyridine (analytical)	4,6 x 250	Methanol	5 - 50 %	9 min
Silica 2-Ethylpyridine (analytical)	4,6 x 250	Methanol	14 - 32 %	9 min
Silica 2-Ethylpyridine (preparative)	10 x 250	Methanol	14 - 32 %	9 min

5.2.4 Mass Spectrometry (MS)

According to Frame et al. (2014), mass spectrometry (MS) is an analytical technique, which aims at offering information on quantity as well as quality regarding the molecular structure of compounds and mass of molecules in samples, respectively. In order to attain this objective, the molecules in a sample have to be transferred into gas phase ions. The ions can consequently be separated by their mass-to-charge-ratio (m/z) and detected in proportion to their abundance (De Hoffmann and Stroobant, 2007).

In the context of this diploma thesis, LC-MS was used for structure elucidation. Therefore, the samples had to be solved in methanol. The mass spectrometry instrumentation contains a linear ion trap and an electrospray ionization source (ESI). Table 9 (page 32) presents which instrumentations, software, stationary and mobile phase were used.

Table 9: MS Instrumentation

HPLC-Instrumentation	DIONEX UltiMate 3000 RS Pump
	DIONEX UltiMate 3000 RS Autosampler
	DIONEX UltiMate 3000 RS Column Compartment
	DIONEX UltiMate 3000 Diode Array Detector
	DIONEX UltiMate 3000 Corona Ultra RS
MS-Instrumentation	THERMO FISCHER LTQ XL Linear Ion Trap Mass Spectrometer
Software	DIONEX Chromeleon XPress
	THERMO FISCHER Xcalibur
Stationary phase	Licrosphere 100 RP 18e 5µm (Internal Number: 098)
	Dimension: 250 x 4 mm
Mobile phase	Solvent A: H2O + 0,1 % FA
	Solvent B: Acetonitrile + 0,1 % FA

5.2.5 Nuclear Magnetic Resonance Spectroscopy (NMR)

Nuclear Magnetic Resonance Spectroscopy (NMR) is a powerful analytical method for structure elucidation offering visualization of atoms and molecules. Due to magnetic interactions between NMR-active nuclei along covalent bindings or through space interactions, using the nuclear Overhauser effect a three-dimensional structure can be provided (Diehl et al., 2008). The product name, the calibration and the software of the used NMR-Instrument are given in Table 10 (page 32).

Table 10: NMR Instrumentation

NMR-Instrumentation	BRUKER 500 UltraShieldTM
	Prodigy CryoProbe (TCI) for enhanced sensitivity
Software	MESTRELAB RESEARCH MestReNova

5.3 Solvents

All solvents used in the context of the thesis at hand are given in Table 11 (page 33).

Table 11: List of all used solvents and their producers

Solvent	Solvent specification	Producer	Location
Acetone	Aceton Normapur	VWR BDH Prolabo®	Vienna
Acetonitril	Acetonitril HiPerSolv Chromanorm	VWR BDH Prolabo®	Vienna
Chloroform	Chloroform Normapur	VWR BDH Prolabo®	Vienna
Dichloromethane	Dichlormethan Normapur	VWR BDH Prolabo®	Vienna
Ethanol	Ethanol 96 %	Brenntag Austria GmbH	Vienna
Formic acid for HPLC	Formic acid Rotipuran	Carl Roth GmbH + Co. KG	Karlsruhe
Formic acid for TLC	Acidum formicum conc.	Gatt-Koller	Absam
Hydorchloric acid 37 %	Salzsäure rauchend 37 % p. a.	Merck	Darmstadt
Methanol	Methanol Normapur	VWR BDH Prolabo®	Vienna
Sodium hydroxide 32 %	Natronlauge 32 %	Merck	Darmstadt
Trifluoroacetic acid	Trifluoroacetic acid 61030	Riedel-de Haën AG	Seelze
Water	Water, double distilled	Deionized water, distilled with GFL 2004	Vienna

5.4 Results

The following chapter presents the results of the experimental work. The employed methods have already been described in the previous chapter. As mentioned before, the task was the depletion of diterpene acids and the phytochemical investigation of the resulting compounds. Thus, the first challenge was to remove resin acids by using ion exchange chromatography. TLC analyses were carried out in order to compare separations and examine the composition and purity of resulting fractions. The phytochemical investigation of the fractions included isolation and identification of pure compounds. This was achieved by the use of FC, SFC, HPLC, NMR and MS methods.

5.4.1 Depletion of diterpene resin acids with IEC

Firstly, the working steps of IEC are thoroughly detailed. Following this, the optimization steps for achieving an almost complete depletion will be demonstrated using several examples.

I. Working steps of IEC

Ion exchange resins need essential preconditions to achieve an effective separation. The first prerequisite includes a **wetting procedure** where resin beads are filled in a suitable beaker and covered with distilled water. After stirring the beads, the mixture rests for about 15 minutes so that the resin has time for swelling. Thereafter, the water is decanted to remove any impurities (Durham, 2006).

The process is repeated once more, but this time with a solvent, which is used for dissolving the sample. In the frame of this work, methanol was used as solvent of the sample and, consequently, an aqueous 90 % MeOH solution was applied.

Before the wet ion exchange resin beads are filled into a column, a piece of cotton has to be put in to prevent the column material from leaking. The column, filled with the ion exchange resin, is then rinsed with a fresh 90 % MeOH solution. If the column material is sufficiently preconditioned, the solved sample (10 % solution of the sample in methanol) can be applied carefully to the ion exchange resin. The drop speed was set to 30-50 drops/min during the whole separation process. In the whole process the column should never run dry to prevent the beads from shrinking.

After the whole sample solution has been applied, it is necessary to let it rest and interact for about 10 minutes by closing the valve of the column. The carboxylic acid group of the resin acid (COOH) protonates the ion exchanger ($-NR_2H^+$) and the carboxylate (COO-) in turn interacts with the protonated ion exchanger. At this point, a clearly visible yellow ring appears which constitutes the bound acids (Figure 7, page 35).

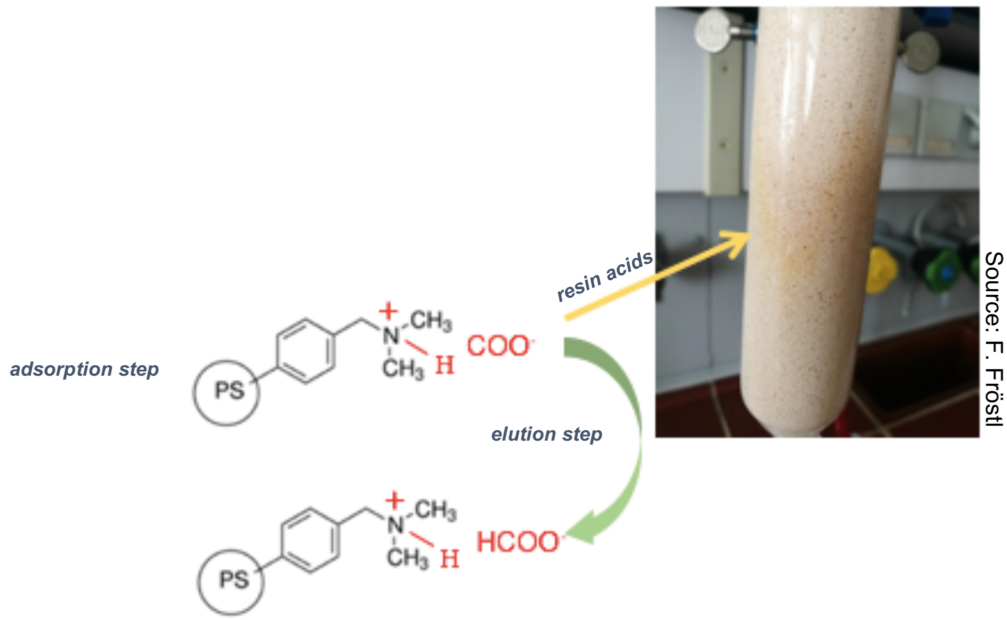


Figure 7: The attached acids in the column (right) and the process of displacement of the resin acid (COO^-) by the formic acid ($HCOO^-$) during the elution step (left)

In this so-called **adsorption step** acids are retained in the column while other substances, like phenols, alcohols and apolar compounds, can elute. The column is flushed with a 90 % MeOH solution. As a result, everything that does not bind to the ion exchange resin is washed off and accumulates in the first fraction, the so-called phenol fraction.

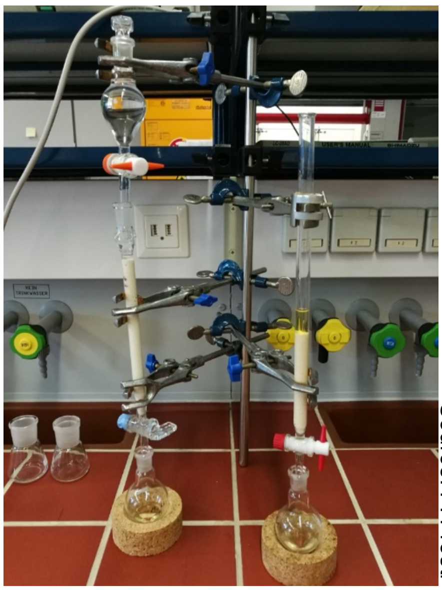
After finishing the adsorption step, the **elution step** follows. With regard to the choice of eluents, different concentrations of formic acid (0.01 % to 5 %), NaCl (1 %) and TFA (0.1 %) in methanol were tested. 5 % formic acid in MeOH turned out to be the most effective one in respect to removing diterpene acids from the ion exchanger. This works by displacement, which is demonstrated in Figure 7, page 35. Hence, the obtained fraction from the elution step contains mainly acidic substances. The used volumes of the solutions in the adsorption and elution steps correspond to the respective sample amounts of each separation.

As a last step a **regeneration process** had to be carried out to restore the starting conditions. This required a deprotonation of the stationary phase, which was realized by washing the column with an aqueous 4 % NaOH solution. To remove residual sodium hydroxide the column was rinsed with distilled water and preconditioned again with 90 % MeOH solution to start another separation cycle. The ion exchange resin can be reused several times before starting to lose its separation capacity.

The resulting fractions from the adsorption and elution step are evaporated by means of a rotary evaporator and subsequently dissolved in acetone and transferred to a sample vial. The fractions are analyzed by TLC to evaluate the degree of depletion. The process of applying the sample solution to the plate and further steps, including the development and derivatization are laid out in detail in chapter 5.2.3.2 on page 27. The results of the separation and the TLC analyses are depicted in the following chapter.

II. Optimization of an IEC method supported by TLC

The working steps of each experiment have been performed in accordance with the workflow in chapter I., page 34. By testing out different solvents, column volumes and sample quantities, the best procedure for the depletion of diterpene acids was strived for.



Source: F. Fröstl

Figure 8: Dowex Monosphere 66 (left), Dowex Marathon WBA (right)

i. In an initial experiment, two weak basic anion exchangers, Dowex Monosphere 66 and Dowex Marathon WBA, were tested and compared. Thus, two columns were used, as demonstrated in Figure 8, page 37.

After following the practical instruction, two fractions from the adsorption step and five fractions from the elution step resulted per each column. The materials that were used are presented in Table 12 on page 37. All fractions from the adsorption and elution step were gravimetrically determined as shown in Table 13 and Figure 9 (page 38).

Table 12: Materials and solvents

	Mo 66	**Ma**
Amount	5.2g	4.35g
Column Volume	11.8ml	7.85ml
Sample:		
NaOH-fraction in MeOH (10 % solution)	1g/10ml	1g/10ml
Adsorption solvent:		
MeOH 90 % /H2O	25ml	15ml
Eluent:		
0.01 % FA in MeOH	40ml	40ml
0.1 % FA in MeOH	45ml	45ml
1 % FA in MeOH	45ml	45ml
NaCl solution (0.8g NaCl, 80ml MeOH, 4ml H2O)	50ml	30ml

Table 13: Determined weights of all fractions

Name of Fraction	Empty weight [g]	Resulting weight [g]	Amount [mg]
Mo66 Phenols (SampApp) 01	6.98182	7.11759	135.77
Mo66 Phenols (SampApp) 02	6.8736	7.30337	429.77
Mo66 Carboxylic acid (FA 0.01 %) 03	6.8798	6.9816	101.8
Mo66 Carboxylic acid (FA 0.1 %) 04	6.86876	6.9156	46.84
Mo66 Carboxylic acid (FA 1 %) 05	6.79001	6.81757	27.56
Mo66 Carboxylic acid NaCl 06	6.81241	6.87215	59.74
Mo66 Carboxylic acid NaCl 07	6.95037	6.96783	17.46
Ma Phenols (SampApp) 01’	6.77217	7.07273	300.56
Ma Phenols (SampApp) 02’	6.84012	7.34388	503.76
Ma Carboxylic acid (FA 0,01 %) 03’	6.80727	6.90102	93.75
Ma Carboxylic acid (FA 0,01 %) 04’	6.87493	6.9002	25.27
Ma Carboxylic acid (FA 1 %) 05’	6.94975	6.95808	8.33
Ma NaCl (Carboxylic acid) 06’	6.78143	6.8146	33.17
Ma NaCl (Carboxylic acid) 07’	6.92188	6.92699	5.11

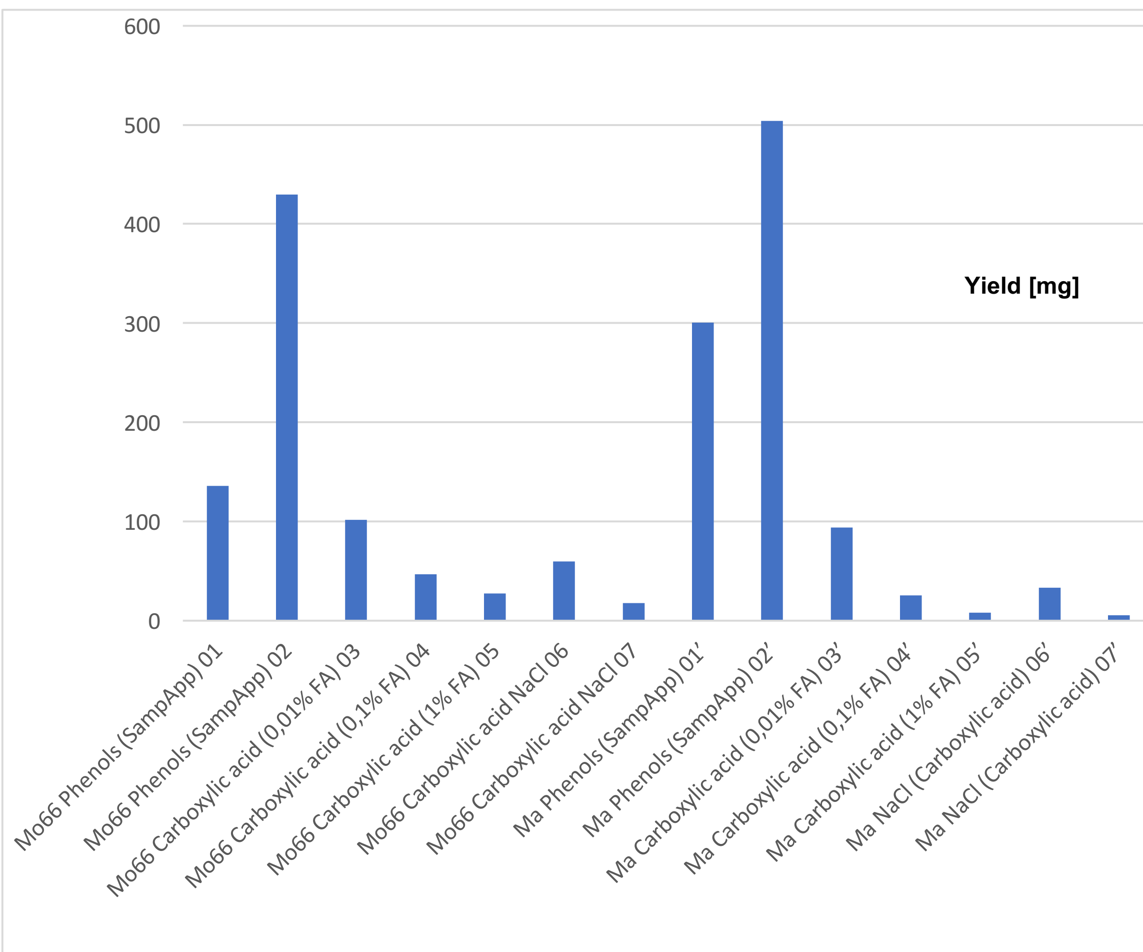


Figure 9: Graphical representation of all determined weights

Further steps, including the transfer of sample solution on TLC fingerprints, are listed in the chapter 5.2.3.2 on page 27.

Analysis: As shown in the TLC comparison (Figure 10, page 40), the results of each anion exchanger were quite similar, although the resin acid bands of the Marathon material tended towards a slightly higher intensity in the carboxylic acid fractions. For this reason, the decision was made to continue with the next experiments using only Dowex Marathon WBA. However, the aim of removing the resin acids was not completely achieved in this experiment. The intensity of the acids in fraction 01, 01', 02 and 02' was fairly equal to fractions 03, 03' to 07, 07' (the enrichment of the violet spot was still visible). If the depletion had been a success, the spots in the first and second fractions would have disappeared.

Further experiments, using different solvent compositions, sample concentrations and various eluents, were carried out, unfortunately without success. The reason for the recurrent ineffective separations was a capacity overload, caused by a column volume that was dimensioned too low.

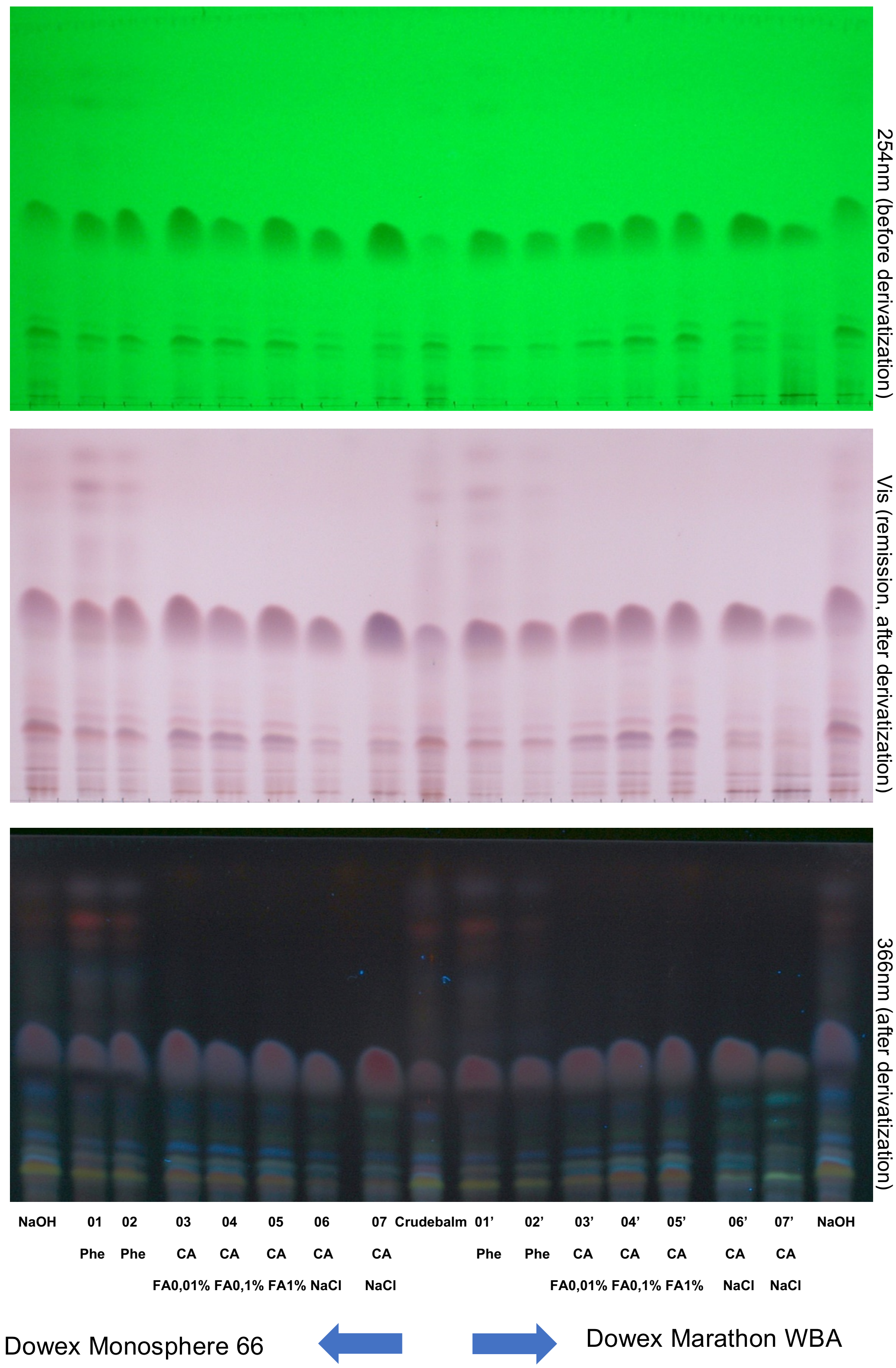


Figure 10: TLC comparison of Dowex Monosphere 66 and Dowex Marathon WBA

Stationary phase: Silica gel 60G F_{254}
Mobile phase: $CHCl_3$ + MeOH + TFA (97 + 3 + 0.1)
Detection: UV_{254}, Anisaldehyde/sulphuric acid solution: Vis, UV_{366}

ii. By choosing a bigger column and a greater amount of the chosen Dowex Marathon WBA, a higher volume and consequently enhanced separation capacity was implemented. The total procedure was carried out on the basis of the description in chapter I. (page 34). The experiment was conducted under the conditions shown in Table 14 (page 41).

Table 14: Materials and solvents

	Ma
Amount	75g
Column Volume	179.613ml
Sample:	
NaOH-fraction in 10ml MeOH (10 % solution)	1g/10ml
Adsorption solvent:	
MeOH 90 % /H2O	400ml
Eluent:	
5 % FA in MeOH	400ml
TFA 0.1 %	200ml
Regeneration solvent:	
1molar NaOH-solution (4 %)	500ml

Table 15: Determined weights of Phenols and Carboxylic acid fractions

DOWEX-MARATHON WBA	Empty weight [g]	Resulting weight [g]	Amount [mg]
Phenols (SampApp) 01	6.93814	7.00238	64.24
Carboxylic acids (FA 5 %) 02	6.86663	7.22854	361.91
Carboxylic acids (TFA 0.1 %) 03	6.85114	7.29578	444.64

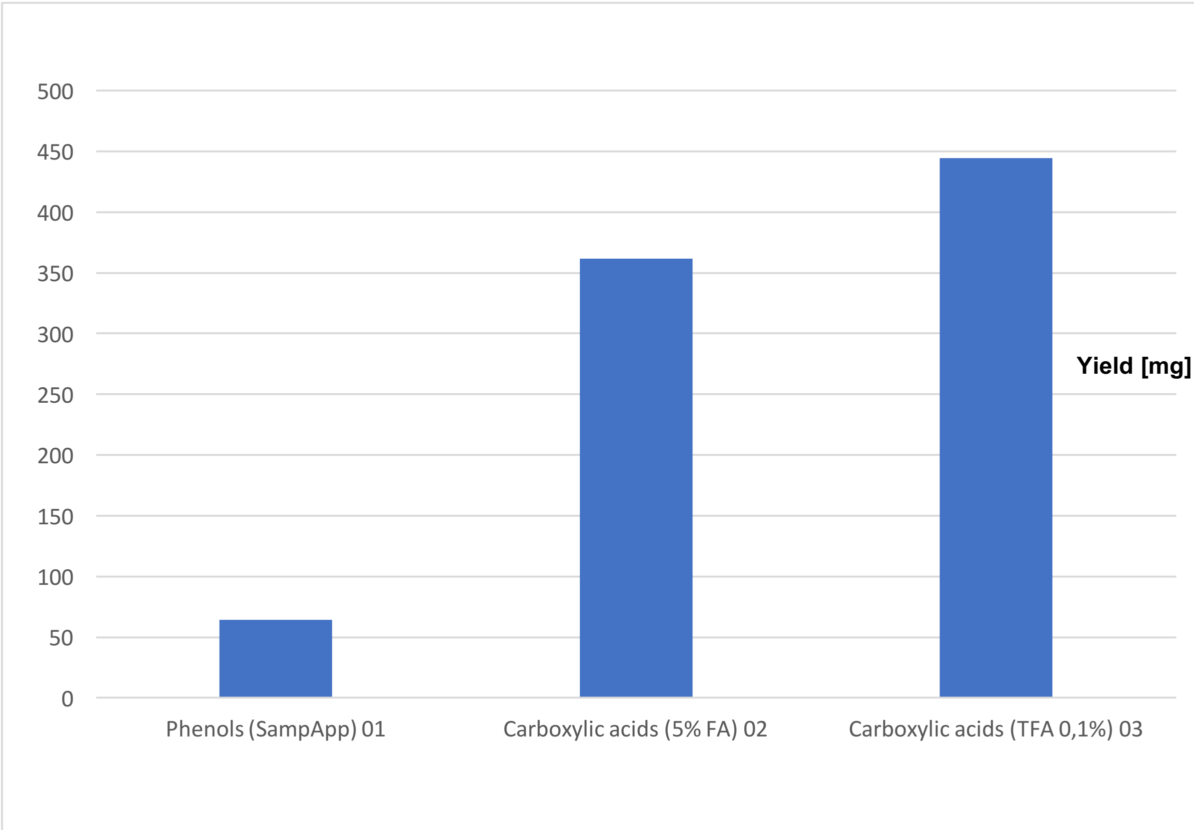


Figure 11: Graphical representation of the determined Phenols and Carboxylic acids weights

Analysis: The depletion of resin acids was successful as shown in the significant weight difference between the phenolic compounds and the carboxylic acids in Table 15 and Figure 11 on page 42. Additionally, the absence of the violet spot (representing the resin acids) in fraction 01 at an R_f value of ~ 0.3 confirmed their depletion (Figure 12 on page 43). To collect more of the desired phenol fraction the depletion process was repeated several times.

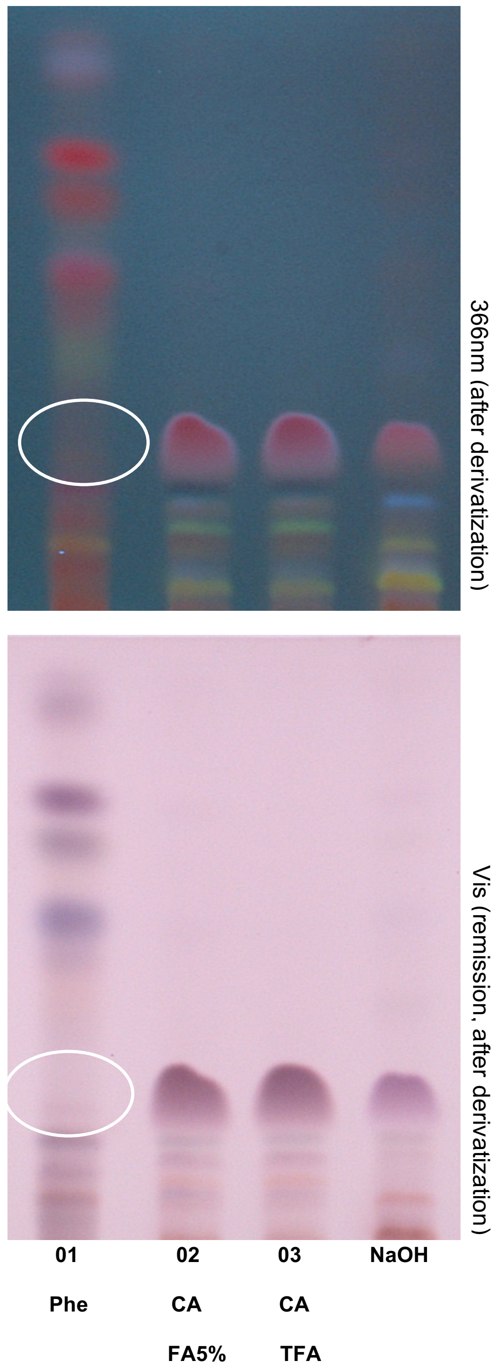


Figure 12: TLC comparison, showing the depletion of resin acids in the phenol fraction (encircled area)

Stationary phase: Silica gel 60G F_{254}
Mobile phase: $CHCl_3$ + MeOH + TFA (97 + 3 + 0.1)
Detection: Anisaldehyde/sulphuric acid solution: Vis, UV_{366}

iii. By raising the amount of the sample, the limit of the ion exchange resin's capacity was tested. After trying to apply quantities from 3-4g of the sample an "upscaling" to 8g was carried out, which showed positive results as well.

The application of big sample amounts should be carried with caution, considering that the visible yellow ring of the attached acids started to move faster than with lower amounts. The quantity of adsorption solvent was reduced and the elution step was optimized by using only one eluent (5 % FA in MeOH). To ensure a quantitative yield of the phenolic compounds, larger volumes of elution solvent were used. The solvents and the used volumes are given in Table 16 on page 44. Since the aim was the accumulation of phenol fraction for further investigations, only their weights were determined (Table 17 and Figure 13, page 45) and applied on a TLC plate (Figure 14, page 46).

Table 16: Solvents

	Ma
Sample:	
NaOH-fraction in MeOH (10 % solution)	8g /80ml
Adsorption solvent:	
MeOH 90 % /H2O	70ml
Eluent:	
5 % FA in MeOH	1000ml
Regeneration solvent:	
1molar NaOH-solution (4 %)	500ml

Table 17: Determined weights of the phenol fractions

Dowex-Marathon WBA	Empty weight [g]	Resulting weight [g]	Amount [mg]
Phenols 1	6.96377	7.10139	137.62
Phenols 2	6.9436	7.13848	194.88
Phenols 3	6.95087	6.99668	45.81

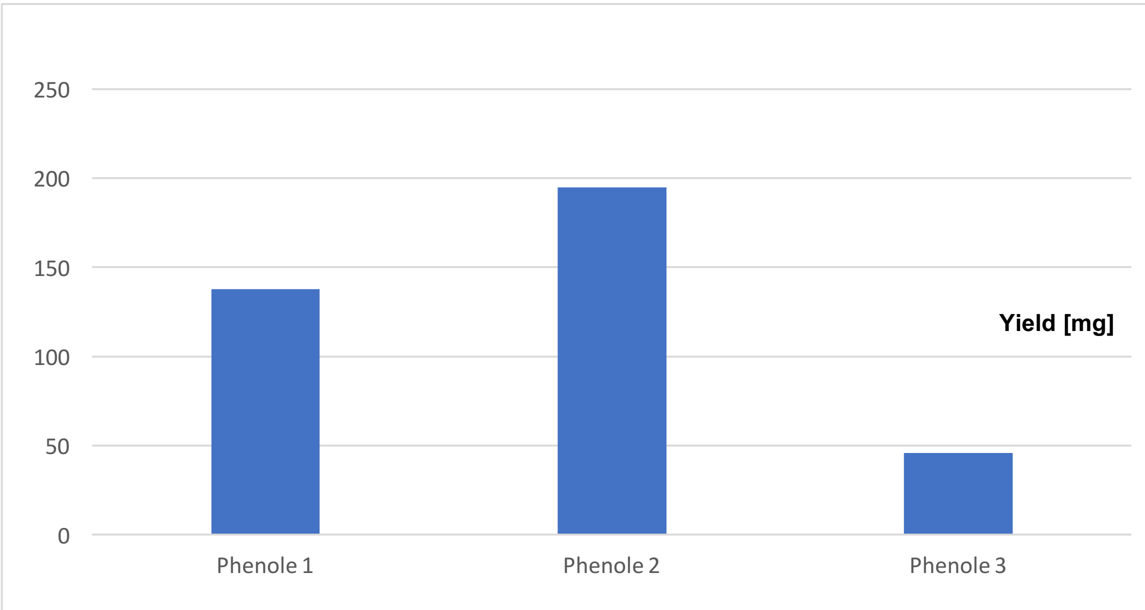


Figure 13: Graphical representation of the determined Phenol weights

Analysis: Applying bigger amounts of the sample resulted in a bigger yield of the phenol fractions and allowed for a more efficient workflow.

By applying amounts from 3 to 8 g of the NaOH-fraction from the liquid-liquid separation (see chapter 5.2.2, page 23), phenol fractions with a yield between 5 and 10 % resulted.

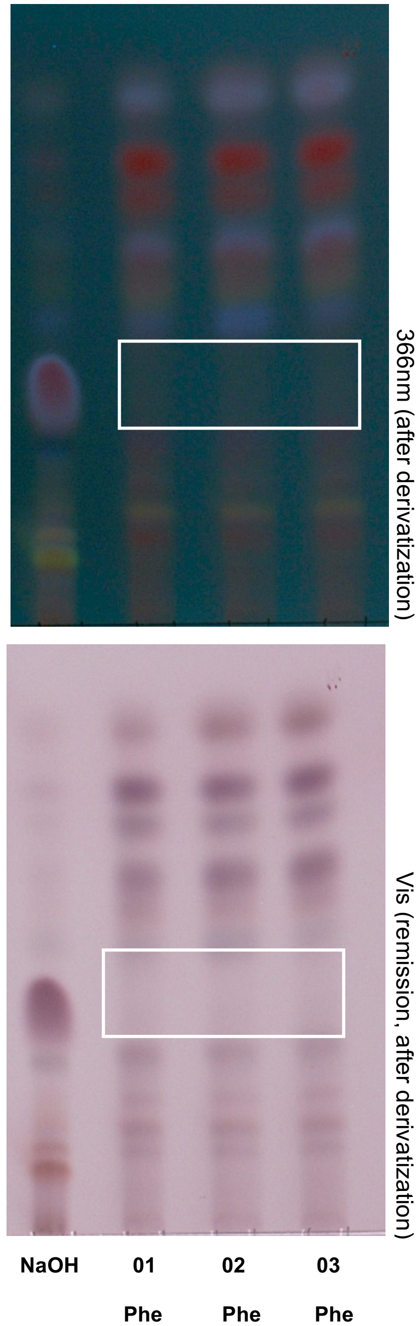


Figure 14: TLC comparison, showing the depletion of resin acids in the phenol fractions (marked area)

Stationary phase: Silica gel 60G F_{254}
Mobile phase: $CHCl_3$ + MeOH + TFA (97 + 3 + 0.1)
Detection: Anisaldehyde/sulphuric acid solution: Vis, UV_{366}

5.4.2 Phytochemical Investigation of the phenol fraction

As soon as a sufficient amount of the phenol fraction was collected via IEC, further fractionations and investigations with the objective of isolating and identifying substances were possible.

I. Fractionation of Phenols via Flash chromatography

The aim of isolating pure compounds from the phenol fraction was realized by the use of FC. This efficient separation method allowed a finer partitioning of the constituents of this fraction, which in turn paved the way to the isolation of pure substances.

The methodology and the workflow can be gathered from chapter 5.2.3.3 (page 28). The resulting fractions from various repeated FC runs were combined, evaporated, weighted and analyzed by TLC.

Due to an insufficient separation of the substance peaks and residual resin acids in the sample, the first two runs of FC (FC01-02) were discarded (Figure A 5 on page 63 and Figure A 6 on page 64; see Appendix). After picking only the resin acid free samples for the fractionation, the resulting FC03b, 04a and 04b were selected for further investigation (see Figure A 7 on page 65, Figure A 8 on page 66, Figure A 9 on page 67 in the appendix). After combining those three FC runs 11 resulting fractions were visualized by a TLC (Figure 15 on page 48). Due to the fact that fraction 7 seemed to be pure, an HPLC analysis was carried out.

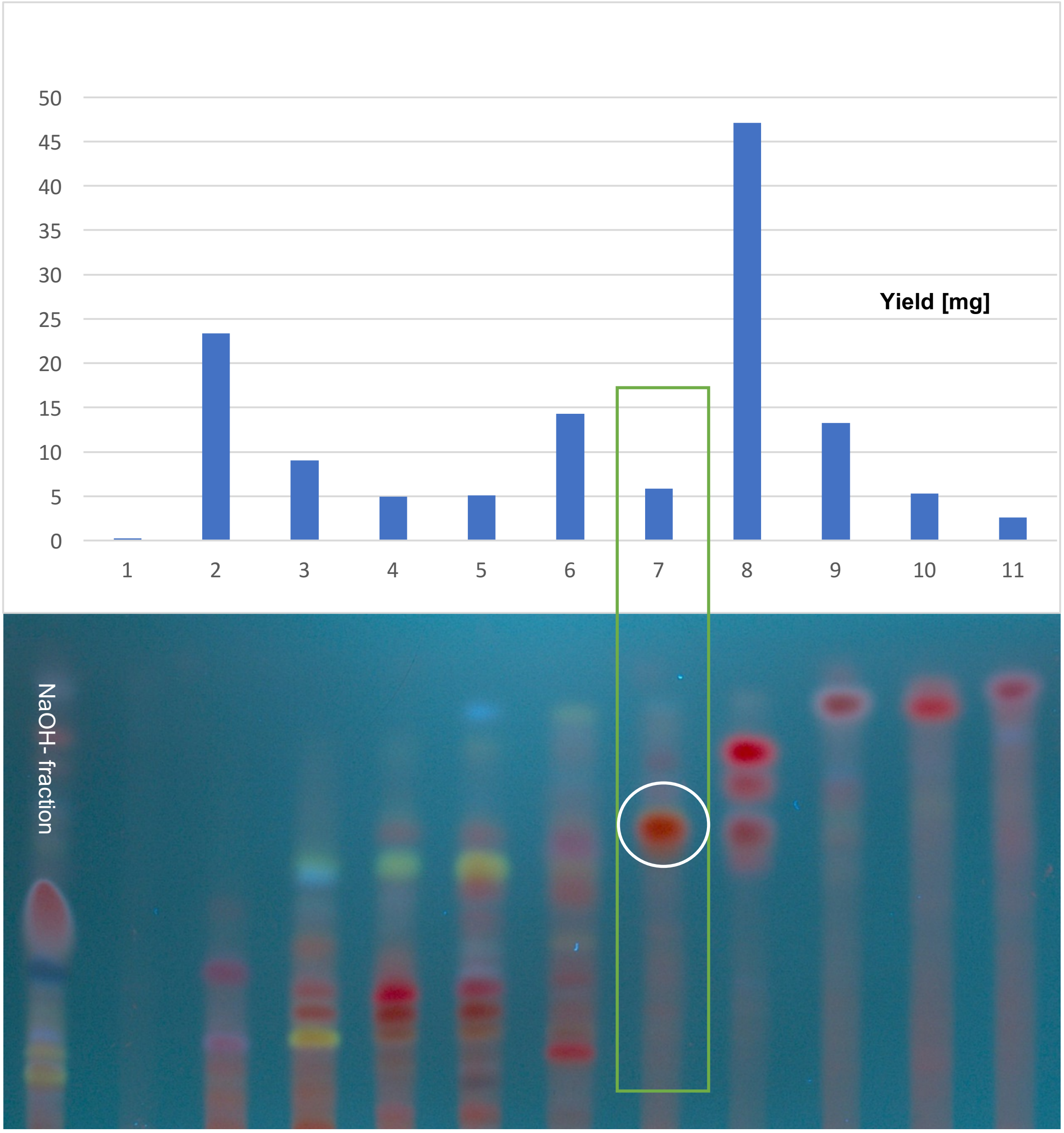


Figure 15 : TLC of combined fractions (1-11) of 03b, 04a and 04b compared to NaOH-fraction and correlated to their respective yields

Stationary phase: Silica gel 60G F_{254}
Mobile phase: Dichloromethane + acetone + FA (80+18+2)
Detection: Anisaldehyde/sulphuric acid solution: UV_{366}

II. HPLC Analysis of fraction 7

After carrying out an HPLC run of fraction 7, the suspicion of already having isolated a pure substance was confirmed (the method and used instruments are described in chapter 5.2.3.4 on page 29). This is shown in Figure 16 on page 49, by the one single big peak in the HPLC chromatogram (listed below). In the FC chromatogram (FC04a in the appendix; Figure A 8 on page 66) the small marked peak represents fraction 7. The structure elucidation of this compound is entrusted to subsequent research due to this thesis being quite comprehensive already at the end of the isolation.

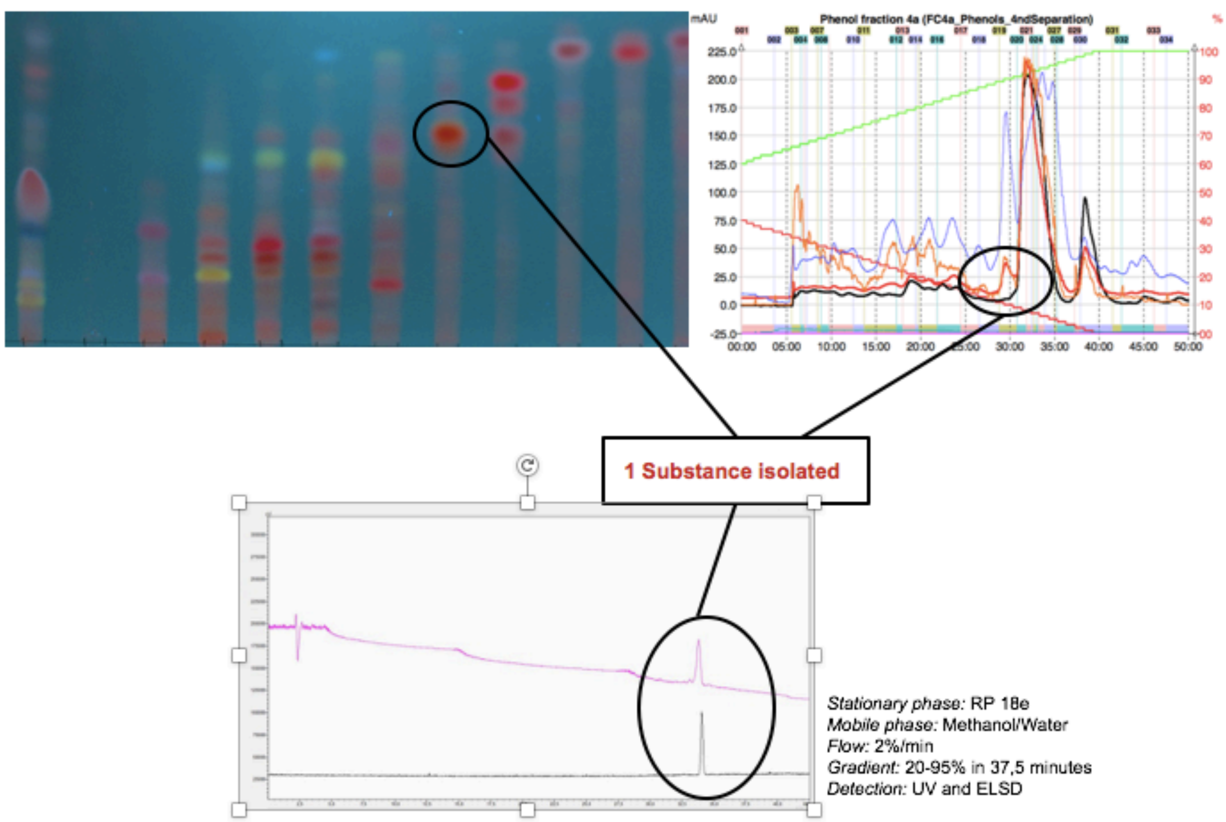


Figure 16: TLC of combined fractions (1-11) of 03b, 04a and 04b (above left), FC04a of Phenols (above right) and HPLC of fraction 7 (below); the circles indicate the pure compound respectively

6 Summary

Norway spruce balm is a resinous excretion, possessing a kneadable and soft texture, of the tree *Picea abies*. Due to its wound healing and antimicrobial properties, which have been confirmed by several studies, it is applied topically to treat wounds, burns and ulcers. After many decades of successful application in traditional folk medicine, a submission of Norway spruce balm as traditional herbal medicinal product is sought.

The first part of the diploma thesis constitutes an in-depth literature review to provide evidence of traditional use. The term “Fichtenfaulpech” was first published 2016 in ÖAB and therefore did not occur in prior research. Consequently, similar terms such as spruce resin or pitch resin were included in the literature review. However, spruce resin is a collective term, which comprises various excretion products by spruces, including the examined resinous excretion.

This literature review showed that there are numerous application areas in the longstanding use of this natural product by different people and nations. Concerning its healing power, exemplary applications are given in a wide variety of indications such as external injuries and diseases, including burns, infected wounds and cuts, but also fractures, sprains, dislocations, rheumatism and smaller injuries, comprising ingrown nails and insect bites.

Considering that Norway spruce balm has yet unidentified constituents, the experimental part analyzed its phytochemical composition, with a focus on the NaOH-fraction. First, diterpene resin acids, one of the main components of Norway spruce balm, were depleted in order to investigate the remaining compounds, like phenols, alcohols or apolar substances. By means of an ion exchange chromatography, the depletion could be performed after a few optimization steps. The success was confirmed through the comparison of TLC fingerprints. After a sufficient amount of the depleted mixture was gathered, further separation steps were implemented. The aim of isolating pure compounds from the phenol fraction was facilitated by using flash chromatography. 11 fractions resulted by combining three separation runs (FC03b, 04a and 04b). These fractions were visualized by means of TLC. Fraction 7 seemed to be pure, which was confirmed with additional support from an HPLC analysis. The structure elucidation of this pure compound is entrusted to subsequent research due to this thesis being quite comprehensive already at the end of the isolation.

Zusammenfassung:

Fichtenfaulpech ist eine harzartige Ausscheidung mit weicher und knetbarer Konsistenz der Gemein-Fichte *Picea abies*. Aufgrund der wundheilenden und antimikrobiellen Eigenschaften, die durch mehrere Studien bestätigt wurden, wird es äußerlich zur Behandlung von Wunden, Verbrennungen und Geschwüren angewendet. Nach langjähriger, erfolgreicher Anwendung in der traditionellen Volksmedizin, wird die Einreichung des Fichtenfaulpechs als traditionelles pflanzliches Arzneimittel angestrebt.

In dieser Hinsicht stellt der erste Teil dieser Diplomarbeit eine profunde Literaturrecherche dar, um den Nachweis einer traditionellen Anwendung zu belegen. Der Begriff Fichtenfaulpech wurde 2016 erstmals im ÖAB veröffentlicht und kam daher in früheren Untersuchungen nicht vor. Folglich wurden ähnliche Begriffe wie „Fichtenharz“ oder „Pechharz“ in die Literaturrecherche einbezogen. Fichtenharz ist jedoch ein Sammelbegriff, der verschiedene Ausscheidungsprodukte von Fichten umfasst, einschließlich der untersuchten Harzausscheidung.

Der Fokus der Recherche liegt daher auf den vielseitigen Anwendungsbereichen und der langjährigen Nutzung dieses Naturprodukts durch verschiedene Menschen in unterschiedlichen Nationen. In Bezug auf seine Heilkraft werden Anwendungsbeispiele für eine Vielzahl von Indikationen angegeben, zum Beispiel äußere Verletzungen und Krankheiten, einschließlich Verbrennungen, infizierte Wunden und Schnittschäden, aber auch Frakturen, Verstauchungen, Luxationen, Rheuma und kleinere Verletzungen, darunter eingewachsene Nägel und Insektenstiche.

In Anbetracht dessen, dass Fichtenfaulpech noch unbekannte Inhaltsstoffe aufweist, beschäftigt sich der experimentelle Teil mit phytochemischen Untersuchungen, unter besonderer Beachtung der NaOH-Fraktion. Vorerst wurden Diterpenharzsäuren, die zu den Hauptbestandteilen von Fichtenfaulpech zählen, abgetrennt, um die resultierenden Verbindungen zu untersuchen, welche einen wesentlich geringeren Anteil ausmachen. Diese Abtrennung konnte nach einigen Optimierungsversuchen mittels Ionenaustauschchromatographie durchgeführt werden. Anhand dünnschichtchromatographischer Untersuchungen konnte dieser Erfolg bestätigt werden. Nachdem genügend Menge der resultierenden Verbindungen (Diterpene bereits abgetrennt) gesammelt wurde, konnten weitere Aufreinigungsschritte durchgeführt werden. Das Ziel, reine Verbindungen zu isolieren, wurde mittels Flash Chromatographie erleichtert. Nachdem drei Auftrennungen (FC03b, 04a und 04b) ausgewählt wurden, resultierten 11 Sammelfraktionen,

welche mit Hilfe von DC Analysen visualisiert wurden. Fraktion 7 schien sehr rein zu sein. Mittels zusätzlicher Unterstützung von HPLC, konnte sichergestellt werden, dass eine Reinsubstanz erfolgreich isoliert wurde. Die Strukturaufklärung dieser Substanz wird folgenden Untersuchungen überlassen, da diese Arbeit zum Zeitpunkt der Isolierung bereits äußerst umfassend war.

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8 Appendix

Figure A 1: An illegible and incomplete copy of the original “Loferer Rezeptbüchl”

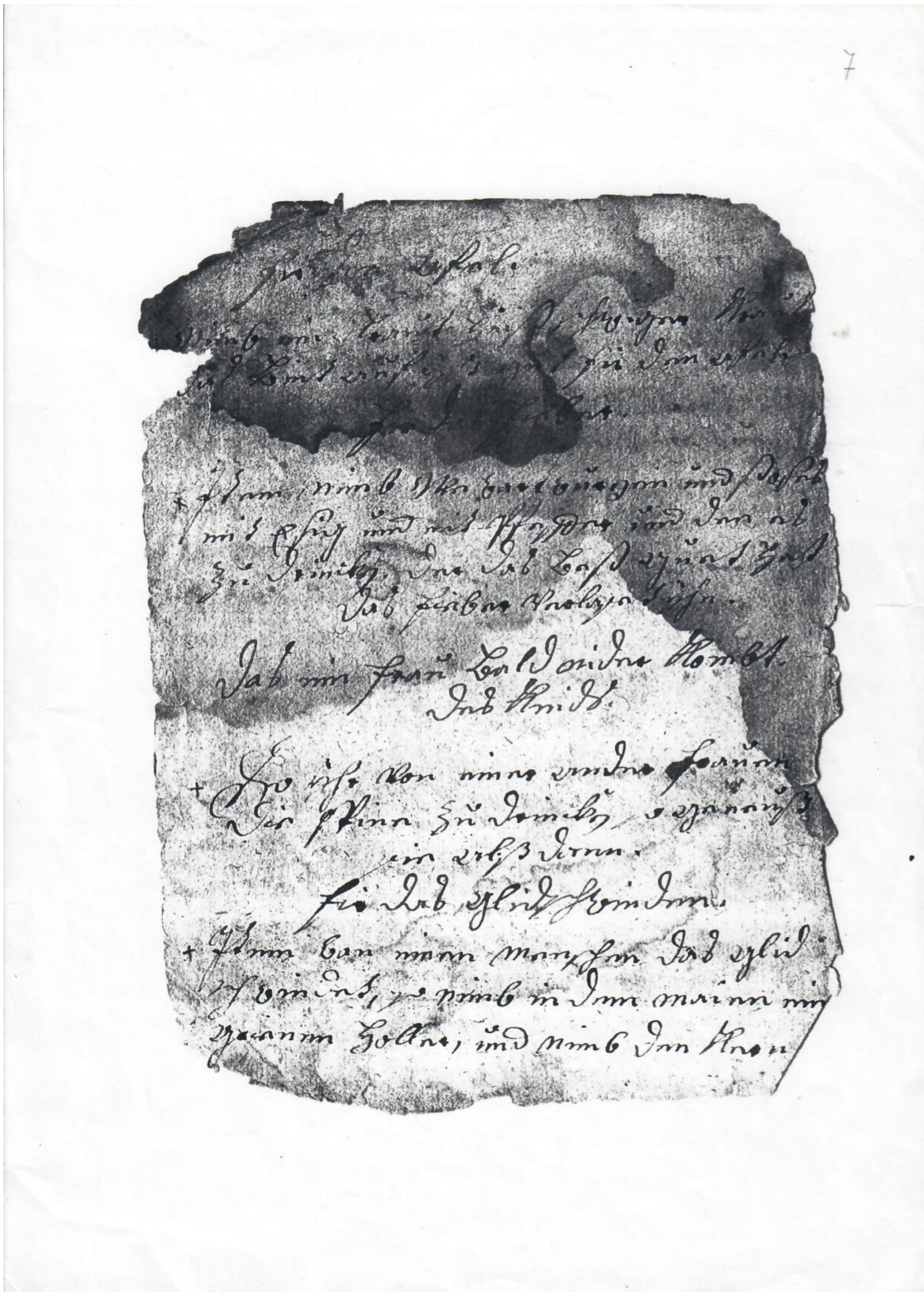

Figure A 2: “Dörrband”-recipe of the East-Styrian Pharmacopoeia

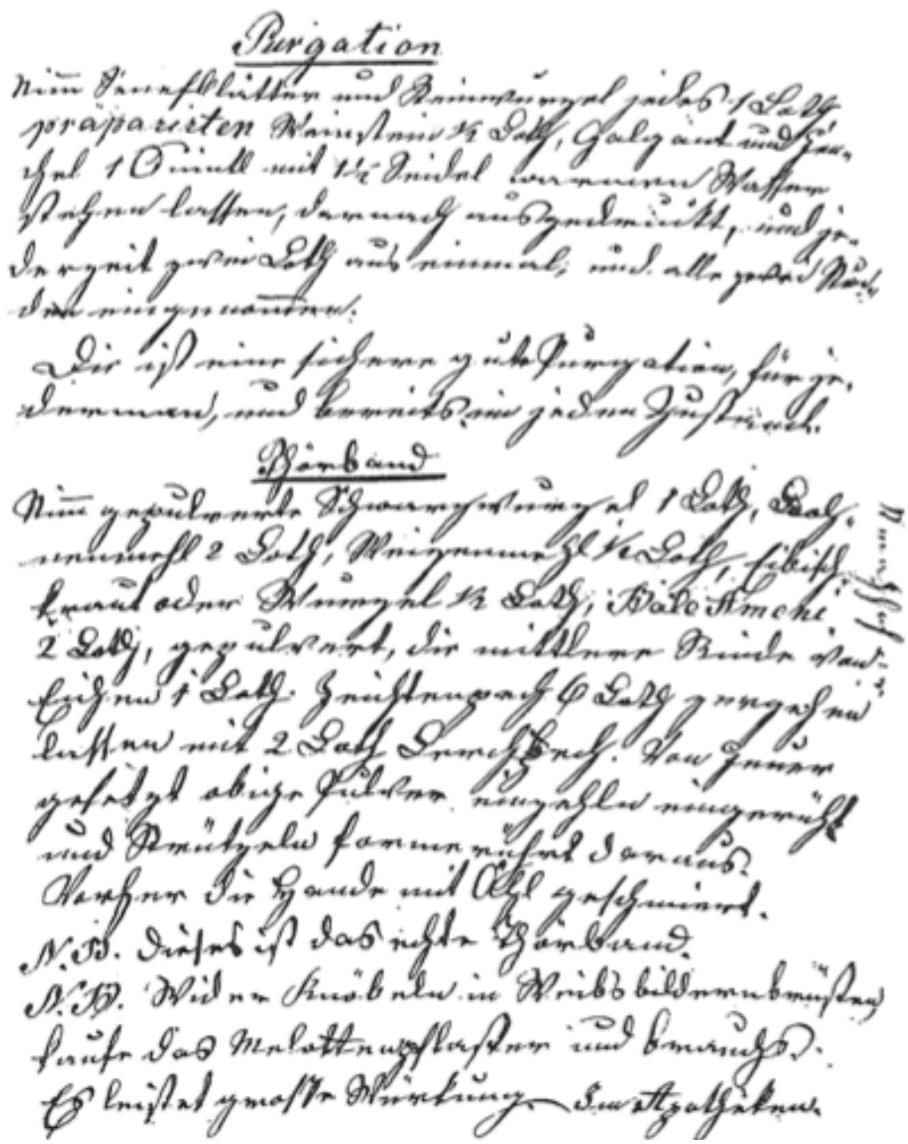
Purgation

Figure A 3: Recipe of the “Steffisalbe”

Steffisalbe	
Ziegen- oder Schweinefett	60 g
Lärchpech	3 g
Fichtenpech	3 g
Kräuterbalsam	3 g
Kampfergeist	10 g
Bienenwachs	6 g
Rosswurzen	2 g
Enzianwurz	2 g
Meisterwurz	2 g
Arnikablüten	3 g
Kamillenblüten	3 g
Sonikelblüten	3 g

Figure A 4: The original detailed preparation of this house salve by Strigl

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HAUSSALBE

Herstellung:

Fichtenharz (weißes Pech)
Bienenwachs
Butter oder Kuhschmalz
evtl. ein wenig Lärchenharz
} zu gleichen Teilen

Alles zusammen langsam kochen, bis es eine klare Brühe ist. Abkühlen lassen. Jetzt gibt man 1 EL Arnikaschnaps dazu (1 TL pro Tiegel), dann Speiseöl für die Streichfähigkeit der Salbe: 1 Tropfen Salbe in kaltes Wasser geben, um zu testen, wie fest es wird. Dann mit Speiseöl verbessern, bis es die richtige Streichfähigkeit hat. In Dosen abfüllen.

Figure A 5: Flash chromatogram 01 of the Phenol fractions

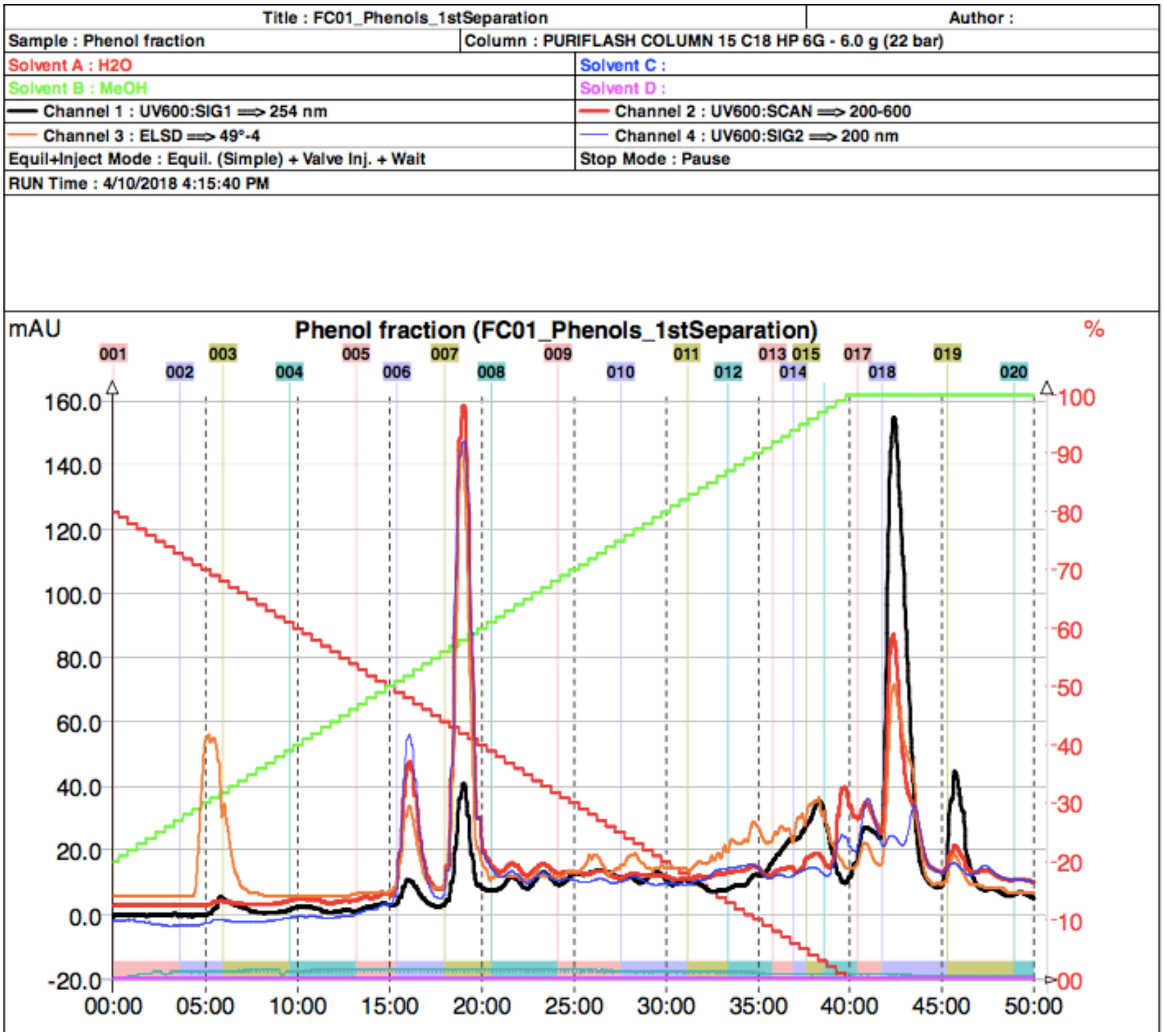

Figure A 6: Flash chromatogram 02 of the Phenol fractions

Title : FC02a_Phenols_2ndSeparation	Author :
Sample : Phenol fraction	Column : PURIFLASH COLUMN 15 C18 HP 6G - 6.0 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°-4	Channel 4 : UV600:SIG2 ==> 200 nm
Equil+Inject Mode : Equil. (Simple) + Valve Inj. + Wait	Stop Mode : Pause
RUN Time : 5/16/2018 4:08:32 PM	

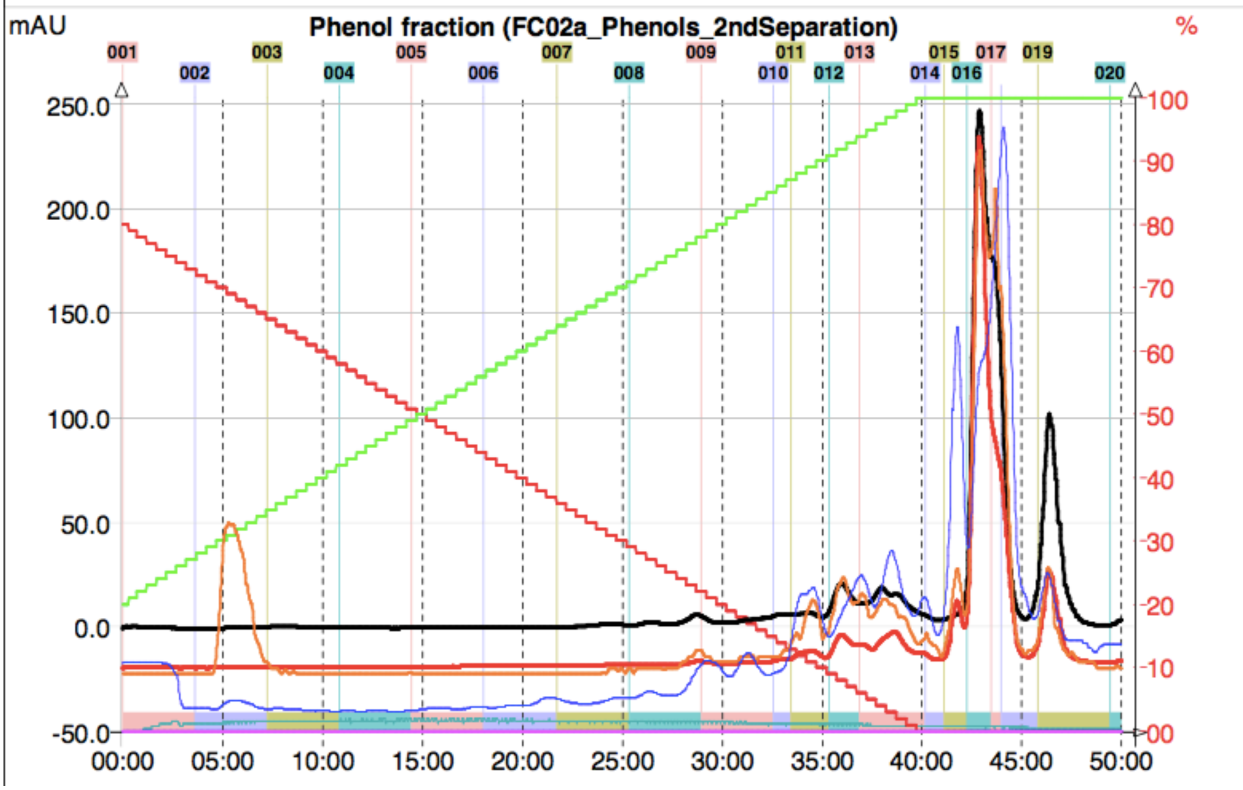

Figure A 7: Flash chromatogram FC 03b of the Phenol fractions

Title : FC3b_Phenols_3ndSeparation	Author :
Sample : Phenol fraction 3b	Column : PURIFLASH COLUMN 15 C18 HP 6G - 6.0 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°-4	Channel 4 : UV600:SIG2 ==> 200 nm
Equil+Inject Mode : Equil. (Simple) + Valve Inj. + Wait	Stop Mode : Pause
RUN Time : 5/23/2018 1:51:54 PM	

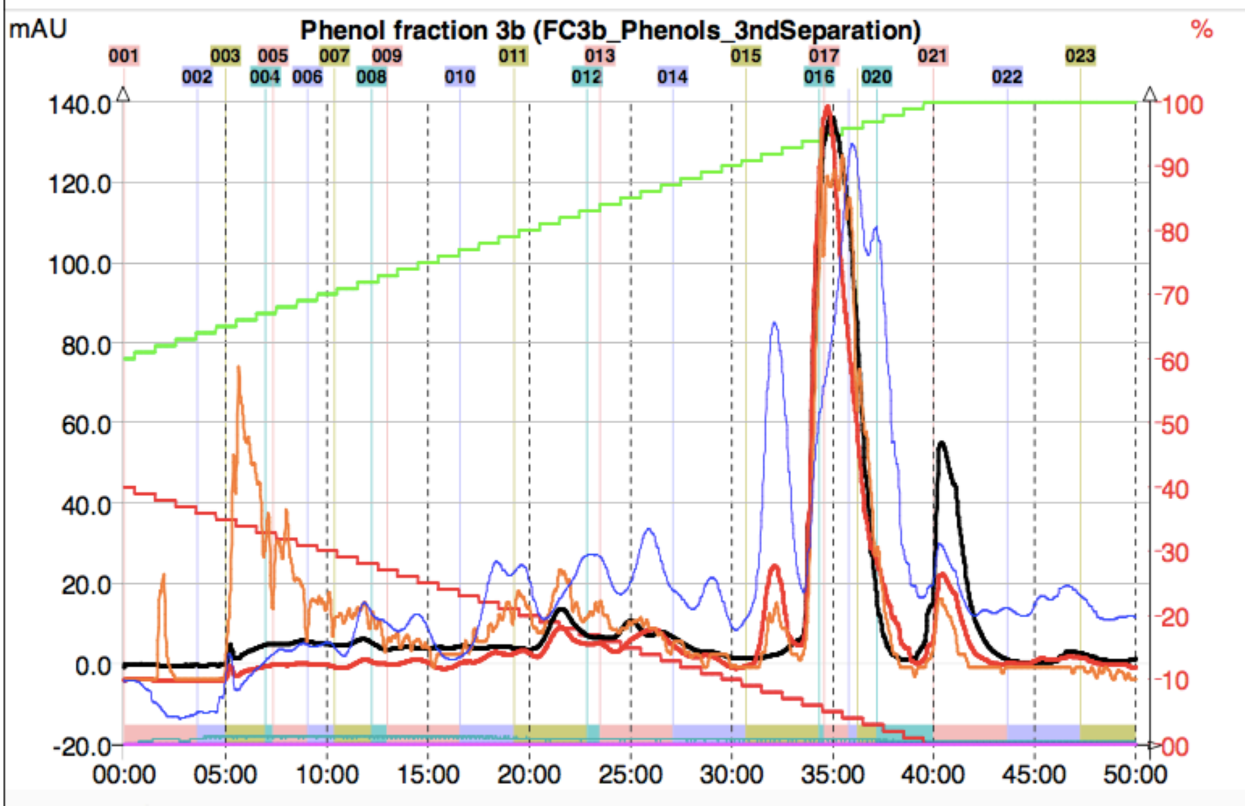

Figure A 8: Flash chromatogram FC04a of the Phenol fractions

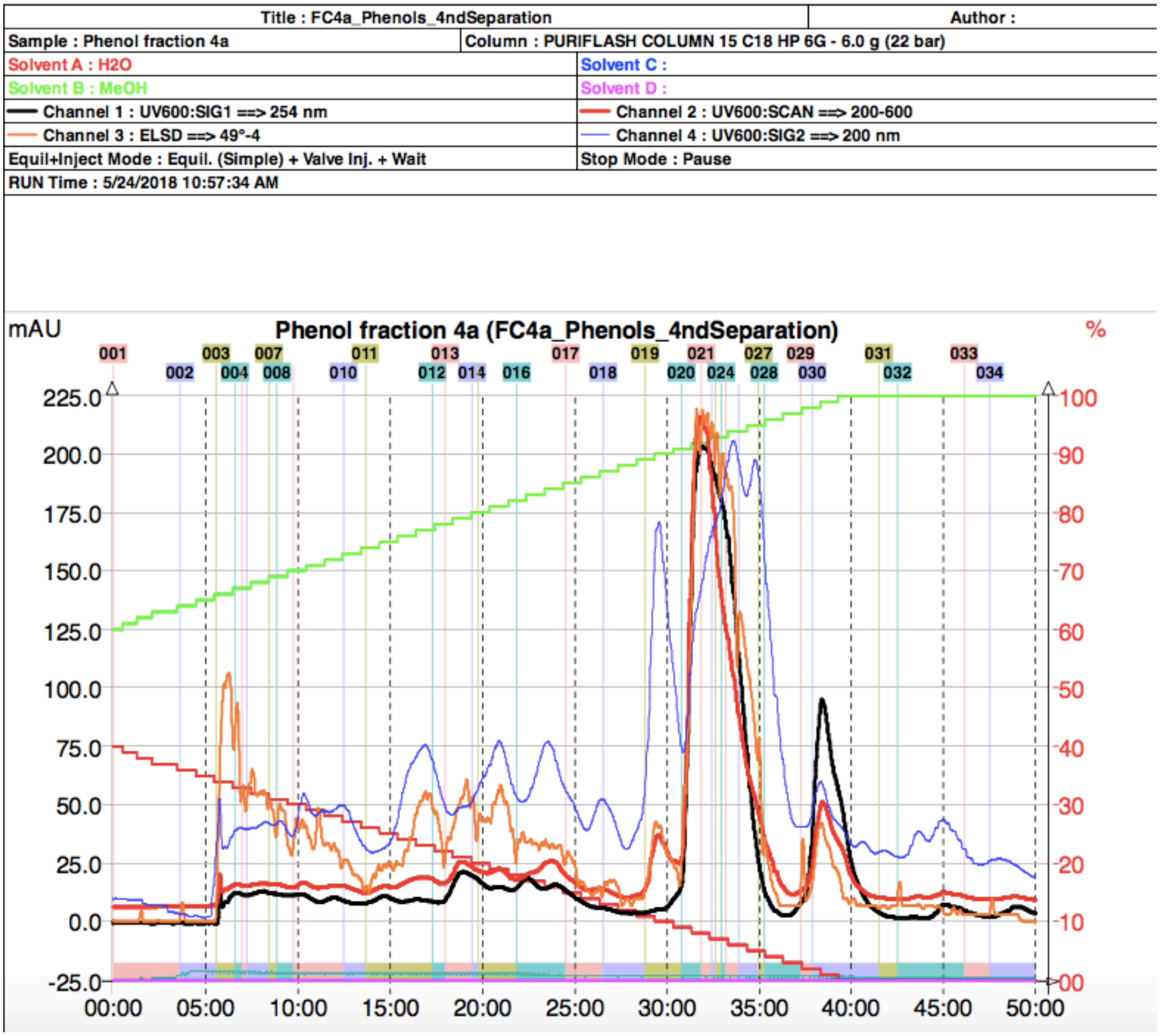

Figure A 9: Flash chromatogram FC04b of the Phenol fractions