universität wien

DIPLOMARBEIT / DIPLOMA THESIS

Titel der Diplomarbeit / Title of the Diploma Thesis

Quantifying resin acids in Norway spruce balm by gas chromatography – contributions to method optimization

verfasst von / submitted by

Georg Fridolin Aichner

angestrebter akademischer Grad / in partial fulfilment of the requirements for the degree of

Magister der Pharmazie (Mag.pharm.)

Wien, 2020/ Vienna, 2020

Studienkennzahl lt. Studienblatt / degree programme code as it appears on the student record sheet:	A 449
Studienrichtung lt. Studienblatt / degree programme as it appears on the student record sheet:	Diplomstudium Pharmazie
Betreut von / Supervisor:	Ao. Univ.-Prof. Mag. Dr. Sabine Glasl-Tazreiter

Acknowledgement

First, I would like to thank my supervisor ao. Univ.-Prof. Mag. Dr. Sabine Glasl-Tazreiter for giving me the opportunity to participate in the project on Norway spruce balm.
It makes me happy that this thesis is also a small part of this very interesting project. Furthermore, I am very grateful that my supervisor always took her time to help me, when I needed it. Her experience and positive mind were essential for my thesis.
In addition I would like to mention her teaching about medicinal plants and her botanical excursions, which gave me a greater insight in the world of medicinal plants.

I would also like to express my gratitude to my co-supervisor Mag. Thomas Göls. I could not have imagined a better co-supervisor. His passion for science and research inspired me and his scientific and organized way to work taught me a lot.

Thanks to both, they were a great team. I will always look back with joy at this time in the laboratory together with them.

Moreover, I want to thank ao. Prof. Mag. Dr. Gottfried Reznicek for sharing his big knowledge about gas chromatography with me. He helped me to solve many technical problems during my work.

Furthermore, I would like to mention ao. Univ.-Prof. Mag. Dr. Johannes Saukel, who brought up the idea of the project about Norway spruce balm. I am very grateful for his teaching about botany.

Last, I would like to thank Univ.-Prof. Mag. Dr. Verena Dirsch, the head of the Department of Pharmacognosy, for providing all the facilities I needed for my work and for her great effort in teaching.

Not to forget I want to thank all the members of the team of Pharmacognosy. I would like to express my deep respect to all of them for creating a place of science, but also of humanity, tolerance and equality. I am proud of the work with you together.

Table of contents

1. Introduction

Picea abies (L.) H. KARST is a coniferous tree and belongs to the family of Pinaceae. It is endemic in northern, eastern and central Europe and especially widespread in Boreal conifer forests of northern and northeastern Europe. Figure 1 p. 3 shows the distribution area of *Picea abies*.

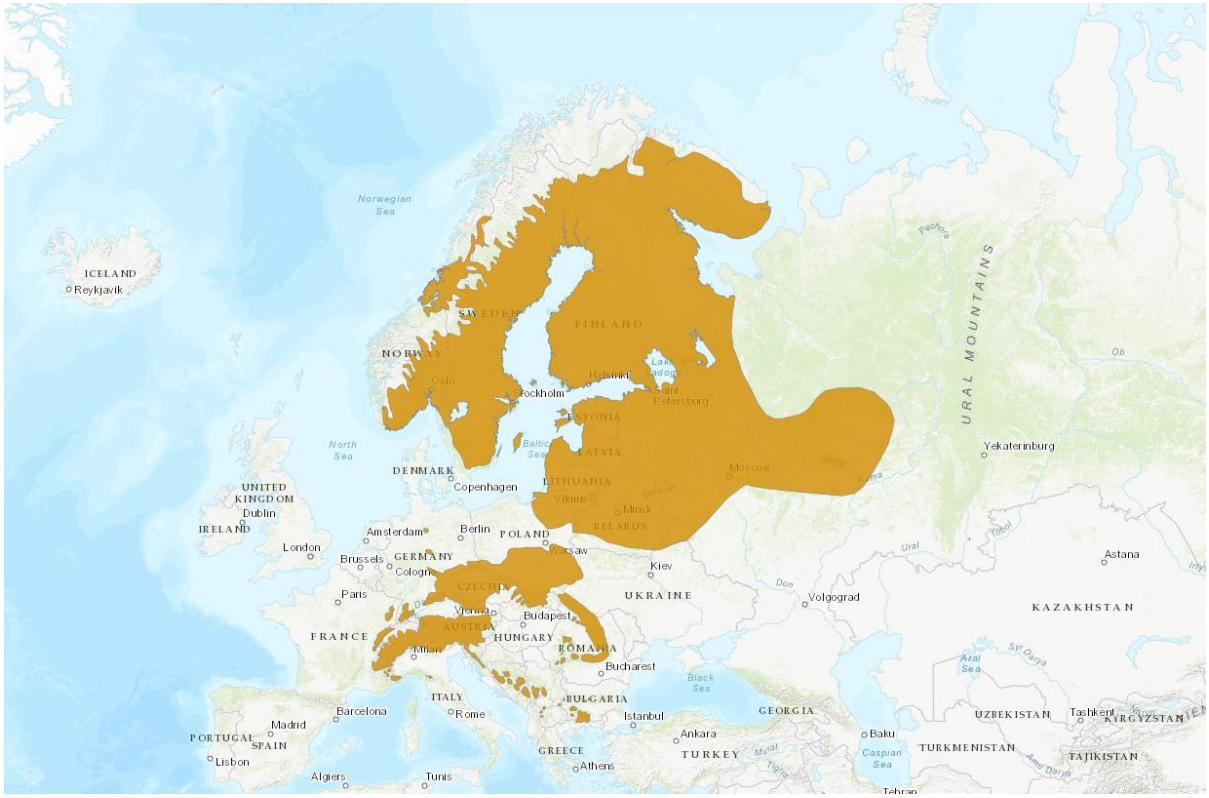

Figure 1: Distribution area of the Norway spruce
Farjon, A. 2017. Picea abies. The IUCN Red List of Threatened Species 2017: e.T42318A71233492. http://dx.doi.org/10.2305/IUCN.UK.2017-2.RLTS.T42318A71233492.en

The Norway spruce is better adapted to places with a lot of rain and as a result more successful in those areas than, for example, *Pinus sylvestris. Picea abies* is characterized by a flat root system. It can be found from sea level up to 2.000 m, depending on latitude and other climatic factors. In case conifer trees are injured they secret oleoresin from their trunks with the purpose to protect themselves from microbial attacks and loss of water. Typical constituents of the oleoresin are monoterpenes and resin acids.[1] Especially the chemical group of diterpene resin acids were important for this thesis. They form a group of carboxylic acids with the same chemical scaffold, which consists of three fused rings with the molecular formula of $C_{20}H_{30}CO_2$. They can be divided into abietic-type or pimaric-type resin acids, depending on the presence of a methyl- and ethenyl- or isopropyl- group in position 13. Resin acids have a sticky and highly viscose texture with a color ranging from transparent to amber-colored. The tree

produces these molecules as protective substances against insects, microbes and as wood preservatives. The resin acids are produced by epithelial cells, which form endothelial cells of resin ducts in the tree.[2] In the Figure 2 p. 4 below the most common resin acids are shown.

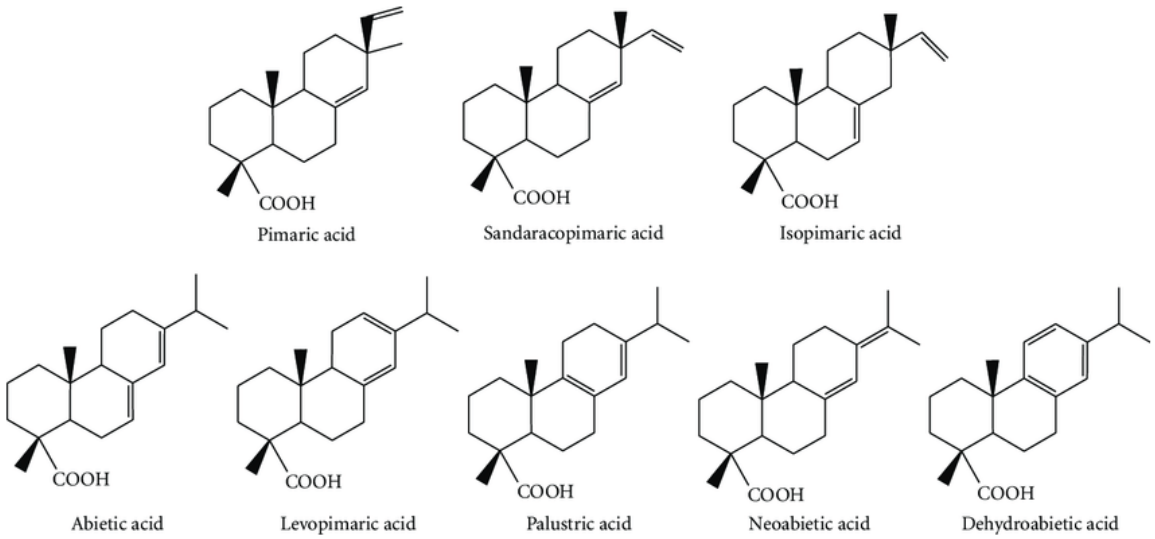

Figure 2: Chemical structures of the eight common resin acids in Picea abies
https://www.researchgate.net/figure/Chemical-structures-of-the-most-common-resin-acids-7_fig1_235347568

Since 2016 the Austrian Pharmacopeia (ÖAB) includes the monograph "*Piceae abietis pix recens*", which describes Norway spruce balm. In 2019 the monograph "Unguentum picis recentis Piceae Abietis officinale" was introduced in the ÖAB characterizing an ointment for wound healing. The pharmacopoeia clearly distinguishes the balm from the resin. According to traditional medicine the latter is excluded from pharmaceutical use. The balm can be harvested by scraping it out of knotholes or injured sites from the trunk of *Picea abies*, whereas the hardened pieces or drops of the clear, amber-colored resin are discarded. The balm has a yellow, white, brown or slightly pink color and a kneadable texture. The consistency resembles chewing gum. Therefore coniferous balm has been introduced recently as chewing gum under the name Alpengummi.[3]

A salve made of Norway spruce balm mixed with butter or lard plays an important role in European folk medicine. We have evidence for its use in the Austrian and South Tyrolean traditional medicine but it is also in use by the Lapp people in Northern Finland and Sweden.[4]This preparation has been used for centuries to treat different kinds of wounds.[5] As previously mentioned the balm protects the tree of microbial attacks, indicating the presence of antimicrobial substances. Microbial tests on agar plates confirmed this activity against

bacteria which commonly occur in infected wounds. The inhibited bacteria species are mainly gram-positive, for example methicillin resistant *Staphylococcus aureus* (MRSA) and vancomycin resistant *Enterococcus* (VRE). In times of rising antibiotic resistances, Norway spruce balm could be a new source for antibacterial compounds, especially for wound infections of the skin.[6] An antifungal activity of the salve is also proven in a study. It is active against dermatophytes and species of the Trichophyton genus.[7] Additionally its positive effect on the healing of pressure ulcers and other chronic injuries suggests that Norway spruce balm also contains substances which improve wound healing.[5]

There is more research needed to properly understand the active principles of this plant exudate and in what ways and by which mechanisms Norway spruce balm affects wound healing.

2. Aim of the work

This thesis was conducted as a part of a current doctoral thesis, which sheds light on the phytochemistry and wound healing properties of Norway spruce balm at the Department of Pharmacognosy of the University of Vienna. This project aims at the identification of yet unknown chemical compounds in Norway spruce balm and at correlating them with their influence on wound healing. The strategy of the project is based on the concept of bioactivity-guided fractionation. Norway spruce balm is separated in fractions and tested for the activity *in vitro*. The most active fractions are further separated and tested again for their activity with the aim to detect pure active compounds.

Since there is little information on the quantitative composition of the compounds in the balm. The goal of this thesis was to establish a quantification method of the most abundant resin acids. These are abietic, neoabietic, dehydroabietic, palustric, pimaric, sandaracopimaric, isopimaric and levopimaric acid. Their chemical structures are shown in Figure 2 ,p. 4. Gas chromatography played a central role in achieving that goal. Still today gas chromatography has an unsurpassed separation performance even compared to UHPLC. However, one major limitation is the required volatility and heat-stability of the analytes. The chemical structures of diterpene resin acids with a carboxylic acid group and the resulting polarity result in a high boiling point.

Therefore, it was necessary to establish a derivatization method, which transforms resin acids to a more volatile substance. Furthermore, this reaction had to proceed quantitatively and yield stable products. Additionally, it was necessary to create a chromatographic method with an optimal separation of the highly similar resin acids in Norway spruce balm. Establishing an adequate derivatization and analysis method were the main objectives of the thesis at hand.

3. Material and methods

This chapter describes the plant material and methods that were used in the course of this thesis.

3.1. Plant material

Basis for the present diploma thesis were two balm samples and an extract enriched in resin acids named RAMIX (see Table 1, p. 7). RAMIX had been proceeded within a previous diploma thesis and originated from Norway spruce balm batch number LU01.[4] It had been collected by hand with metal spoons in Lungau, Salzburg, in collaboration with the Austrian Federal forests. An initial purification step was followed by acid/base-liquid-liquid separation yielding the NaOH-fraction (see Figure 3, p. 8). The NaOH-fraction had been subjected to ion exchange chromatography yielding the so called "RAMIX" fraction, which was used as a starting point for this thesis. These working steps were performed in a previous diploma thesis.[4]

Table 1: Overview of the plant material

Resin acid mix (RAMIX, see Figure 3), an extract from Norway Spruce balm (batch LU01)
Norway spruce balm (batch GA02)
Norway spruce balm (batch FP2018)

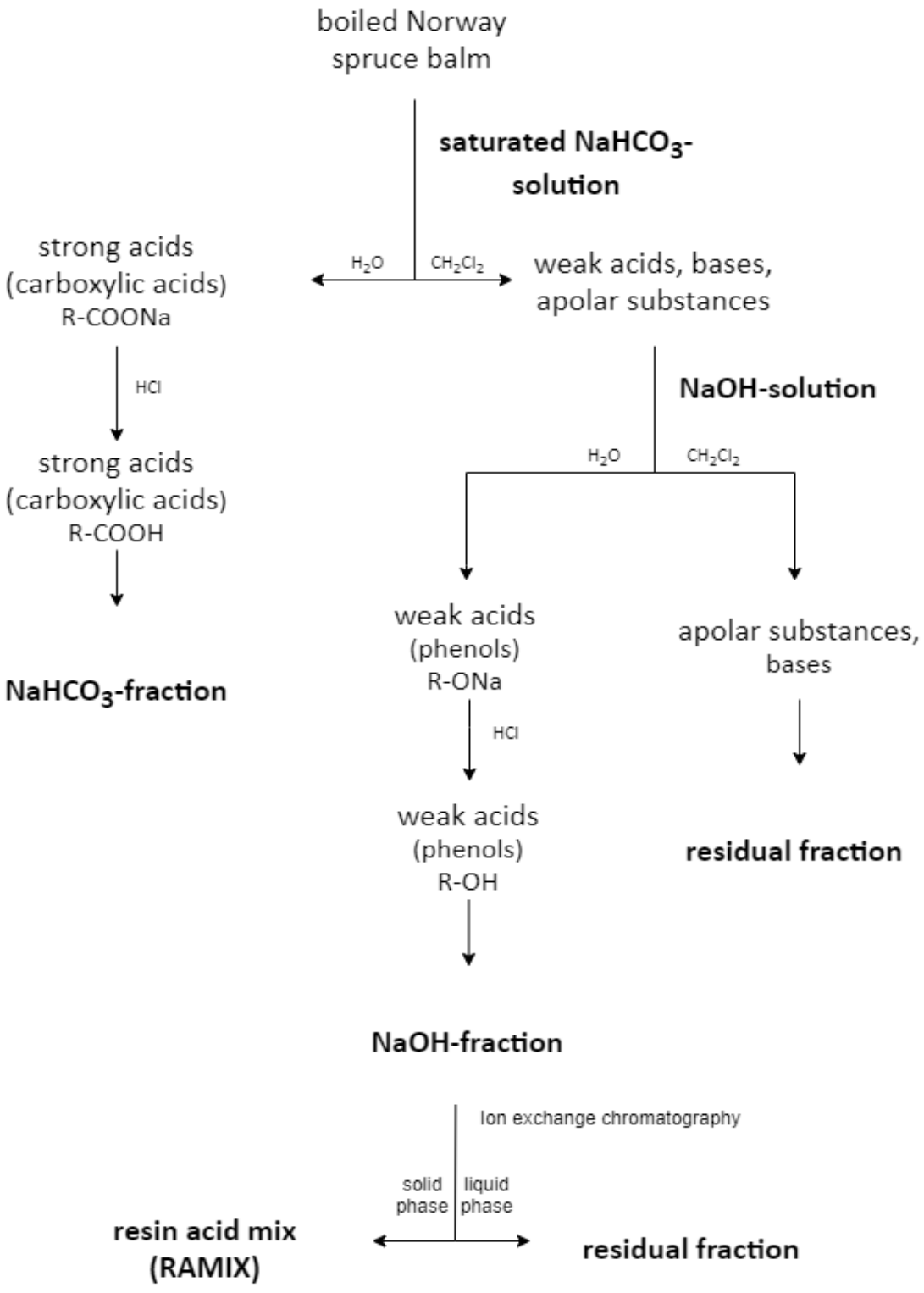

Figure 3: Liquid-Liquid extraction steps that are necessary for the extraction of RAMIX

3.2. Thin layer chromatography (TLC)

Thin layer chromatography was used to check the purity or composition of flash chromatography fractions. TLC is a simple chromatographic separation method. The stationary phase is coated in a thin layer on an aluminum or glass plate. Common layer materials are silica gel, chemically modified silica gel (alkylated, amino-, diol- or nitrile- silica gel), cellulose or aluminum oxide. As mobile phase various solvents of different polarity can be used. An important advantage of TLC is that the mobile phase can be easily changed.[8]

The first step is to apply the sample material on the TLC plate with a glass capillary. After the solvent of the sample is evaporated the plate is placed in the development chamber that contains the mobile phase. A piece of filter paper placed parallel to the TLC plate helps to guarantee a more homogeneous saturation of the chamber with mobile phase solvent. Via capillary forces the mobile phase migrates towards the top of the plate, separating the substance mixture in the process. The substances are separated because of their differences in polarity which cause different intensities of adsorption to the stationary phase, and, therefore, different migration distances.

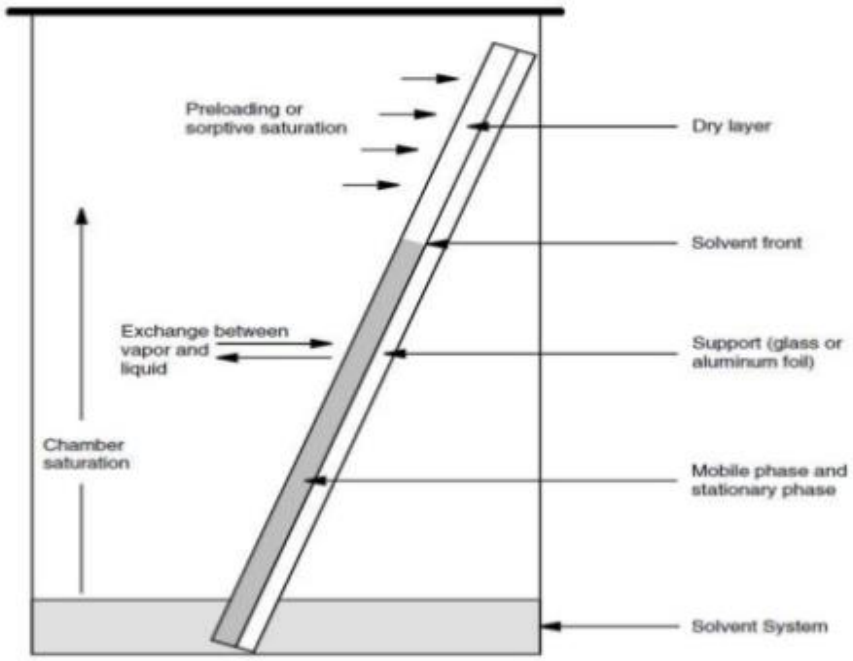

Figure 4: Scheme of a thin layer chromatographic analysis
https://www.slideshare.net/LavakusaBanavatu/thin-layer-chromatography-43293607

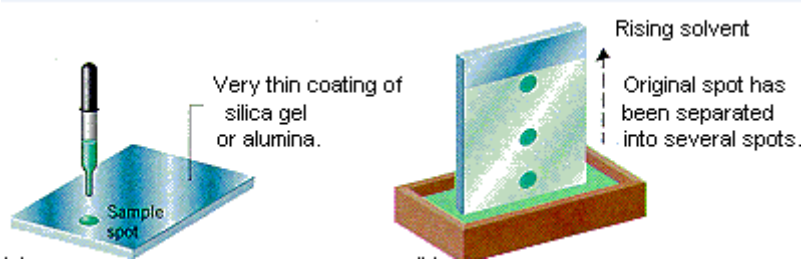

Figure 5: Application of different samples with a glass capillary on a line
https://chemed.chem.purdue.edu/genchem/topicreview/bp/ch15/separate.php

In general TLC is mainly used for qualitative and semi-quantitative analyses. An important qualitative parameter is the retention factor (R_f), which is the quotient of the distance from the start zone and the compound spot (z_R) and the distance from the start zone and the solvent front (z_M).

$$R_f = \frac{z_R}{z_M}$$

Figure 6: Formula of the retention factor[8]

After the separation of the sample, UV light of different wave lengths (254nm, 365nm[8]) was used to visualize the substance spots. The detection reagent anisaldehyde/sulphuric acid solution[9] was used to derivatize the substances on the TLC-plate in order to get refined results. The plates were dipped into the derivatization reagent and heated up for 2-3 min on 100-105°C. A CAMAG-TLC visualizer allowed to photograph the developed TLC-plates before and after the derivatization under 254 and 365 nm UV-light.

3.3. Flash Chromatography (FC)

The method of flash chromatography is a form of liquid chromatography. The stationary phase with intermediate sized particles (5-10 µm) is packed in a column. Mostly silica gel, either modified (reversed phase column) or unmodified, is used as stationary phase material.[10] The mobile phase is a mix of solvents, which is often applied in a gradient. Due to the smaller particle size pressure is needed to force the mobile phase through the packed column.

After the mobile phase passed through the column it is collected in different fractions. PDA and ELSD are possible detectors, which are used to analyze the composition of collected fractions. Additionally, TLC can be used to analyze every single obtained fraction.[8]

In the work at hand FC was used in combination with TLC to enrich dehydroabietic acid from a purchased standard (80%) to obtain a purity of 99%. PDA and ELSD detectors were used to detect the fractions containing the demanded substance. The fractions containing the pure substance without impurities were combined and the solvent was evaporated under reduced pressure.

Table 2: FC instrumentation

FC-Instrumentation	Interchim puriFlash®4250
Stationary phase	Column 15 C18 HP 6G (22 bar)
Mobile phase	Methanol 60 %
Detection	PDA Detector (254 nm and SCAN 200-600 nm)
	Preparative-integrated ELSD
Software	Interchim Software

3.4. High performance liquid chromatography (HPLC)

HPLC is one of the most common chromatographic methods. The stationary phase consists of very small particles (<3-5 µm) densely packed in a column. The same material as in flash chromatography is used, with reversed phase columns (e.g. octadecylated silica gel) being most frequently employed. In the case of reversed phase HPLC the mobile phase is a mixture of an intermediately polar solvent (ACN or MeOH) and water. Pressure up to 200 bar is applied to force the mobile phase through the packed column. Depending on the sample molecules, adsorption and desorption interactions between the stationary and mobile phase occur differently, allowing the separation to take place.[8]

Compared to gas chromatography, HPLC has a lower separation efficiency, but it allows a wider range of different molecules to be analyzed, because the sample must not be volatile and heat stable.[11] [12]

In this work a Shimadzu HPLC instrumentation was used to check isolated compounds and commercially acquired substances for their purity. For detection ELSD and PDA (190 nm and 254 nm wavelength) were utilized.

Table 3: HPLC Instrumentation

HPLC-Instrumentation	SHIMADZU Degasser DGU-20A5
	SHIMADZU Auto Sampler SIL-AC 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 5 µm (Number: 098)
	Dimension 250 x 4 mm
Mobile Phase	Solvent A: H_2O + 0,1 % formic acid
	Solvent B: Acetonitrile + 0,1 % formic acid

3.5. Supercritical fluid chromatography (SFC)

The SFC can be used for analytical and preparative applications. The SFC works with supercritical gas with added modifiers (MeOH, EtOH, IPA, ACN, etc.) as mobile phase. A common mobile phase is supercritical CO_2. If pressure and temperature are high enough gas and liquid state cannot be distinguished anymore. The gas is in its supercritical fluid state. Figure 7, p.13 illustrates this state and its required conditions. Supercritical fluids are characterized by their low density and viscosity. The diffusion coefficient of substances in this state is ten times higher than in the liquid state and allows a faster separation.[8] The fractions are analyzed via an ELSD and a PDA detector and collected with an automatic fraction collector.

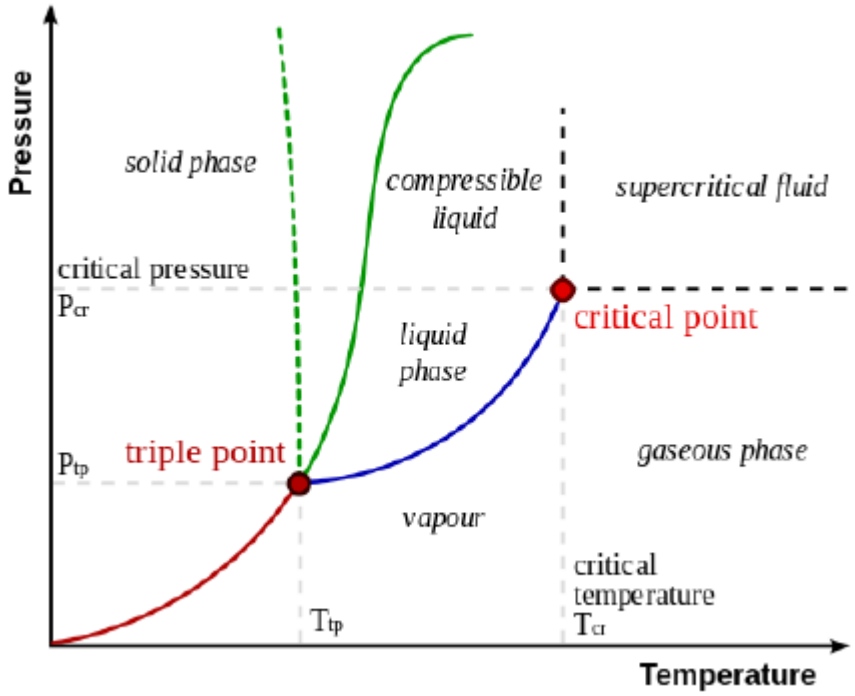

Figure 7: Phase diagram of CO_2
https://commons.wikimedia.org/wiki/File:Phase-diag2.svg

Table 4: 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 5: Stationary phases and solvent gradients for SFC

Column	**Dimension (mm)**	**Modifier**	**Gradient (%modifier)**	**Run Time**
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

3.6. Gas chromatography

Gas chromatography is an analysis method for volatile and heat-stable molecules. It has a very high separation efficiency even compared to more recent techniques like High Performance Liquid Chromatography (HPLC). The central part of a gas chromatograph is the capillary column with a microscopic polymer layer (stationary phase) inside. An inert gas (mobile phase) flows through the column under regulated conditions. The whole column is located in an oven, allowing an exact temperature setting inside the column. As with all chromatographic systems the separation takes place due to a difference in intensity of the interactions of the respective sample molecule with the stationary phase. Temperature programs in the column oven allow the evaporation of the molecules according to their boiling points at different time points which adds another separation dimension. Consequently, temperature is crucial parameter that influences the quality of the separation.

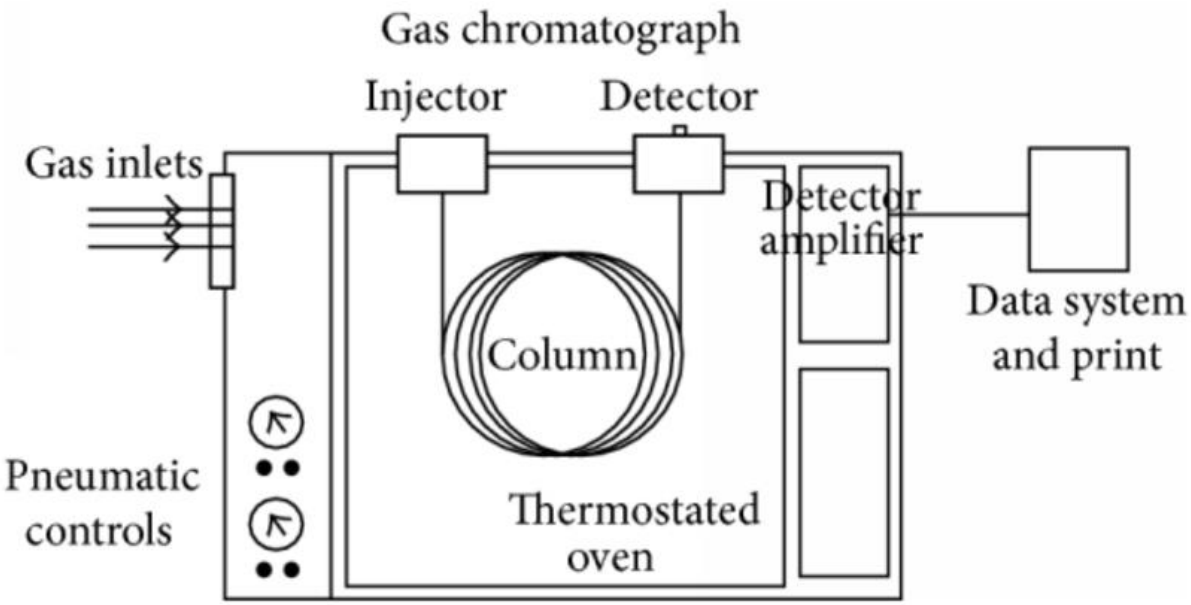

Figure 8: Schematic setting of a gas chromatograph
https://www.researchgate.net/figure/Structure-diagram-of-gas-chromatograph_fig4_275472097

The sample is injected by an autosampler syringe through a septum into the injector. The injector is connected by a glass liner to the column, the temperature must be high enough so that all sample substances evaporate at the same time. The mobile phase flow carries the vaporized molecules on to the head of the column, where the temperature is lower and therefore the substances condense at the beginning of the column. The injection can take place in splitless or in split mode. In splitless mode the whole sample volume reaches the column, because the split valve is closed (see Figure 9, p. 16).

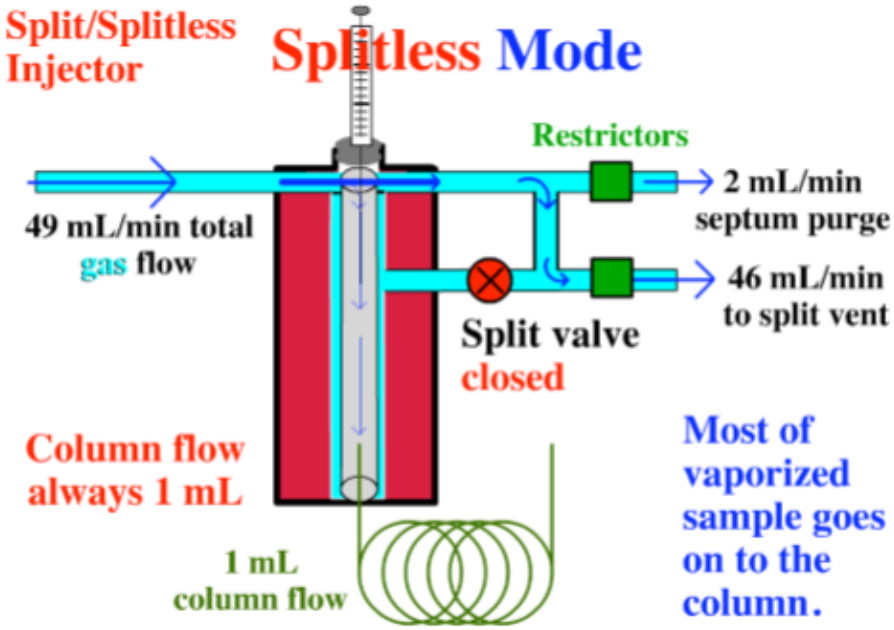

Figure 9: Injector in splitless mode
https://www.shsu.edu/~chemistry/primers/pdf/GC.pdf

A highly concentrated sample can be disadvantageous for the quality of the separation. The peak shape deteriorates, they become broader, tailing may occur and they may overlay with other substance peaks. In this case split mode may be a promising option. In this setting just an adjustable fraction of the sample reaches the column, since the split valve is open. It is possible to control the split ratio via the GC-Software. A split ratio of 1:100 was applied in this thesis. This means that 100 parts are injected in the injector and just 1 part reaches the column (see Figure 10, p. 17).

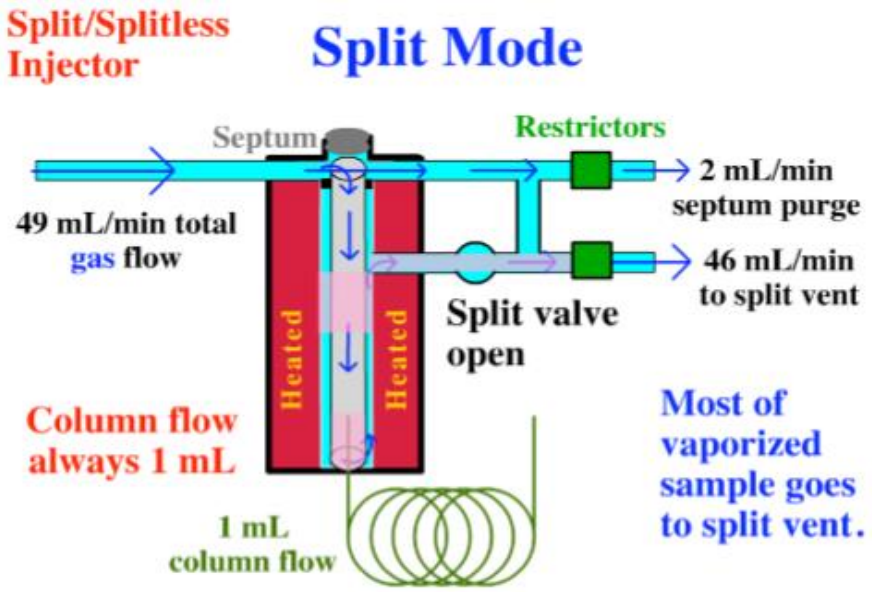

Figure 10: Injector in split mode
https://www.shsu.edu/~chemistry/primers/pdf/GC.pdf

If a molecule has reached the column head, the gas flow transports it further through the column and finally to the detector. As mentioned before, physicochemical properties of the molecule are the determining factor for the extent of adsorption and desorption of the analyte between mobile and stationary phase resulting in distinct retention times.

The two most important detector types in gas chromatography are flame ionization detector and mass spectrometer. The flame ionization detector (see Figure 11, p. 18) is known for its low detection limit and its wide linear range of detection. Furthermore, it has a high process robustness.[8] The central part of a flame ionization detector is a nozzle, where hydrogen and oxygen create a flame. An ignition coil is necessary to start the burning process in a controlled way. When hydrogen is burnt no ions are formed, so only a low basic current (3 pA) is measured. When organic compounds of the sample exit the column and reach the detector flame, ions are formed and the change in electric voltage is measured. A flame ionization signal

is formed when cations and electrons from the organic compounds in the flame reach the collector electrode.[8]

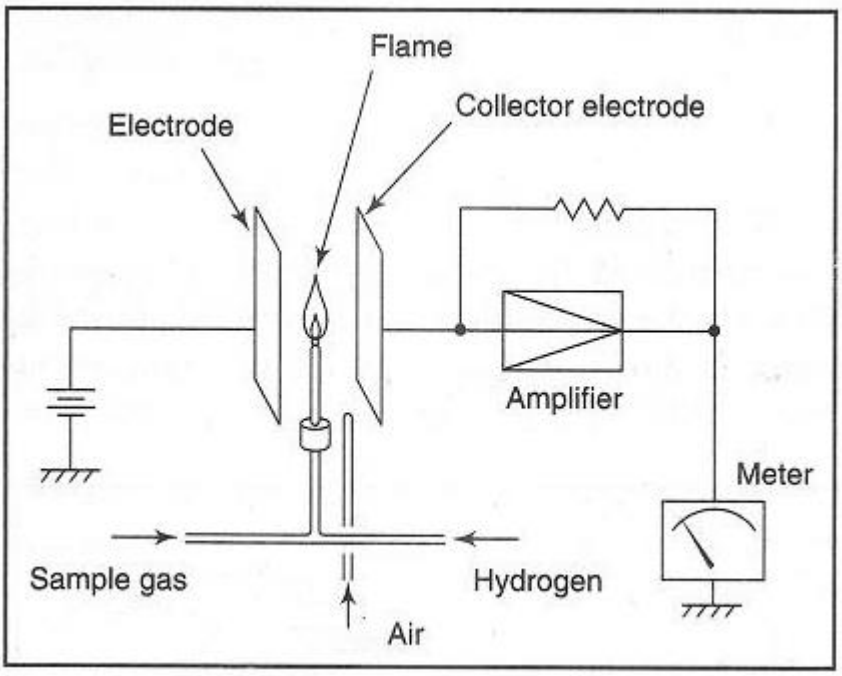

Figure 11: Flame ionization detector
https://automationforum.in/t/hydrocarbon-analyzer-flame-ionization-and-thermal-conductivity-detector-method/5023

The molecular properties of a substance determine the intensity of its signal. Different molecules produce different amounts of ions, which causes a different signal intensity.

To quantify substances accurately, it is important to establish a calibration curve to make sure that detected signals lie within the linear range of the detector. Furthermore, only a stable baseline allows to detect signals of high quality.[8]

In this thesis the gas chromatography instrumentation and parameters given in Table 6 and Table 7, p. 19 were used.

3.6.1. General GC-FID parameters

Two different GC-FID instrumentations were used because of technical problems with Perkin Elmer XL.

Table 6: Perkin Elmer XL Instrumentation for GC-FID

GC-System	Perkin Elmer XL gas chromatograph
Stationary phase	ZB-5 column Zebron (5% Phenyl, 95% Dimethylpolysiloxane) 60 m length, 0.25 mm diameter, 0.25 µm coating
Pre-column	1 m deactivated capillary pre-column
Mobile phase	Nitrogen (purity 5.0); Flow: 50 ml/min;
Temperature program	Startplateau of 5 min 100°C, phase 1 100-200°C, rate 20°C/min, phase 2 200-270°C, rate 1°C/min
Injection volume	1 µl, split ratio ?
Detection	Flame ionization detector Synthetic air (purity 5.0), Hydrogen (purity 5.0)

Table 7: Shimadzu GC-2010 Instrumentation for GC-FID

GC-System	Shimadzu GC-2010
Column	ZB-5 column Zebron (5% Phenyl, 95% Dimethylpolysiloxane) 60m length, 0.25mm diameter, 0.25 µm coating
Pre-column	1m deactivated capillary pre-column
Mobile phase	Helium (purity 5.0)
Temperature program	Startplateau of 5 min 100°C, phase A 100-200°C, rate 15°C/min, phase B 200-250°C, rate 2°C/min
Applied volume	1µl, split ratio 1:100

Detection	Flame ionization detector Synthetic air (purity 5.0), Hydrogen (purity 5.0)

3.6.2. Derivatization

Derivatization has the purpose of making sample components analyzable with a particular analytical system. In gas chromatography it is often used to increase the lipophilicity and therefore volatility of substances. Common derivatization methods are silylation, acylation and methylation. Silylation is often used to modify active hydrogen groups. Methylation can be used to esterify carboxylic groups. All these methods can help to widen the range of substances analyzable by gas chromatography. To increase the yield of the derivatization product it is important to find a suitable reagent for the particular sample.[13] At the beginning of the work it was not clear which derivatization reagent is the best option to derivatize resin acids, so different derivatization methods were tested. The experiments began with silylation agents, because there was already gained experience at the department with silylation of resin acids.[14] In the end Methylation agent Methyl8-reagent (N,N-Dimethylformamide dimethyl acetal) produced by the company Thermo Fisher Scientific turned out to be the best option. It was used for derivatization to obtain methyl esters of resin acids.

To find the ideal reaction setting different reaction times and temperatures were tested. See Table 11, p. 25 for the details about the different tested derivatization methods. The sample substances (RAMIX, abietic acid, glucose) were solved directly in Methyl8-reagent or pyridine in heat dried V-vials. The filled V-vials were heated in an electrical oven for a certain reaction time. Storage vials with septum caps were used to protect the derivatization agents of moist, moreover all derivatization agents were stored in a desiccator. HPLC-syringes were used to take out the derivatization agents of the storage vials.

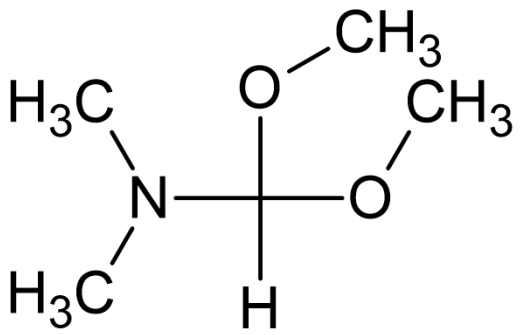

Figure 12: Chemical structure of Methyl8-reagent

3.7. Validation

The limit of detection is the lowest concentration of a substance in a sample that can be detected but not quantified by a chromatographic method. The limit of quantification is the lowest concentration of a substance in a sample that can be quantified reproducibly. The signal-to-noise method is an established way to determine LOD and LOQ. Accordingly, LOD is the concentration with a signal-to-noise ratio of 3:1, this means that the concentration of the substance must result in a peak with a height three times higher than the average noise peak height in the area around the retention time of the analyte peak. LOQ generally is the concentration with a signal-to-noise ratio of 10:1.[15]

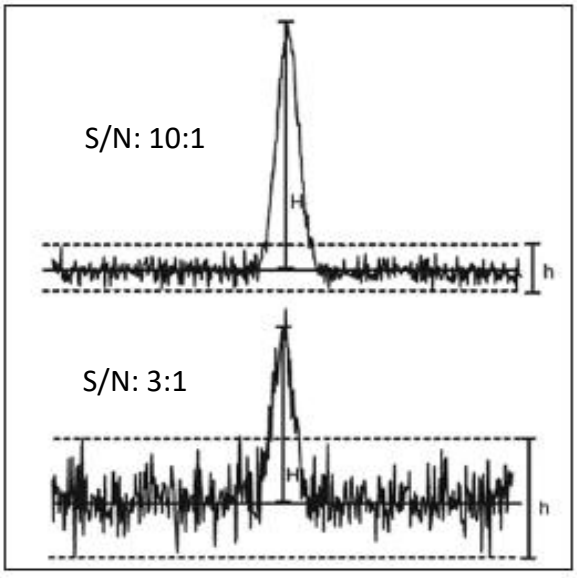

Figure 13: Signal-to-Noise ratio (S/N) examples for LOD and LOQ [15]

3.8. Pure substances and solvents

In the following tables, all solvents and chemicals are listed with their technical specifications.

Table 8: Solvents used

Solvent	Solvent specification	Producer	Location
Acetone	Acetone Normapur	VWR BDH Prolabo®	Vienna
Methanol	Methanol Normapur	VWR BDH Prolabo®	Vienna
Formic acid for TLC	Acidum formicum conc.	Gatt-Koller	Absam
Pyridine	Pyridine	Merck	Darmstadt
Triflouroacetic acid	Trifluoroacetic acid 610630	Riedel-de Haen AG	Seelze
Water	Water, double distilled	Deionized water, distilled with GFL 2004	Vienna

Table 9: Derivatization reagents used

Derivatization reagent	Reagent specification	Producer	Location
BSTFA	N,O-Bis(trimethylsilyl)trifluoroacetamide	Pierce Chemical Co	Dallas
HMDS	Hexamethyldisilazane	Sigma Aldrich	Steinheim
TMCS	Trimethylchlorosilane	Sigma Aldrich	Steinheim
M8R-reagent	N,N-Dimethylformamide dimethyl acetal	Thermo Fisher Scientific	Göteborg

Table 10: Pure substances used

Pure substance	Producer	Location
Arachidic acid	Merck	Darmstadt, Germany
Abietic acid	ChemCruz	USA

Dehydroabietic acid	Purified at the department of pharmacognosy	Department of Pharmacology, Faculty Life Sciences, University of Vienna (provided by Dr. Marco Stadler), Austria
D (+)-Glucose, anhydrous	Merck	Darmstadt, Germany
Methyl palmitate	Fluka	Steinheim, Germany
Palmitic acid	Serva	Heidelberg, Germany
Pimaric acid	MP Biomedicals	Eschwege, Germany

4. Results

The derivatization method of the sample and the temperature gradient of the column oven of the gas chromatography system were the two parameters, which influenced the most the success of the quantification of the resin acids in Norway spruce balm.

The sample preparation required derivatization to make resin acids volatile enough for gas chromatography: silylation and methylation were evaluated. At the department of Pharmacognosy of the University of Vienna was gained already experience with silylation of resin acids for qualitative gas chromatography analysis, thus the search began with different silylation agents.[14]In the first experiments the pure substance abietic acid was analyzed. When it became clear that it was necessary to test different derivatization reagents at different conditions, RAMIX (see Table 1, p. 7) was used in order to safe the highly pure abietic acid.

After a suitable derivatization method was found it was necessary to develop a temperature program for the GC-FID analysis, because only the right temperature gradient of the column oven allowed to separate the different chemically similar resin acids. Separated peaks in the chromatogram were an important goal to make quantification possible.

4.1. Development of a derivatization method

As mentioned before the objective of this thesis was to establish a method for the quantification of resin acids in Norway spruce balm. The resin acids themselves were not volatile enough to be analyzed by gas chromatography. Therefore, it was necessary to find a suitable derivatization method for the generation of stable, volatile products. Furthermore, the derivatization reaction should proceed quantitatively. Different derivatization methods were tested (see Table 11, p. 25), but Methyl8-reagent (N,N-Dimethylformamide dimethylacetal) was the only reagent which met the expectations. Only after the derivatization with Methyl8-reagent it was possible to generate reproducible gas chromatograms (see Figure 17, p. 29). For the quantification it was very important that the peak areas remained constant for at least some hours (see Table 12, p. 30). Table 6, p. 19 contains a more detailed description of the gas chromatographic system and its temperature program.

Table 11: Derivatization methods b-h, method h generated stable reaction products(see Figure 17, p.29);
The reaction products were analyzed with GC-FID system in Table 6 p. 19;
See Table 9, p.22 for reagents used; See Table 10, p.22 and Table 1, p. 7 for sample substances;

ID letter & result	**Sample**	**Derivatization agent**	**Reaction time and temperature**
b no peak	0,1 mg AA + 0,1 ml Pyridine	9,9 µl BSTFA + 0,1 µl TMCS	5 min, room temperature
c no peak	0,1 mg AA + 0,1 ml Pyridine	9,9 µl BSTFA + 0,1 µl TMCS	10 min, 60°C
d no peak	1,0 mg AA + 0,1 ml Pyridine	9,9 µl BSTFA + 0,1 µl TMCS	10 min, 60°C
e no peak	1,0 mg AA + 0,1 ml Pyridine	99 µl BSTFA + 10 µl TMCS	20 min, 60°C
f glucose peak	1,3 mg Glucose + 0,1 ml Pyridine	99 µl BSTFA + 10 µl TMCS	20 min, 60°C
g unstable peaks	500 µg RAMIX +0,1 ml Pyridine	4 µl HMDS + 4 µl TMCS	injected instantly
h stabel peaks	**1 mg RAMIX**	**200 µl Methyl8-reagent + 200 µl pyridine were added after the reaction**	**30 min, 85 °C**

4.1.1. Silylation

As mentioned before it was already shown at the department that it is possible to silylate resin acids for a qualitative gas chromatography analysis, but until to this point there was not performed any silylation method, which generates stable enough products for a quantification analysis. There was found a silylation method for the quantification of resin acids in literature.[1] Therefore, the first idea was to derivatize abietic acid with different silylation agents to understand, if some of them generate stable reaction products.

A mixture of 99µl BSTFA (N,O-Bis(trimethylsilyl)trifluoroacetamide) and 1µl TCMS was used as derivatization reagent for the silylation of the resin acids. Its composition was slightly varied in order to improve the reaction. The mixture was prepared under the hood taking care that the derivatization reagents did not get in contact with moisture, because this would have caused a useless silylation of H_2O. The storage glass vials were dried with heat before use. Furthermore, the filled storage glass vials where closed with a septum cap. HPLC-syringes were used to take out derivatization reagent of the capped storage vials. The reagent vials were stored in a desiccator (not longer than one week) to avoid the ingress of moisture.

i) In the first experiment 0.1mg of abietic acid and 1mg of abietic acid were dissolved in 0.1ml of pyridine in a septum capped V-vial. 10µl of a solution of 9,9 µl BSTFA and 0,1 µl TMCS were added with a HPLC-syringe. The first sample was analyzed after a reaction time of 5min at room temperature and a second sample was analyzed after 10min at 60°C in an electrical oven. From previous GC-FID experiments it was known that the derivatives of the resin acids had retention times of more than 10 min. But after analysis of before mentioned samples no peaks of the derivatized resin acids were detected in the expected retention time interval (see Figure 14, p. 26, chromatograms b, c and d).

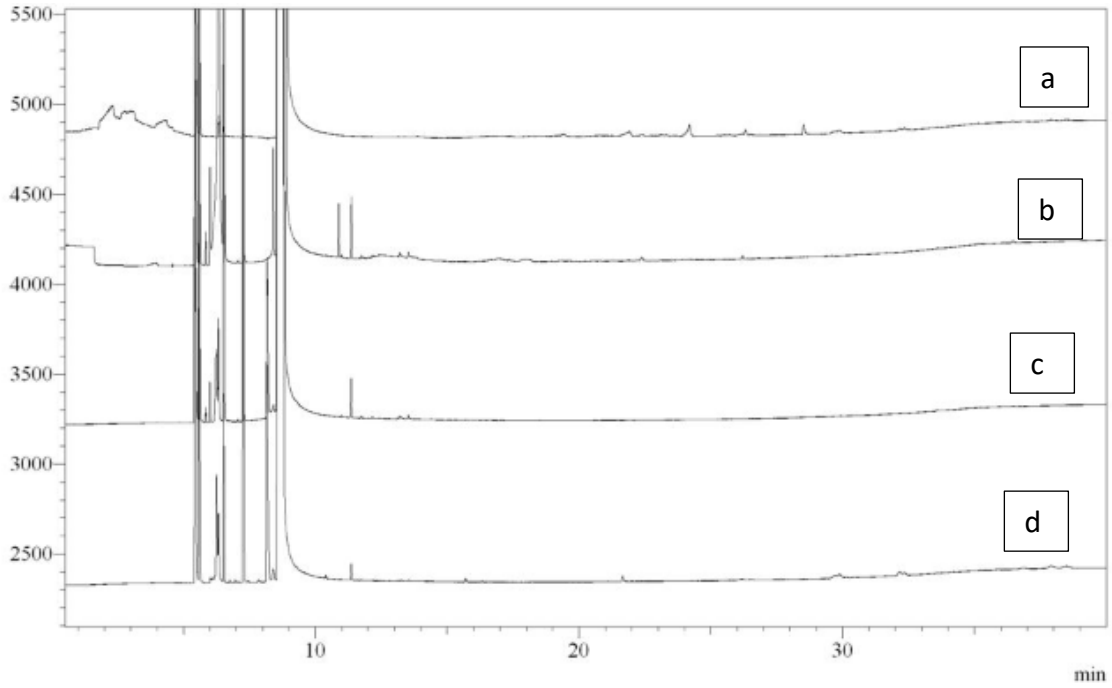

Figure 14: GC-FID analysis of abietic acid after derivatization with 10µl silylation reagent using different amounts and derivatization parameters (see Table 11, p. 25 ; b, c, d); GC-FID system: Table 6;
a) pyridine blank
b) 0.1mg abietic acid (see Table 10, p. 22) 5min room temperature
c) 0.1mg abietic acid (see Table 10, p. 22)10min 60°C
d) 1.0mg abietic acid (see Table 10, p. 22)10min 60°C

ii) After this first experiment the reaction conditions were changed in so far as the volume of the silylation reagent was increased to the tenfold and its composition was changed by increasing the TMCS concentration. This time 1 mg of abietic acid was diluted in 0.1 ml pyridine in a V-vial, 99 µl BSTFA + 10 µl TMCS were added with a HPLC-syringe. The sample was analyzed after 20 min at 60°C, to prolong the chemical reaction time. Still no visible peaks of silylated abietic acid were detected (see Figure 15, p. 27, chromatogram e).

To test derivatization method and analysis system, the same experiment was repeated with anhydrous D(+)-glucose instead of a resin acid, knowing that glucose is readily silylated under these conditions. As expected, glucose was silylated and detected at a retention time of about 34min (see Figure 15, p. 27, chromatogram f). The conclusion was drawn that the BSTFA-TMCS combination is not able to silylate carboxylic groups of resin acids as, while it works with the functional groups of glucose. For this reason, another silylation agent was tested.

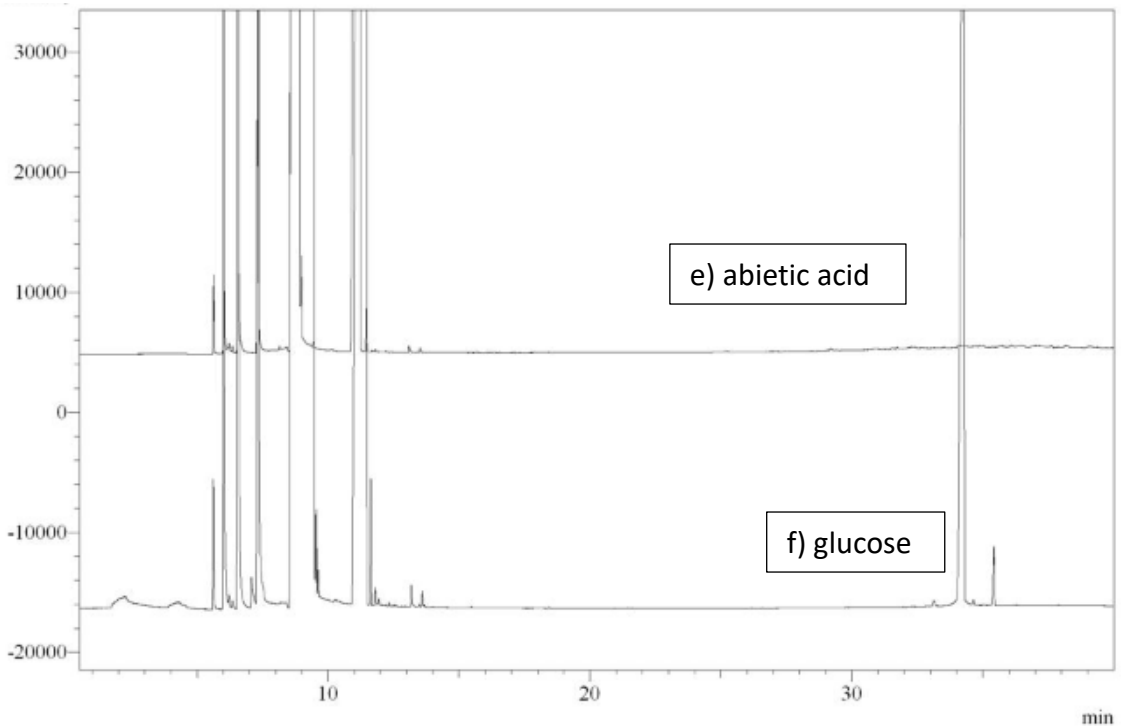

Figure 15: GC-FID analysis of abietic acid and glucose after derivatization with silylation reagent BSTFA-TMCS 10% (see Table 11, p. 25, methods e, f); GC-FID system: Table 6, p. 19;
e) 1.0mg abietic acid (see Table 10, p. 22), only solvent peaks
f) 1.3mg glucose (see Table 10, p. 22), 34min clear glucose peak

ii) At this point of the work RAMIX (see Table 1, p. 7) was used instead of the pure abietic acid, to save the pure substance for subsequent analysis. 500 µg of RAMIX were dissolved in 0.1 ml pyridine. 4 µl HMDS (Hexamethyldisilazane) and 4 µl TMCS (Trimethylchlorsilane) were added, the resulting solution was mixed and immediately injected and analyzed by GC-FID. This reagent resulted in peaks with a satisfactory peak shape (see Figure 16, p. 28) but on the other hand, the products revealed a low stability. It was not possible to generate a chromatogram with the same peak shape again one hour after the derivatization. This observation ruled

out batch analyses and renders the validity of the quantification results questionable.

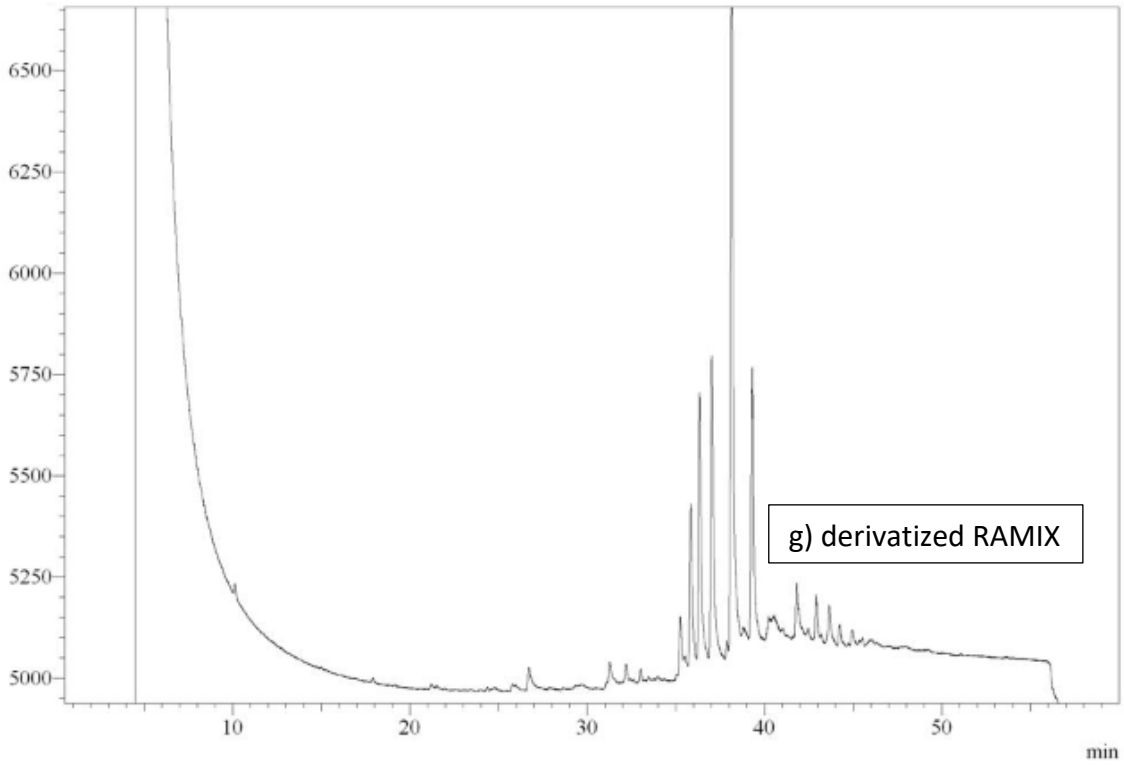

Figure 16: GC-FID analysis of RAMIX sample (see Table 1, p. 7) after derivatization with silylation reagent 4µl HMDS + 4µl TMCS (see Table 11, p. 25, method g); GC-FID system: Table 6, p. 19;

4.1.2. Methylation

Silylation was not successful in derivatizing resin acids, therefore the next step was to try methylation. Methyl-8 reagent (see Table 9, p. 22) was used for this reaction. 1mg of RAMIX (see Table 1, p. 7) was dissolved in 200 µl Methyl-8 reagent. This solution was incubated for 30 min at 85°C. For a time span of 10 h the resulting solution was injected hourly in the gas chromatographic system to get an idea of the stability. During those 10 h the respective peak pattern seemed to be stable. The area under the curve of the peak at 36 min varied with a standard deviation of 11.4 %. Two outliers were observed, with the possible cause of an autosampler variability or in connection with concentration changes of the methylated resin acids, caused by evaporation of the solvent. The autosampler variability was considered acceptable, because in any case it would have been necessary to use an internal standard for an exact quantification. To make the method more robust, considering uncontrollable evaporation of the solvent during the 30 min 85°C heating step of the derivatization method, 200 µl of pyridine were added after each methylation reaction. The intention was to increase the pyridine volume to guarantee, that the sample solution was not saturated by derivatized

resin acids and to make small volume changes of the solvent pyridine less relevant for the measurement.

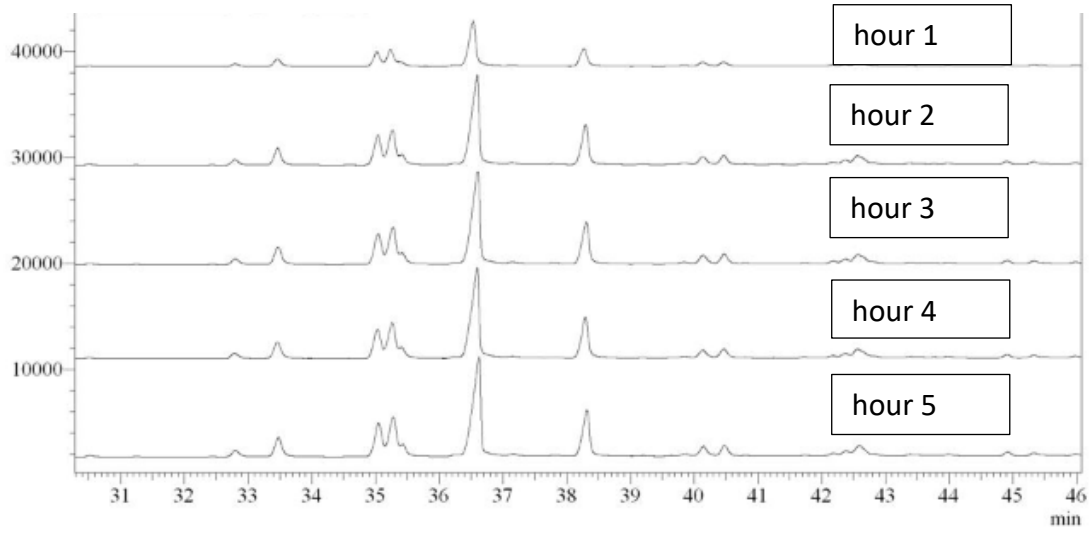

Figure 17: GC-FID analysis of 1mg RAMIX (see Table 1, p. 7) after methylation with Methyl-8 reagent (see *Table 11*, p. 25*, method h); GC-FID system: Table 6, p.19;*
Derivatized RAMIX was injected every hour to show the stability of the reaction products: hour 1-5

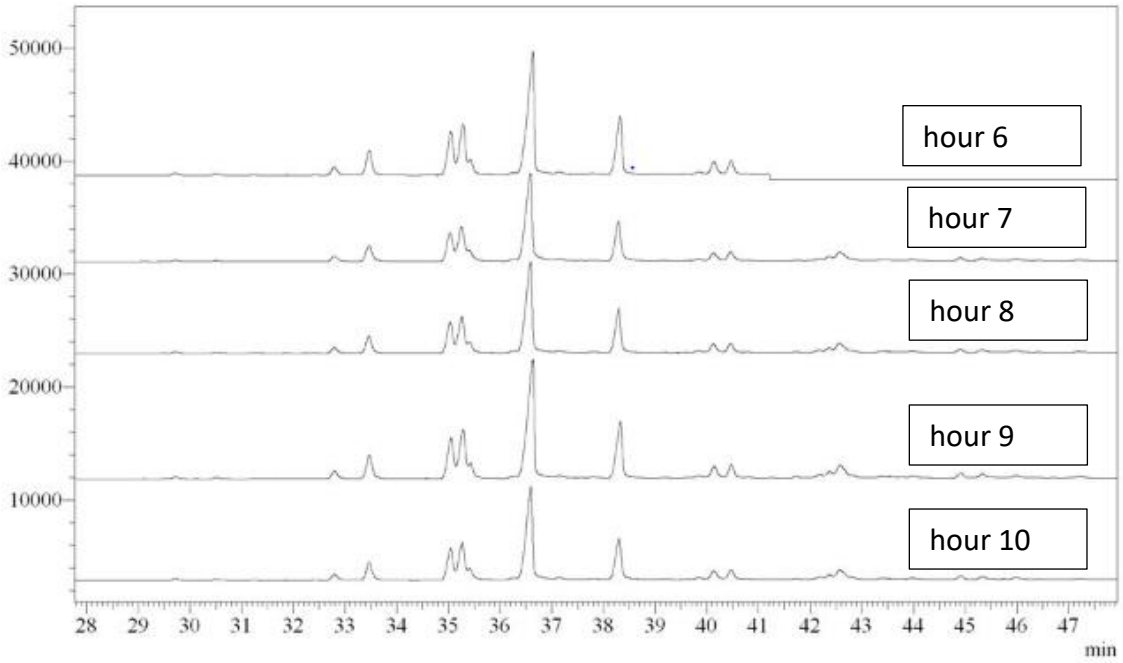

Figure 18: GC-FID analysis of 1mg RAMIX (see Table 1, p. 7) after methylation with Methyl-8 reagent (see Table 11, p.25, method h); GC-FID system: Table 6, p. 19;
Derivatized RAMIX was injected every hour to show the stability of the reaction products: hour 6-10

Table 12:Comparison of the AUC parameter of the highest peak at retention time 36.5 min (see Figure 17 and Figure 18);

	Ret.Time	AUC	AUC%		
hour 1	36.571	65778	100		**SD (AUC%)**
hour 2	36.534	33827	**51**	outlier	11,4
hour 3	36.598	70995	108		
hour 4	36.596	76903	117		
hour 5	36.627	86073	131		
hour 6	36.627	86073	131		
hour 7	36.589	70107	107		
hour 8	36.591	70561	107		
hour 9	36.636	98538	**150**	outlier	
hour 10	36.594	73717	112		

Figure 17 and Figure 18 p. 29 show that the shape of the chromatogram remained constant for 10 hours, therefore it was concluded that the derivatization products could be stable enough for a quantification analysis. The comparison of the AUC parameter of the peak with the retention time 36.5 min in Table 12, p. 30 should evidence that the amount of methylated resin acid remains the same for at least 10 hours. There are no signs of an increase or a decrease of the derivatization product for 10 hours. The 36.5 min peak was chosen because it had the highest AUC and was separated from all other peaks. The AUC values varied with a standard deviation of 11,4 %, this seemed to be acceptable, since no internal standard was yet applied.

The outcome of this chapter was that it is possible to derivatize the resin acids in RAMIX with Methyl8-reagent in the setting of Table 13, p. 30. RAMIX was dissolved directly in Methyl8-reagent in septum capped V-vials using HPLC-syringes:

Table 13: See Table 9, p.22 for reagents used; See Table 1, p. 7 for sample substances; The reaction products were analyzed with GC-FID system in Table 6 p. 19;
Details of the final derivatization method with Methyl8-reagent;

ID	Sample	Derivatization agent	Reaction time and temperature
h	1 mg RAMIX	200 µl Methyl8-reagent + 200 µl pyridine were added after the reaction	30 min, 85 °C

The next step was to optimize the temperature program, to separate the peaks as far as possible to make the AUC quantification more precise. Moreover, it was time to choose an internal standard substance to avoid biased values caused by the autosampler.

4.2. Optimization of the temperature program

Many of the resin acids in Norway spruce balm have a very similar chemical structure (see Figure 2 p. 4) and as a result similar retention times. This required an optimization of the GC-method in order to achieve baseline separation for accurate quantitative analysis. One parameter that influences the resolution of the peaks and the quality of the separation is the temperature program of the column oven. Variation of the starting temperature and heating rate resulted in most notable improvements considering the separation of the peaks in the chromatogram.

All analyses started with an initial plateau phase of 5 min at 100°C, during which the resin acids condensed at the head of the GC-column, after being evaporated in the injector. Further all analyses started with a first heating phase (heating phase 1) revealing a steep increase in temperature (8-20°C/min) followed by heating phase 2 with a flat heating rate (1-2°C/min), and a final heating step (see Table 14, p. 31)

Table 14: The different tested temperature gradients for GC-FID analysis; Program G had the best separation power with RAMIX (Table 1, p. 7); GC-system: Table 7, p. 19;

Program name	Plateau phase	Heating phase 1	Heating phase 2	Final heating step
A	initial 5min 100°C	100-200°C, rate 20°C/min	200-270°C, rate 1°C/min	10 min of the final temperature
B	initial 5min 100°C	100-170°C, rate 20°/min	170-250°C, rate 1°C/min	10 min of the final temperature
C	initial 5min 100°C	100-200°C, rate 10°C/min	200-270°C, rate 2°C/min	10 min of the final temperature
D	initial 5min 100°C	100-170°C, rate 10°/min	170-250°C, rate 2°C/min	10 min of the final temperature
E	initial 5min 100°C	100-150°C, rate 10°C/min	150-250°C, rate 2°C/min	10 min of the final temperature
F	initial 5min 100°C	100-200°C, rate 20°C/min	200-270°C, rate 2°C/min	10 min of the final temperature
G	**initial 5min 100°C**	**100-200°C, rate 15°C/min**	**200-250°C, rate 2°C/min**	**10 min of the final temperature**
H	initial 5min 100°C	100-200°C, rate 10°C/min	200-270°C, rate 2°C/min	10 min of the final temperature
I	initial 5min 100°C	100-190°C, rate 8°C/min	190-260°C, rate 2°C/min	10 min of the final temperature

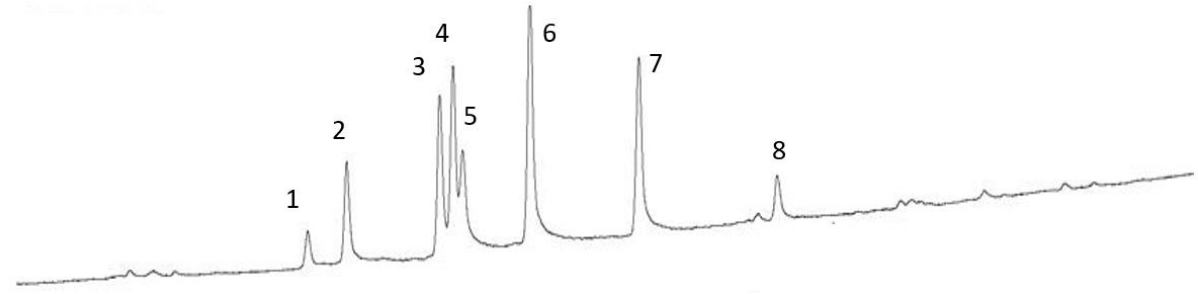

Figure 19: GC-FID analysis of RAMIX (see Table 1, p. 7) providing the resin acids 1-8; GC system: Table 7, p. 19; Derivatization method: Table 13, p. 30;

To characterize the different resin acid peaks of Norway spruce balm, the retention times of the resin acid peaks were compared with the retention times of commercially obtained pure resin acids. Figure *20*, p. 32 shows the chromatogram of Norway spruce balm GA02 (see Table 1, p. 7) and two chromatograms of resin acid standards. In that manner dehydroabietic acid and pimaric acid were identified. It was not possible to identify all peaks because not all resin acids were available in a satisfying pure form.

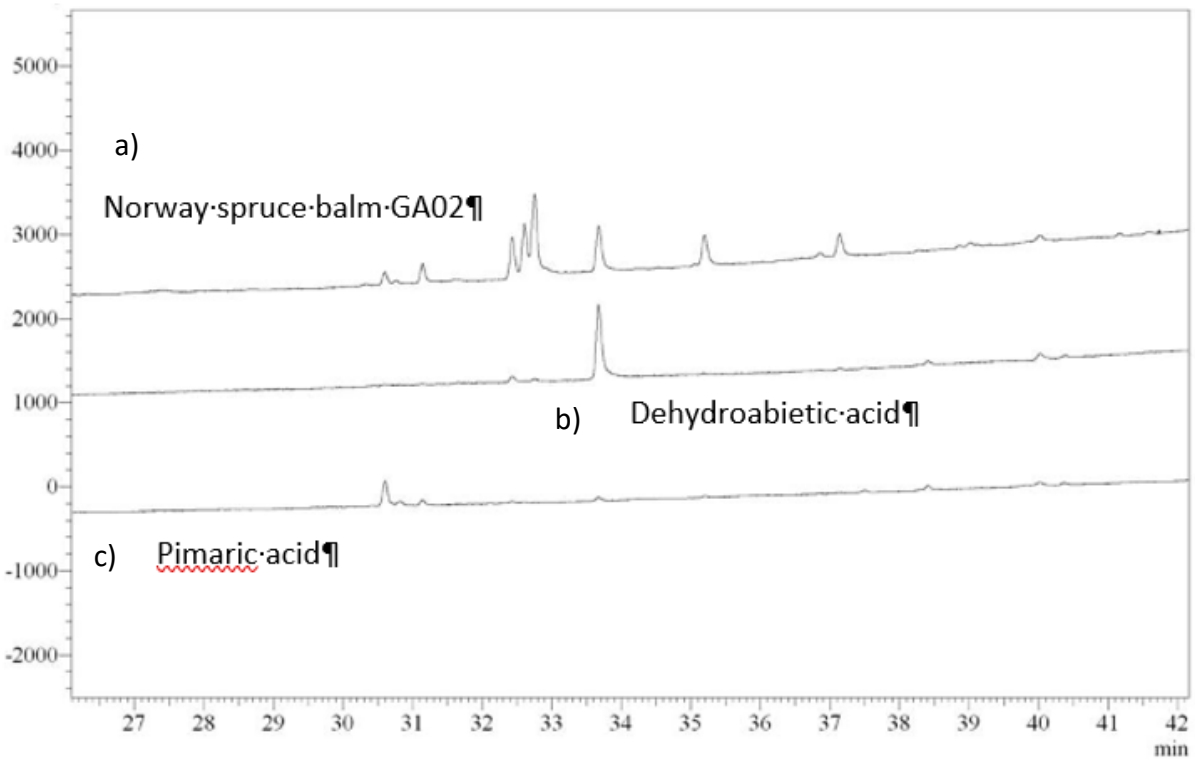

Figure 20: GC-FID analysis of different methylated samples; GC system: Table 7, p. 19; Derivatization method: Table 13, p. 30;
a) Norway spruce Balm GA02 (see Table 1, p. 7)
b) Dehydroabietic acid (see Table 10, p. 22)
c) Pimaric acid (see Table 10, p. 22)

The following Figure 21, p. 33 shows the chromatograms of the methylated RAMIX sample, analyzed with temperature program A and temperature program B (see Table 14, p. 31). Especially the separation of the cluster of peaks 3, 4 and 5 (see Figure 19, p.32) was not satisfying with the applied temperature programs.

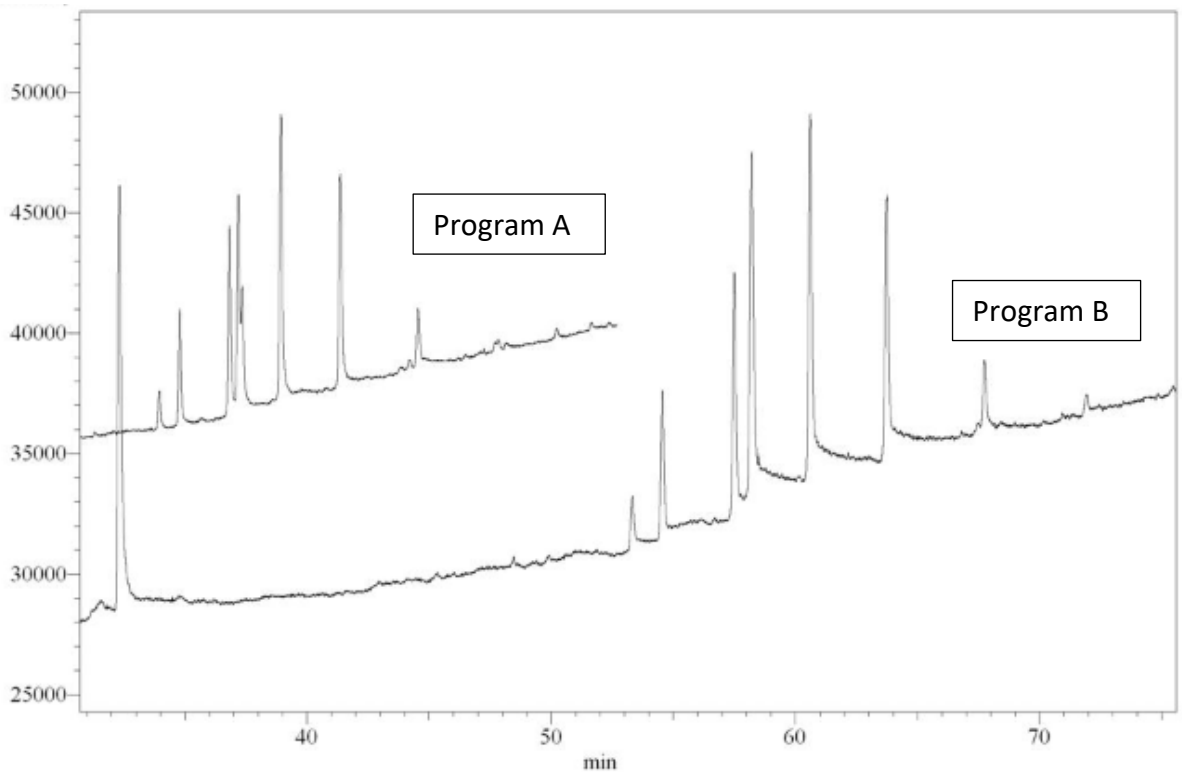

Figure 21: GC-FID analysis of RAMIX (see Table 1, p. 7); GC system: Table 7, p. 19; Derivatization method: Table 13, p. 30; Program A: Start-plateau of 5min 100°C, phase 1 100-200°C, rate 20°C/min, phase 2 200-270°C, rate 1°C/min (Table 14, p. 31)
Program B: Start-plateau of 5min 100°C, phase 1 100-170°C, rate 20°C/min, phase 2 170-250°C , rate 1°C/min (Table 14, p. 31)

To gain a better separation power the temperature gradient of phase 1 was reduced from 20°C/min to 10°C/min heating rate, while the gradient of heating phase 2 was increased from 1°C/min to 2°C/min (see following Figure 22, p. 34, program C, D, E). Program C (see Table 14, p. 31) seemed promising but was still not satisfying.

At this point it was decided to try different temperature gradients in heating phase 1 (20°C, 15°C, 10°C, 8°C), while heating phase 2 was not changed (see following Figure 23 p. 34, program F, G, H, I). Program G (see Table 14, p. 31) showed the best separation power of all tested gradients, it had to be accepted that the peak cluster 3,4,5 (see Figure 19, p. 33) was still not perfectly baseline separated.

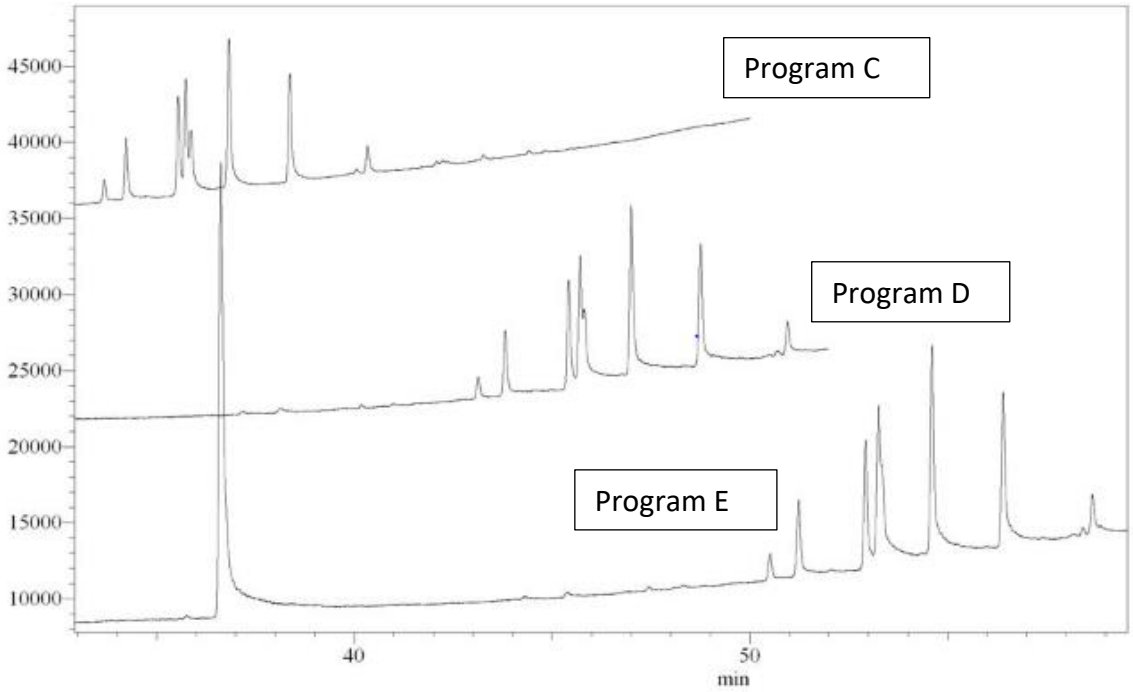

Figure 22: GC-FID analysis of RAMIX (see Table 1, p. 7); GC system: Table 7, 19; Derivatization method: Table 13, p. 30;
Program C: Start-plateau of 5 min 100°C, phase 1 100-200°C, rate 10°C/min, phase 2 200-270°C, rate 2°C/min (Table 14, p. 31)
Program D: Start-plateau of 5 min 100°C, phase 1 100-170°C, rate 10°C/min, phase 2 170-250°C, rate 2°C/min (Table 14, p. 31)
Program E: Start plateau of 5 min 100°C, phase 1 100-150°C, rate 10°C/min, phase 2 150-250°C, rate 2°C/min (Table 14, p. 31)

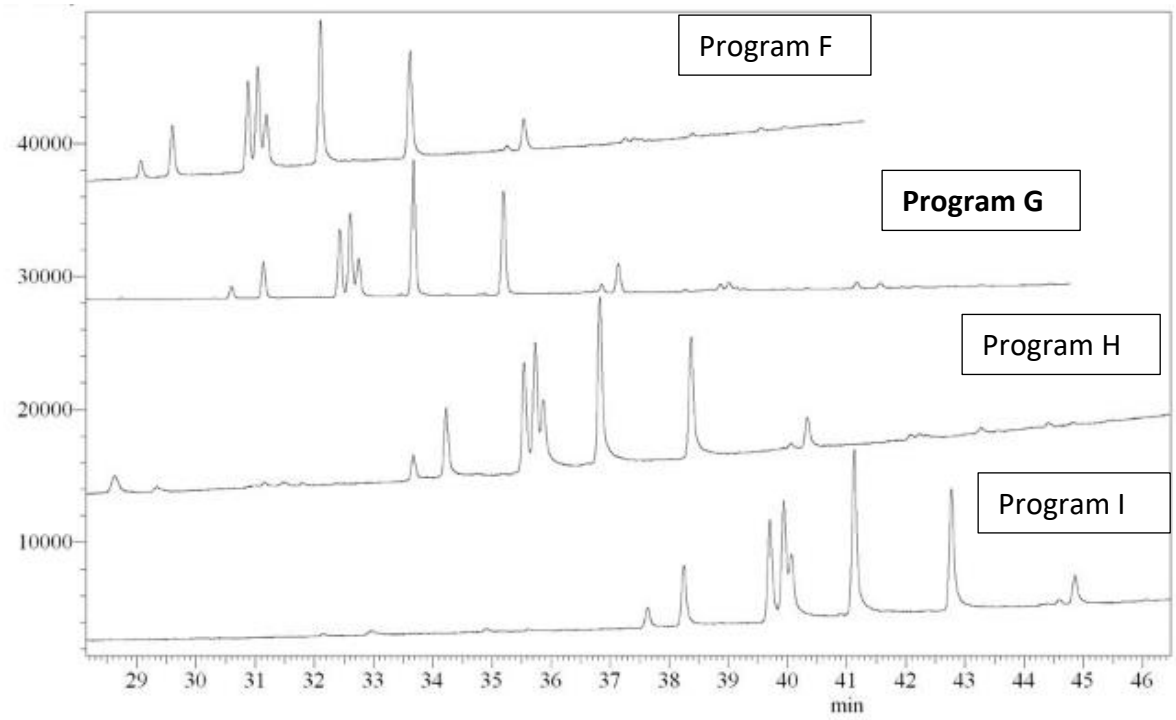

Figure 23: GC-FID analysis of RAMIX (see Table 1, p. 7); GC system: Table 7, p. 19; Derivatization method: Table 13, p. 30;
Program F: Startplateau of 5min 100°C, phase 1 100-200°C, rate 20°C/min, phase 2 200-270°C, rate 2°C/min (Table 14, p. 31)
***Program G: Startplateau of 5min 100°C, phase 1 100-200°C, rate 15°C/min, phase 2 200-250°C, rate 2°C/min** (Table 14)*
Program H :Startplateau of 5min 100°C, phase 1 100-200°C, rate 10°C/min, phase 2 200-270°C, rate 2°C/min (Table 14)
Program I: Startplateau of 4min 100°C, phase 1 100-190°C, rate 8°C/min, phase 2 190-260°C, rate 2°C/min ((Table 14)

4.3. Development of a column purge program

In the course of the method establishment it became apparent, that peaks of resin acids sometimes appeared even when injecting pure solvent. One reason could be due to contaminations of the autosampler syringe because of an insufficient purge program. But this was ruled out after injections of pure dehydroabietic acid by hand showed the same phenomenon (see Figure 25, p. 37).

Figure 24, p. 36 shows that with increasing numbers of injections of pure dehydroabietic acid the peak height ratio between dehydroabietic acid and the so called “ghost peaks” increased. The peak height of dehydroabietic acid increased, while the peak height of the “ghost peaks” decreased. It was assumed that the injection of the dehydroabietic acid sample had purged the GC-system. Therefore it was concluded that the injection of pure solvent between GC-analysis should help to get rid of resin acid residues as well. It required the implementation of a regularly applied purge program.

For the development of such a purge step different purge programs were tested (see Table 15, p. 35 program A - program E): Norway spruce balm GA02 (see Table 1, p. 7) was derivatized (see Table 13, p. 30) and injected in the GC-FID system (see Table 7, p. 19). The whole balm was used instead of the RAMIX extract to bring the analysis setting closer to the practical work in the lab. After the GC-FID analysis of Norway spruce balm GA02 different purge programs (see Table 15, p. 35) were executed and subsequently pure pyridine was analyzed the same way as Norway spruce balm before. If the chromatogram of pure pyridine was free of “ghost peaks” the purge step was successful.

Table 15: Overview of purge programs PA-PE

Purge programs			
Program name	**Number of 1 µl pyridine injections**	**Duration of 320 °C heating period**	
PA	4	3 min	
PB	3	5 min	
PC	2	10 min	
PD	3	10 min	
PE	**2**	**20 min**	**best effect**

All of the purge programs consisted of an isothermal temperature program at 320°C. They differed in duration of the heating period and the number of solvent injections. Figure 26,

Figure 27, Figure 28, p. 38 show GC-FID chromatograms of methylated resin samples and of a subsequently analyzed pyridine blank. Between the resin analysis and the pyridine injection a certain purge program was performed. The chromatograms demonstrate that only the purge program PE had a sufficient effect.

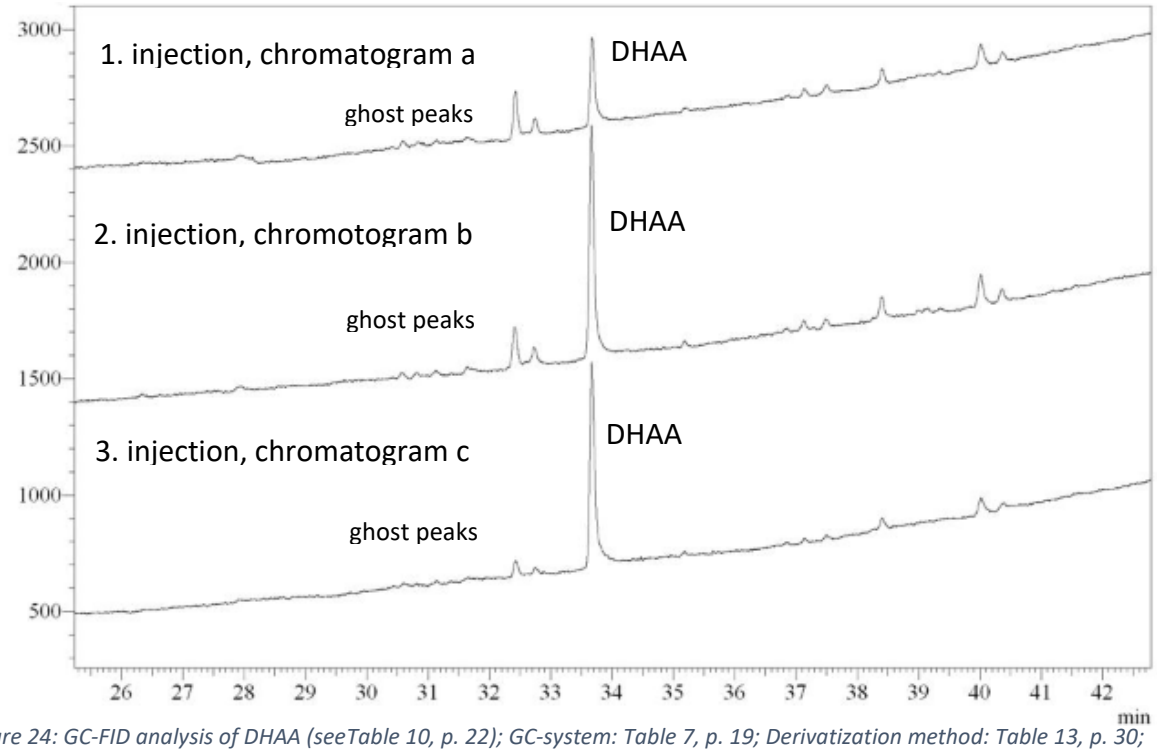

Figure 24: GC-FID analysis of DHAA (seeTable 10, p. 22); GC-system: Table 7, p. 19; Derivatization method: Table 13, p. 30; The same sample solution of dehydroabietic acid was analyzed three times in a row to show the changing ratio between the peak height of DHAA and ghost peaks.

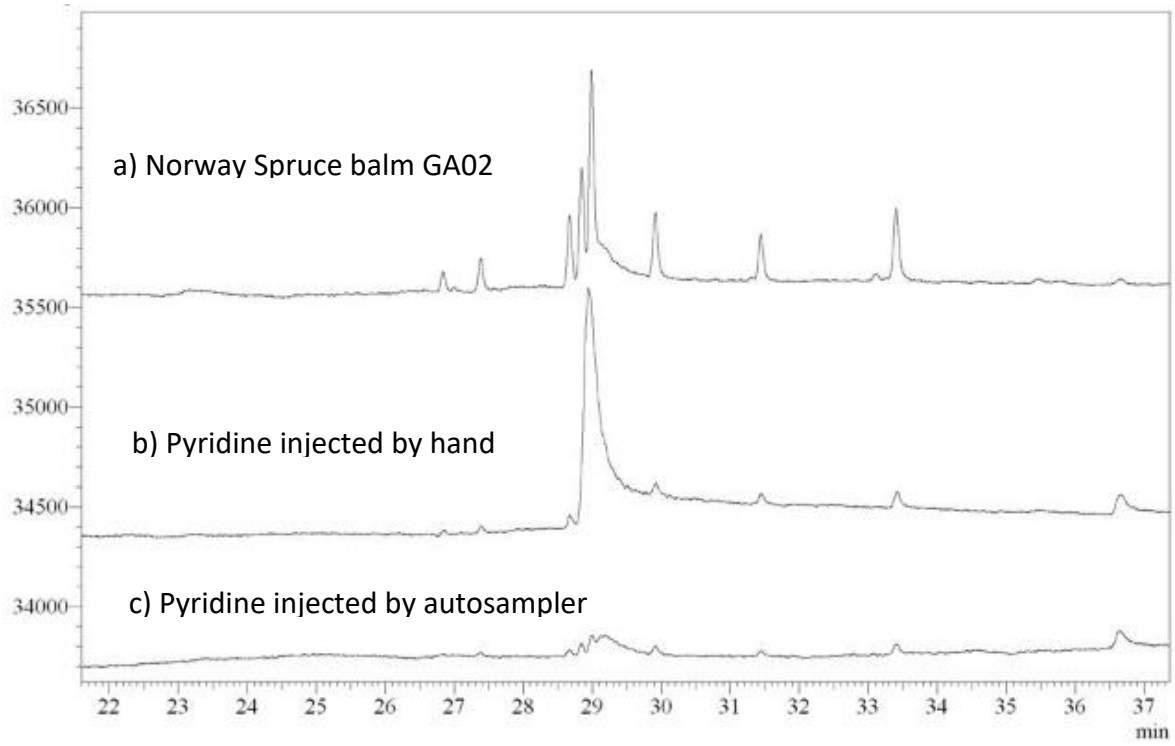

Figure 25: GC-system: Table 7, p. 19;
a) M8R derivatized (Table 13, p. 30) GA02 Norway spruce balm (Table 1, p. 7)
b) Pyridin injected by hand
c) Pyridin injected by autosampler

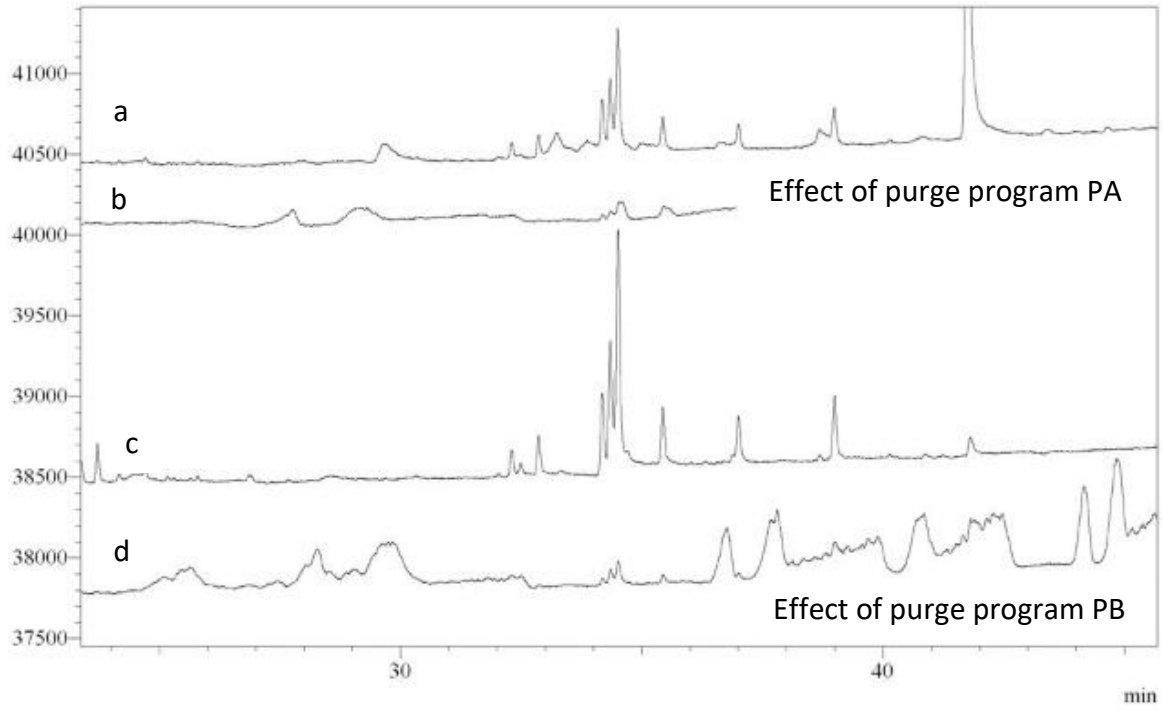

Figure 26: GC-FID system: Table 7, p. 19;
a) M8R derivatized (Table 13, p. 30) Norway spruce balm GA02 (Table 1, p. 7)
b) Pyridine injection after a purge program 4 times 3min 320°C (PA)
c) M8R derivatized (Table 13, p. 30) Norway spruce balm GA02 (Table 1, p. 7)
d) Pyridine injection after a purge program 3 times 5min 320°C (PB)

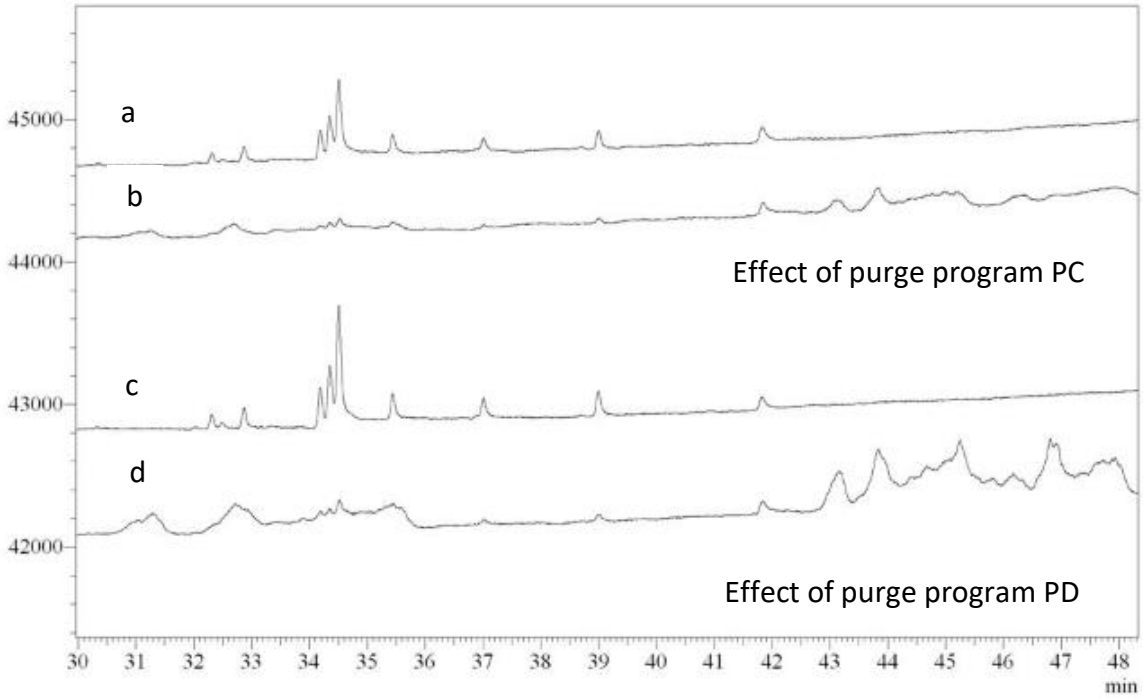

Figure 27: GC-system: Table 7, p. 19;
a) M8R derivatized (Table 13, p. 30) Norway spruce balm GA02 (Table 1, p. 7)
b) Pyridine injection after a purge program 2 times 10min 320°C (PC)
c) M8R derivatized (Table 13, p. 30) Norway spruce balm GA02 (Table 1, p. 7)
d) Pyridine injection after a purge program 3 times 10 min 320°C (PD)

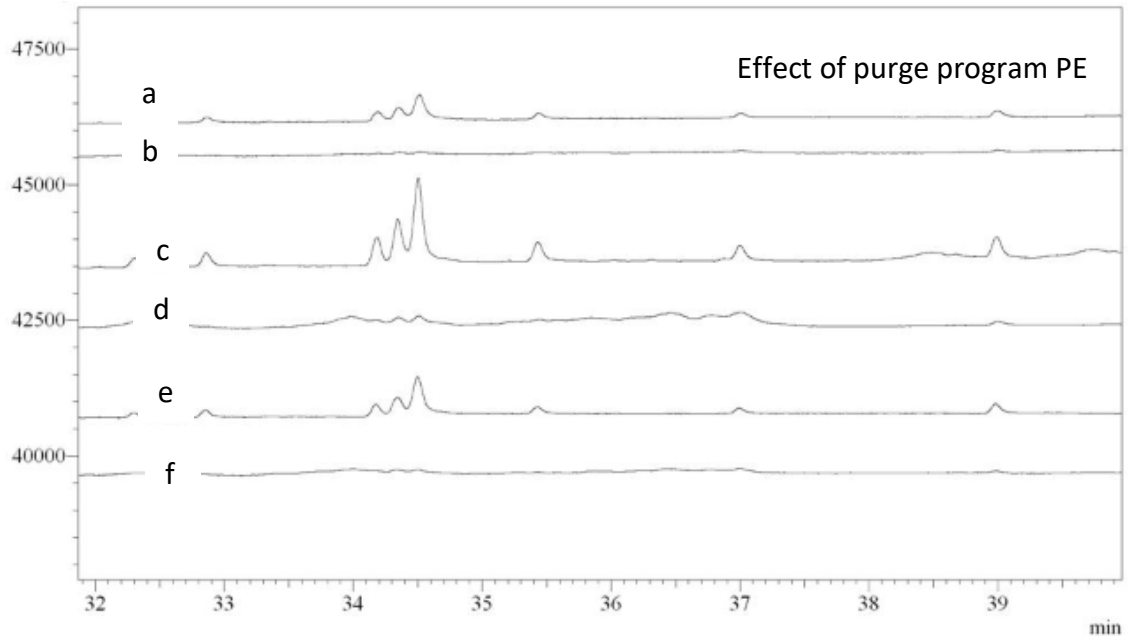

Figure 28: Effect of Purge program PE:
GC-system: Table 7, p. 19;
a) M8R derivatized (Table 13, p. 30) Norway spruce balm GA02 (Table 1, p. 7)
b) Pyridine injection after purge program of 2 times 20min 320°C (try1)
c) M8R derivatized (Table 13, p. 30) Norway spruce balm GA02 (Table 1, p. 7)
d) Pyridine injection after purge program of 2 times 20min 320°C (try2)
e) M8R derivatized (Table 13, p. 30) Norway spruce balm GA02 (Table 1, p. 7)
f) Pyridine injection after purge program of 2 times 20min 320°C (try3)

Developing a purge program that helps to avoid “ghost peaks” was important for a precise quantification method. It had to be accepted that it would almost double the analysis time, because of the additional purge step. At this point the M8-reagent derivatization method (see Table 13, p. 30), the temperature program G of the column oven (Table 14, p. 31) and the purge program PE formed the basis for a GC-FID quantification method adapted to the special setting of resin acids in Norway spruce balm. The next step was to choose a fitting internal standard substance for the quantification of resin acids.

4.4. Finding of a suitable internal standard

For the quantification of resin acids in Norway spruce balm the selection of an internal standard was necessary. The internal standard had the purpose to make the quantification of resin acids more robust and precise. It was assumed that it could compensate bias factors as variations of the autosampler injection volume or concentration changes caused by vaporizing solvents. At the beginning of the quantification analysis the internal standard was added to the sample, thus the standard substance is affected by the same interferences as the analyte. The substance had to be easily available, analyzable in the same GC-FID system as Norway spruce balm and it should possess a retention time close to the resin acids without overlapping.

The tested internal standard substances arachidic acid, palmitic acid and methyl palmitate were diluted in acetone. The concentration of this solution was exactly known, so it was possible to add an exact amount of internal standard to the resin acid sample using variable volume pipettes. After the acetone of the standard solution was vaporized the M8-reagent was added and the derivatization started (see Table 13, p. 30). Subsequent the derivatized sample mix was analyzed with the established GC-FID system of Table 7, p. 19.

Arachidic acid was considered as an standard substance option, but as the following Figure 29, p. 40 shows, it turned out that it overlaps with the peaks of the resin acids in Norway spruce balm.

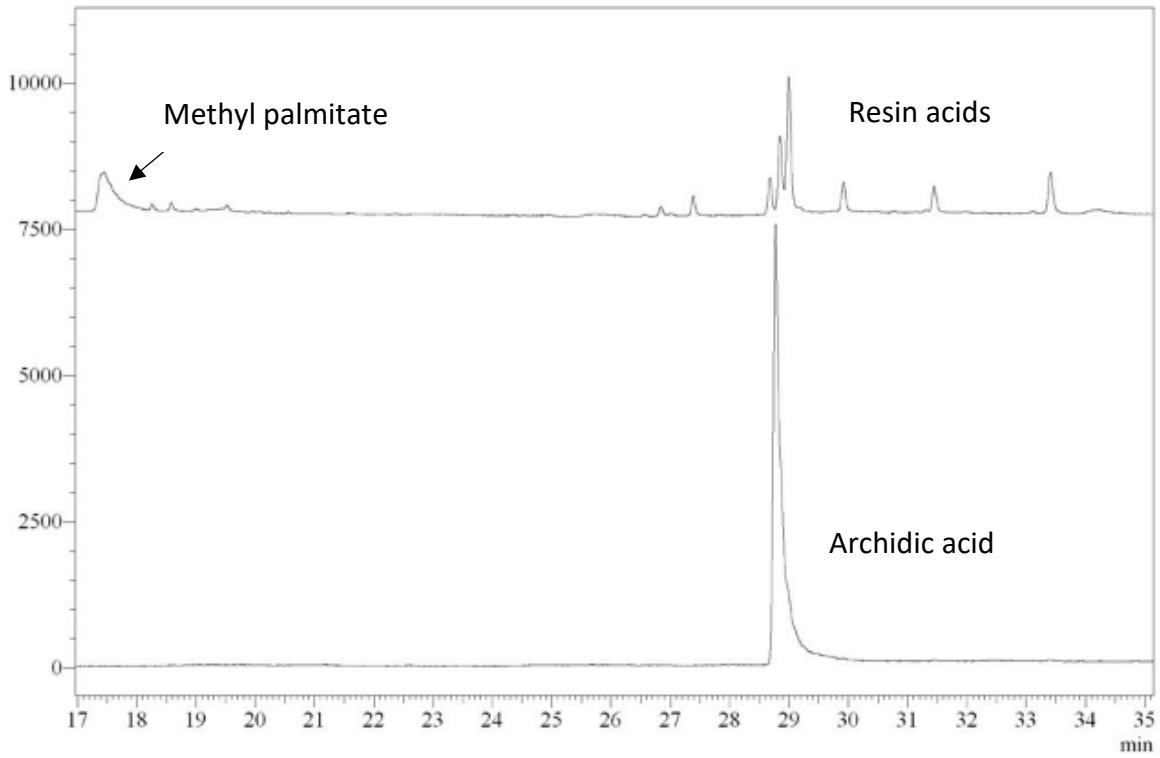

Figure 29:GC system: Table 7, p. 19; Derivatization method: Table 13, p. 30
c) Norway spruce balm GA02 (see Table 1, p. 7) + Methyl palmitate (see Table 10, p. 22)
b) 2mg/ml arachidic acid (see Table 10, p. 22)

The other two options were free palmitic acid and methyl palmitate, which is the methyl ester of palmitic acid. Both were mixed with the resin sample before the derivatization reaction with M8-reagent (see Table 13, p. 30). Free palmitic acid was methylated by M8-reagent. Methyl palmitate was not affected by the derivatization agent. As Figure 29, p. 40 shows, the peak of methyl palmitate fits well in the peak sequence of the Norway spruce balm chromatogram. A mass ratio of 1:10 of internal standard to Norway spruce balm showed similar areas under the curve of internal standard and resin acids. To find an optimal mass ratio between Norway spruce balm and internal standard, in Figure 30, p. 41 two different batches of balm with supposed different contents of resin acids were used (GA02 and FP2018, see Table 1, p. 7).

Table 16 p. 41 depicts the standard deviation of standard correction factors of methyl palmitate and palmitic acid. By using methyl palmitate instead of palmitic acid a lower standard deviation of the standard correction factors was achieved. For that reason methyl palmitate was more favorable as internal standard compared to palmitic acid. A potential explanation of the superior results of methyl palmitate compared to the un-esterified acid could be that the derivatization is not reproducible for fatty acids under the same reaction

setting as resin acids. However, other research groups have successfully worked with free fatty acids as internal standard and Methyl8-reagent as derivatization reagent before.[16]

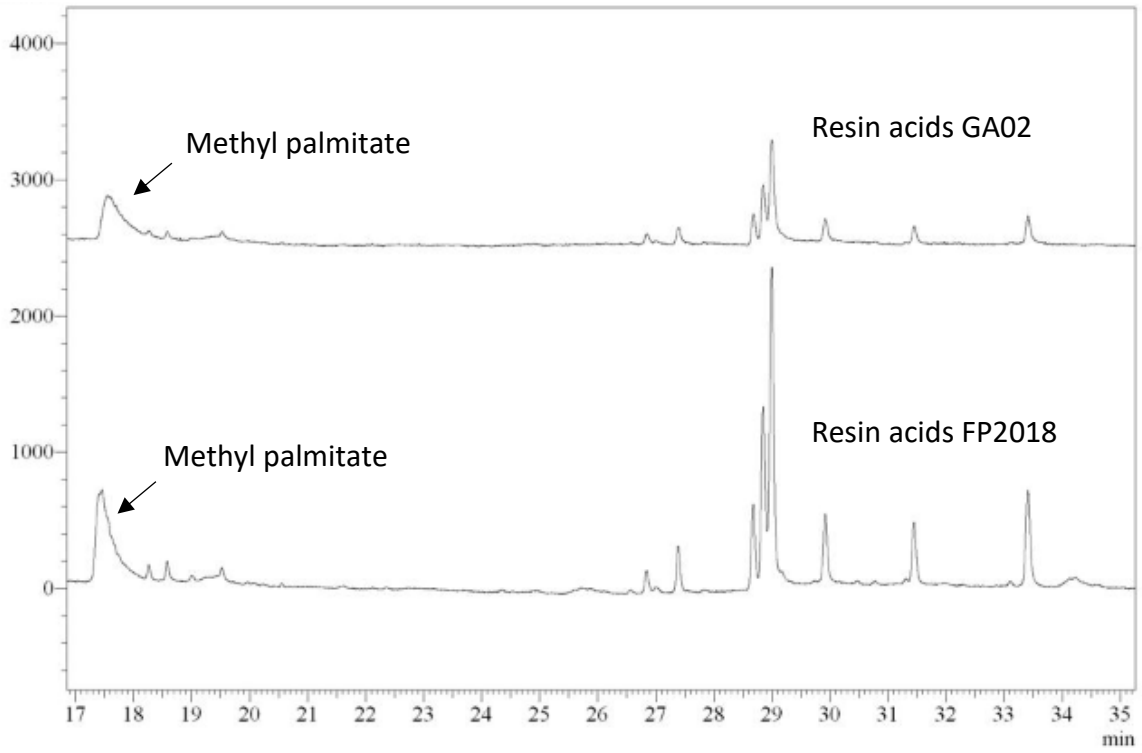

Figure 30: Derivatization reaction see Table 13; GC-FID system see Table 7;
GC-FID analysis of 1mg methyl palmitate (Table 10) + 10mg Norway spruce balm (GA02 or FP2018 in Table 1);

Table 16: Data analysis and comparison of the two different internal standard substances palmitic acid and methyl palmitate (see Table 10)

Palmitic acid				
mg of palmitic acid	**AUC IS**	**mg of DHAA**	**AUC DHAA**	**Standard correction factor**
0,1	408.788	0,05	71.946	2,84
0,1	397.790	0,5	1.468.068	1,35
0,1	419.982	0,5	1.545.134	1,36
0,1	275.597	1	3.235.891	0,85
0,1	429.502	1,5	5.203.052	1,24
0,1	403.094	1,5	4.737.706	1,28
0,1	568.589	2,8	11.195.418	1,42
			Standard deviation of standard cor. f.	
			16,50%	

Methyl palmitate				
mg of methyl palmitate	**AUC IS**	**mg of DHAA**	**AUC DHAA**	**Standard correction factor**
0,3	26.788	0,05	3.148	1,42
0,3	23.840	0,1	6.199	1,28
0,3	13.095	0,5	18.312	1,19
0,3	17.289	1	49.779	1,16
0,3	25.565	1,5	99.932	1,28
			Standard deviation of standard cor. f.	
			8,10%	

The parameter standard correction factor describes the ratio between the AUC of resin acid and internal standard. Calculating the standard correction factor, it has to be considered that the analyzed amount of standard substance and resin acid is not the same.

$$\frac{AUC\ internal\ standard}{AUC\ DHAA * \frac{mg\ IS}{mg\ DHAA}} = Standard\ \ correction\ \ factor$$

The choosen internal standard methyl palmitate allowed to define a standard correction factor for the resin acid dehydro abietic acid. Therefore, it was concluded that it should work with other resin acids as well, but further work is necessary to define standard correction factors for all different resin acids in Norway spruce balm. The SD variation of 8,1% and the asymmetrical peak form of the internal standard peak (see Figure 30, p. 41) had to be accepted. It was not possible to find a temperature gradient for the column oven, which generated a sharp methyl palmitate peak and separated the resin acids satisfyingly. The last step was to determine the concentration range, where the quantification method can be applied. Thus, a standard curve for dehydro abietic acid had to be generated.

4.5. Generation of a standard curve: Calibration of dehydroabietic acid

A standard curve was prepared as a starting point for the quantitative determination of the resin acids in various samples. Different weights of dehydroabietic acid were tested to examine the linear range of the area under the curve. In addition, the limit of detection (LOD) and the limit of quantification (LOQ) had to be determined. However, this intent could not be accomplished due to an issue with reproducibility of the AUC at concentrations below 0.05mg resin acid. Peaks were still detected and their height was measurable, but their AUC varied

too much. One attempt at an explanation could be that in spite of a purge program being performed, small amounts of resin residue from previous analysis biased the subsequent results. Especially analyses of samples with low concentrations of DHAA would be affected strongly. The following Table 17 p. 43 demonstrates the high deviation of the standard correction factor at concentrations below 0.05 mg and over 1.5 mg. Over 2.0 mg of DHAA solubility was a limiting factor. Conclusively the method works reproducibly between 0.05 and 1.5 mg of dehydroabietic acid. This range was marked green in Table 17, p. 43. In the green linear range the standard deviation of the standard correction factor was 7,98 %.

Table 17: Calculation of the standard correction factor for DHAA (see Table 10, p, 22) with internal standard methyl palmitate(see Table 10, p. 22)

AUC IS	AUC DHAA	mg DHAA	mg of methyl palmitate	Standard correction factor
43.075	484	0,002	0,3	0,59
10.408	446	0,003	0,3	0,23
10.850	898	0,005	0,3	0,2
23.686	1.166	0,01	0,3	0,68
26.788	3.148	0,05	0,3	1,42
23.840	6.199	0,1	0,3	1,28
13.095	18.312	0,5	0,3	1,19
17.289	49.779	1	0,3	1,16
25.565	99.932	1,5	0,3	1,28
45.698	352.442	2	0,3	0,86
11.296	214.124	4	0,3	0,7
3.946	48.105	8	0,3	2,19
3.848	95.107	16	0,3	2,16
			Average standard cor. factor	
			1,27	
			Standard deviation of st. cor. f.	
			7,98%	

Figure 31 shows the calibration curve of dehydroabietic acid.

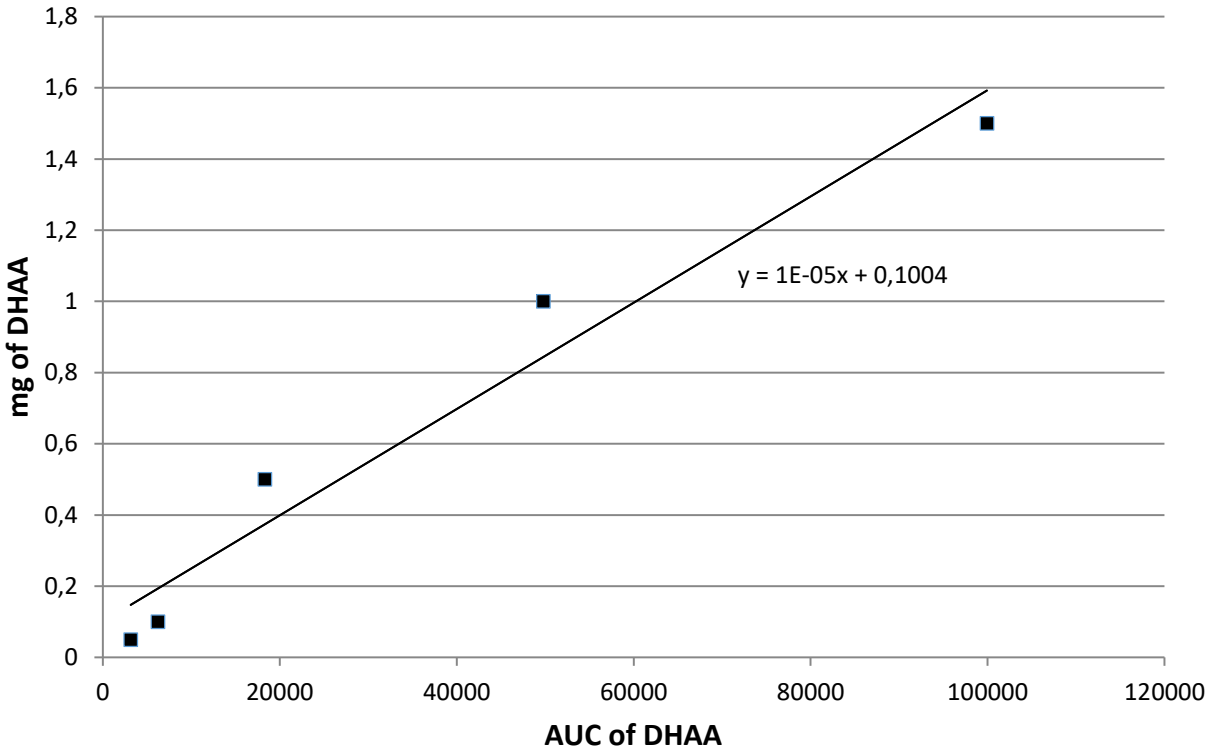

Figure 31: Calibration curve of dehydroabietic acid (see Table 10, p. 22) generated with the green marked values from Table 17, p. 43;

Accordingly to the presented results in Table 17, p. 43 it is possible to quantify dehydro abietic acid in a concentration range between 0,05 mg and 1,5 mg in different batches of Norway spruce balm. Further work is necessary to generate calibration curves for the other resin acids in Norway spruce balm.

5. Discussion and Conclusion

Norway spruce balm is a plant material, which has been traditionally used to treat skin wounds.[7] To substantiate the traditional knowledge the examination of the balm concerning its active components, their mechanism of action and also the quantitative composition is necessary.

The objective of this thesis was the establishment of a method to quantify resin acids from Norway spruce balm batches, which was successfully implemented. A derivatization method with a methylation reagent was established (see Table 13, p. 30). A gas chromatography column oven temperature gradient was developed (see Table 14, p. 31) and a purge step between the analysis was implemented in the quantification method (see Table 15, p. 35). Methyl palmitate was chosen as internal standard for quantification and a standard curve for dehydro abietic acid was generated. Naturally the analysis and especially the quantification of single substances in a complex mixture come with their own challenges. Even though a method could be successfully established some questions still remain:

The most important open questions relate to the accumulation of resin acids in the gas chromatograph. Resin acid peaks were registered after previous injections of Norway spruce balm, even when solely pure solvent was injected. Literature does not report about this phenomenon with resin acids. After every sample injection the execution of a purge program, which consumed more than 40min measuring time (see program PE in Table 15, p. 35), was necessary. Here in this work that purification step was essential to guarantee the absence of resin acid residue, while a publicized method exists, which quantifies resin acids without any purge program[1]. The source of the contamination remains unclear. It was possible to exclude the autosampler as culprit (see Figure 25, p. 37) and also the column could be eliminated as potential origin of pollution, since the resin acid peaks in balm samples and in blank injections appeared always with the same retention time. If the place of accumulation would have been the column, at least a little shift of the peaks would have been the logical consequence. Further investigations are needed to find the reason and the place of the impurity in the analytical setup. Maybe the understanding of this impurity would also lead to a more exact quantification, because even though the purge program PE was applied, the chromatogram of the pure solvent still displayed very low resin acid peaks (see Figure 28, p. 38). Longer purge

programs could be helpful, but in the practical laboratory work this would cost even more time and resources.

Another uncertainty concerns the methods of derivatization. Maybe other derivatization methods would generate products that are quantified more easily. During this work different methods of silylation and methylation were tested (see Table 11, p. 7): It was not possible to derivatize Norway spruce balm with the combination of BSTFA and TMCS, although there is literature mentioning this method[1]. HMDS + TMCS (1:1) is another known method of trimethylsilylation, but its products were not stable enough for a reasonable quantification. Regarding this thesis Methyl8-reagent proved to be the best suited derivatization method. Figure 17 and Figure 18 p. 29 demonstrate the stability of methylated resin acids over a period of ten hours.

In conclusion a method for derivatizing and quantifying resin acids in unprocessed Norway spruce balm samples as well as samples of other conifer balms like *Larix decidua* MILL, *Pinus nigra* J.F.ARNOLD or *Tetraclinis articulate* (Vahl) Mast. was successfully established.

6. Abstract

Norway spruce balm is a resinous excretion of *Picea abies* (L.) H. KARST. Due to its antimicrobial and wound healing effect, preparations from Norway spruce balm play an important role in the traditional medicine of Austria, of South Tyrol and Scandinavia. The antimicrobial effect has already been demonstrated for Methicillin resistant Staphylococcus aureus (MRSA) or Vancomycin resistant Enterococcus (VRE). However, the potential mechanism of action of the wound healing effect has not yet been elucidated. Nevertheless, this plant exudate could be helpful in times of increasing antibiotic resistances. As an alternative to conventional topical antibiotics, Norway spruce balm could maybe play a role in the prevention of wound infections, antibiotic resistances and the treatment of chronic injuries.

To understand the properties of this plant material more precisely, it is necessary to know its qualitative and quantitative composition. For the latter objective this diploma thesis was initiated. The goal was to develop a gas chromatographic method, which allows the

quantification of the group of diterpene resin acids such as abietic acid, dehydroabietic acid or pimaric acid in different Norway spruce balm batches.

Resin acids have apolar backbones and contain a carboxylic acid function, which requires derivatization to transfer them into volatile substances. Whereas silylation failed, the derivatization with methyl8-reagent was successful to enable the analysis via gas chromatography. The different diterpene resin acids of the abietic- and pimaric-type are very similar to each other (see Figure 2, p. 4), which made the chromatographic separation difficult and required an optimal methodology. It was necessary to carry out additional column purge programs between all the measurements in order to prevent the spruce balm constituents from accumulating in the gas chromatograph. However, this carry over effect is assessed as limitation of this method and has to be taken carefully into consideration regarding future quantification development. As a final step, a calibration curve for the quantification of dehydroabietic acid was generated. Methyl palmitate served as an internal standard.

7. Zusammenfassung

Fichtenfaulpech ist eine harzartige Ausscheidung von *Picea abies* (L.) H. KARST. Aufgrund seiner antimikrobiellen und wundheilungsfördernden Wirkung hat es einen hohen Stellenwert in der traditionellen Medizin Österreichs, Südtirols und Skandinaviens. Der antimikrobielle Effekt wurde an bestimmten Bakterienkulturen wie Methycillin resistenten Staphylococcus aureus (MRSA) oder Vancomycin resistenten Enterococcus (VRE) bereits gezeigt, aber besonders der Wirkmechanismus, welcher die Wundheilung beschleunigt, wurde noch nicht aufgeklärt. In Zeiten von wachsenden Antibiotikaresistenzen könnte es sich bei diesem, in der Volksmedizin bewährtem Pflanzenmaterial, um ein hilfreiches Werkzeug handeln, um den Einsatz von topisch angewendeten Antibiotika zu verringern. Dies wiederum könnte dazu beitragen das vermehrte Auftreten von nosokomialen Keimen zu verringern.

Um die Wirkung dieses Pflanzenmaterials genauer zu verstehen ist es notwendig, dessen qualitative und quantitative Zusammensetzung. Letzteres war das Hauptaufgabengebiet dieser Diplomarbeit. Es sollte eine gaschromatographische Methode erarbeitet werden, welche es erlaubt Vertreter der Stoffgruppe der Harzsäuren wie Abietinsäure,

Dehydroabietinsäure oder Pimarsäure in verschiedensten Fichtenfaulpechproben möglichst genau zu quantifizieren.

Bei Harzsäuren handelt es sich um sauerstoffhaltige Diterpene, somit war es notwendig diese schwer flüchtigen Moleküle mit Methyl8-Reagenz zu derivatisieren, um diese für die Methode der Gaschromatographie flüchtig genug zu machen. Die einzelnen Säuren dieser Molekülklasse sind einander sehr ähnlich, was die chromatographische Trennung erschwerte und nach einem speziellen Temperaturprogramm verlangte. Außerdem stellte sich heraus, dass zusätzliche Ausheizvorgänge des Säulenofens zwischen den einzelnen Messungen notwendig waren, um zu verhindern, dass sich Fichtenfaulpechbestandteile im Gaschromatographen anreicherten.

Nachdem die Vorgangsweise der Derivatisierung und das Temperaturprogramm feststanden, konnte eine Eichgeraden zur Quantifizierung der Harzsäure Dehydroabietinsäure erarbeitet werden. Als interner Standard wurde Palmitinsäuremethylesther ausgewählt. Die größte Hürde im Laufe dieser Diplomarbeit waren die Akkumulation von Harzsäuren im Gaschromatographen und die dadurch verfälschten Ergebnisse. Der genaue Ort und der Grund dieser Anreicherung konnten noch nicht genau definiert werden, allein ein verlängerter Ausheizschritt des Säulenofens konnte Abhilfe schaffen. Vielleicht kann in Zukunft eine zeit- und energie-sparendere Methode gefunden werden, welche eine Verfälschung durch Probenrückstände verhindert. Die Eichgeraden zu den restlichen Harzsäuren und weiterführende Untersuchungen werden als Teil von zukünftigen Diplomarbeiten ausgearbeitet.

List of figures

References

1. Holmbom, T., Reunanen, M. & Fardim, P. Composition of callus resin of Norway spruce, Scots pine, European larch and Douglas fir. *Holzforschung* **62**, 417–422 (2008).
2. Keeling, C. I. & Bohlmann, J. Diterpene resin acids in conifers. *Phytochemistry* **67**, 2415–2423 (2006).
3. https://www.alpengummi.at/ accessed 05.09.2020.
4. Fröstl, F. DIPLOMARBEIT / DIPLOMA THESIS, Titel der Diplomarbeit / Title of the Diploma Thesis: Ion exchange chromatography for the depletion of resin. *Univ. Vienna, Fac. Life Sci. Dep. Pharmacogn.* (2019).
5. Sipponen, A., Jokinen, J. J. & Lohi, J. Resin salve from the Norwegian spruce tree: a 'novel' method for the treatment of chronic wounds. *J. Wound Care* **16**, 72–74 (2014).
6. Rautio, M., Sipponen, R., Peltola, R., Lohi, J., Jokinen, J., J., Papp, A., Carlson, P., Sipponen, P. . Antibacterial effects of home-made resin salve from Norway spruce (Picea abies). *Apmis* **115**, 335–340 (2007).
7. Rautio, M., Sipponen, A., Lohi, J., Lounatmaa, K., Koukila-Kähkölä, P., Laitinen, K. In vitro fungistatic effects of natural coniferous resin from Norway spruce (Picea abies). *Eur. J. Clin. Microbiol. Infect. Dis.* **31**, 1783–1789 (2012).
8. Manfred H. Gey. *Instrumentelle Analytik und Bioanalytik, p. 310, p. 93, p. 62, p. 155*. (2008).
9. Jork, H., Funk, W., Fischer, W., Wimmer, H. *Thin-Layer Chromatography - Reagents and Detection Methods*. vol. 1a (1990).
10. Poole, C. F. The Essence of Chromatography. *J. Chem. Educ.* **80**, 883 (2003).
11. Ahuja, S. Derivatization in gas chromatography. *J. Pharm. Sci.* **65**, 163–182 (1976).
12. LaCourse, W. R. Column Liquid Chromatography: Equipment and Instrumentation. *Anal. Chem.* **74**, 2813–2832 (2002).
13. Sellers, K. & Corporation, R. Why Derivatize ? Improve GC Separations with

Derivatization. *www.restek.com* 2–3 www.restek.com/Patents-Trademarks (2010).

14. Göls, T., Saukel, J., Glasl, S. Separation and charachterization of resin acids in Norway spruce balm. *First Austrian Summit Nat. Prod. January 14th-16th, Seefeld, Austria* (2018).

15. Alankar Shrivastava, V. B. G. Methods for the determination of limit of detection and limit of quantitation of the analytical methods. *Chronicles of Young Scientists* vol. 2 21–25 (2011).

16. Peng, G. & Roberts, J. C. Solubility and toxicity of resin acids. *Water Res.* **34**, 2779–2785 (2000).

List of abbreviations

AA	Abietic acid (see Table 10, p.22)
ACN	Acetonitrile
AUC	Area under the curve
BSTFA	N,O-Bis(trimethylsilyl)trifluoroacetamide
DHAA	Dehydroabietic acid (see Table 10, p.22)
ELSD	Evaporative Light Scattering Detector
EtOH	Ethanol
FC	Flash Chromatography
FID	Flame ionization detector
FP2018	See Table 1, p. 7
GA02	See Table 1, p. 7
GC	Gas chromatography
GC-FID	Gas chromatography - Flame Ionization detector
HMDS	Hexamethyldisilazane
HPLC	High performance liquid chromatography
IPA	Isopropanol

LOD	Limit of detection
LOQ	Limit of quantification
MRSA	Methicillin-resistant Staphylococcus aureus
MS	Mass spectrometry
M8R	Methyl8-Reagent
MeOH	Methanol
$NaHCO_3$	Sodium bicarbonate
ÖAB	Austrian Pharmacopeia (Österreichisches Arzneibuch)
PDA	Photo Diode Array detector
RAMIX	Resin acid mix generated by Fanny Fröstl during the work for her thesis (See Table 1, p. 7)
R_f	Retention factor
SD	Standard deviation
SFC	Supercritical Fluid Chromatography
S/N	Signal-to-Noise ratio
St. cor. fac.	Standard correction factor
TLC	Thin Layer Chromatography
TMCS	Trimethylsilyl chloride
VRE	Vancomycin-resistant Enterococcus