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Quantitation of Bioactive Substances
in Different Norway Spruce Balm Samples

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"It is in the still silence of nature where one will find true bliss."

Unknown

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Abstract

The treatment of chronic wounds can be considered a complex and demanding process. In times of progressive antibiotic resistance, traditional phytotherapeutical approaches regain importance. The exudates of the Norway Spruce (*Picea abies* (L.) H.Karst.) represent a natural remedy with multi-targeted effects, which has been commonly used in folk medicine for impaired wound healing. To ensure consistent pharmaceutical quality, a method to determine the chemical composition is needed. Aim of this master's thesis was to analyse different Norway Spruce Balm samples, collected in various regions of Austria, and quantify their active components via high performance liquid chromatography coupled to UV-diode array detection (HPLC-UV-DAD). Therefore, three main components were chosen: the lignan pinoresinol and two diterpene resin acids, dehydroabietic acid and neoabietic acid. To ascertain the fundamental parameters necessary for quantitation, this research started with analysis of the pure substances. Subsequently, six spruce balm samples were examined, and the above mentioned components were quantified employing the validated method. Because of its natural origin, a heterogenous chemical composition of the balms had to be expected. Quantitation substantiated this assumption and confirmed diterpene resin acids as the main components, making up the largest amount in spruce balm.

Zusammenfassung

Die Behandlung chronischer Wunden stellt einen komplexen und herausfordernden Prozess dar. In Zeiten progressiver Antibiotikaresistenzen, rücken herkömmliche Ansätze der Phytotherapie erneut in den Fokus. Die Exsudate der Gemeinen Fichte (*Picea abies* (L.) H.Karst.) haben sich in der Volksmedizin bei schlecht heilenden Wunden bewährt und stellen ein natürliches Therapeutikum mit komplexer Wirkung dar. Um eine gleichbleibende pharmazeutische Qualität gewährleisten zu können, muss eine Methode entwickelt werden, um die chemische Zusammensetzung des Fichtenbalsams zu überprüfen. Ziel dieser Masterarbeit war es, mittels High Performance Liquid Chromatography und UV-Diode Array Detection (HPLC-UV-DAD) die wirksamkeitsbestimmenden Inhaltsstoffe unterschiedlicher Fichtenbalsamproben Österreichs zu analysieren und zu quantifizieren. Hierfür wurden drei wesentliche Komponenten ausgewählt: das Lignan Pinoresinol, sowie die Diterpenharzsäuren Dehydroabietinsäure und Neoabietinsäure. Diese kamen vorerst als Reinsubstanzen zum Einsatz, um die für die Quantifizierung notwendigen Parameter zu eruieren. Im Anschluss wurden sechs Fichtenbalsame analysiert und besagte Substanzen quantifiziert. Da die Balsame natürliche Produkte darstellen, war eine heterogene chemische Zusammensetzung zu erwarten. Dies hat sich im Zuge der Quantifizierung bestätigt, wobei Diterpenharzsäuren den bei weiten größten Anteil in Fichtenbalsam ausmachen.

List of Abbreviations

AUC	**A**rea **U**nder the **C**urve
DAD	**D**iode-**A**rray-**D**etector
HPLC	**H**igh **P**erformance **L**iquid **C**hromatography
HPLC-UV-DAD	**H**igh **P**erformance **L**iquid **C**hromatography with **UV-D**iode **A**rray **D**etection
LOD	**L**imit **O**f **D**etection
LOQ	**L**imit **O**f **Q**uantitation
NSB	**N**orway **S**pruce **B**alm
PSB	**P**urified **S**pruce **B**alm
ROS	**R**eactive **O**xygen **S**pecies
RP	**R**eversed **P**hase
SNR	**S**ignal-to-**N**oise **R**atio
TLC	**T**hin **L**ayer **C**hromatography
UHPSFC	**U**ltra **H**igh **P**erformance **S**olid **F**luid **C**hromatography

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1 Introduction

1.1 Human Skin and the Formation of Chronic Wounds

Our skin is one of the most essential parts of the human body. It protects us from harmful environmental damages, ensures thermoregulation, sensory perception and even helps us to produce Vitamin D. Anatomically, this largest organ of ours, can be divided into different layers, containing various cell-types and structures (1, 2).
The outermost layer, the epidermis, mainly consists of keratinised dead cells, building a solid physical barrier for protection. Straight beneath lies the dermis, made up of vascular structures and nerves embedded in a collagen matrix, providing nourishment and elasticity for the epidermis. The innermost part of the skin, the subcutis, holds loose connective tissue and fat, crucial for isolation purposes (1).

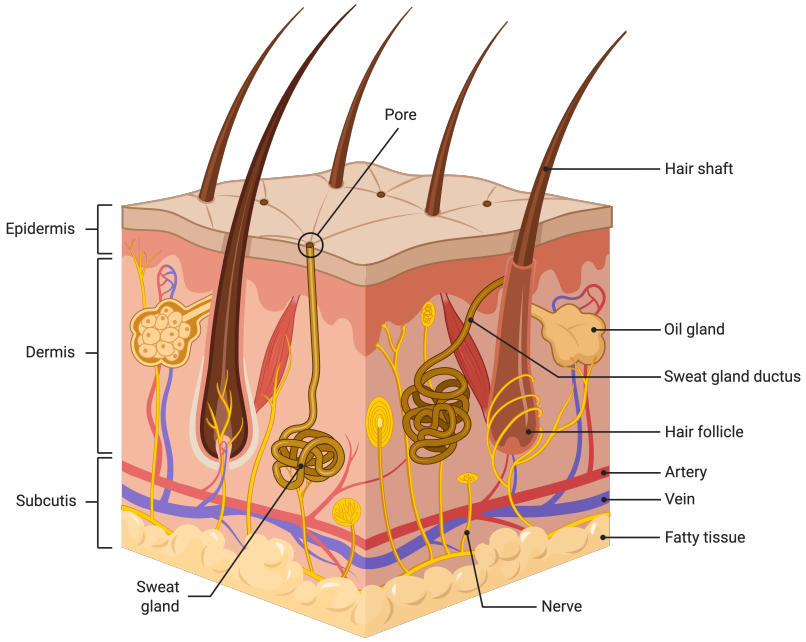

Figure 1: Anatomy of the Human Skin

When damaged, our skin shows the ability to repair itself. The physiological process of wound healing represents one of the most complex signalling pathways in our body. Hence, tight cooperation between cells and second messengers is required (2, 3). Depending on the type and size of the lesion, skin repair may require days or even weeks. Various risk factors like chronic diseases, infections or antibiotic resistance, can have a negative effect on wound healing processes, resulting in the

formation of chronic wounds. Since our population is progressively getting older, insufficient blood circulation and comorbidities contribute to this issue. As a result, patients with chronic wounds suffer from chronic pain, loss of limb function, poor sleep quality and require constant wound care, which is related to increased financial costs (1, 3).

Before investigating promising treatments, a fundamental understanding of the wound healing process is needed. Right after injury, hemostasis occurs, intending to stop the bleeding and prevent pathogens from distributing throughout our vascular system. Therefore vasoconstriction, the activation of platelets and synthesis of fibrin, help to create a local clot. Inflammation emerges, resulting in rapid production of cytokines, as well as reactive oxygen species (ROS) and various growth factors. Through the increased migration of immune cells, pathogens can be eliminated effectively, and angiogenesis is reinforced. Lastly, novel tissue is formed due to chemical stimulation of epithelial cells and fibroblasts, restoring the physiological skin barrier (2, 4).

1.2 The Role of Medicinal Plants in Wound Healing

In traditional medicine, plants have always been of significant importance. One of the most common issues, the treatment of wounds, turned out to be among the first indications in ancient phytotherapy (1).The benefits of medicinal plants are manifold: Not only are they derived from nature itself, but also show a complex phytochemical composition, resulting in multi-targeted pharmacological effects. Since there is no medicinal plant producing only one single active ingredient a broad spectrum of metabolites is responsible for their activity (1, 5).

In addition, phytotherapeutical treatments obviate drug-resistance mechanisms, since they influence many different endogenous pathways simultaneously. Nowadays, numerous *in vitro* and *in vivo* studies substantiate the use of medicinal plants for better wound healing (1, 5).

Prof. Johannes Saukel, who worked as a long-time professor at the Division of Pharmacognosy (University of Vienna), initiated a database for native plants used in Austrian folk medicine. The so called VOLKSMED-database (6-8) contains commonly known species, as well as unusual medical approaches, that were passed on for many generations. Elisabeth Eichenauer studied the myriad entries of this database as part of her PhD-research. In her doctoral thesis "*Traditional Austrian Herbal Medicines for Wound Healing - Analytics and Bioactivity*", she mentions the most used species for wound healing: *Hypericum sp.*, *Calendula sp.* and *Arnica sp.* The majority belong to the family of Asteraceae. Pinaceae, however, prevailed to be one of the rather atypical plant families with remarkable potential for treating wounds (1), as described in the following chapter.

1.3 Norway Spruce Balm (NSB)

The Norway Spruce, also called *Picea abies* (L.) H.Karst., is one of the most common native trees in large parts of Europe. The species of *Picea* belongs to the family of Pinaceae, together with other well-known conifers, such as the European Silver Fir (*Abies sp.*) or the European Larch (*Larix sp.*). It thrives in relatively cool to moderate climates and requires consistent moisture from the surrounding soil. Some specimens can reach hights up to 50 meters, making it one of the largest trees in our latitudes (1).

The use of conifers for treating wounds has a long-standing history. Ancient Greeks and Romans were one of the first to take advantage of their healing effects. Over the last century, many traditional sources reported the use of spruce exudates in folk medicine. To protect itself from dangerous pathogens and fungi, the tree produces different substances, referred to as resins and balms. The difference between the two might seem vague prima facie but can be explained simply: Resin is a glutinous fluid of amber colour, running down the tree bark in thin streaks before hardening. The balm however is of sticky, soft and mouldable consistency, additionally containing substances like essential oil and various other active ingredients, explained later in this chapter (3, 9).

When comparing it to the human body, resin could be seen as an equivalent of blood, whereas the balm serves as a self-produced wound sealant, protecting the lesion. Prior to use, natives reportedly purified the spruce balm with boiling water, before preparing an ointment with lard. The crude plant material (shown in Figure 5) still containing elements of the bark, needles or living organisms, was put in a linen cloth and hung in a pot of boiling water. Purpose of this step was to purify the balm, as well as to eliminate some of the essential oil, that could be too aggressive on the wounded area. After a few minutes, the balm changed its consistency from solid to viscous, penetrated through the fabric and gathered at the bottom of the pot. When cooled down, the remaining water was decanted exposing the solid "purified spruce balm" (PSB) as illustrated in Figure 2, which was further on gently dried at 40°C (1, 5).

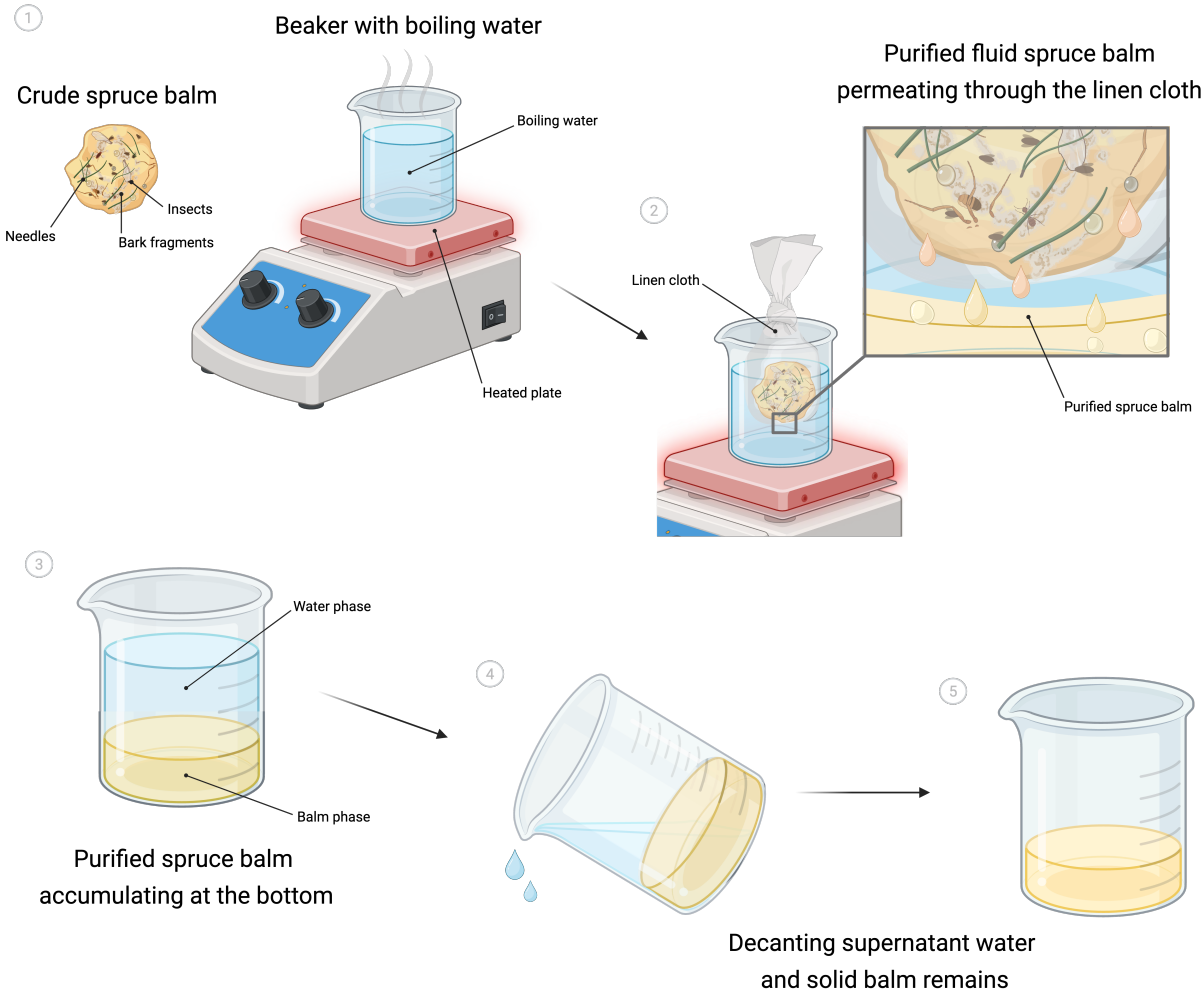

Figure 2: Traditional Purification Process of the Norway Spruce Balm

The Norway spruce balm shows a diverse chemical composition, covering a wide range of polarities. Hydroxycinnamic acids (like ferulic acid, caffeic acid or p-coumaric acid) and lignans (pinoresinol) are some of the polar substances, while diterpene resin acids (DRAs) represent the apolar structures, making up the main part of constituents (Figure 3). The occurring DRAs differ in their stereochemistry and position of their double bounds. Seven of them even show the same molecular weight (306 amu), making further analytical separations challenging (1, 3). This work focused on the three substances pinoresinol, dehydroabietic acid and neoabietic acid, with neoabietic acid representing the beforementioned seven DRAs with equal amu.

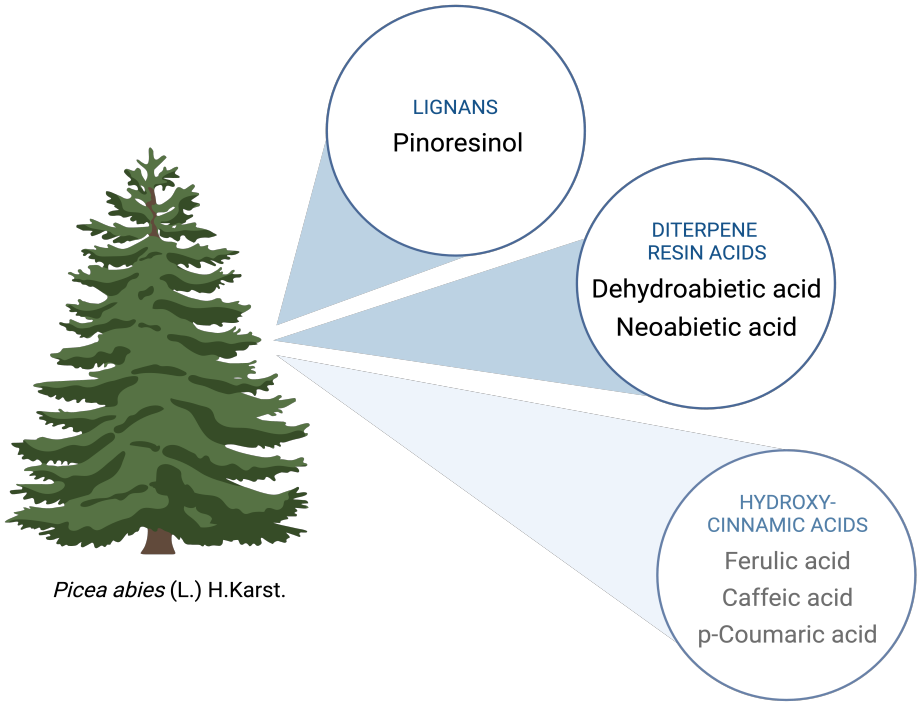

Figure 3: Chemical Composition of the Norway Spruce Balm

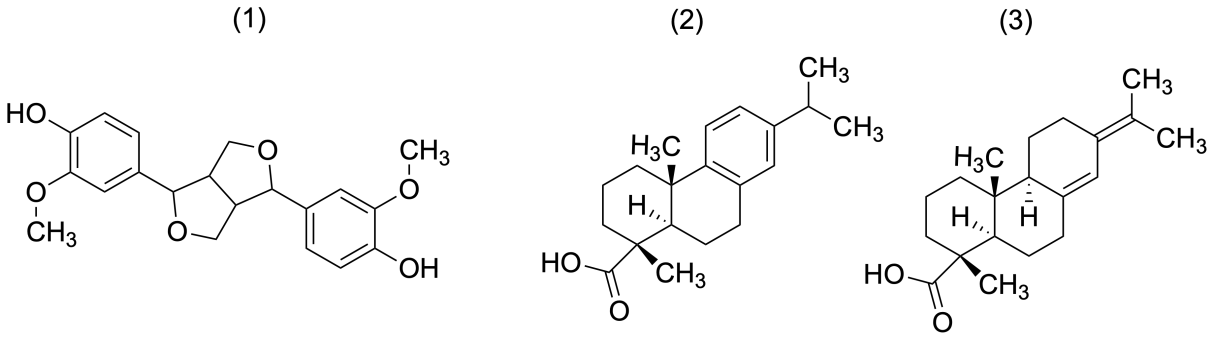

Figure 4: Chemical Structures of Pinoresinol (1), Dehydroabietic Acid (2) and Neoabietic Acid (3)

2 Aim of this Work

The Norway spruce balm, a resinous exudate of *Picea abies* (L.) H. Karst., has a long-standing use in traditional medicine to treat wounds. This research aimed to assess the varying composition of active components in the spruce balm, providing a useful tool for pharmaceutical quality control. This is particularly relevant, since natural products display a heterogenous chemical composition, requiring predefined quantity limits for consistent pharmaceutical quality. Three substances were chosen for analysis and quantitation: pinoresinol, dehydroabietic acid and neoabietic acid. The method of choice was high performance liquid chromatography with UV-diode array detection (HPLD-UV-DAD) which was validated with the pure substances pinoresinol, dehydroabietic acid, and neoabietic acid employing isoferulic acid as internal standard. Validation analyses were followed by quantitation of six spruce balm samples, collected in various regions of Austria.

3 Materials and Methods

3.1 Plant Material

Different Norway spruce balm samples were available at the division of Pharmacognosy, University of Vienna. They had been collected from various locations in Austria over the time period of 2010-2017, by Johannes Saukel, Thomas Göls, Sabine Glasl-Tazreiter and the Austrian Federal Forests. The samples are summarised in Table 1 - six of them were selected for quantitation analyses within the frame of this thesis. The crude plant material was stored in plastic and glass receptacles under light-protection at 4°C temperature.

Table 1: Origin of the Investigated Spruce Balm Samples

Sample number	Harvest location	Date of harvest
NSB01-NSB08	Promau Kitzhütte, Hollenstein, Lower Austria	09.07.2016
NSB09-NSB12	Gaflenz Heiligenstein, Upper Austria	03.07.2016
NSB13	Lungau, Salzburg	2015
NSB14	Zillingtaler Wald, Burgenland	15.05.2016
NSB15	Zillingtaler Wald, Burgenland	15.05.2016
NSB16	Pinsdorf, Upper Austria	Spring 2016
NSB17	unknown (Austria)	10.07.2015
NSB18	Lungau, Salzburg	24.08.2016
NSB19	unknown (Austria)	2010-2016
NSB20	unknown (Austria)	2010-2016
NSB21	unknown (Austria)	2010-2016
NSB22	Lungau, Salzburg	23.08.2017
NSB23	Schellgaden, Lungau, Salzburg	10.07.2021

Figure 5: Excreted Spruce Balm by the Tree´s Trunk, Harvesting and Harvested Crude Plant Material (10, 11)

Before usage, the balms were acclimated to room temperature, ensuring easier handling, since the cooled plant material maintained too solid in consistency. As shown in Figure 5, the crude material contains various impurities, such as fragments of the bark, needles or insects, resulting in varying appearances and colours. Therefore, centrifugation prior to analysis was required (see 3.2.2).

3.2 Sample Preparation

3.2.1 Dilutions of Internal Standard and Reference Substances

For the internal standard, a stock-solution containing 6,0 g isoferulic acid in 30,0 ml acetone was prepared to reach a concentration of 0,2 mg/ml. When used for analyses together with reference substances or spruce balms, 500 µl of the stock-solution got diluted to reach a final concentration of 0,1 mg/ml internal standard in acetone.

For determination of the response factors, stock-solutions of each of the three pure substances pinoresinol, dehydroabietic acid and neoabietic acid in acetone were prepared. Concentrations used in previous work (1) were considered as reference values. The respective stock-solutions were used for different dilution stages (see Appendix I-III) containing various amounts of the pure substance and a constant concentration of the internal standard isoferulic acid (0,1 mg/ml). 15 different dilution stages within the range of 0,075-0,00001mg/ml for pinoresinol and 0,3-0,00001 mg/ml for dehydroabietic acid, as well as 9 concentrations within the range of 1-0,001 mg/ml for neoabietic acid were prepared. Detailed information can be found in the attachments (I-III). Each sample got injected into the system and analysed for 3 times in a row, with acetone-blank measurements in between, to ensure steady conditions for all measurements. The whole cycle was repeated for another 2 times, resulting in 9 analyses (three cycles in triplicates) of each dilution stage (see Appendix I-III). Subsequently, peak areas could be determined with the resulting chromatograms, serving as important data for the calculation of the average noise, necessary for the evaluation of the later explained limits for detection and quantitation (4.2).

3.2.2 Dilutions of Balm Samples

To examine the composition of relevant bioactive substances, the following preparations of the balms were necessary: After weighing 1,0 g of crude balm material in Erlenmeyer flasks, 10,0 ml acetone served as the organic solvent. Sufficient dissolutions were provided by manual shaking of the mixture for about 1-2 min at room temperature. As shown in Figure 7, complete dissolution could not be achieved. Some impurities of the samples remained undissolved and were removed by centrifugation, resulting in a supernatant of dissolved crude spruce balm. This solution was regarded as corresponding to 1,0 g crude balm in 10,0 ml acetone. Based on this concentration, aliquotes were taken (25 µl) to yield an amount of 2,5 mg/ml crude spruce balm, to which the 500 µl stock-solution of internal standard (0,2 mg/ml isoferulic acid in acetone, see 3.2.1) and 475 µl acetone were added. The sample analysis finally contained 2,5 mg crude spruce balm and 0,1 mg isoferulic acid in 1 ml acetone. This procedure was repeated for all six investigated balm samples (NSB03/04/06/ 12/13/23, see Table 1). Detailed information about the exact weighed amounts, can be found in Appendix IV. Figure 6 illustrates the described workflow used for the phytochemical analysis of spruce balm material via HPLC-UV-DAD.

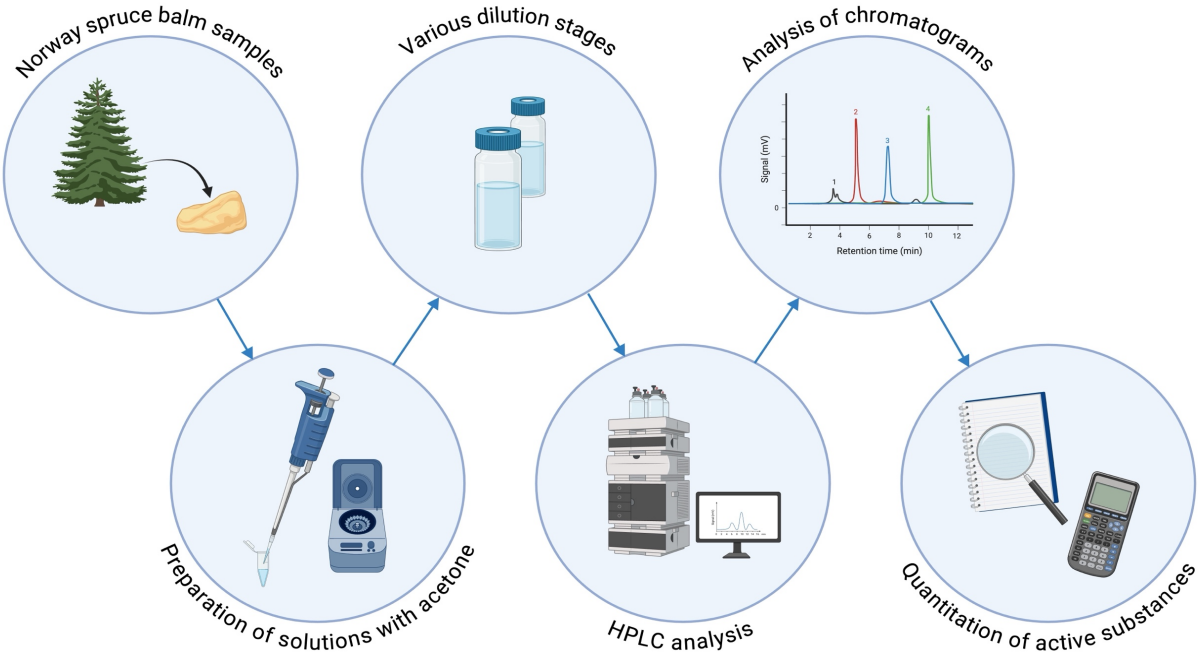

Figure 6: Display of the Workflow Used for Analysing Norway Spruce Balm Samples via HPLC-UV-DAD

Figure 7: Spruce Balm Samples Before and After Dilution

Since acetone is highly volatile, all tubes and vials had to remain closed after usage. After undergoing analysis, perforated caps needed to be changed to prevent future deviations of concentration due to the evaporation of acetone. Due to the tempered HPLC rack, the tested vials remained cooled while undergoing analysis and had to be refrigerated afterwards (under 4°C), to preserve stability if used again. Isoferulic acid, a substance of natural origin, was chosen as the internal standard, even though its stability at room temperature had been proven to be moderate (see below). The main reasons for its use include its accurate detectability, chemical similarity to some of the investigated components (pinoresinol), as well as its proper retention time, preventing overlaps with other peaks in the chromatogram. However, empirical findings during this work indicated a temperature dependent instability of isoferulic acid in acetone. When stored at room temperature for too long (over hours), degradation processes were detected, resulting in peak splitting, as visualised in the following HPLC chromatogram (Figure 8). Hence, immediate and persisting cooling of the solutions is of significant importance. Careful storage and handling at 4°C prevent degradation and warrant stability.

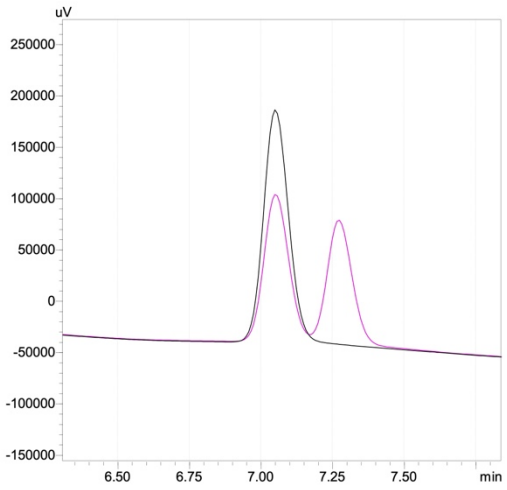

Figure 8: HPLC-UV-DAD Chromatogram of Stable (black, stored at 4°C, analysed immediately) and Instable (pink, stored at room temperature, analysed after 24 hours) Isoferulic Acid at 190nm

3.3 High Performance Liquid Chromatography (HPLC)

For this work, high performance liquid chromatography was chosen as the analytical method. Owing to its capacity to accurately analyse a wide range of compounds in minimal sample amounts, it is commonly used for separation, identification and quantitation purposed in various fields of science. The principal of HPLC is simple: A solvent, also known as the mobile phase, gets pushed through a column, representing the stationary phase and filled with packing material (mostly modified silica gel). Following the laws of chromatography, the injected analyte can be separated due to chemical interactions with the material in the column. (12)

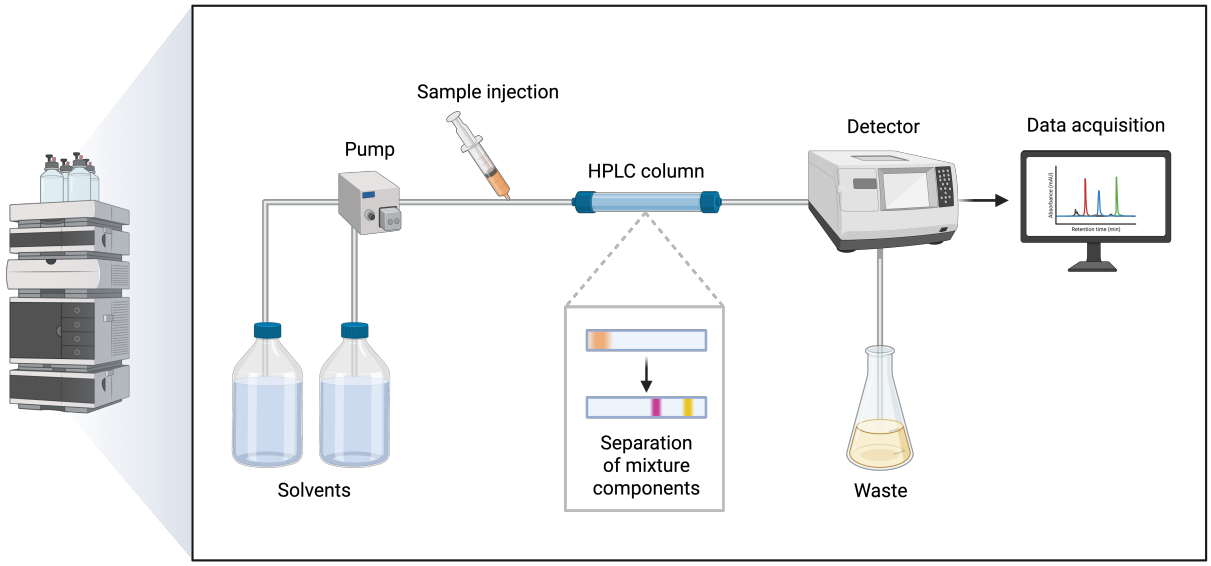

Figure 9: Setup of a High Performance Liquid Chromatography (HPLC) Instrument

Considering the types of columns and solvents, normal-phase and reversed-phase analyses can be distinguished. Reversed-phase (RP) HPLC uses polar solvents together with an unpolar solid phase (RP-18/C18 columns), holding back unpolar molecules for much longer. In contrast, normal-phase systems consist of unpolar solvents and a polar solid phase, chemically modified with amino, cyano or diol groups to retain polar molecules. (13) After passing the stationary phase, the mobile phase reaches the detector, connected to a computer station. Depending on the analysis, different detector types can be used. In the present work, a UV/Vis-diode array detector (UV-Vis-DAD) was used, since it can detect a wide range of substances and additionally record UV-spectra. After reaching the detector, an electric signal is

produced and transferred to the computer, creating the desired chromatogram. (12) To improve peak shape and separation, as well as to reduce the time of analysis, gradient elution can be performed. In this process, two mobile phases, differing in polarity, help to create a concentration gradient with higher elution power. The changing composition of the two solvents can be navigated using a computer software. A reversed-phase system was used for this work, considering its optimal compatibility with a UV-Vis-DAD and various solvents. Moreover, better reproducibility is ensured, making reversed phase more appropriate for quantitation purposes. The exact software and parameter configurations used, are shown in Table 2.

Table 2: HPLC Instrument and Parameter Configuration

Instruments	SHIMADZU Auto Sampler SIL-20AC HAT SHIMADZU Column Oven CTO-20AC SHIMADZU Communications Bus Module CBM-20A SHIMADZU Degasser DGU-20A5 SHIMADZU Diode Array Detector SPD-M20A SHIMADZU Liquid Chromatograph LC-20AD
Software	SHIMADZU Lab Solutions
Temperature	25°C column oven
Stationary Phase	Acclaim© 120 C18, 3 µm, Size: 150 mm x 2,1 mm
Mobile Phases	Solvent A: double distilled water + 0,1 % formic acid Solvent B: acetonitrile + 0,1 % formic acid
Injected Volume	1 µl
Analysis Time	25 min
Gradient Elution	15-95 % Mobile Phase B
Flow Rate	0,3 ml/min
Detecting Wavelength	190 nm

3.4 Solvents, Reagents, Substances

For this work, acetone was used as the solvent. Due to its high solubility properties, a wide range of compounds with different polarities can be dissolved, substantiating its common use in phytochemical analytics. Especially phenolic substances, such as hydroxycinnamic derivatives, as well as diterpene resin acids and lignans can be

extracted sufficiently. Since those compounds are particularly present in the Norway spruce balm, acetone turned out to be the solvent of choice (1, 14).

For the chromatographic analysis, gradient elution was performed, as described in the previous chapter 3.3. The two mobile phases consisting of double distilled water with 0,1% formic acid (A) and acetonitrile with 0,1% formic acid (B) ensured an effective separation. Acetonitrile is representing one of the most used mobile phases in RP-HPLC, due to its low UV absorbance and high elution strength (15).
Formic acid has proven to refine peak shapes and establish a better peak separation, when added in small concentration, especially if the analytes exhibit acidic character as this is the case with diterpene resin acids (16).

The pure substances pinoresinol, dehydroabietic acid and neoabietic acid, dissolved in acetone, were analysed beforehand to evaluate fundamental HPLC parameters, such as the limit of detection (LOD) and the limit of quantification (LOQ), necessary for the validation of the method and explained in the subsequent chapter 4.

Table 3: List of Reagents Used in This Thesis

Solvent	LOT	Manufacturer	Purity
Acetone	22A074021	VWR Chemicals	
Acetonitrile	23G244009	VWR Chemicals	
Dehydroabietic acid	---	Division of Pharmacognosy, University of Vienna	99,3%
Formic acid	297256288	Carl Roth GmbH + Co. KG	≥ 98%
Isoferulic acid	55610	Funka Analytical	
Neoabietic acid	C1518	Santa Cruz Biotechnology, Inc.	99%
Pinoresinol	CQV894	BLD PHARMATECH GmbH	95%
Water	double distilled water	distilled with GFL 2004, Division of Pharmacognosy, University of Vienna	

4 Results

4.1 Calculation of the background noise level

To determine the detection limits the detector's level of background noise must be calculated. Therefore, nine acetone blank runs were analysed under previously described conditions (Table 2), as to measure the changes in baseline height during random intervals. Three random intervals with 1 minute of each run were determined (see Table 4) and all 27 values were averaged. The calculations yielded a mean value of approximately 46,0 mV. Usually UV-Vis detectors show a measurable noise level, clearly visible in the enlarged chromatogram. Since this was not the case with the instrument used, height differences during these intervals were identified:

Table 4: Determination of the Mean Background Noise-Level

Run	Interval [min]	Height [mV]	Run	Interval [min]	Height [mV]	Run	Interval [min]	Height [mV]
1	13:00-14:00	31	4	08:00-09:00	57	7	11:00-12:00	48
1	18:00-19:00	50	4	17:00-18:00	34	7	18:00-19:00	34
1	25:00-26:00	42	4	24:00-25:00	41	7	28:00-29:00	55
2	08:00-09:00	62	5	09:00-10:00	62	8	05:00-06:00	68
2	12:00-13:00	37	5	12:00-13:00	41	8	17:00-18:00	40
2	17:00-18:00	38	5	24:00-25:00	43	8	26:00-27:00	63
3	10:00-11:00	35	6	11:00-12:00	45	9	08:00-09:00	63
3	16:00-17:00	40	6	20:00-21:00	32	9	10:00-11:00	57
3	24:00-25:00	33	6	24:00-25:00	43	9	25:00-26:00	49
							Mean	**46,0**
							Standard deviation	± 10,93

4.2 Limit of Detection (LOD) and Limit of Quantification (LOQ)

Before measuring the respective amounts of active substances in the spruce balm samples, the system's limitations had to be verified. The *limit of detection* or LOD represents the minimal concentration of a substance, that can be detected by the

instrument. However, this term should not be confused with the methods *sensitivity*, which is an indicator for the capability to detect minor differences in data. On the other hand, the *limit of quantitation*, LOQ, illustrates the minimal concentration of a substance allowing for quantitation with an adequate degree of certainty (17, 18). LOD and LOQ are determined by an essential parameter, the signal-to-noise ratio (SNR). It defines the relation between the level of a desired signal in correlation to its background noise, as illustrated in the following Figure 10.

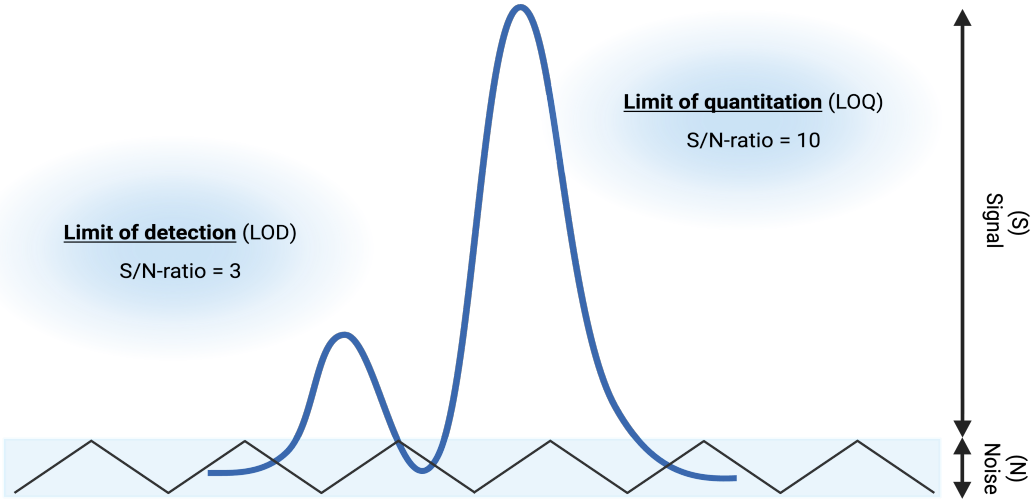

Figure 10: Visualisation of the Signal-to-Noise Ratio (SNR) to Determine LOD and LOQ

The LOD should result in a peak three times higher than the noise level, whereas LOQ should be at least 10 times higher than the baseline-noise (17, 18). For each tested substance, the parameters of LOD and LOQ are different. Hence, it is crucial to determine those values before defining the lowest concentrations to be analysed.

In the present work, the analysis began with preparing dilutions of each pure substance in acetone, as described in chapter 3.2. Each vial contained a volume of 1 ml, whereof 1 µl got injected into the HPLC system. The HPLC method used, performed the previously described gradient elution from 15-95% within a time frame of 25 minutes (Table 2). Various dilution ranges depending on the reference substances (see Appendix I-III) were prepared to figure out the exact limits for each substance. For detection, a UV-Vis-DAD was opted at a wavelength of 190 nm. Each concentration got injected 3 times in a row with acetone blank measurements in between to maintain steady conditions. The following tables (Table 5-6) contain the forementioned limits for the investigated substances pinoresinol, dehydroabietic acid and neoabietic acid.

Table 5: Limit of Detection (LOD) and Limit of Quantitation (LOQ) for Pinoresinol

	c [mg/ml]	Height	S/N factor	Mean
LOD	**0,00003**	134	2,910	**2,94** ± 0,057
		133	2,888	
		138	2,997	
		135	2,932	
		132	2,867	
		139	3,019	
		135	2,932	
		139	3,019	
		132	2,867	
LOQ	**0,00015**	558	12,120	**12,20** ± 0,088
		557	12,098	
		568	12,337	
		564	12,251	
		555	12,055	
		566	12,294	
		562	12,207	
		561	12,185	
		564	12,251	

Table 6: Limit of Detection (LOD) and Limit of Quantitation (LOQ) for Dehydroabietic Acid

	c [mg/ml]	Height	S/N factor	Mean
LOD	**0,00003**	140	3,041	**2,96** ± 0,087
		131	2,845	
		132	2,867	
		137	2,975	
		142	3,084	
		137	2,975	
		140	3,041	
		136	2,954	
		130	2,823	
LOQ	**0,000035**	502	10,904	**10,99** ± 0,052
		509	11,056	
		503	10,925	
		507	11,012	
		505	10,969	
		505	10,969	
		506	10,991	
		506	10,991	
		510	11,078	

Table 7: Limit of Detection (LOD) and Limit of Quantitation (LOQ) for Neoabietic Acid

	c [mg/ml]	Height	S/N factor	Mean
LOD	**0,003**	139	3,019	**2,99** ± 0,052
		140	3,041	
		139	3,019	
		137	2,975	
		134	2,910	
		142	3,084	
		137	2,975	
		135	2,932	
		136	2,954	
LOQ	**0,0035**	556	12,294	**12,22** ± 0,072
		559	12,142	
		560	12,164	
		560	12,164	
		559	12,142	
		567	12,316	
		566	12,294	
		560	12,164	
		566	12,294	

Table 8: Overview of the Measured Limits of Detection (LOD) and Limits of Quantitation (LOQ)

	c LOD [mg/ml]	**c LOQ [mg/ml]**	**LOD S/N (mean)**	**LOQ S/N (mean)**
Pinoresinol	0,00003	0,00015	2,94 ± 0,057	12,20 ± 0,088
Dehydroabietic acid	0,00003	0,000035	2,96 ± 0,087	10,99 ± 0,052
Neoabietic acid	0,003	0,0035	2,99 ± 0,052	12,22 ± 0,072

4.3 Determination of the Response Factor

A detector signal differs when detecting and comparing two different substances – an analyte and the internal standard – depending on the chromophore and the UV-maxima of the two compounds. Therefore, a correlation factor is needed to compensate this effect. The *response factor* compares the signals between analyte and internal standard, ensuring the calculation of the true value. To calculate this parameter, areas under the curve (AUCs) and amounts of the both substances are needed (1, 17).

$$Response\ Factor\ (RF) = \frac{AUC_{internal\ standard} \cdot Concentration_{analyte}}{Concentration_{internal\ standard} \cdot AUC_{analyte}}$$

The following table shows the computed response factors for pinoresinol, dehydroabietic acid and neoabietic acid, calculated from 15 dilution stages of pinoresinol and dehydroabietic acid and 9 dilution stages of neoabietic acid, as described in 3.2.1.

Table 9: Calculated Response Factors (RF) for Pinoresinol, Dehydroabietic Acid and Neoabietic Acid

	Pinoresinol	**Dehydroabietic acid**	**Neoabietic acid**
Response factors (mean)	**0,465** ± 0,0100	**0,587** ± 0,0119	**5,898** ± 0,0498

The response factors of pinoresinol and dehydroabietic acid range around 0.5, which seems plausible, since both compounds reveal an aromatic moiety. At 190 nm detection wavelength they are obviously detected with double intensity compared to isoferulic acid. In contrast, the signal of neoabietic acid is almost six times weaker than that of isoferulic acid. This is due to a lack of any aromatic ring or conjugated double bonds.

4.4 Quantitation of Pinoresinol and Diterpene Resin Acids (DRAs)

After evaluating LODs, LOQs and response factors, quantitation was performed with six selected spruce balm samples (NSB03/04/06/12/13/23, see Table 1). Therefore,

dilutions were prepared, each containing 2,5 mg NSB and 0,2 mg isoferulic acid (internal standard) in 1 ml acetone (see chapter 3.2.2). Based on previous analyses, a concentration of 2,5 mg spruce balm proved to be the optimal amount for quantitation purposes, since the peak areas could be measured within the linear range of the detector. Using the same HPLC method as previously described (Table 2), each sample was analysed in triplicate, with acetone blank measurements before every new sample. After recording the respective chromatograms, peak areas of pinoresinol, dehydroabietic acid and neoabietic acid were determined and used for calculation. The stacked chromatograms of the samples are shown in Figure 12, page 26.

Together with the calculated response factor, each substance could be quantified in milligram and percentage using the formulas below. The resulting contents of pinoresinol, dehydroabietic acid und neoabietic acid are given as bar charts in Figure 11.

$$Amount\ of\ analyte\ [mg] = \frac{Amount_{internal\ standard} \cdot AUC_{analyte} \cdot RF}{AUC_{internal\ standard}}$$

$$Amount\ of\ analyte\ [\%] = \frac{Amount_{internal\ standard} \cdot AUC_{analyte} \cdot RF \cdot 100}{AUC_{internal\ standard} \cdot Amount_{analyte}}$$

Table 10: Summary of the Quantitation Results

	Pinoresinol		Dehydroabietic acid		Neoabietic acid	
	mg	*%*	*mg*	*%*	*mg*	*%*
NSB03	**0,017** ± 0,0001	**0,70** ± 0,006	**0,114** ± 0,0002	**4,56** ± 0,006	**1,490** ± 0,0037	**59,63** ± 0,147
NSB04	**0,017** ± 0,00004	**0,71** ± 0,002	**0,145** ± 0,0003	**5,81** ± 0,010	**1,370** ± 0,0024	**54,81** ± 0,095
NSB06	**0,055** ± 0,0002	**2,20** ± 0,010	**0,159** ± 0,0062	**6,38** ± 0,248	**0,941** ± 0,0371	**37,65** ± 1,483
NSB12	**0,000** ± 0,000003	**0,01** ± 0,0001	**0,225** ± 0,0015	**9,02** ± 0,059	**0,941** ± 0,0069	**37,65** ± 0,274
NSB13	**0,036** ± 0,0016	**1,48** ± 0,067	**0,175** ± 0,0080	**7,03** ± 0,321	**0,666** ± 0,0295	**26,67** ± 1,180
NSB23	**0,014** ± 0,00004	**0,57** ± 0,002	**0,269** ± 0,0005	**10,80** ± 0,021	**0,528** ± 0,0013	**21,15** ± 0,053

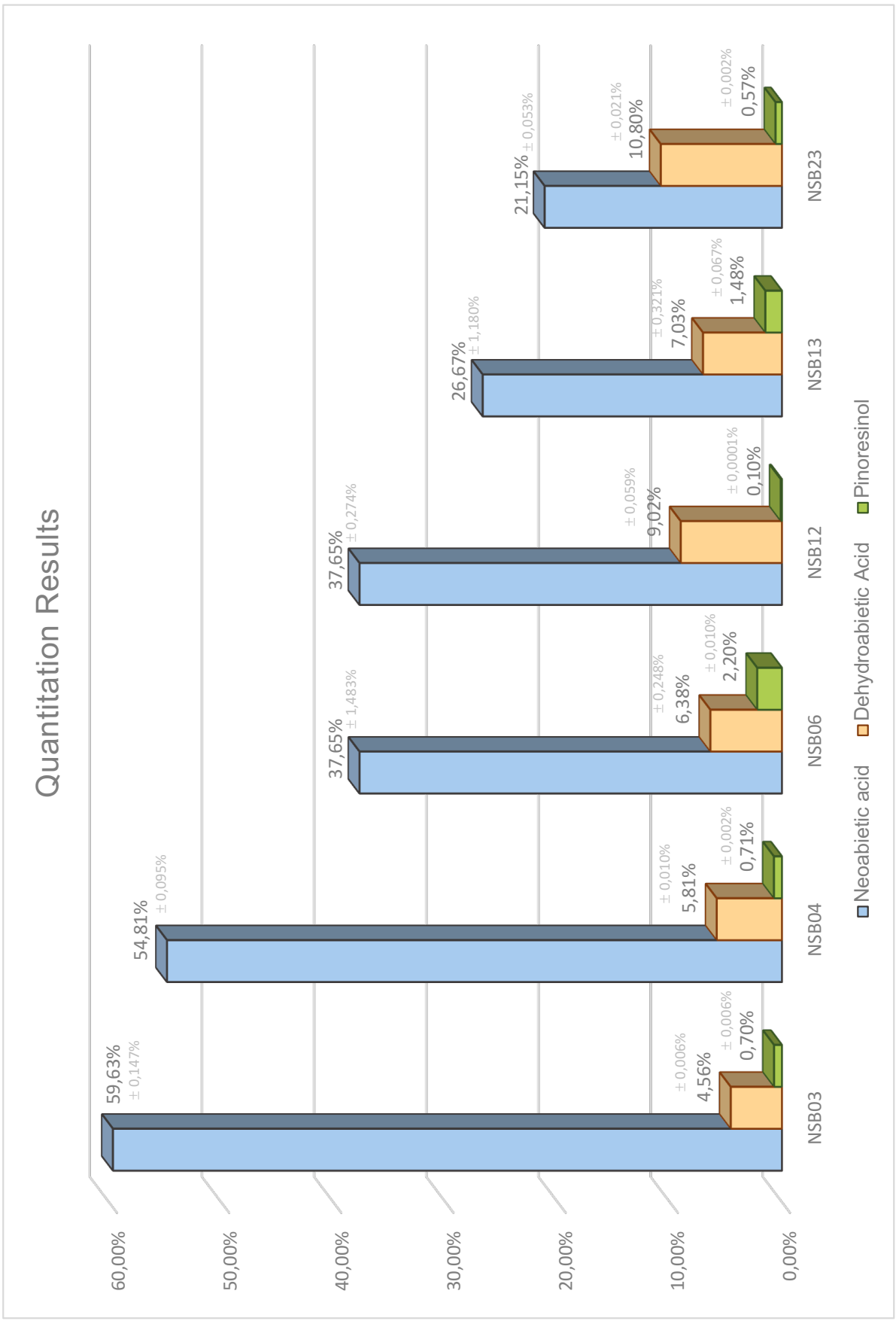

Figure 11: Graphic Display of the Quantitation Results in Percentage Originating from Analyses Shown in Figure 12

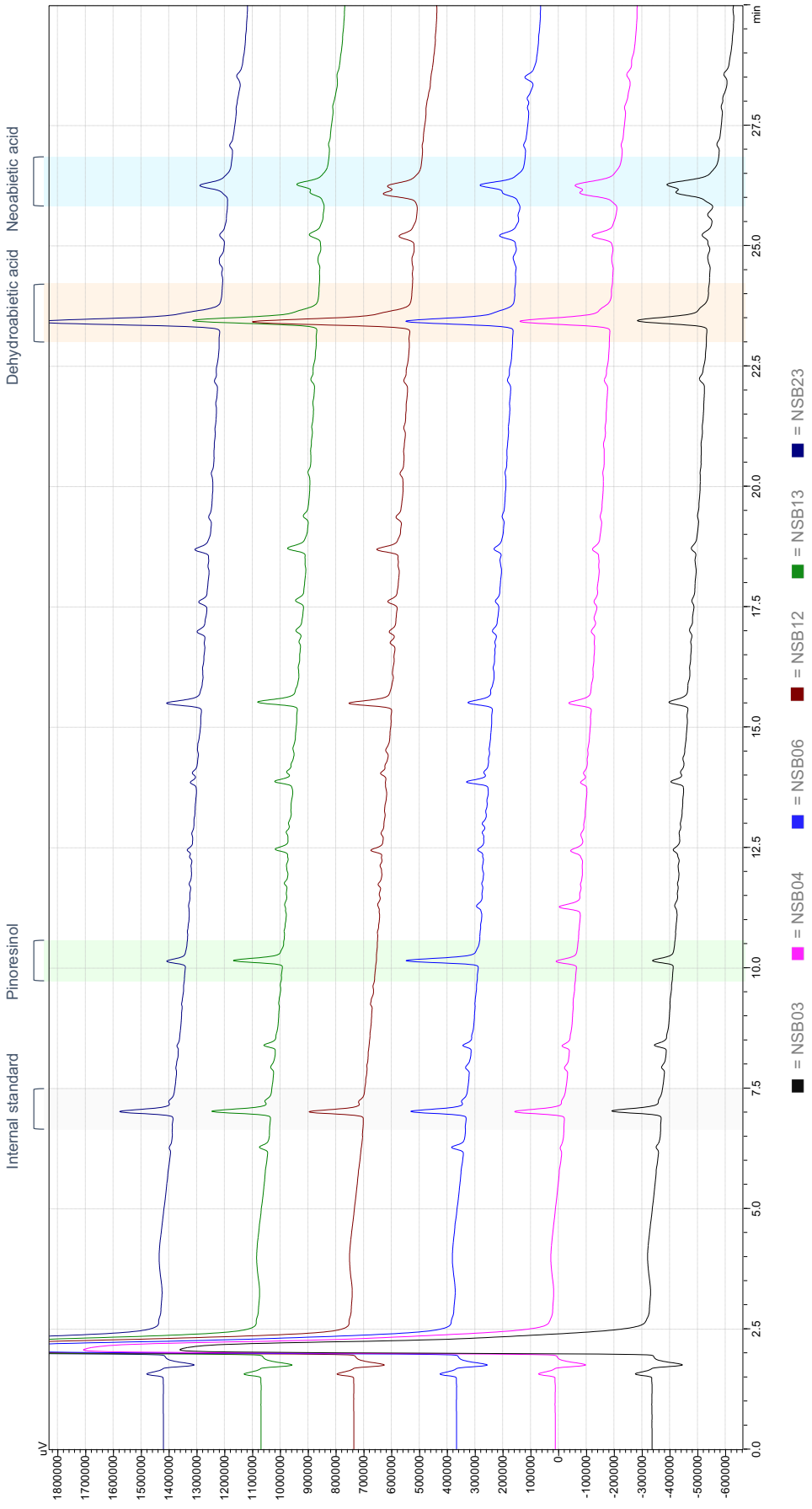

Figure 12: HPLC-UV-DAD Chromatograms of NSB-Samples (2,5 mg/mL, see Table 1) with Internal Standard Isoferulic acid (0,1 mg/mL) (HPLC-system see Table 2) Detection Wavelength: 190 nm

5 Discussion

Norway spruce balm, the exudate of *Picea abies* (L.) H.Karst., has been used for decades to treat wounds. Due to its longstanding therapeutic success, Sico-Pharma (a pharmaceutical company in Austria) created *Picea Salbe®*, a commercial product for wound care, available on the Austrian pharmaceutical market as a medical device. Picea balm as well as an ointment containing picea balm are officinal and included in the Austrian Pharmacopoeia (19). The required quality parameters in the monograph are restricted to qualitative composition to be determined by TLC, but lack any specifications regarding the quantity of marker compounds. Nevertheless, quantitation of the balm's main ingredients is a prerequisite for its pharmaceutical use. Thus, in this thesis an HPLC-method with an internal standard was employed and validated to provide a useful tool for the investigation of various spruce balm samples regarding their quantitative composition. Pinoresinol, dehydroabietic acid and neoabietic acid were selected as marker compounds, since they represent major constituents in the balm on the one hand. On the other hand previous work has shown abietic acids to induce keratinocyte proliferation (20, 21). Numerous dilution stages of those pure substances were analysed and their LODs and LOQs were determined. The recognisably low concentrations in the microgram and nanogram range indicate a precise analysis and a high sensitivity, attributable to the instrument. The response factors were calculated to identify differences in the detector's response, compared to the internal standard. Pinoresinol showed the lowest response factor, followed by dehydroabietic acid, whereas neoabietic acid yielded higher values. A possible reason for this might be the presence of aromatic substructures found in pinoresinol, resulting in a higher detectability via UV-Vis detection compared to non-aromatic DRAs. Subsequently, quantitation of the crude spruce balm samples was performed. Determination of accuracy is still outstanding and has to be validated by recovery experiments. Due to the fact that those spruce exudates originate from natural sources, a heterogeneous chemical composition has to be expected. The quantitation results substantiate those presumptions but clearly state that neoabietic acid and its analogous diterpene resin acids with a molecular weight of 306 can be considered as the major components among the tested substances. However, it must be noted that other diterpene resin acids in the balm, which have the same molecular weight but differ in

structure, eluate at the same time as neoabietic acid. A separation of these compounds on RP-18 material cannot be achieved, therefore these diterpene resin acids were recorded as one peak and are calculated as neoabietic acid. Separation of the diterpene resin acids with 306 amu was achieved by Göls et al. (5, 22) using ultra high performance supercritical fluid chromatography (UHPSFC-MS). Since this device is rather rare in laboratories, this thesis aimed to employ a system which is easily accessible and generally used. The presented method provides the basis for prospective quality control of Norway spruce balm samples and represents the first quantitative analysis for picea balms using HPLC.

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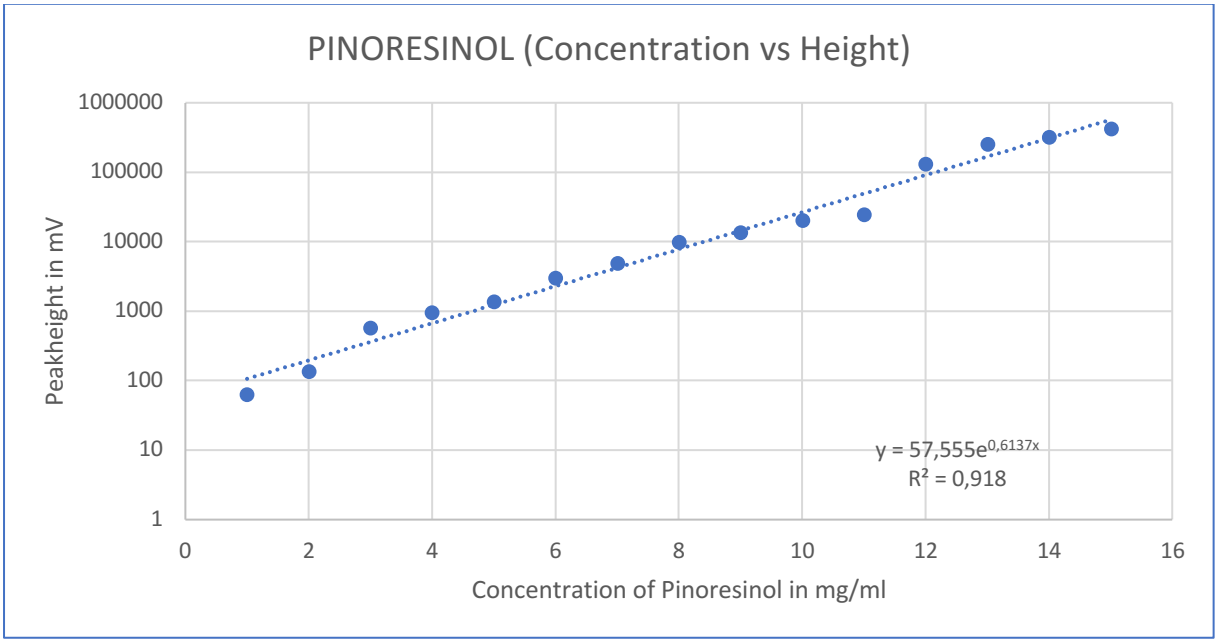
The Illustrations in this thesis were created using BioRender.

Chemical structures were created with Chemdraw 23.1.2.

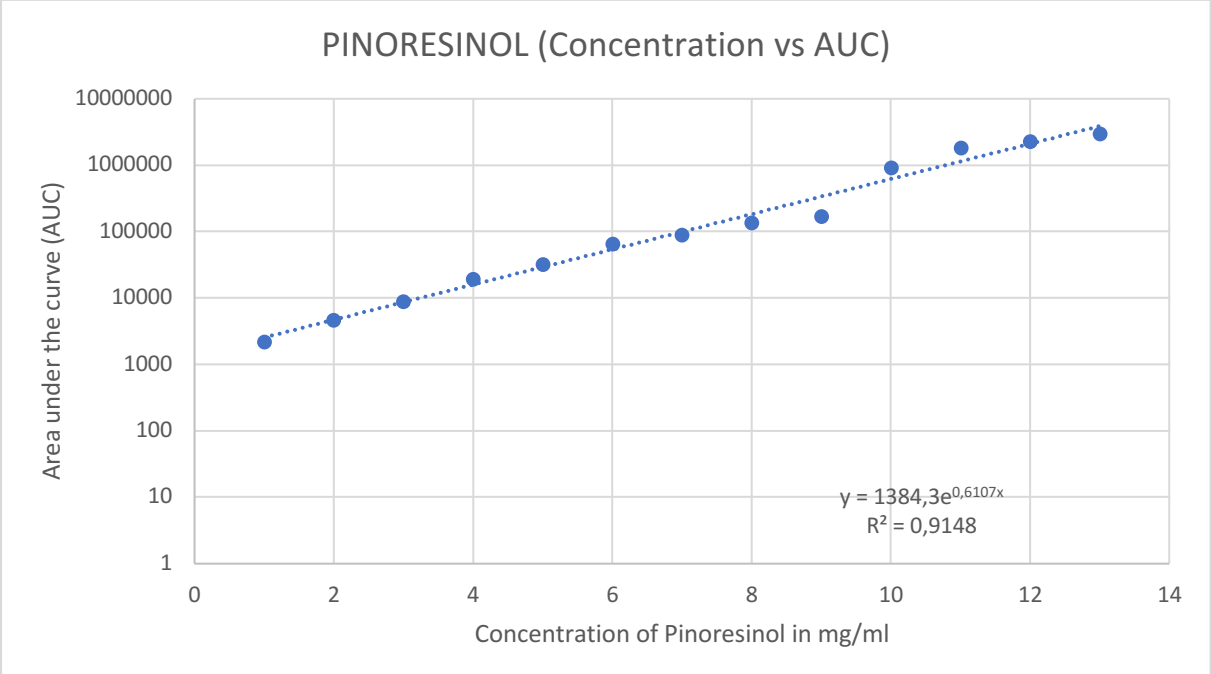
Appendix

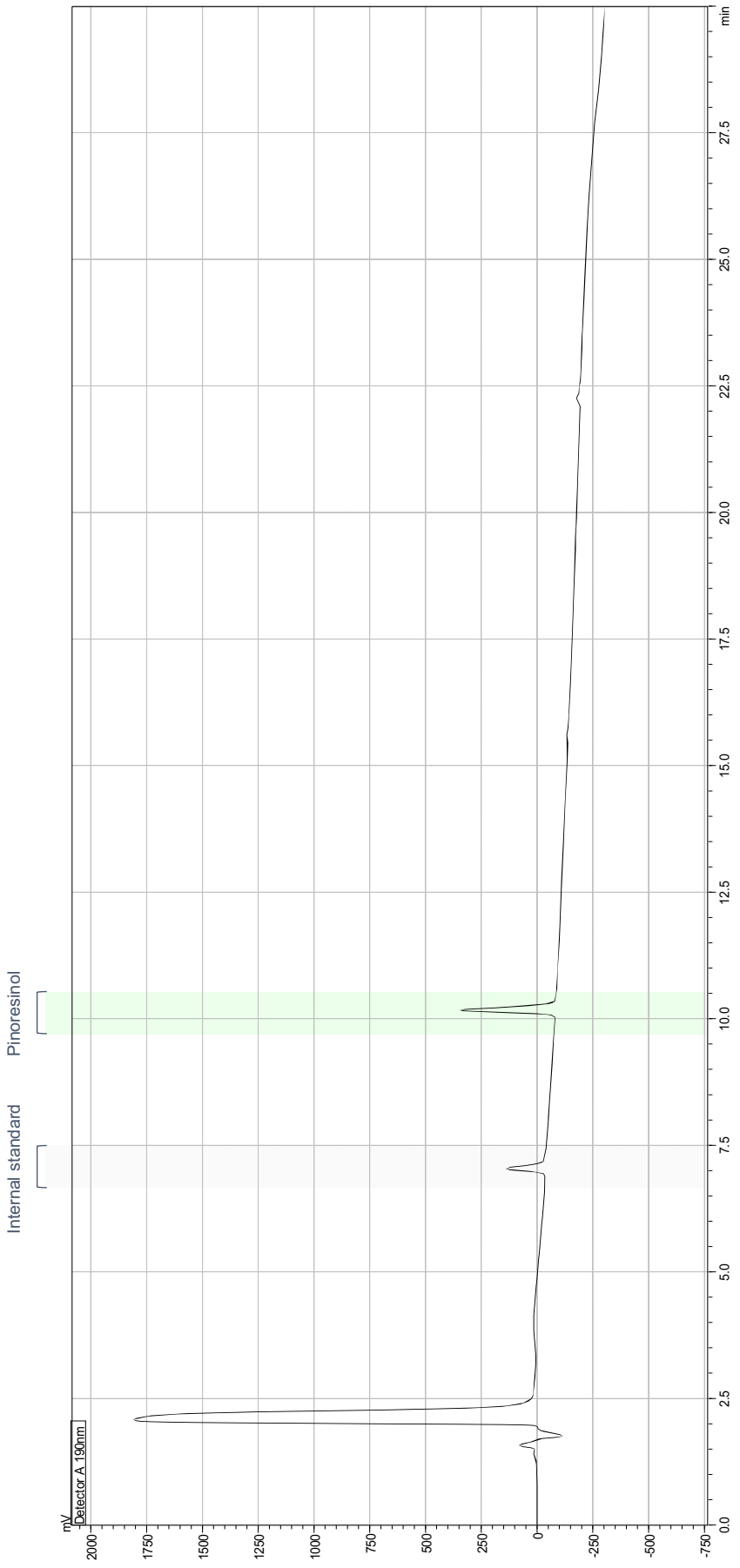
I. Data of Pinoresinol

Dilution Stages of Pinoresinol	
Concentration (mg/ml)	*Height (mV)*
0.00001	62
0.00003	135
0.00015	562
0.00035	951
0.0003	1362
0.0006	2989
0.001	4859
0.002	9815
0.003	13417
0.0045	20192
0.006	24354
0.03	129260
0.06	251797
0.075	315035



Dilution Stages of Pinoresinol	
Concentration (mg/ml)	*Area Under the Curve (AUC)*
0.00001	---
0.00003	---
0.00015	2218
0.00035	4685
0.0003	8862
0.0006	19517
0.001	32340
0.002	65891
0.003	90312
0.0045	136921
0.006	170684
0.03	928854
0.06	1823327
0.075	2282399

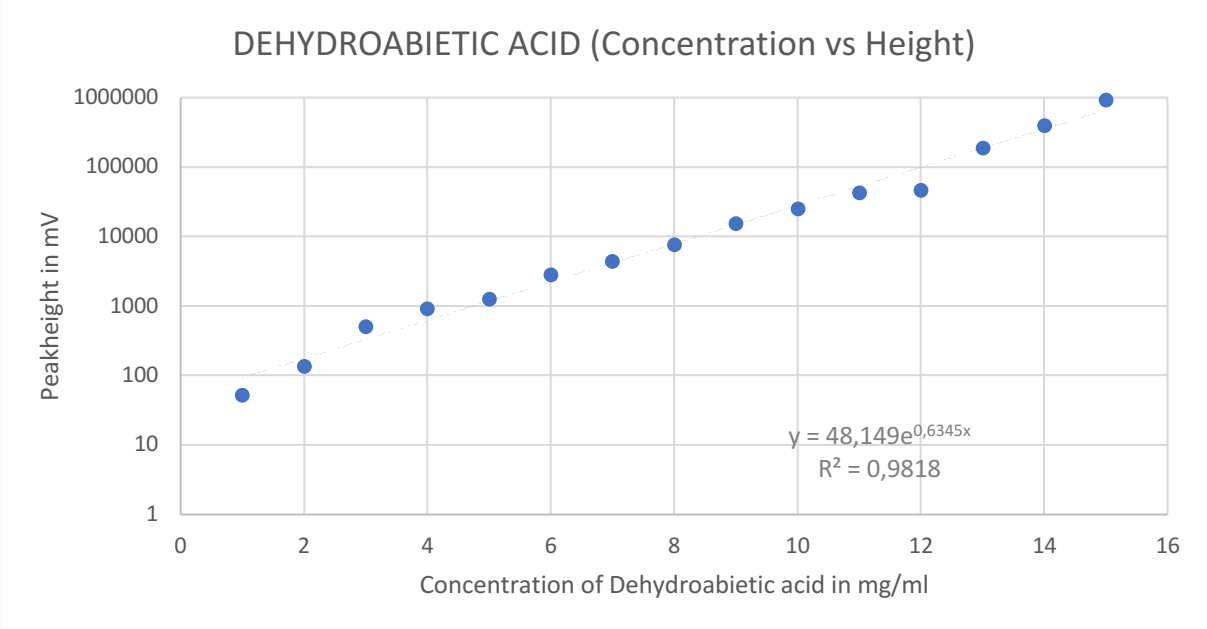




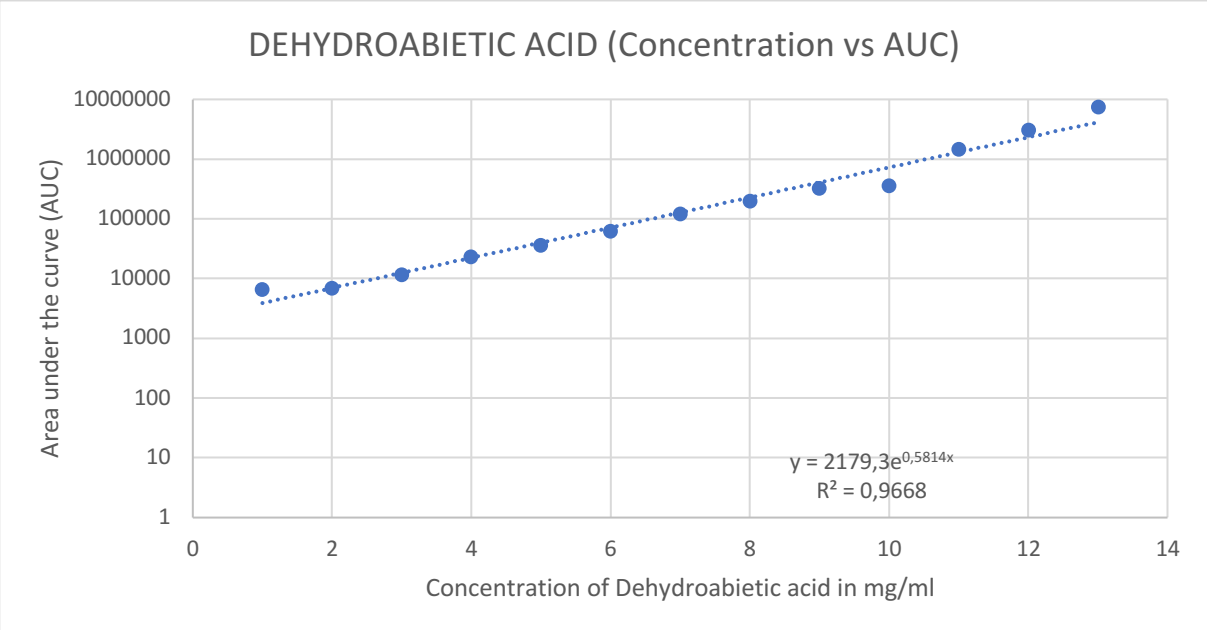
HPLC-UV-DAD Chromatogram of 0,1 mg/ml Pinoresinol at 190 nm (Stationary Phase: RP-18; Mobile Phases: Double Distilled Water + 0,1% Formic Acid, Acetonitrile + 0,1% Formic Acid; Internal Standard: 0,1 mg Isoferulic Acid)

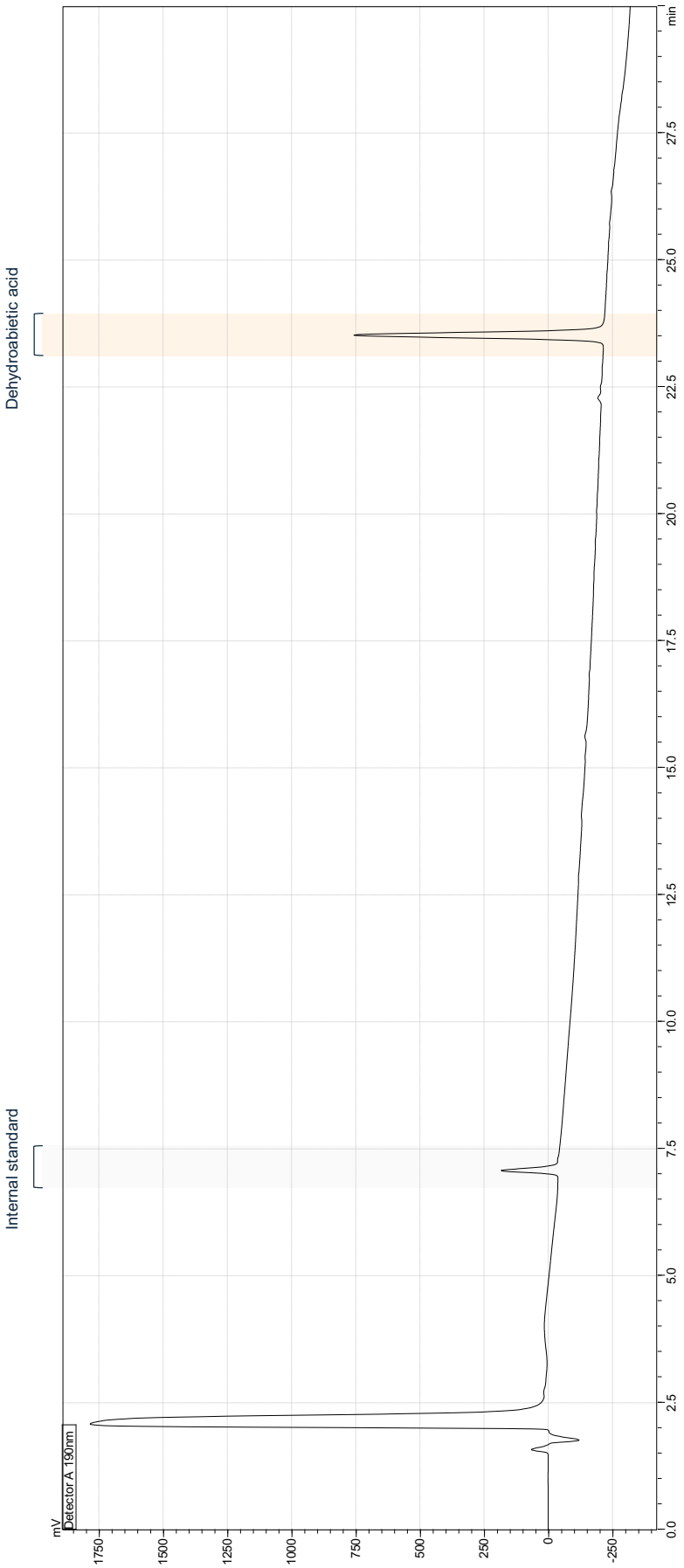
II. Data of Dehydroabietic Acid

Dilution Stages of Dehydroabietic Acid	
Concentration (mg/ml)	*Height (mV)*
0.00001	52
0.00003	136
0.000035	506
0.00005	924
0.0001	1272
0.0003	2796
0.0005	4394
0.001	7619
0.003	15492
0.005	25209
0.009	42194
0.01	46280
0.05	187498
0.1	391919
0.3	922215



Dilution Stages of Dehydroabietic Acid	
Concentration (mg/ml)	*Area Under the Curve (AUC)*
0.00001	---
0.00003	---
0.000035	506
0.00005	924
0.0001	1272
0.0003	2796
0.0005	4394
0.001	7619
0.003	15492
0.005	25209
0.009	42194
0.01	46280
0.05	187498
0.1	391919
0.3	922215

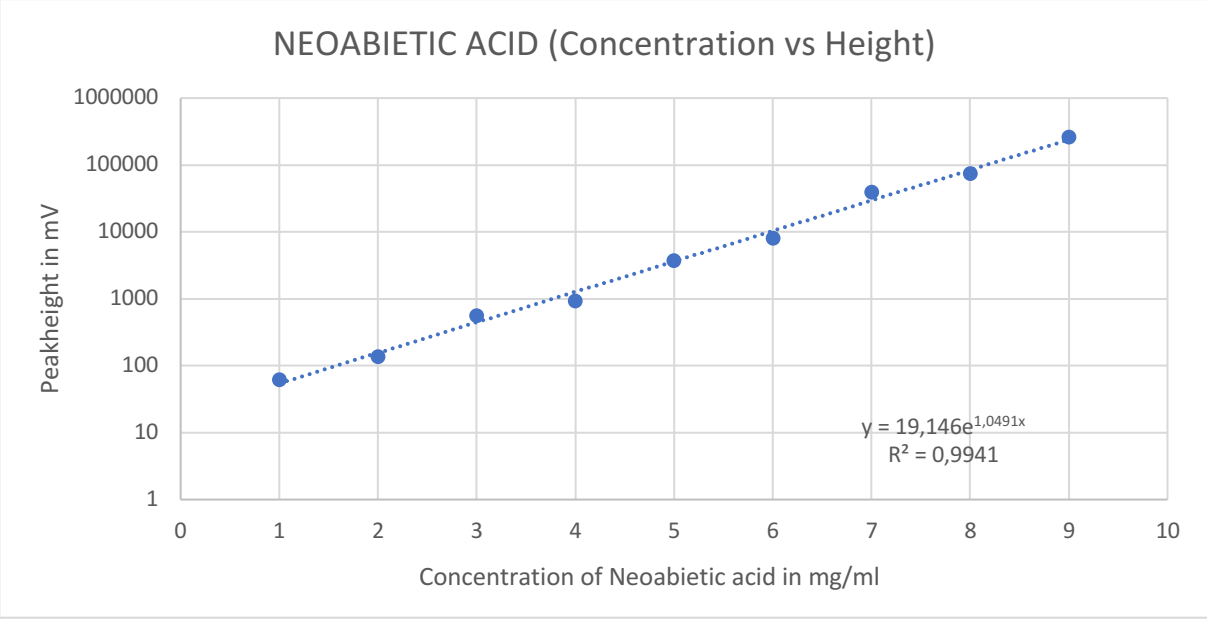




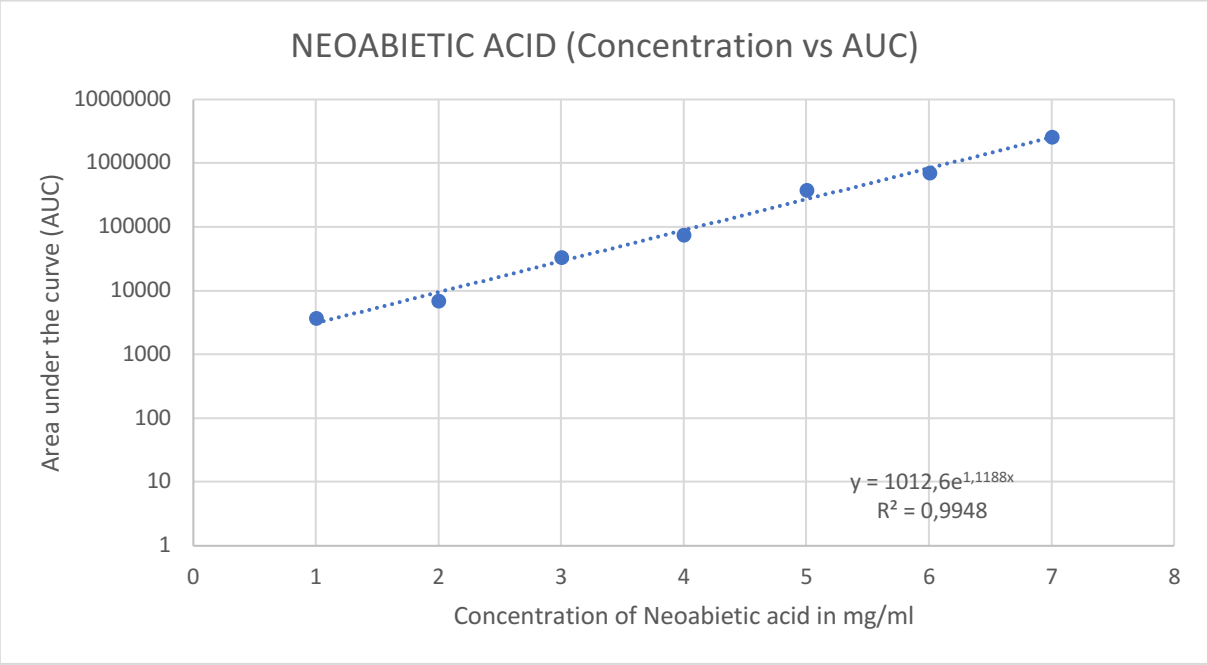
HPLC-UV-DAD Chromatogram of 0,3 mg/ml Dehydroabietic Acid at 190 nm (Stationary Phase: RP-18; Mobile Phases: Double Distilled Water + 0,1% Formic Acid, Acetonitrile + 0,1% Formic Acid; Internal Standard: 0,1 mg Isoferulic Acid)

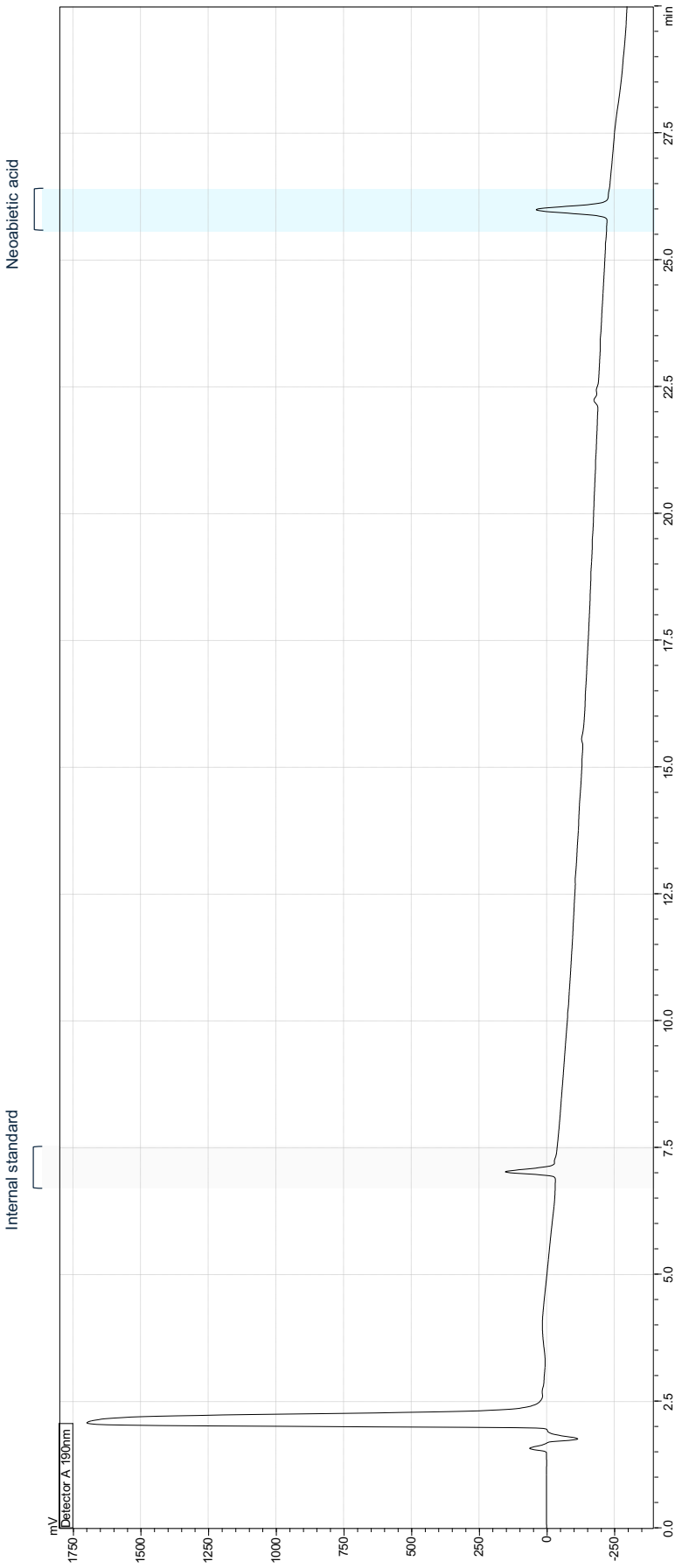
III. Data of Neoabietic Acid

Dilution Stages of Neoabietic Acid	
Concentration (mg/ml)	*Height (mV)*
0.001	63
0.003	138
0.0035	563
0.005	932
0.015	3726
0.03	8208
0.15	39965
0.3	74637
1.0	264184



Dilution Stages of Neoabietic Acid	
Concentration (mg/ml)	*Area Under the Curve (AUC)*
0.001	---
0.003	---
0.0035	3672
0.005	6919
0.015	33502
0.03	75585
0.15	375589
0.3	706561
1.0	2573704





HPLC-UV-DAD Chromatogram of 1,0 mg/ml Neoabietic Acid at 190 nm (Stationary Phase: RP-18; Mobile Phases: Double Distilled Water + 0,1% Formic Acid, Acetonitrile + 0,1% Formic Acid; Internal Standard: 0,1 mg Isoferulic Acid)

IV. Weighed Amount of Substances

Pure substances	Amount weighed [mg]	Initial dilution [mg/ml]
Pinoresinol	0,25	**0,1**25
Dehydroabietic acid	10,09	**0,3**03
Neoabietic acid	16,03	**1,0**02
Isoferulic acid (internal standard)	6,03	**0,2**01

Spruce balm samples	Amount weighed [mg]	Initial dilution [mg/ml]
NSB03	1000,07	**2,5**000175
NSB04	1000,00	**2,5**0000
NSB06	1000,06	**2,5**0015
NSB12	1000,06	**2,5**0015
NSB13	1000,00	**2,5**0000
NSB23	1000,00	**2,5**0000

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