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Titel der Diplomarbeit / Title of the Diploma Thesis

„Isolation and Characterization of Bioactive Constituents from *Pterocarpus santalinus* L.“

verfasst von / submitted by

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LIST OF ABBREVIATION

^{1}H-NMR	Hydrogen-1 Nuclear Magnetic Resonance
ACN	Acetonitrile
CAD	Charged Aerosol Detector
CC	Column Chromatography
Col.	Column
Config.	Configuration
CX3CL1	Fractalkine, Chemokine Ligand 1
DCM	Dichloromethane
DMSO	Dimethyl Sulfoxide
DT_{50}	Death Time; Time Point, when 50% of the Observed Nematodes of one Population died
ELINA	Eliciting Natures Activities
ELSD	Evaporative Light Scattering Detector
et al.	et alii (lat.: and others)
H_2O	Water
H_2SO_4	Sulfuric Acid
HEMWat	*n*-Hexane, Ethyl Acetate, Methanol, Water
Hep3B	Epithelial Liver Cells
HetCA	Hetero Covariance
HPCCC	High-Performance Counter-Current Chromatography
HUVEC	Human Umbilical Vein Endothelial Cells
IC_{50}	Half Maximal Inhibitory Concentration
InterHPCCC	PuriFlash Interchim and High-Performance Counter-Current Chromatography
ISM	Isocratic Solvent Manager
LL	Lower Layer
MeOH	Methanol
MF	Micro-fraction
MS	Mass Spectrometry

PCR	Polymerase Chain Reaction
PDA	Photodiode Array
QDa	Quadrupole Dalton
SSo	Stock Solution
TLC	Thin Layer Chromatography
TNF-α	Tumor Necrosis Factor Alpha
t_R	Retention time
UL	Upper Layer
UPLC	Ultra-Performance Liquid Chromatography
UV	Ultraviolet
Vis	Visible

1 ABSTRACT

The growing proportion of the elderly population and the associated chronic diseases pose an ever-increasing challenge to health care systems in countries all over the world. Therefore, one focus of today's research is on deciphering the mechanisms of the aging process and finding new targets that positively influence the lifespan (i.e., health span). The nematode *Caenorhabditis elegans* (*C. elegans*) has become very popular in aging research, as many cellular pathways that regulate the aging process in humans are conserved in the small nematode. Measuring the lifespan of the worm is a tool to evaluate the life-extending effect of natural products.

In a previous study, a lead-like enhanced extract generated from the heartwood of *Pterocarpus santalinus (i.e.* red sandalwood or red sanders*;* PtesanXDM)*,* the DCM fraction of PtesanXDM (PtesanXDM_DCM) and one sub-fraction, namely PSD01_03, showed promising effects in *C. elegans* by significantly prolonging the nematodes' lifespan. Apart from this, the samples were also screened in a cell-based *in vitro* assay in HUVECs to assess their anti-inflammatory property. PSD01_03 showed promising results in both test systems and was phytochemically examined in the scope of this diploma thesis. Sub-fractions of PSD01_03 were generated using a Puriflash Interchim column chromatography. The resulting ten sub-fractions, namely PSD04_01 – PSD04_10, were tested for their lifespan prolonging effects in *C. elegans*, as well as 35 generated micro-fractions (MFs, i.e., PSD-01 – PSD-35) were tested for their *in vitro* anti-inflammatory properties in HUVECs. Hence, this work was thematically split into two subject areas:

The first part of this work was dedicated to the phytochemical processing of PSD04_01 – PSD04_10. Sub-fractions that significantly affected the lifespan of *C. elegans* were further investigated. From six sub-fractions, which increased the nematodes' lifespan, i.e., PSD04_02, PSD04_04, PSD04_05, PSD04_07, PSD04_08 and PSD04_09, three were selected for an in-depth phytochemical work-up, namely PSD04_02 (DT_{50} extension of 20.63%; $p < 0.01$), PSD04_04 (DT_{50} extension of 17.97%; $p < 0.01$), and PSD04_07 (DT_{50}

extension of 15.87%; $p < 0.01$). These three sub-fractions were further separated with using a combination of a Puriflash Interchim and a High-Performance Counter-Current Chromatographic system (HPCCC). The use of this hyphenated method followed by a further purification step with Sephadex column chromatography resulted in the isolation of eight red sandalwood constituents (i.e., PSD06_01, PSD06_02, PSD06_04, PSD07_01, PSD07_02, PSD09_01, PSD09_02, and PSD09_03), which were subjected to 1D- and 2D NMR structure elucidation.

The second part of this work was dedicated to the targeted isolation of *in vitro* anti-inflammatory constituents from the most active fraction PSD01_03. Therefore, a biochemometric approach called ELINA (Eliciting Nature's Activities) was applied: MFs PSD-01 – PSD-35 were forwarded to ^{1}H NMR analysis and LC-MS-ELSD measurements to acquire chemical information of the metabolite pattern. In parallel, HUVECs treated with the 35 MFs at a concentration of 25 μg/mL were evaluated by quantifying the mRNA of the pro-inflammatory chemokine CX3CL1. Biological and chemical datasets from 35 MFs were then correlated via heterocovariance (HetCA) analysis and statistical total correlation spectroscopy (STOCSY). Packages of 3-4 MFs with a clear increase/decrease in activity were compiled. In this way, NMR and MS signals from compounds correlating to the observed activity can be localized in the respective MFs, which enables a subsequent targeted isolation of bioactive compound(s). In this diploma thesis, exemplarily, package 1 embracing the increasingly active MFs PSD-01 to -04 was probed with the biochemometric ELINA approach. This enabled the targeted identification and isolation of two potentially active agents, PSD10_02 and PSD10_03. To prove the predictability of ELINA, a bioactivity testing of PSD10_02 and PSD10_03 will be part of further investigations at the Division of Pharmacognosy, University of Vienna and by our cooperation partner Dr. Rainer de Martin from the Medical University of Vienna.

2 ZUSAMMENFASSUNG

Der wachsende Anteil der älteren Bevölkerung und die damit verbundenen chronischen Erkrankungen stellen die Gesundheitssysteme in Ländern der ganzen Welt vor immer größere Herausforderungen. Daher liegt ein Fokus der Forschung auf der Entschlüsselung der Mechanismen des Alterungsprozesses und der Suche nach neuen Zielproteinen, welche die Lebensspanne positiv beeinflussen können. Der Fadenwurm *Caenorhabditis elegans* (*C. elegans*) ist in der *in vivo* Alterungsforschung sehr verbreitet, da viele zelluläre Signalwege, die an der Regulation von Alterungsprozessen beim Menschen beteiligt sind, äquivalent in *C. elegans* vorkommen. Die Messung der Lebensdauer der Nematoden ist ein Werkzeug, um die lebensverlängernde Wirkung von Naturstoffen zu bewerten.

In früheren Studien zeigten ein Extrakt („Lead-Like Enhanced Extract“) aus dem Kernholz von *Pterocarpus santalinus*, dem Roten Sandelholz (PtesanXDM), die DCM-Fraktion dieses Extrakts (PtesanXDM_DCM) und eine daraus abgeleitete Fraktion (d.h. PSD01_03) in *C. elegans* eine vielversprechende Wirkung, da sie die Lebensdauer der Nematoden signifikant verlängerten. Darüber hinaus wurden die Proben in einem zellbasierten In-vitro-Assay in HUVECs auf ihre entzündungshemmende Eigenschaft geprüft. PSD01_03 zeigte in beiden Testsystemen vielversprechende Ergebnisse und wurde daher in der vorliegenden Diplomarbeit phytochemisch analysiert. Fraktionen von PSD01_03 wurden mittels Puriflash Interchim-Säulenchromatographie generiert. Zehn Fraktionen, nämlich PSD04_01 - PSD04_10, wurden in *C. elegans* auf ihre lebensverlängernde Wirkung untersucht; 35 hergestellte Mikrofraktionen (MF, d.h. PSD-01 - PSD-35) wurden in HUVECs auf ihre entzündungshemmenden Eigenschaften *in vitro* getestet. Daher wurde die Arbeit in zwei Themenbereiche eingeteilt.

Der erste Teil dieser Diplomarbeit war der phytochemischen Aufarbeitung von PSD04_01 - PSD04_10 gewidmet. Aus sechs Fraktionen, die die Lebensdauer der Nematoden signifikant verlängerten, d.h., PSD04_02, PSD04_04, PSD04_05, PSD04_07, PSD04_08 und PSD04_09, wurden drei für eine eingehende phytochemische Untersuchung ausgewählt, nämlich PSD04_02 (DT_{50}-Verlängerung um 20.63%; $p < 0.01$), PSD04_04 (DT_{50}-

Verlängerung um 17.97%; $p < 0.01$) und PSD04_07 (DT_{50}-Verlängerung um 15.87%; $p < 0.01$). Diese drei Fraktionen wurden mittels einer Kombination aus einem automatischen System zur präparativen Fraktionierung (Puriflash Interchim) und einem Hochleistungs-Gegenstromchromatographie (HPCCC) System weiter aufgetrennt. Durch den Einsatz dieses kombinierten Verfahrens und gefolgt von weiteren Reinigungsschritten mittels Sephadex-Säulenchromatographie konnten acht Inhaltsstoffe aus dem roten Sandelholz isoliert werden (d.h. PSD06_01, PSD06_02, PSD06_04, PSD07_01, PSD07_02, PSD09_01, PSD09_02, PSD09_03). Diese wurden einer 1D- und einer 2D-NMR-basierte Strukturaufklärung unterzogen.

Der zweite Teil dieser Diplomarbeit widmete sich der gezielten Isolierung entzündungshemmender Inhaltsstoffe aus der aktiven Fraktion PSD01_03. Hierfür wurde ein biochemometrischer Ansatz namens ELINA (Eliciting Nature's Activities) verwendet: Die MF PSD-01 – PSD-35 wurden für eine ^{1}H NMR-Analyse und LC-MS-ELSD-Messungen weitergeleitet, um chemische Informationen über das Metabolitenprofil zu erhalten. Parallel dazu wurden in HUVECs, die mit den 35 MFs in einer Konzentration von 25 μg/ml behandelt wurden, durch Quantifizierung der mRNA des proinflammatorischen Chemokins Cx3CL1, die biologischen Effekte evaluiert. Biologische und chemische Datensätze dieser 35 MF wurden anschließend mittels Heterokovarianzanalyse (HetCA) und statistischer Gesamtkorrelationsspektroskopie (STOCSY) korreliert. Pakete von 3-4 MF mit einer eindeutigen Zunahme bzw. Abnahme der Aktivität wurden erstellt. Auf diese Weise können NMR- und MS-Signale von Verbindungen, die mit einer beobachteten Aktivität korrelieren, in den jeweiligen MF lokalisiert werden, was eine anschließende gezielte Isolierung bioaktiver Substanz(en) ermöglicht. In der vorliegenden Diplomarbeit wurde exemplarisch Paket 1, welches die zunehmend aktiven MF PSD-01 bis PSD-04 umfasst, mit dem biochemometrischen ELINA-Ansatz untersucht. Dies ermöglichte die gezielte Identifizierung und Isolierung zweier potenziell aktiver Verbindungen, PSD10_02 und PSD10_03. Um die Vorhersagekraft von ELINA nachzuweisen wird eine Austestung der Bioaktivität dieser beiden Isolate Teil weiterer Untersuchungen in der Abteilung für Pharmakognosie der Universität Wien und von unserem Kooperationspartner Dr. Rainer de Martin von der Medizinischen Universität Wien sein.

3 AIM OF THE WORK

3.1 Isolation and Characterization of Bioactive Compounds from *Pterocarpus santalinus*

The aim of this work was to investigate (i) the biological effects of selected fractions generated from the heartwood extract of *Pterocarpus santalinus* (L.f.) on lifespan prolongation in the nematode *Caenorhabidits elegans* and its *in vitro* anti-inflammatory properties in HUVECs and (ii) the application of the biochemometric approach ELINA to enable a targeted isolation of putative bioactive compounds through phytochemical processing. Previous diploma theses showed a significant lifespan prolonging effect of the heartwood extract and its dichloromethane fraction, i.e., PtesanXDM_DCM in *C. elegans.* Sub-fractions of PtesanXDM_DCM (i.e., PSD01_01 – PSD01_04) were further investigated on lifespan prolongation in *C. elegans* and anti-inflammatory effects in HUVECs. The third sub-fraction, namely PSD01_03, showed the highest bioactivity in both systems. Based on these results, this sub-fraction was selected for an in-depth investigation resulting in two subject areas in this diploma thesis:

(i) Phytochemical investigation of a bioactive fraction from *P. santalinus* affecting the health span of *C. elegans*, and
(ii) Application of a biochemometric approach for the targeted isolation of *in vitro* anti-inflammatory compounds in *P. santalinus*

4 INTRODUCTION

4.1 Isolation and Characterization of Bioactive Compounds from *Pterocarpus santalinus*

4.1.1 Classification and Botanical Description of Pterocarpus santalinus

P. santalinus (L.f.) [synonym: *Lingoeum santalinum* (L.f.) Kuntze; (theplantlist.org, 2021)], known as red sanders or red sandalwood, belongs to the family of Fabaceae. The deciduous tree is small to medium size and most common in south India, where it grows in dry rocky ground. The heartwood, which can be seen in Figure 1, is very hard and has a characteristic purple color, due to the natural dye santalins A-C. The leaves are three foliated and 10-18 cm long, broadly egg-shaped or orbicular (Figure 2). The bisexual flowers are yellow and 2 cm long. In season the tree produces thousands of fragrant flowers yet the natural fruit set is very low (Azamthulla et al., 2015).

Figure 1 - Heartwood of *P. santalinus* [indiamart.com; accessed on December 2020]

Figure 2 - Yellow flowers and trifoliated leaves of *P. santalinus* [wikimedia.org; accessed on March 2021]

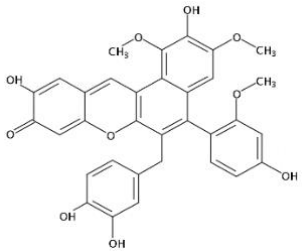
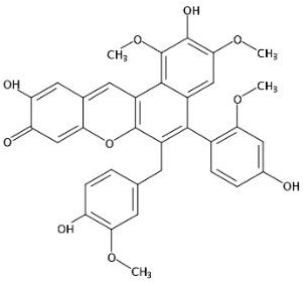
4.1.2 Traditional Use of Pterocarpus santalinus

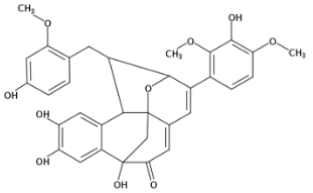
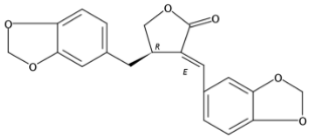
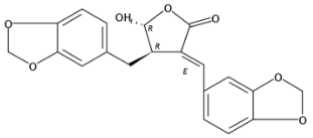
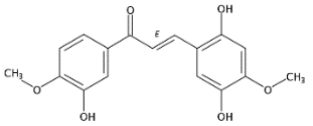
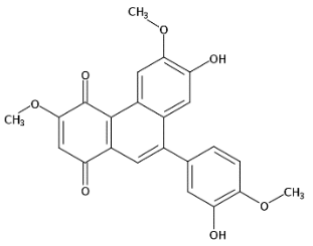
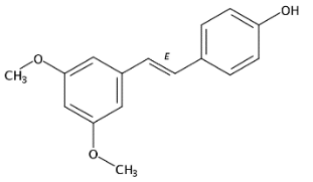
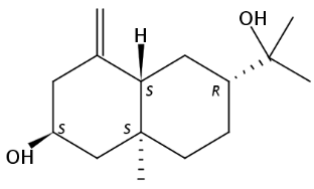
In addition to the use of the heartwood as a natural dye for furniture in the craft industry and as a coloring agent in cosmetics, food and pharmaceutical industries, different parts of the plant are widely used in folk medicine. Various types of wood preparations have been described in literature: A paste (i.e. a powdered wood soaked in water) or the powder is used for burning rash, infections, inflammation as well as on the forehead to relieve headache and for hemorrhage control. A stem bark extract has been reported as effective for the treatment of diabetes, fever, snake bites, and due to its hepatoprotective effects also for the therapy of jaundice. A brew out of the wood is applied orally at chronic dysentery, mental aberration, vomiting but also as an anthelminthic. Other plant organs such as the fruits or the leaves are used against psoriasis or hyper nervous activity. In literature there are much more herbal preparations and application areas described, reflecting not only the great importance of *P. santalinus* as a traditional remedy but also as a prolific research topic of still undisclosed molecular insights (Arunkumar et al., 2014; Walpola et al., 2011).

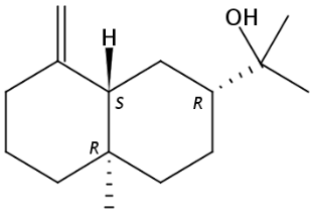
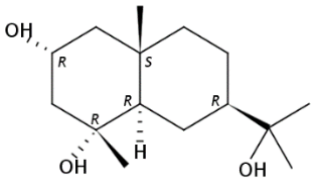
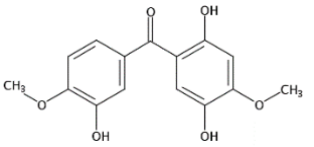
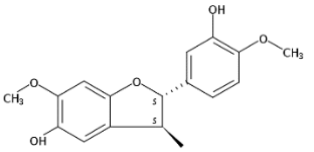
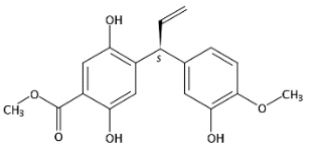
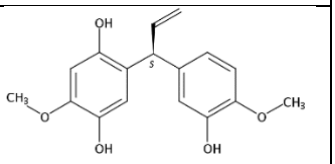
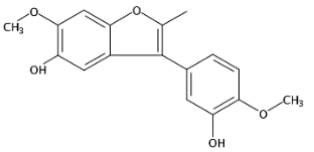
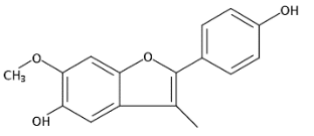
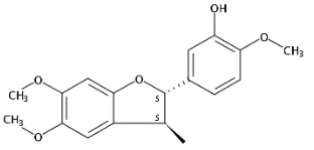
4.1.3 Known Constituents from Literature and their Bioactivities

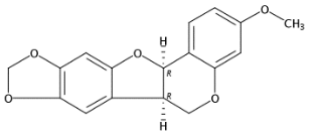
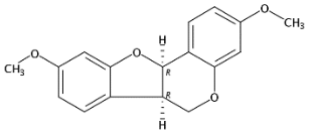
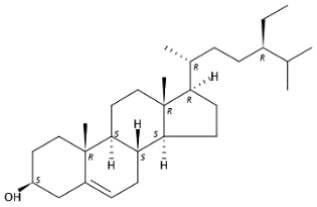
Previous phytochemical investigations have revealed that *P. santalinus* contains several phytochemical classes such as phenolic compounds, triterpenoids, saponins, tannins, alkaloids, sterols and glycosides (Azamthulla et al., 2016). Often described compounds from the heartwood are santalin A, B and Y, benzofurans, isoflavonoides, neoflavonoides (melanoxoin, dalbergin), lignans (savinin and calcoderin), phenanthrenedione (pterolinus K), chalcones (pterolinus L), sesquiterpenes (pterocarpol, pterocarptriol…) and further compounds such as β-sitosterol, aurone glycosides and pterostilbene, a structural analog of resveratrol (Bulle et al., 2016; Wu, S. F. et al., 2011). Table 1 gives an overview of often reported compounds identified in the heartwood of red sanders (Bulle et al., 2016; Walpola et al., 2011).

Table 1 - Known compounds of *P. santalinus* heartwood from literature (Scifinder)

Substance class	**Substance name**	**CAS**	**Molecular weight [g/mol]**	**Structure**
Benzoxanthenon derivates	Santalin A	3818 5-48-7	582.55	
	Santalin B	5103 3-46-6	596.58	

	Santalin Y	16742 5-77-6	586.59	
Lignans	Savinin	49 3-95-8	352.34	
	Calocedrin	9889 1-33-9	368.34	
Chalcone	Pterolinus L	133451 1-43-1	316.31	
Phenanthrene-dione	Pterolinus K	133451 1-41-9	406.38	
Stilbene	Pterostilbene	53 7-42-8	256.30	
Sesquiterpene	Pterocarpol	2167 7-80-5	238.37	

	β-Eudesmol	47 3-15-4	222.37	
	Pterocarptriol	5280 1-07-7	256.38	
Neoflavonoids	Melanoxoin	5811 5-05-2	290.27	
	Melanoxin	2508 9-37-6	301.32	
	Pterolinus F	129731 5-16-2	330.33	
	Pterolinus G	129731 5-17-3	302.32	
Benzofurans	Pterolinus A	129731 5-11-7	300.31	
	Pterolinus B	129731 5-12-8	270.28	
	Pterolinus C	129731 5-13-9	316.35	

Pterocarpane (Isoflavonoids)	Pterocarpin	52 4-97-0	298.29	
	Homopterocarpin	60 6-91-7	284.31	
Sterols	β-Sitosterol	83-46-5	414.71	

4.1.4 Reported Bioactivities in Literature

Until now several reports regarding antibacterial, anticancer/cytotoxic, hepatoprotective effects but also wound healing, anti-inflammatory, antidiabetic, and antioxidant properties have been reported for *P. santalinus*. These potential health benefits have been observed in many *in vitro* but also some *in vivo* studies. Since many active compounds have often been isolated from the heartwood of *P. santalinus*, this plant organ is of special interest for health-related research topics (Bulle et al., 2016). The following summary provides an overview of the most stated activities from literature.

4.1.4.1 Anti-inflammatory activity:

The benzofurans pterolinus A, B and D, isolated from a heartwood extract, showed a significant inhibition of superoxide anion generation in human neutrophils with an IC_{50} value of 0.35, 0.19 and 0.29 μg/mL, all three were more potent than the positive control phosphatidylinositol-3-kinase inhibitor (Wu, S.-F. et al., 2011). Savinin and calocedrin, both lignans isolated from the heartwood, were active in an anti-inflammatory assay by inhibiting the TNF-α production and T cell proliferation in lipopolysaccharide-stimulated RAW264.7 cells (Cho et al., 2001). Pterolinus L and pterolinus K have also been screened for their anti-inflammatory activity in cancer cell lines and showed significant effect in inhibiting superoxide anion generation or elastase release (Wu, S. F. et al., 2011).

4.1.4.2 Cytotoxic activity:

Pterolinus K and pterolinus L were also screened for cytotoxic activity in six cancer cell lines, where pterolinus K had a selective effect against Hep3B and pterolinus L had a moderate activity in all six cancer lines (Wu, S. F. et al., 2011). Melanoxoin, a neoflavonoid isolated from the heartwood, had the highest cytotoxic effect in comparison to other neoflavonoids and benzofurans against Ca9-22 with an IC_{50} value of 0.46 μg/mL (Wu, S.-F. et al., 2011). Effects of pterostilbene, a structural analog form resveratrol, are documented in a wide range of cancers with different mechanisms of anticancer actions. For instance, pterostilbene inhibited the adrenocortiocotropic hormone production in the brain of mice. This revealed in an increased sensitivity to oxidative stress in melanoma and pancreatic cancer (Obrador et al., 2021).

4.1.4.3 Antibacterial activity:

Methanolic extracts from the leaf and the stem bark showed a concentration dependent antibacterial activity against several organisms. The stem bark extract showed a broad-spectrum activity hence it was active against *Enterobacter aerogenes, Escherichia coli, Pseudomonas aeruginosa, Staphylococcus aureus, Bacillus subtilis* etc. The leaf extract was active against *Escherichia coli, Alcaligenes faecalis, Enterobacter aerogenes and Pseudomonas aeruginosa* (Manjunatha, 2006).

4.1.4.4 Antidiabetic effect:

There are already some early studies on anti-diabetic effects with extracts of *P. santalinus.* To get a more concrete insight into the mechanism of bioactivity there are more studies needed using isolated compounds from the plant (Bulle et al., 2016).

4.1.4.5 Hepatoprotective effect:

Albino rats with galactosamine induced acute hepatotoxicity were treated with a suspension of MeOH extracts from the heartwood in two different doses (200 and 400 mg/kg) against silymarin as positive control. The functional state of the organ was monitored by biochemical parameters specific for the liver. In rats, treated with the

extract from *P. santalinus,* these parameters were significant decreased, which could be an indication for hepatoprotective effects (Dhanabal et al., 2006).

However, investigations on a molecular level of compounds comprised in *P. santalinus* is still in its infancy and further investigations are required to get a better understanding on the plant's bioactivities. In order to assess the possible health benefits of red sandalwood, a characterization of the active molecules together with an evaluation of the interactions with cellular systems is necessary.

4.2 Phytochemical Investigation of a Bioactive Fraction from *Pterocarpus santalinus* affecting the Health Span of Caenorhabditis *elegans*

4.2.1 Caenorhabditis elegans as a Model Organism for Aging Research

For the first time in 2020 the number of the global population aged 60 years or older was greater than the population younger than 5 years. The enormous pace of an increasingly aging population around the world is one of the greatest challenges of the 21st century. This shift in the age distribution towards the elderly goes along with an increase to a wide diversity of age-related chronic diseases. For prevention, the key target for individuals could be a healthy lifespan. The cellular processes which are associated with affecting the healthspan are still being researched. However, it can be assumed that a higher concentration of glycemic, lipid- and inflammatory biomarkers are correlating with senescence. Natural product (NP) research can contribute to finding new potential geroprotective agents that have an affection on these biomarkers and decrease the rate of aging-process (Bulterijs et al., 2020; Li et al., 2021; who.int, 2021).

The nematode *C. elegans* was first introduced as a model organism in 1963. Since then, it has become very popular in *in vivo* aging research, as it has been proven that the mechanism of several pathways that regulate the aging process in humans were equal in the free-living nematode. Accordingly, a hypothesis in geroscience is, that interventions that delay the rate of aging will in turn lead to the prevention and/or reduction of the

severity of age-related diseases. With many other advantages the worm is often used as an *in vivo* model (Bulterijs et al., 2020):

(i) Benefits like small size (adults: ~ 1.5 mm length), high fertility (up to 300 eggs/adult worm), fast generation cycle (~ 2 - 3 days form egg to fertile adult) and cheap culturing conditions make the nematode an economically favorable model.

(ii) 41% of the completely sequenced genome and up to 75% of disease-related genes of *C. elegans* have human orthologs. In addition, gene mutations can easily be engineered, which facilitates the observation of targets and mechanisms.

(iii) The optical transparency of the worm's body allows the monitoring of the morphology, pathophysiology and fluorescent or luminescent reporters during *in vivo* observations.

4.3 A Biochemometric Approach for the Targeted Isolation of *in vitro* Anti-Inflammatory Compounds in *Ptercarpus santalinus*

4.3.1 ELINA: A ^{1}H NMR-MS-based Drug Discovery Tool for Identifying Bioactive Compounds in Complex Mixtures

NPs research is still one of the most important discovery tool for new drug leads. Nevertheless, the great diversity of the secondary metabolite profile in plants (and fungi) poses an enormous challenge for the isolation and identification of pure compounds. Classical bioactivity-guided fractionation/isolation is one of the most frequently used strategies in NP research but can be hampered by various factors: (i) because of the repetitive fractionation steps, it is a time-consuming process and bioactivity can get lost due to adhesion of bioactives to chromatographic materials, (ii) bioactive molecules in low quantity are easily overlooked (especially when they are overshadowed by highly abundant but inactive compounds) and (iii) it can leads to the re-discovery of already known but inactive molecules. Efficient strategies are necessary to make bioactive

constituents of complex mixtures accessible for further investigations. ELINA, which stands for Eliciting Nature's Activities, is a biochemometric tool for identifying bioactive compounds in a complex mixture prior to isolation. The workflow is based on a statistically heterocovariance analysis (HetCA) which combines bioactivity and chemical data from micro-fractions (MFs) generated from one bioactive extract (Grienke et al., 2019; Zwirchmayr, Julia et al., 2020) . A schematic overview of the single steps performed during an ELINA procedure is given in Figure 3.

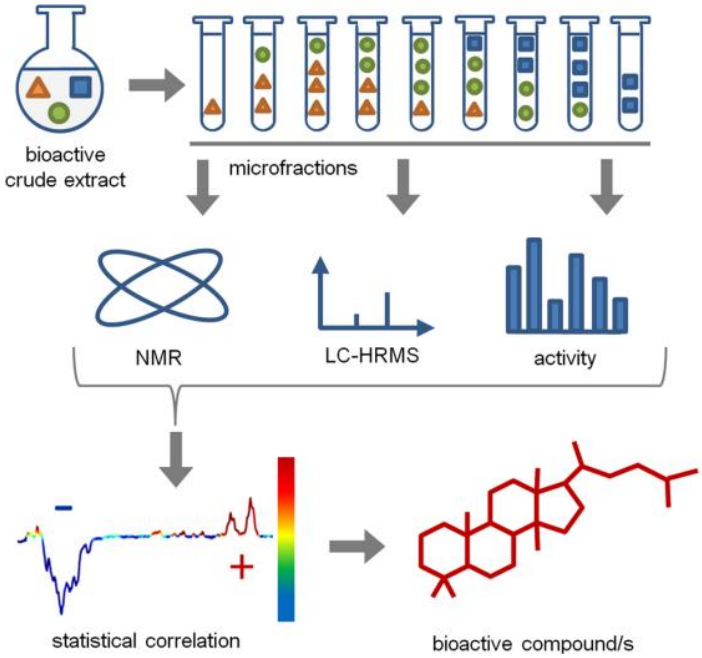


Figure 3 - Schematic overview of the drug discovery tool "ELINA" workflow (Grienke et al., 2019)

The first step in an ELINA procedure is the micro-fractionation. Here, the goal is to create a variability of secondary metabolites over several contiguous fractions, e.g., individual compounds are spread out over several MFs. This also enables identifying synergistic and/or additive interactions between the molecules, since they are not separated from each other (which corresponds more to the "natural effect" of an extract). After the fractionation procedure, the generated MFs are forwarded to ^{1}H NMR and LC-MS measurements (hyphenated to a semi-quantitative detector such as ELSD or CAD), to get structural and quantitative information of the MFs. Apart from this, the MFs are forwarded to bioactivity testing, e.g., in an *in vitro* assay to evaluate quantitative variances of activity related to the variances in the secondary metabolite profile of the MFs.

Packages of three to four MFs, demonstrating an increase or decrease in activity, are generated and the corresponding ^{1}H NMR and bioactivity data are forwarded to heterocovariance (HetCA) analysis. Here, the quantitative ^{1}H NMR and bioactivity data are correlated, so that the resonances given by a bioactive constituent can be distinguished from the resonances given by an inactive compound. In addition, the integration of LC-MS-ELSD data allows a semi-quantitative characterization and a dereplication of potential active molecules prior isolating them. A targeted isolation and identification of predicted compounds can be performed and can accelerate and improve the NP drug discovery process (Grienke et al., 2019).

5 RESULTS AND DISCUSSION

5.1 Isolation and Characterization of Bioactive Compounds from *Ptercarpus santalinus*

5.1.1 Fractionation of the Bioactive Fraction PSD01_03 to ten Sub-Fractions PSD04_01 – PSD04_10

In Figure 4 the hierarchy of the fractionation process starting from a large-scale dichloromethane (DCM)/methanol (MeOH) heartwood extract is pictured. The bioactive fraction PSD01_03, deriving from the DCM fraction PtesanXDM_DCM, was selected for a large-scale fractionation using a Puriflash Interchim column chromatography device with a dry load. This process was repeated in total for four times with ~1000 mg of PSD01_03 (mixed 1:2 with silica gel) in a reversed phase mode with gradient elution. All parameters of the chromatographic method and the composition of the gradient are shown in Table 4, and Table 5, respectively. Per run 90 – 100 fractions were collected and applied on TLC. Based on the chromatograms of the ELSD/PDA detectors and a TLC analysis, the fractions were combined to ten sub-fractions, i.e., PSD04_01 – PSD04_10. Fractions yields are shown in Table 22. ~ 1 mg of each fraction was dissolved in DMSO at a final concentration of 10 mg/mL and forwarded to bioactivity testing in a *C. elegans* lifespan assay.

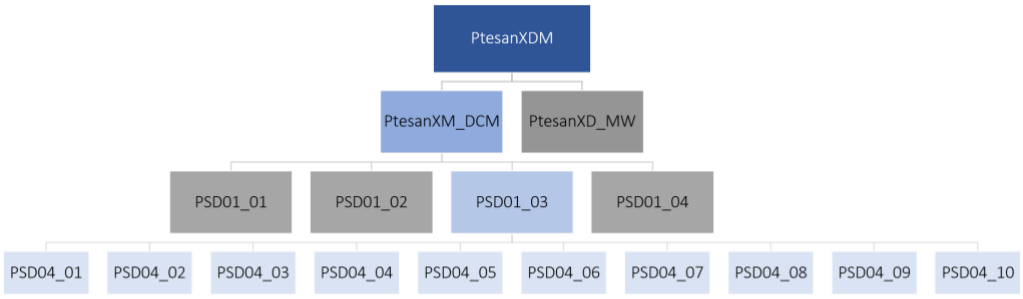


Figure 4 - Fractionation hierarchy starting from a *P. santalinus* heartwood extract to bioactivity guided fractionation of PSD01_03 to ten Sub-Fractions

5.1.1.1 TLC and UPLC Analysis of PSD04_01 - PSD04_10

The generated Interchim fractions PSD04_01 – PSD04_10 were investigated with a collective TLC analysis. 8 μL of each fraction were applied at a concentration of 1 mg/mL. TLC parameters are given in Table 3. Figure 5 shows the collective TLC of the ten sub-fractions. The fractions PSD04_01 – PSD04_10 were further chromatographed over UPLC at a concentration of 1 mg/mL and an injection volume of 1 μL. Detection of compounds using PDA, ELSD and QDa detector. All parameters are given in Table 18 and Table 21. A collection of all UPLC chromatograms is shown in the Appendix, Chapter 8.1.

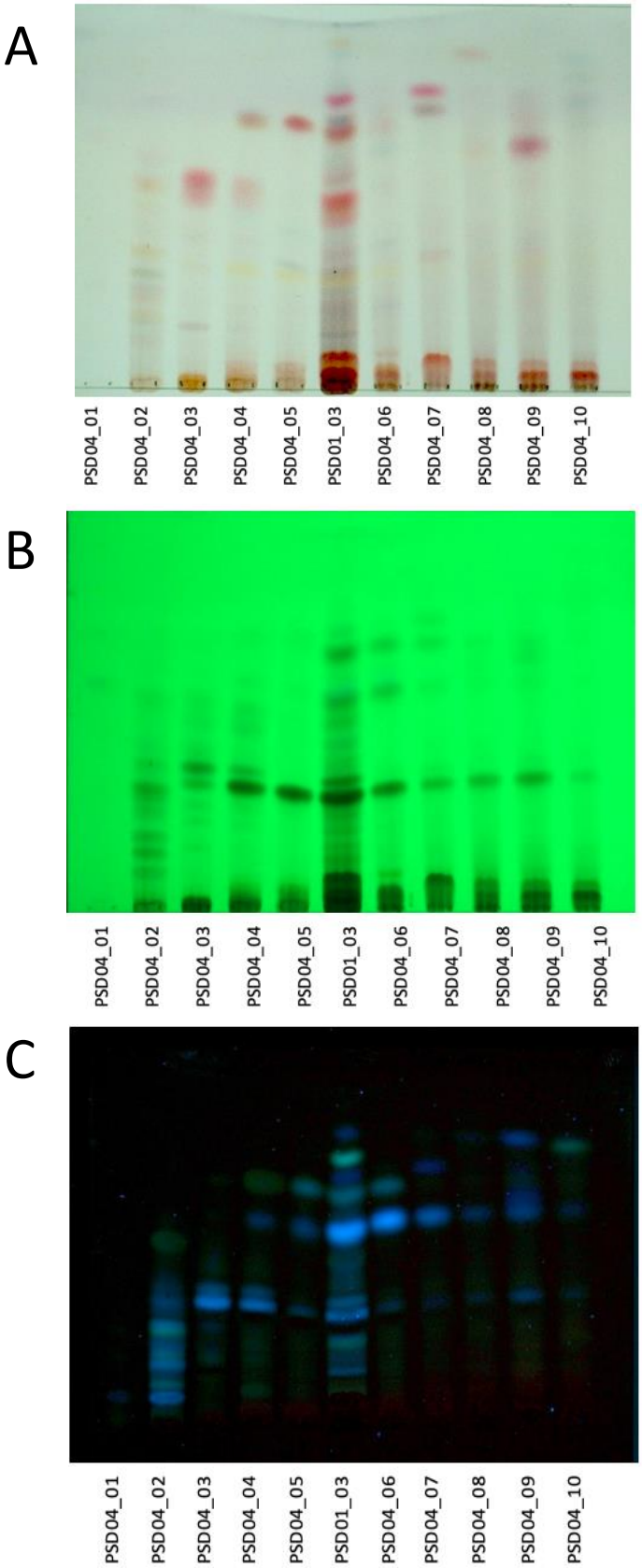


Figure 5 - Collective TLC of PSD04_01 - PSD04_10, A: detection in Vis after using spraying reagent Vanillin/H_2SO_4, B: detection at 254 nm, C: detection at 366 nm; TLC parameters are given in 7.1.3

5.1.2 Bioactivity Results of PSD04_01 – PSD04_10 in a Caenorhabditis elegans Life Span Assay

A *C. elegans* lifespan assay with the ten fractions PSD04_01 – PSD04_10 (all at 25 μg/mL) and the well-known positive control reserpine at 30 μM (Saharia et al., 2016) was performed. In Figure 6 and Table 2 the results of the screening are shown. Worms treated with the positive control reserpine showed a significant increase of the death time 50% (DT_{50}; timepoint, when 50% of the nematodes were dead/alive) in comparison to the vehicle control (DMSO 1%). Reserpine treatment resulted in a DT_{50} extension of 31.24 % ($p < 0.01$). Apart from that, six fractions were able to increase the nematodes' lifespan significantly, i.e.: PSD04_02 (DT_{50} extension of 20.63%; $p < 0.01$), PSD04_04 (DT_{50} extension of 17.97%; $p < 0.01$), PSD04_05 (DT_{50} extension of 19.05%; $p < 0.01$), PSD04_07 (DT_{50} extension of 15.87%; $p < 0.01$), PSD04_08 (DT_{50} extension of 16.38%; $p < 0.01$) and PSD04_09 (DT_{50} extension of 16.38%; $p < 0.05$). The first five fractions were prioritized and selected for further phytochemical investigations. PSD04_09 was prioritized least because of the high standard deviation due to discriminative results between the replicates. Especially PSD04_02 was particularly interesting, since this fraction increased the worm's lifespan by 20.63 % compared to the vehicle control.

The *C. elegans* lifespan experiment was performed according to the protocol described in (Zwirchmayr, J. et al., 2020), adapted from (Solis et al., 2011).

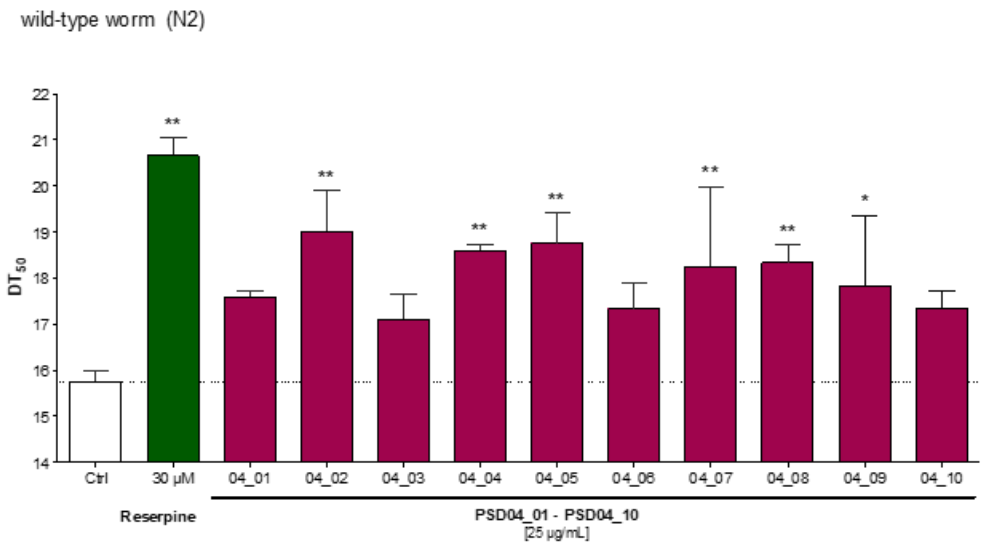


Figure 6 - Results of PSD04_01-PSD04_10 (all at 25 μg/mL) in a *C. elegans* Lifespan Assay (zoomed); Bars represent the mean DT50 ± SD of three parallel experiments. Significance was assessed by One-Way ANOVA and Dunnett's post-test (* $p < 0.05$; ** $p < 0.01$).

Table 2 - Results of PSD04_01-PSD04_10 (all at 25 μg/mL) in a *C. elegans* Lifespan Assay

	Mean DT_{50}	**SD**	**DT_{50} Increase/Decrease [%]**	***p*-value**
Ctrl	15.75	0.25		
Reserpine 30 μM	20.67	0.3819	31.24	$p < 0.01$
PSD04_01	17.58	0.1443	11.62	n.s.
PSD04_02	19.00	0.9014	20.63	$p < 0.01$
PSD04_03	17.08	0.5774	8.44	n.s.
PSD04_04	18.58	0.1443	17.97	$p < 0.01$
PSD04_05	18.75	0.6614	19.05	$p < 0.01$
PSD04_06	17.33	0.5774	10.03	n.s.
PSD04_07	18.25	1.732	15.87	$p < 0.01$
PSD04_08	18.33	0.3819	16.38	$p < 0.01$
PSD04_09	17.83	1.528	13.21	$p < 0.05$
PSD04_10	17.33	0.3819	10.03	n.s.

5.1.3 Fractionation of the Bioactive Fraction PSD01_03 to 35 Micro-fractions PSD-01 – PSD-35

For the biochemometric ELINA approach, micro-fractions of PSD01_03 were generated with PuriFlash Interchim column chromatography. Therefore, ~1000 mg of PSD01_03 was applied once with a dry-load cartridge. Apart from the fraction volume, the same method as for the fractionation of PSD04_01 – PSD04_10 was used. Here, more fractions were collected per minute. After this single fractionation run 350 fractions were analyzed with TLC. Based on that monitoring, 35 final MFs were pooled, i.e., PSD-01 – PSD-35 (see Figure 7). A collective TLC of PSD-01 to PSD-35 is shown in Figure 8 and the fraction yields are shown in Table 23. ~ 1 mg of each fraction was dissolved in DMSO at a final concentration of 10 mg/mL and forwarded to bioactivity testing by Dr. Rainer de Martin from the Medical University of Vienna, Department of Vascular Biology and Thrombosis Research (see Chapter 5.1.3.3).

Figure 7 - Fractionation hierarchy starting from a heartwood extract to fractionation of PSD01_03 to 35 micro-fractions for ELINA approach

5.1.3.1 TLC and UPLC Analysis of PSD-01 – PSD-35

Again, a quantitative TLC analysis of the 35 MFs was performed. 3 *µL* of PSD-01 to PSD-35 were applied at a concentration of 1.39 mg/mL. Figure 8 shows the collective TLC analysis with the initial fraction PSD01_03. Here, the aim of micro-fractionation was to spread the compounds of a complex mixture over several consecutive MFs in different concentrations and combinations. As an example, in the TLC comparison in Figure 9, the derivatized chromatogram of the fraction PSD04_03 shows two pink-red bands at a R_f = 0.6. These compounds/bands spread out over the MFs PSD-08 to PSD-13.

The 35 MFs were further chromatographed over UPLC at a concentration of 3 mg/mL and an injection volume of 1 μL. Detection of compounds using PDA, ELSD and QDa detector. All parameters are given in Table 18 and Table 21. A collection of all UPLC chromatograms is shown in the Appendix, Chapter 8.1. A dereplication with UPCL-MS was performed for selected fractions used in the biochemometric ELINA approach (PSD-03 – PSD-05) see Figure 29.

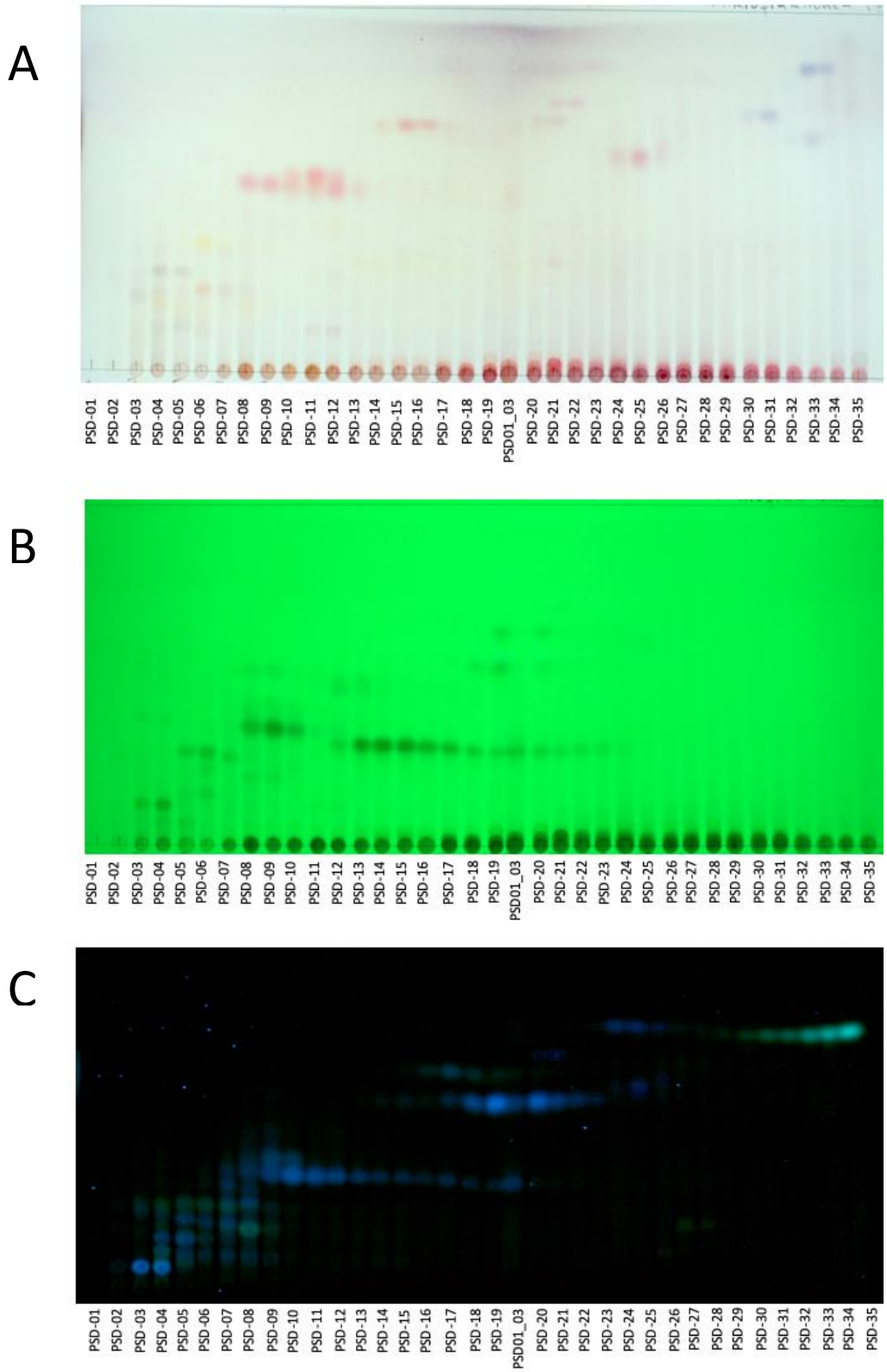


Figure 8 - Collective TLC of PSD-01 - PSD-35, A: detection in Vis after using spraying reagent Vanillin/H_2SO_4, B: detection at 254 nm, C: detection at 366 nm; TLC parameters are given in 7.1.3

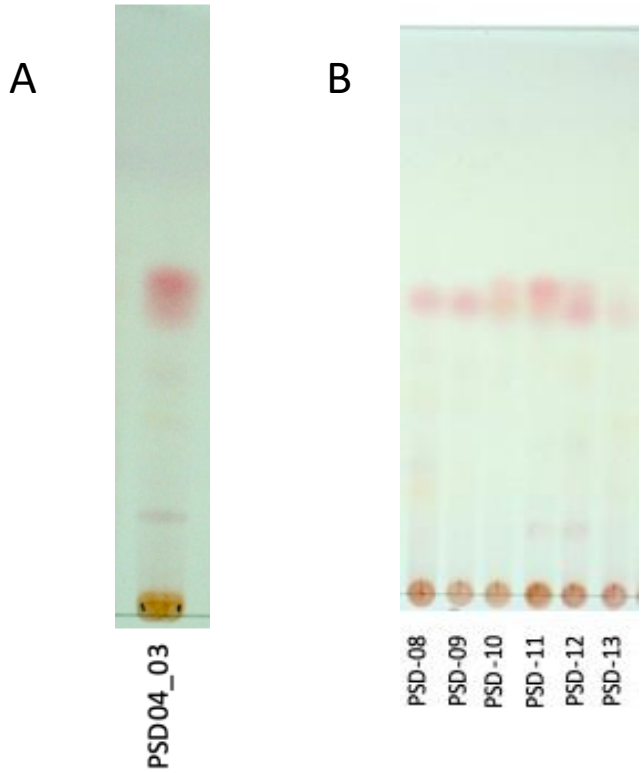


Figure 9 - TLC comparison of PSD04_03 and the micro-fractions PSD-08 - PSD-13, detection in Vis after using spraying reagent Vanillin/H_2SO_4; TLC parameters are given in 7.1.3

5.1.3.2 NMR Measurements of PSD-01 – PSD-35

For the quantitative ^{1}H NMR measurement all 35 MFs were dissolved in methanol-d4 to reach a concentration of 3 mg/mL. 750 μL of each fraction was transferred into an Eppendorf tube and centrifuged ad 3000 rpm for 5 min to avoid precipitation in the NMR tube. 650 μL of the supernatant were transferred to NMR tubes. Detailed information on the NMR measurements is reported in (Grienke et al., 2019; Zwirchmayr, Julia et al., 2020). A quantitative variance of ^{1}H NMR signals over consecutive MFs was desired. Figure 10 shows the stack plot of the obtained *P. santalinus* MFs PSD-01 to PSD-35.

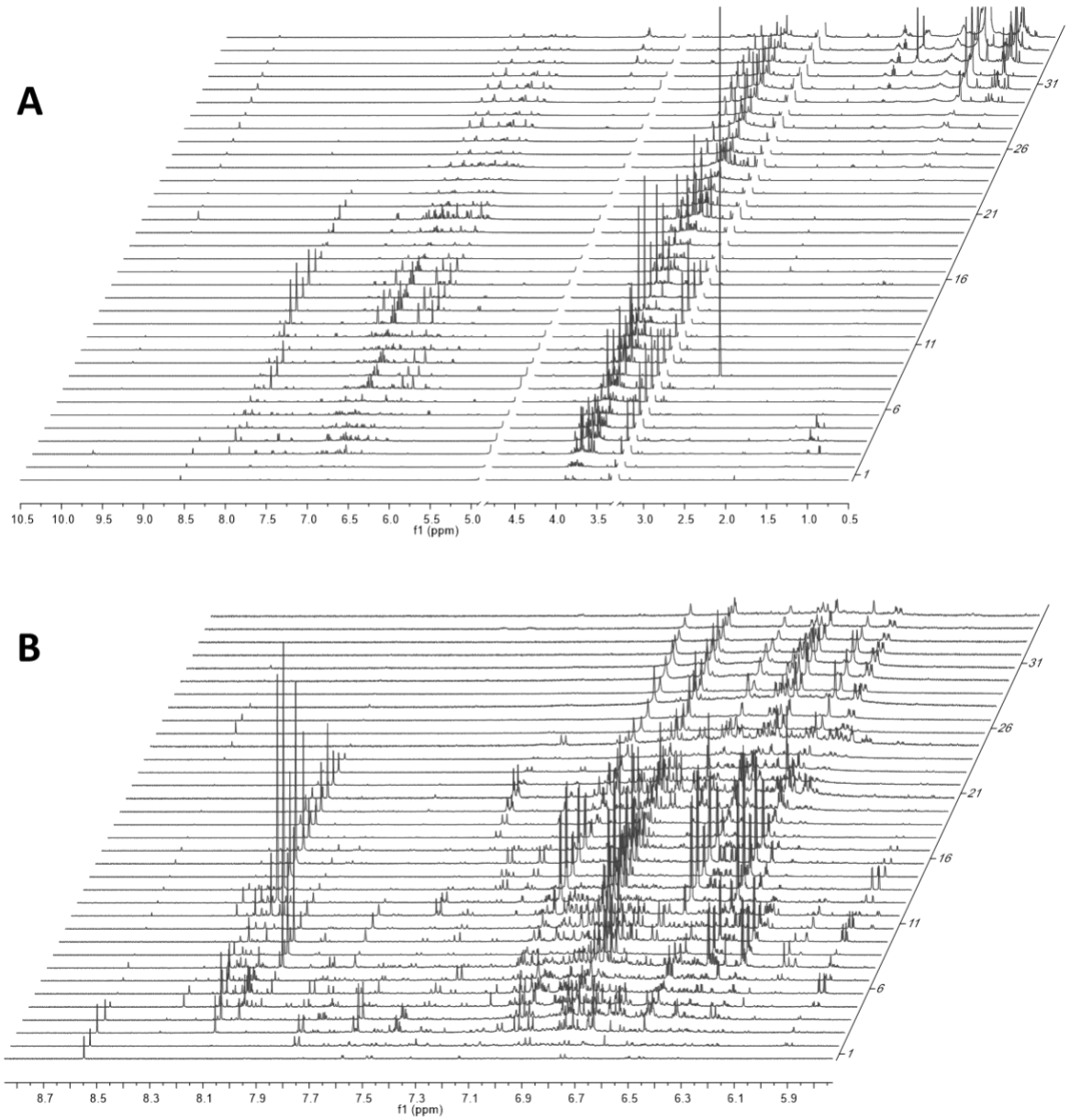


Figure 10 - Stack Plot ^{1}H NMR spectra of PSD-01 - PSD-35; A: δH 0.50 - 10.50 ppm; B: 5.80 - 8.80 ppm; The water signal at 4.9 ppm and the signal of the solvent at 3.31 ppm are not shown.

5.1.3.3 Bioactivity Results of PSD-01 to PSD-035 in a Cell-Based Assay Addressing the Pro-*Inflammatory Chemokine CX3CL1*

Bioactivity patterns of a cell-based assay quantifying mRNA expression of the chemokine CX3CL1, relating to the variance of constituents, were obtained for the *P. santalinus* MFs. In Figure 11 the bioactivity results of the MFs PSD-01 – PSD-12 are shown. It can be seen

that over the course of fraction PSD-01 to PSD-04 an increase in activity is given (i.e., Package 1; red square). On the contrary, Package 2 showed a decreasing activity over the course of PSD-06 to PSD-08. The results of PSD-13 to PSD-35 are still pending (26.02.2021).

Figure 11 - Bioactivity data of PSD-01 - PSD-12 in a real-time PCR analysis of the pro-inflammatory chemokine CX3CL1; the red squares represent package 1 and 2 employed for the biochemometric ELINA approach; PSD-01 – PSD-12 (25 μg/mL), the initial fraction PSD01_03 (50 μg/mL) and the vehicle control were assayed for CX3XL1 expression in primary human venous endothelial cells (HUVEC). HUVECs were either untreated (DMSO) or pre-treated (30 min) with 25 µg/mL PSD-01 – PSD-12 or 50 μg/mL PSD01_03. Bar charts represent relative mRNA levels of CX3CL1 expression ± SD; n = 3.

5.1.3.4 ELINA

Packages 1 and 2 were selected for the ELINA approach. Further investigations concerning to this MFs are detailed in chapter 5.3. Due to time limitations, a targeted isolation of ELINA predicted active constituents was only performed for package 1 in the course of this diploma thesis.

5.1.4 Fractionation Tree

For an overview, all fractions deriving from the extract PtesanXDM are shown in the fractionation tree in Figure 12. The figure shows the hierarchy of the extraction and fractionation process. In the dark-blue box is the first extract made (PtesanXDM) (Thrakl, 2019), which was separated into two fractions by liquid-liquid fractionation (i.e. PtesanXM_DCM and PtesanXDM_MW; diploma thesis Ptak, 2020). Because PtesanXM_DCM showed significant activity in a *C. elegans* life span assay and a cell-based assay from Dr. Rainer de Martin in past works, this fraction was selected for a further separation with the Puriflash Interchim in a normal phase mode. As described in Chapter 7.1, the sub-fraction PSD01_03 was fractionated with Puriflash Interchim in a reversed phase mode. In the fractionation tree the deriving ten sub-fractions can be seen. PSD04_02, PSD04_04 and PSD04_07 were fractionated with Puriflash Interchim hyphenated to High-Performance Counter-Current Chromatography (HPCCC), designated in the following as InterHPCCC. The fraction with the red background (PSD05_16), was selected as package 1 (see 5.3.2.1) and was fractionated with InterHPCCC. The fractions with the grey background (PSD06_03; PSD08_05) were fractionated with a Sephadex column chromatography. For fraction yields see Chapter 7.1.7.

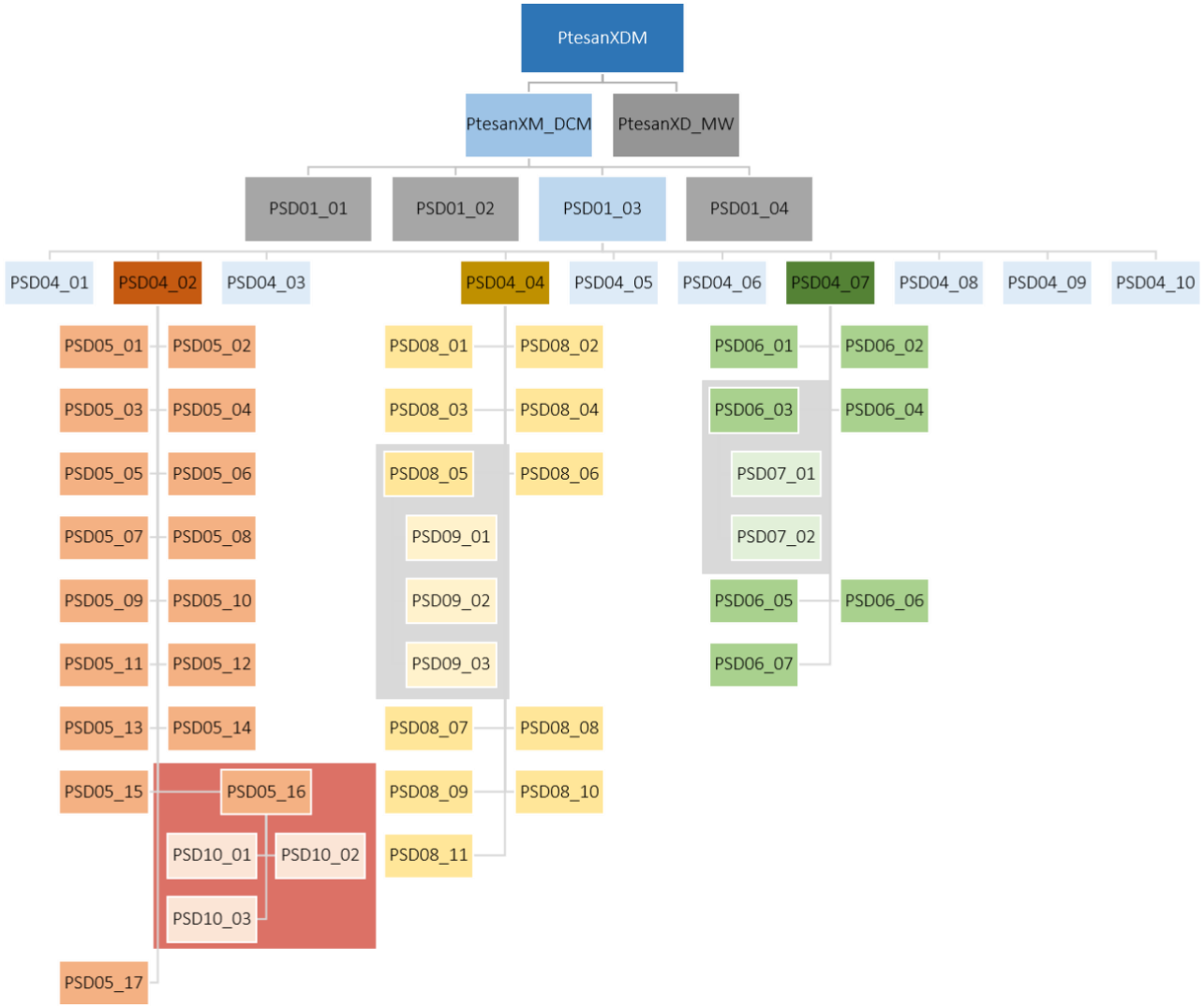


Figure 12 - Fractionation tree of the heartwood extract PtesanXDM

5.2 Phytochemical Investigation of a Bioactive Fraction from *Pterocarpus santalinus* Affecting the Health Span of *Caenorhabditis elegans*

5.2.1 Purification and Isolation of selected Fractions PSD04_01 to PSD04_10

5.2.1.1 Workflow

As obvious from the TLC plate depicted in Figure 5, the only fraction without red polar pigments is PSD04_01. Within this diploma thesis, our focus was to

(i) Deplete the fractions with pigments as they are very persistent concerning e.g., adhesion on solid columns and to

(ii) isolate the predicted bioactive compounds,

(iii) identify the isolated compounds via NMR structure elucidation and

(iv) forward the isolates to bioactivity testing.

Over time it became evident that the red pigments even covered up visible compounds. Also, the TLC plate shows, how complex the composition of the sub-fractions still is. Related to the dereplication of the selected active sub-fractions, we saw that the molecular weight of the constituents was too similar to separate them based on their weight, e.g., by size exclusion chromatography. Therefore, the method of choice was a chromatographic system that separates by differences in polarity, such as the High-Performance Countercurrent Chromatography (HPCCC).

From the five active sub-fractions (i.e., those extending the DT_{50} significantly in the *C. elegans* lifespan assay), three were selected for further purification as part of this work, namely PSD04_02, PSD04_04 and PSD04_07. The other active sub-fractions and further purification steps of the extract PSD01_03 will be part of future experiments at the Division of Pharmacognosy, Department of Pharmaceutical Sciences, University of Vienna.

5.2.1.2 “InterHPCCC”: A Hyphenated Method for Optimization of the Chromatographic Separation

The basic concept of the HPCCC is to separate the analytes by distribution between two immiscible layers, similar to a separating funnel. The benefits are:

(i) no adsorption on a solid stationary phase takes place (as both phases are liquids), hence, there is no loss of sample subjected to chromatographic separation.

(ii) the separation depends on the relative distribution of the single compounds, so this also works very well when the compounds have a similar molecular mass.

(iii) The use of different HEMWat systems within one separation, i.e., gradient elution, allows for an optimal separation suited for each applied material and facilitated the depletion of polar, persistent pigments.

To optimize this chromatographic system, we decided to combine the HPCCC with an automatic column chromatography, the Puriflash Interchim. Basically, the HPCCC replaces the column of the Puriflash Interchim. Therefore, the channel of the flash that usually is placed before the column was attached to the solvent inlet of the HPCCC and vice versa; the channel that is placed after the column was attached to the solvent exit of the HPCCC. With these combined systems designated as “InterHPCCC” the benefits of both chromatographic systems are united. Through the touch screen display of the Flash, it is possible to write and save the method and monitor parameters like pressure, run time etc. during the fractionation process. This guarantees the reproducibility of the InterHPCCC run(s); also, more control is given as the flash has four inlets for mobile phases, so a quaternary gradient elution is possible. Because of the pump-system, the pressure is more stable, which is very important as the maximum pressure for the HPCCC is set to 13 bar. Further, the Puriflash instrument provides a PDA and ELSD detector triggered collection, hence, this supports the monitoring during the separation process by the chromatogram of the Interchim software. Anyway, as not all compounds are visible in both detectors all the time, a time-based fractionation was performed.

Further detailed information about the InterHPCCC is provided in Chapter 7.1.1.

5.2.1.3 HEMWat Screening for Selected Bioactive Fractions

5.2.1.3.1 HEMWat Screening of PSD04_02

For the solvent system screening ~20 mg of the fraction PSD04_02 was diluted in 7 ml MeOH (stock solution, SSo). The biphasic HEMWat systems 13, 14, 15, 16 and 17 were prepared in a test tube. The compositions of the systems are shown in Table 7 in 7.1. In each system one part of pure MeOH was replaced by one part of the stock solution. After the phase separation, one aliquot from upper and lower phase of each system was taken and transferred in glass vials. The dilutions were applied on a TLC plate for a monitoring of the separation (Figure 13). This analysis revealed that the systems 17 and 14 are well suited for the separation with the HPCCC. In system 17 the fraction had a uniform affinity to both phases, except for the red pigments at the bottom of the plate in the upper phase of this system. Therefore, it was possible to separate the pigments from the other compounds. The compounds with a R_f of 0 – 0.4 could elute with the more polar upper phase of system 14.

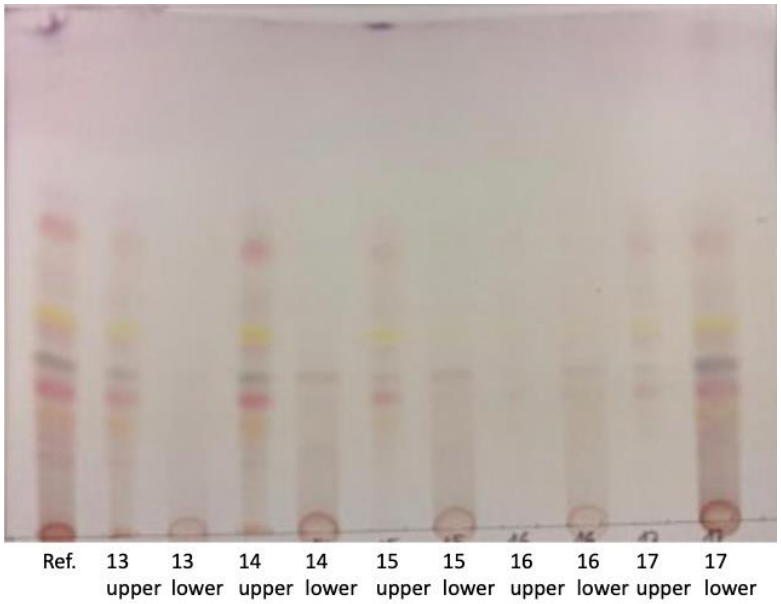


Figure 13 - HEMWat screening of PSD04_02, detection in VIS after using spraying reagent Vanillin/H_2SO_4; TLC parameters are given in 7.1.3

5.2.1.3.2 HEMWat Screening of PSD04_04

For the SSo 12.7 mg of PSD04_04 was diluted in 10 mL MeOH. The biphasic HEMWat systems 14, 15, 16, 17, 18 and 19 were prepared in a test tube (for composition see Table 10). In each system one aliquot of the in MeOH diluted sub-fraction was applied, which in turn replaced on part of pure MeOH. The further process was executed as in the HEMWat screening described before. The distribution of the compounds can be seen in the TLC of the tested HEMWat systems in Figure 14. From the TLC it can be observed that the compounds of this fraction have a bright range of polarity, as the spots on the TLC plate are present at R_f 1.0 – 0.1. So, a HPCCC run with gradient elution, using several HEMWat solvent systems with finer gradations (starting more apolar) was necessary. Again, a normal phase run with gradient elution was performed. The lower layer (LL) of HEMWat system 17 was used as stationary phase; as mobile phases we used the upper layer (UL) of system 17 to start with and further used the upper layers of HEMWat systems 16, 15 and 14. Hence the property of the red pigments showing a good affinity to the LL was exploited in order to deplete them from the other compounds present.

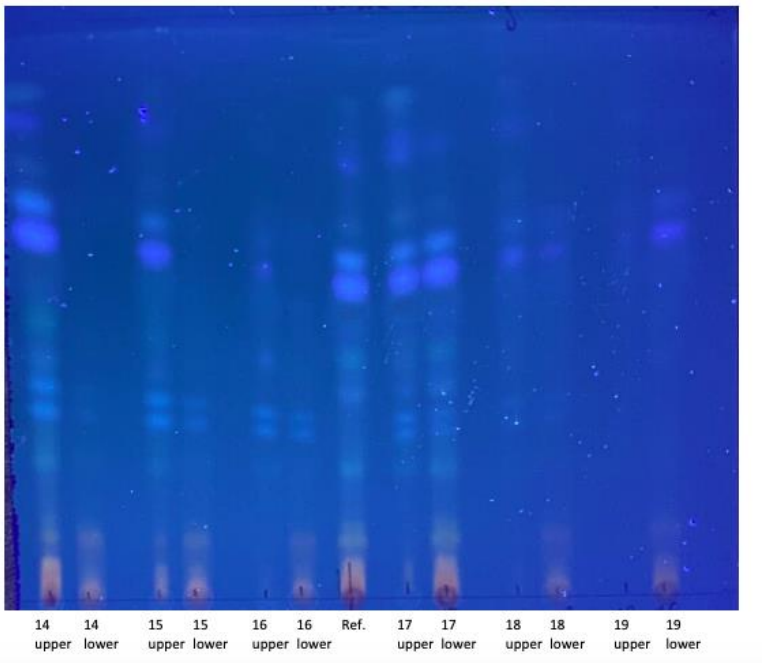


Figure 14 - HEMWat screening PSD04_04, detection at 366 nm; TLC parameters are given in 7.1.3

5.2.1.3.3 HEMWat Screening of PSD04_07Σ

The screening for the last selected fraction was carried out the same way as described above. A stock solution was prepared at a concentration of 10.78 mg/mL. Compared to the other selected fractions, PSD04_07 is more non-polar. Therefore, we chose the systems 19, 20, 21 and 22 for the HEMWat screening. The biphasic solutions were again prepared in test tubes and 1 mL of the stock solution replaced one part of MeOH. The TLC results of the screening can be seen in Figure 15 and Figure 16. Referring to the figures, the bands at the top were evenly distributed in both phases of the HEMWat system 20; the spots at the bottom, again with the red pigments, had only an affinity to the LL. Accordingly, the LL of system 20 was selected as stationary phase and the UL of system 20, 18 and 16 was chosen for the normal phase gradient elution run.

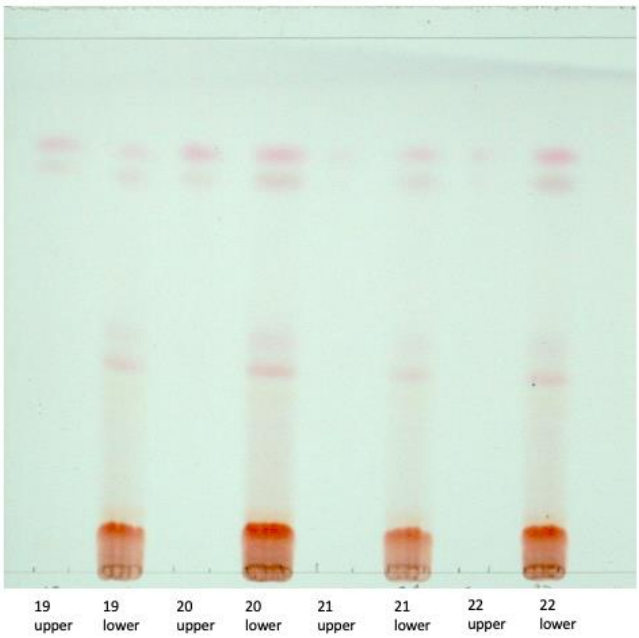


Figure 15 - HEMWat screening PSD04_07, detection in VIS after using spraying reagent Vanillin/H_2SO_4; TLC parameters are given in 7.1.3

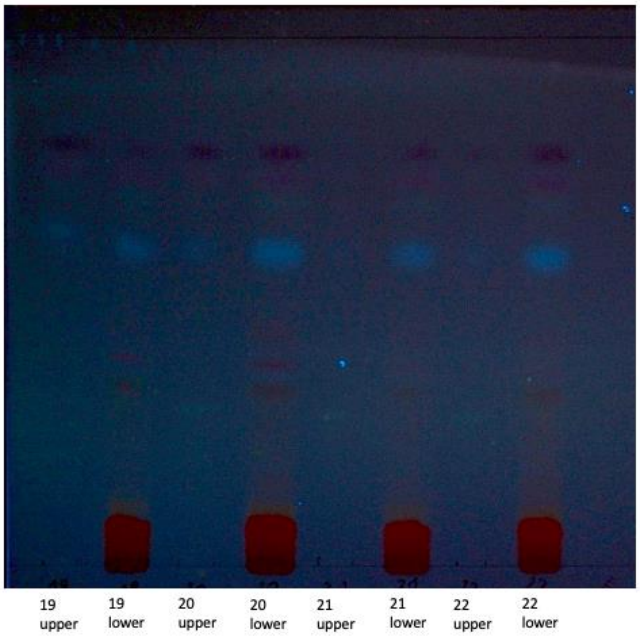


Figure 16 - HEMWat screening PSD04_07, detection in 366 nm after using spraying reagent Vanillin/H_2SO_4; TLC parameters are given in 7.1.3

5.2.1.4 Fractionation of PSD04_02

Prior to each of the three semi-preparative InterHPCCC performances, an adequate method was screened by a test run in an analytical mode (data not shown).

After finding of a suitable HEMWat solvent system and the ideal gradient, the semi-preparative InterHPCCC run was performed. Therefore, ~ 250 mg of the fraction was diluted in 5 mL of each, the stationary phase (17 LL) and the mobile phase (17 UL) because the injection loop can hold a total of 10 ml. After filling of the column with the stationary phase with a flow rate of 10 mL/min, the HPCCC was set to 1600 rpm and the mobile phase was pumped through the connected devises with a flow rate of 6 mL/min. After reaching the equilibrium (i.e., when the mobile phase replaced half of the stationary phase), the diluted fraction was injected with a flat-end Hamilton syringe. At this point the fraction manager of the automated Interchim system was started, collecting 3 mL per fraction (i.e., 2 fractions per minute). Detailed information about the parameters used, see chapter 7.1.5 Table 8 and Table 9. After this single fractionation run, 3 μL of each fraction has applied on a TLC plate for monitoring. Fractions that appeared similar on TLC were pooled together in round-bottom flasks and evaporated under reduced pressure. 17 fractions were pooled, namely PSD05_01

to PSD05_17 (yields are shown in Table 24 and collective TLC is shown in Figure 17) and further chromatographed over UPLC at a concentration of 1 mg/mL and an injection volume of 1 μL. The method we used is shown in Table 19. Selected UPLC chromatograms are shown in the appendix in Chapter 8.1 in Figure 34 and Figure 35.

As described in chapter 5.3.2.1, the obtained polar fractions of PSD04_02, namely PSD05_15 – PSD05 to _17, were considered for a targeted isolation as they were predicted with ELINA to contain the active compounds with anti-inflammatory properties in HUVECs.

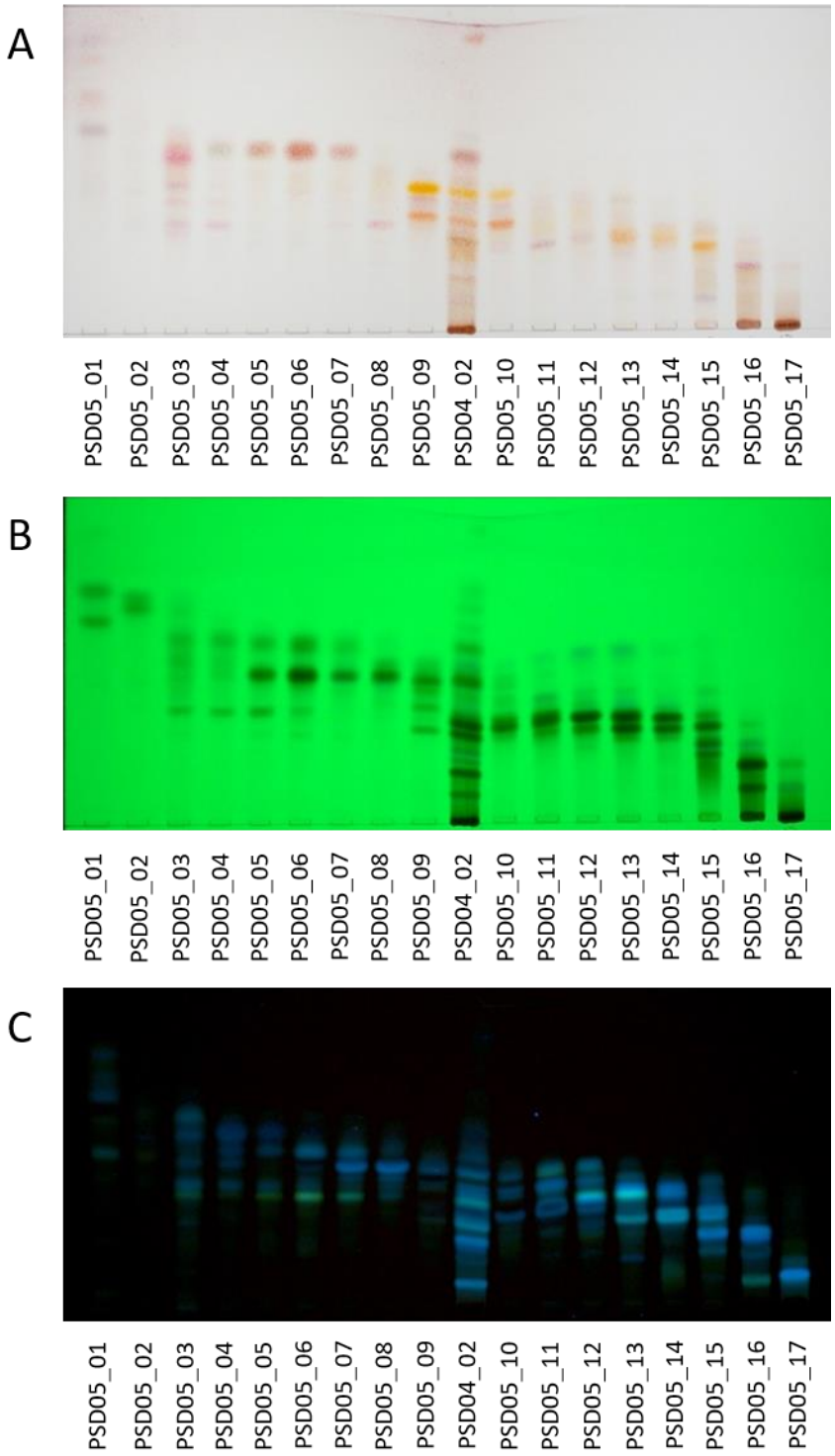


Figure 17 - Collective TLC of PSD04_02 fractions obtained by InterHPCCC fractionation; A: detection in Vis after using spraying reagent Vanillin/H_2SO_4, B: detection at 254 nm, C: detection at 366 nm; TLC parameters are given in 7.1.3

5.2.1.5 Fractionation of PSD04_04

For chromatographic separation ~ 250 mg of PSD04_04 was diluted in 5 mL of the stationary phase (17 LL) and in 5 mL of the mobile phase (17 UL). The column was filled with 17 LL at a flow rate of 10 mL/min for 30 min. After filling the column, the flow rate was reduced to 6 mL/min within 2 minutes, the HPCCC was set to 1600 rpm and the mobile phase was pumped in to reach the equilibrium. The diluted fraction was injected and 1 fraction per minute was collected with the automated fraction manager. In Table 11 and Table 12 the parameters of this method can be seen in detail. After fractionation 3 μL of each fraction was applied on a TLC plate and the compounds were visualized before and after derivatization. Fractions that appeared similar were combined in round-bottom flasks and evaporated under reduced pressure. Through this procedure, 11 fractions were pooled and labeled using the already known method (PSD08_01 – PSD08_11, collective TLC is given in Figure 18). Fraction yields are shown in Table 28. PSD08_01 – PSD08_11 were prepared for UPLC analysis at a concentration of 1 mg/mL and an injection volume of 1 μL. The method is shown in Table 18 and the UPLCs are given in the appendix in Figure 36 and Figure 37.

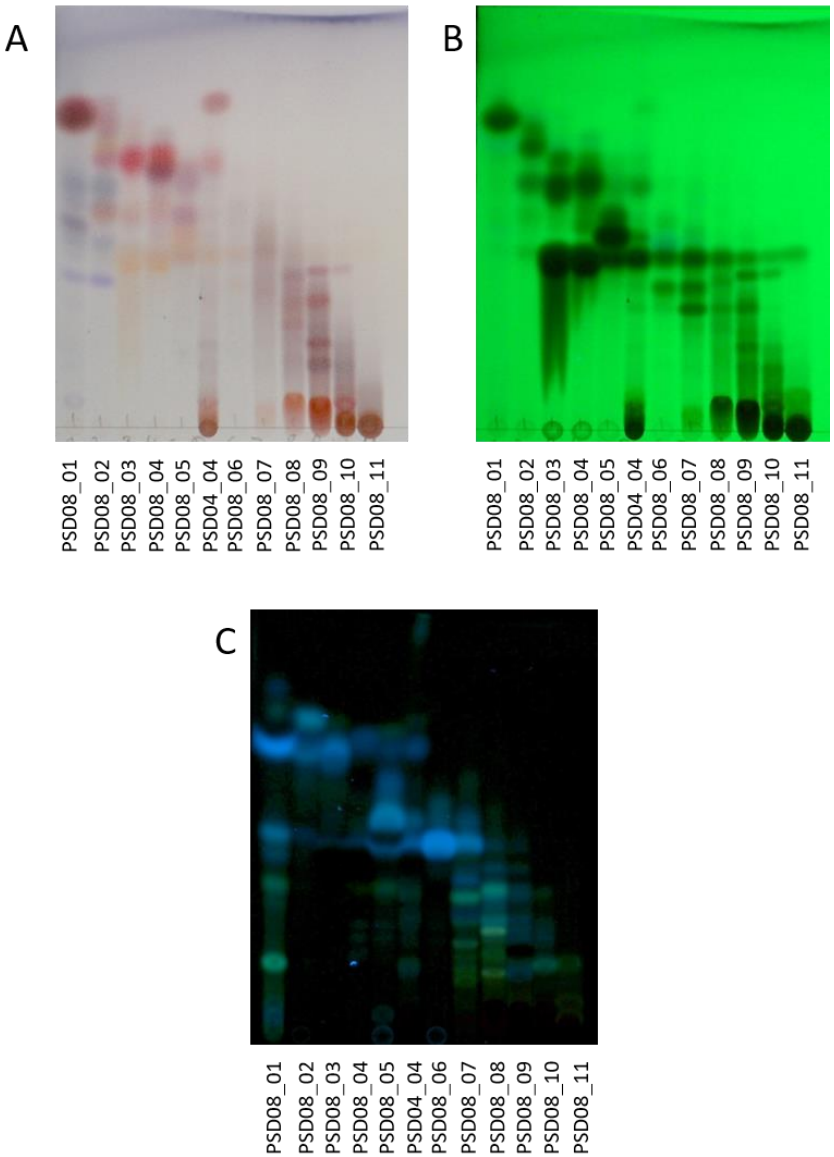


Figure 18 - Collective TLC of PSD04_04 fractions derived by InterHPCCC fractionation; A: detection in Vis after using spraying reagent Vanillin/H_2SO_4, B: detection at 254 nm, C: detection at 366 nm; TLC parameters are given in 7.1.3

Based on MS dereplication of the fractions derived by InterHPCCC fractionation of PSD08_05, a chromatographic separation with Sephadex (i.e., size exclusion chromatography) was performed as there were sufficient differences in the molecular weight of the constituents. Through this purification three compounds could be isolated, namely PSD09_01, PSD09_02 and PSD09_03. The molecular mass is annotated in the compiled UPLC shown in Figure 19; the fraction yields are given in Table 29. A 2D NMR structure elucidation of the isolated pure compounds is ongoing.

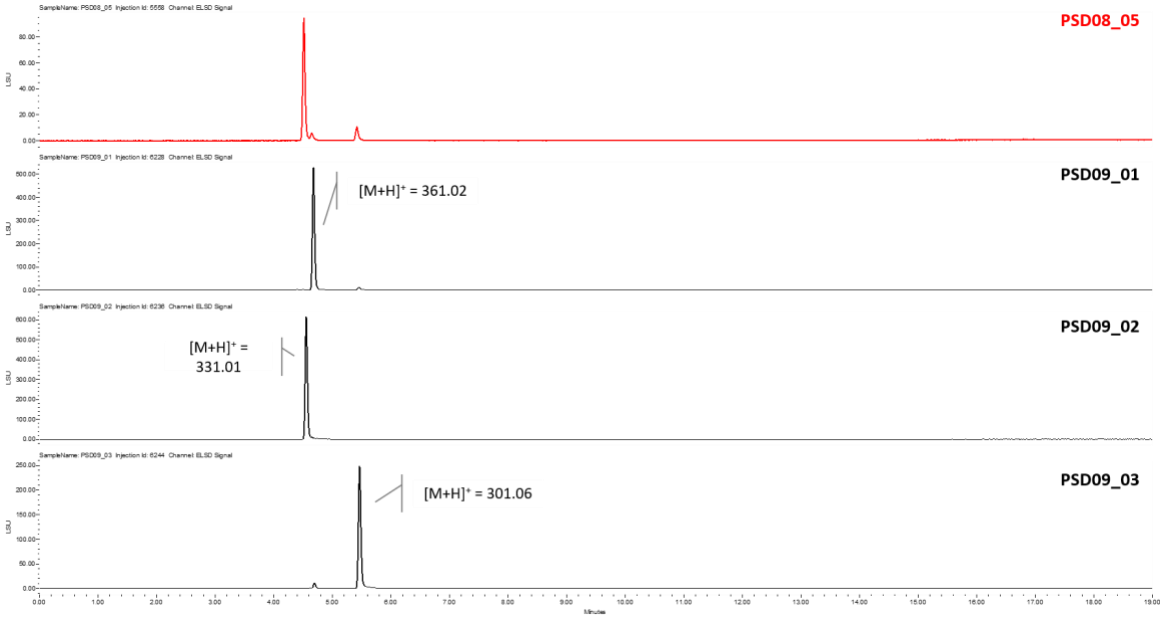


Figure 19 – UPLC analyses of PSD08_05 together with its Sephadex fractions PSD09_01 - PSD09_03 using ELSD and a MS-based dereplication; UPLC parameters are given in 7.1.6

5.2.1.6 Fractionation of PSD04_07

Fraction PSD04_07 yielded 538.72 mg, which required 2 separation runs. For the first run ~ 270 mg were diluted in 5 mL of the stationary phase (20 LL) and in 5 mL of the mobile phase (20 UL). Again, after reaching of the equilibrium, the diluted fraction was injected, and the collection started with 1 fraction per minute. The same procedure was conducted with ~ 250 mg of PSD04_07 for the second run. In total 404 fractions were collected and monitored by TLC. 7 final fractions were pooled out and united in round-bottom flasks, namely PSD06_01 – PSD06_07 (collective TLC is given in Figure 20). The yields are shown in Table 26. PSD06_01 – PSD06_07 were further chromatographed over UPLC at a concentration at 1 mg/mL and an injection volume of 1 μL. Method parameters are shown in Table 20 and UPLCs are given in the appendix in Figure 38.

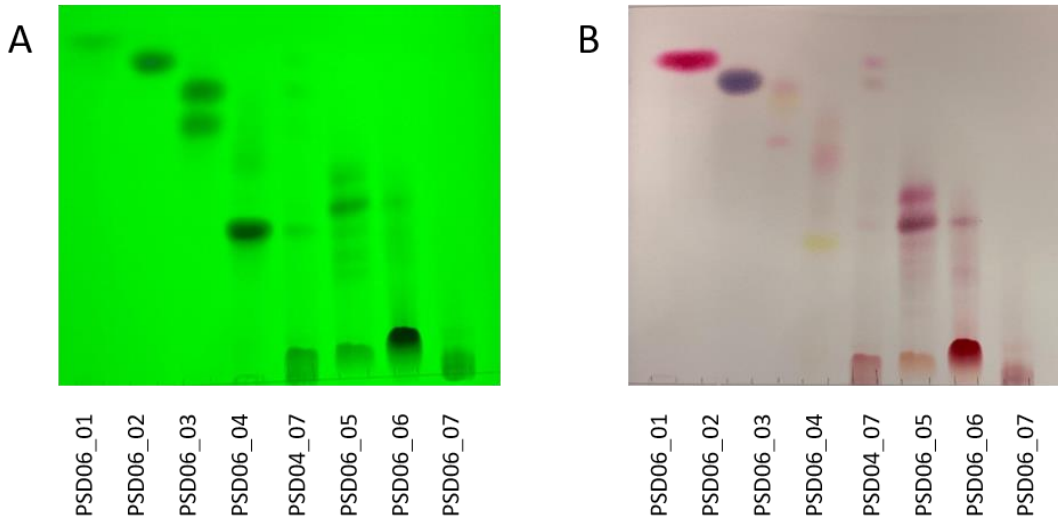


Figure 20 - Collective TLC of PSD04_07 with the obtained InterHPCCC fractions, A: detection at 254 nm, B: detection in Vis after using spraying reagent Vanillin/H_2SO_4; TLC parameters are given in 7.1.3

According to the collective TLC (Figure 20) and the compiled UPLCs (Figure 38) it can be seen that PSD06_01, PSD06_02 and PSD06_4 have been successfully separated from the other molecules in PSD04_07, as they appear as one spot or peak in the TLC and UPLC analyses. An MS dereplication was performed for the first four fractions, the molecular mass is annotated in the UPLCs in Figure 21. A further purification with Sephadex was performed with PSD06_03, hence the two peaks had a difference in their molecular weight. This chromatographic separation resulted in two additional compounds, namely PSD07_01 (*m/z* value of 314.05) and PSD07_02 (*m/z* value of 284.06). Yields are shown in Table 27. To identify these compounds as pure isolates a 2D NMR measurement was performed. A 2D NMR structure elucidation of the isolated pure compounds is ongoing.

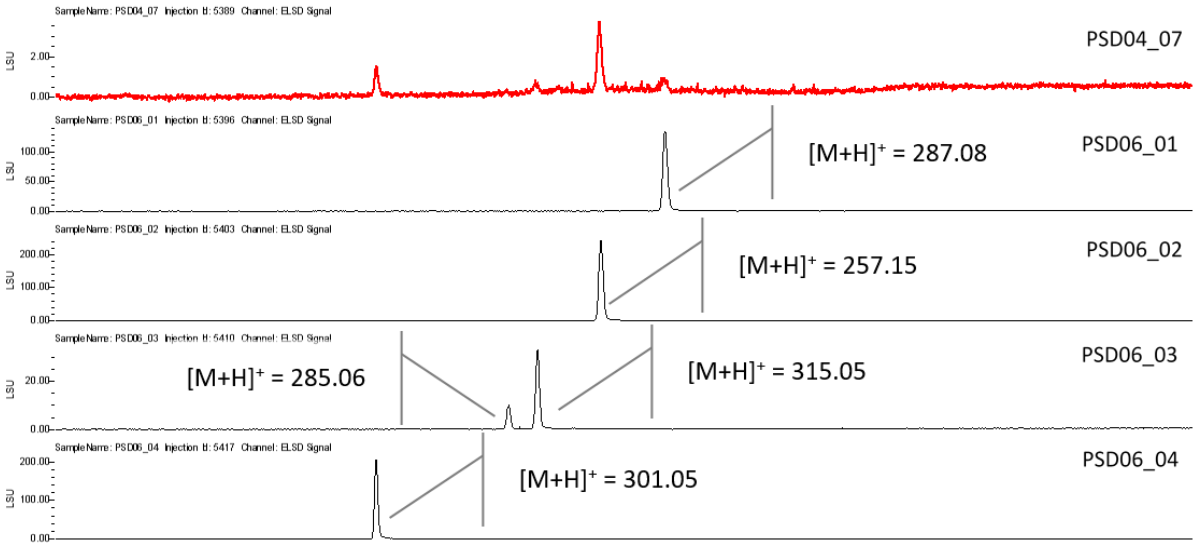


Figure 21 – UPLC analyses of PSD04_07 with its obtained InterHPCCC fractions using ELSD and an MS-based dereplication; UPLC parameters are given in 7.1.6

5.3 A Biochemometric Approach for the Targeted Isolation of *in vitro* Anti-Inflammatory Compounds from *Ptercarpus santalinus*

5.3.1 Correlation of Structural Data with Bioactivity Data to Structurally Identify the Bioactive Constituent(s) from PSD01_03

5.3.1.1 Package 1

For the biochemometric approach ELINA, bioactivity data were correlated with ^{1}H NMR data of a chosen package by using the statistical tool HetCA as described before (Grienke, Foster et al. 2019, Zwirchmayr, Grienke et al. 2020). HetCA plots visualize the correlation between ^{1}H NMR spectra and bioactivity results. In Figure 22 the HetCA plot of package 1 (PSD-01 – PSD-04) is shown. Unfortunately, the correlation did not work for the selected package, as all signals were positively correlated with bioactivity (red signals). Apart from the water and solvent signal at 4.90 and 3.31 ppm, respectively, no signal was negatively correlated with bioactivity (blue signals). Therefore, the semi-quantitative ELSD was used to detect the molecule(s) with potential activity. As obvious from Figure 23, a peak at the retention time (t_R) 2.02 min increased over the course of PSD-01 to PSD-04 (and is not present in the inactive MF PSD-05; chromatogram not shown). According to a dereplication (for more details see chapter

5.3.2) of the selected peak at t_R 2.02 min, the molecule with a *m/z* value of 330.01 could be responsible for the observed bioactivity of package 1. A targeted isolation was performed – see chapter 5.3.2. The identification of the pure compounds by 2D NMR structure elucidation is ongoing.

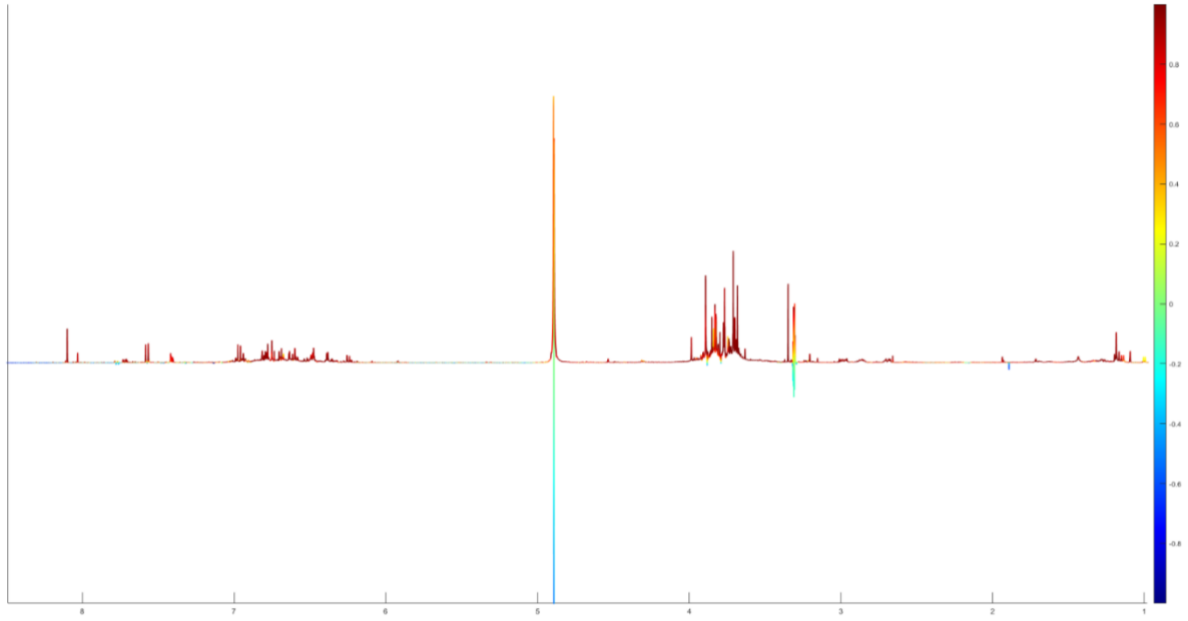


Figure 22 - HetCA plot of package 1. The color code is based on the correlation coefficient: red signals are positively, and blue signals negatively correlated with bioactivity.

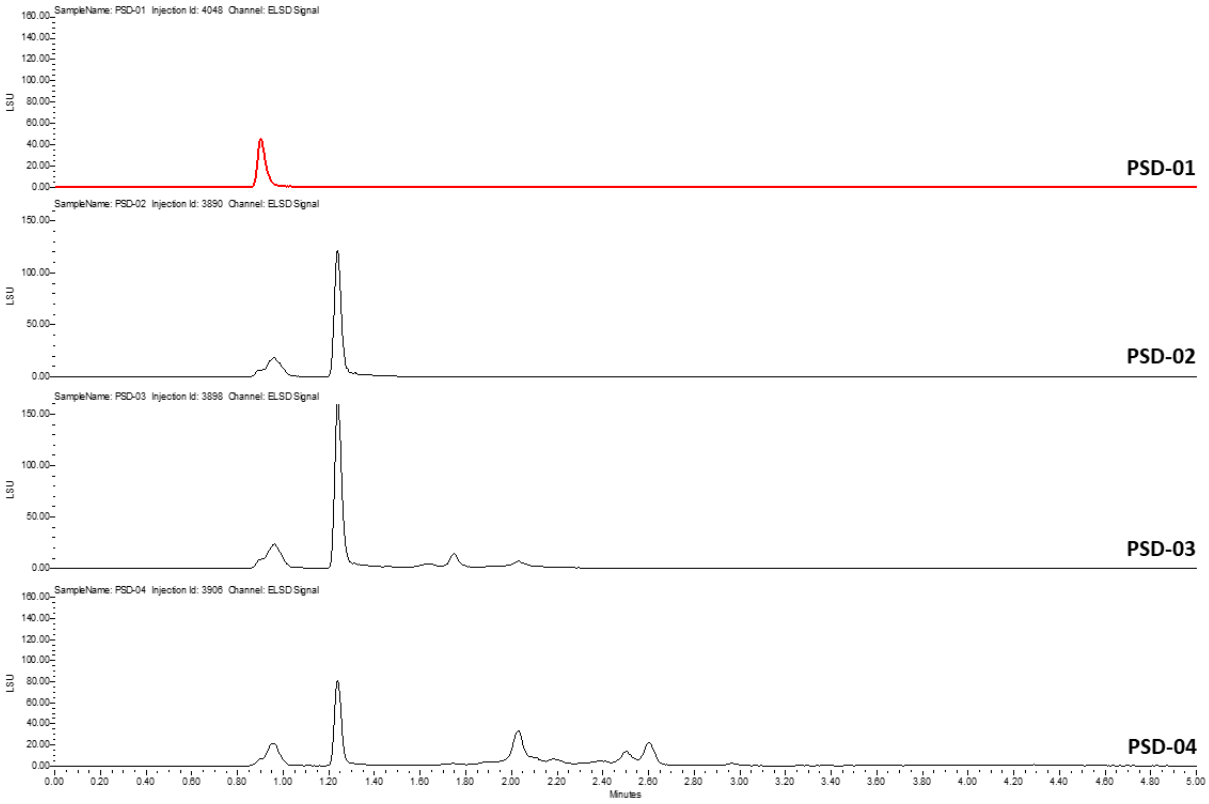


Figure 23 - UPLC-ELSD chromatograms of package 1; zoomed in 0.00 - 5.00 min; UPLC parameters are given in 7.1.6

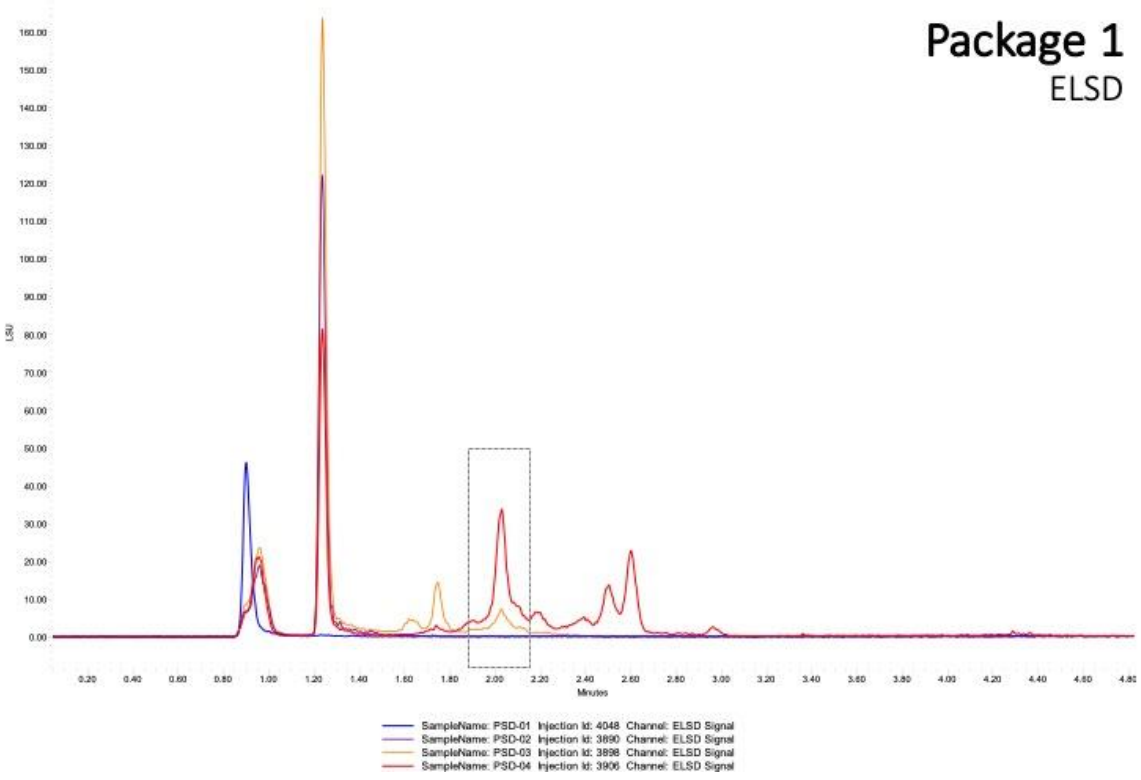


Figure 24 - superimposed UPLC – ELSD chromatograms of Package 1; framed: selected peak at t_R 2.02 min

5.3.1.2 Package 2

In Figure 25 the HetCA plot of package 2, consisting of MFs PSD-06 to PSD-08, is shown. Here, ^{1}H NMR signals that are positively (red signals) as well as negatively (blue signals) correlated with bioactivity can be seen. In the related UPLC-ELSD chromatograms of PSD-06 to PSD-08 (Figure 26), two peaks at t_R = 3.60 min and t_R = 3.80 min with decreased AUC over the course of PSD-06 to PSD08 are present. A dereplication via UPLC-ISM-QDa revealed masses of 314.04 g/mol or 344.01 g/mol for the peak at t_R = 3.60 min and molecular masses of 284.08 g/mol, 344.01 or 374.05 g/mol for the peak at t_R = 3.80 min. A targeted isolation of the compounds for (i) an identification via 2D NMR structure elucidation and (ii) testing of the putative bioactive isolates would be necessary to confirm the predictability of ELINA.

Unfortunately, for reasons of time an isolation of possible bioactive compounds, regarding this package, was not part of this thesis and will be implemented in further experiments at the Department of Pharmaceutical Science in the Division of Pharmacognosy.

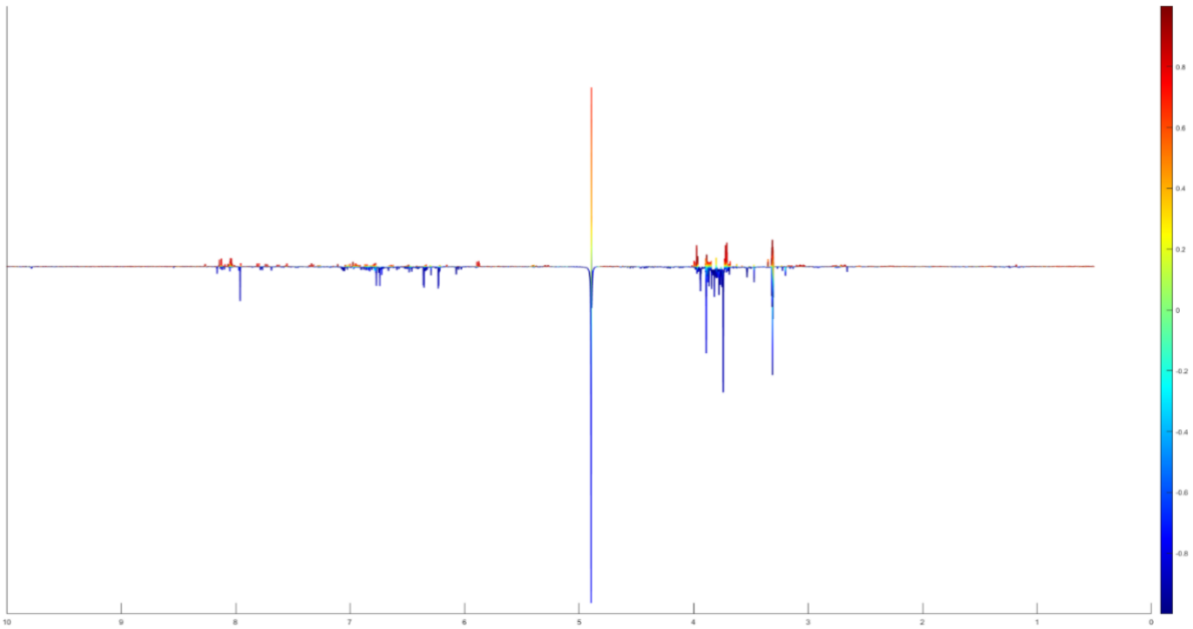


Figure 25 - HetCA plot of package 2. The color code is based on the correlation coefficient: red signals are positively, blue signals negatively correlated with bioactivity.

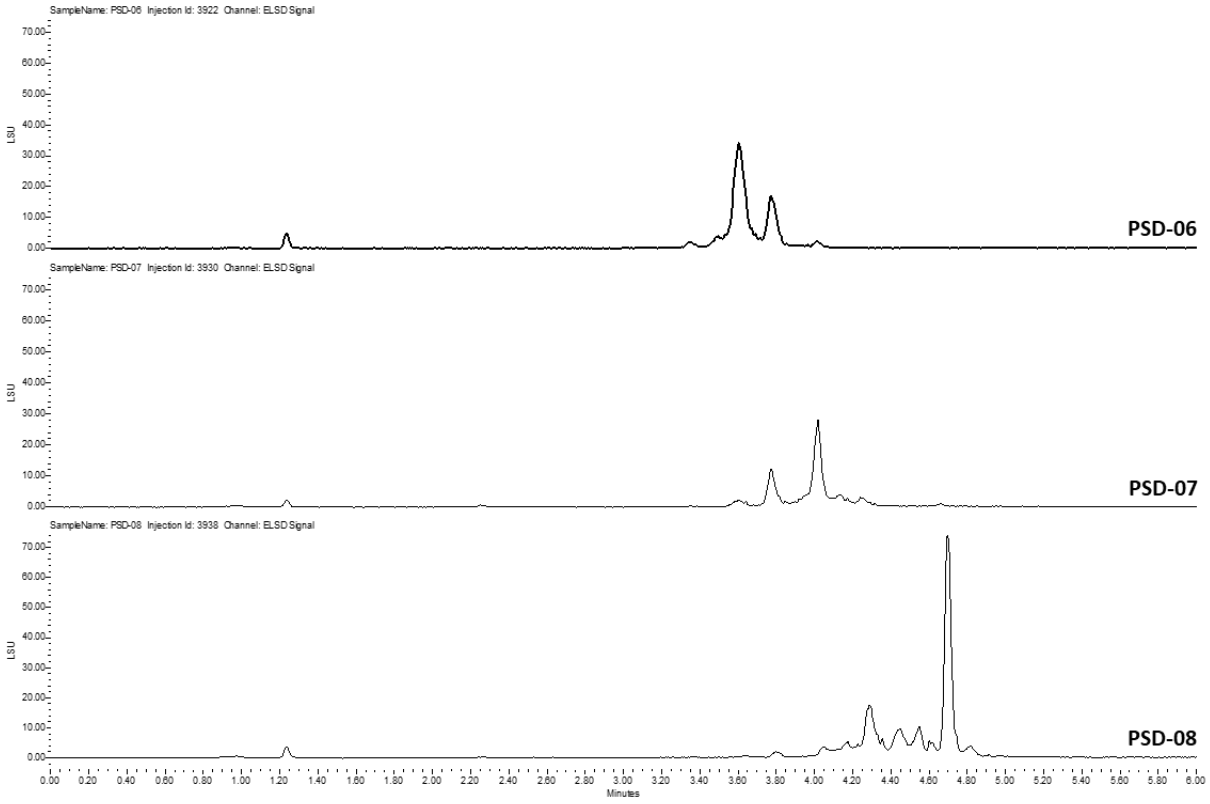


Figure 26- UPLC-ELSD chromatograms of package 2; zoomed in 0.00 - 6.00 min; UPLC parameters are given in 7.1.6

5.3.2 Targeted Isolation and Identification of ELINA Predicted Active Constituent(s)

5.3.2.1 Isolation of Bioactive Compound(s) in Package 1

5.3.2.1.1 Comparison of package 1 and its Sub-fractions of PSD01_03

For a targeted isolation of the predicted bioactive compound(s) in package 1 (consisting of PSD-01 to PSD-04), TLCs of the selected MFs were compared with the ten thereof obtained sub-fractions of PSD01_03. As it can be seen in Figure 27 (B) and Figure 28 (B), the fluorescent as well as the erasing compounds in the MFs with R_f = 0.0 – 0.4 were found in PSD04_02 with a comparable retention time and appearance. Based on the information that PSD-04 was the MF with the highest bioactivity in package 1, the changing of the intensity and the appearance of the TLC spots of this fraction was examined more closely. In Figure 28 (B) the spot of the erasing compound (framed) with R_f = 0.25 gets more intense over PSD-01 to PSD-04 and is gone in PSD-05. Also, a blue, fluorescent compound with R_f = 1.5 (Figure 27) gets more enriched from PSD-02 to PSD-04. Compared with the bioactivity results this could be a

reference to identify the bioactive compound(s), as the activity increased over PSD-01 to PSD-04 and decreased rapidly in PSD-05.

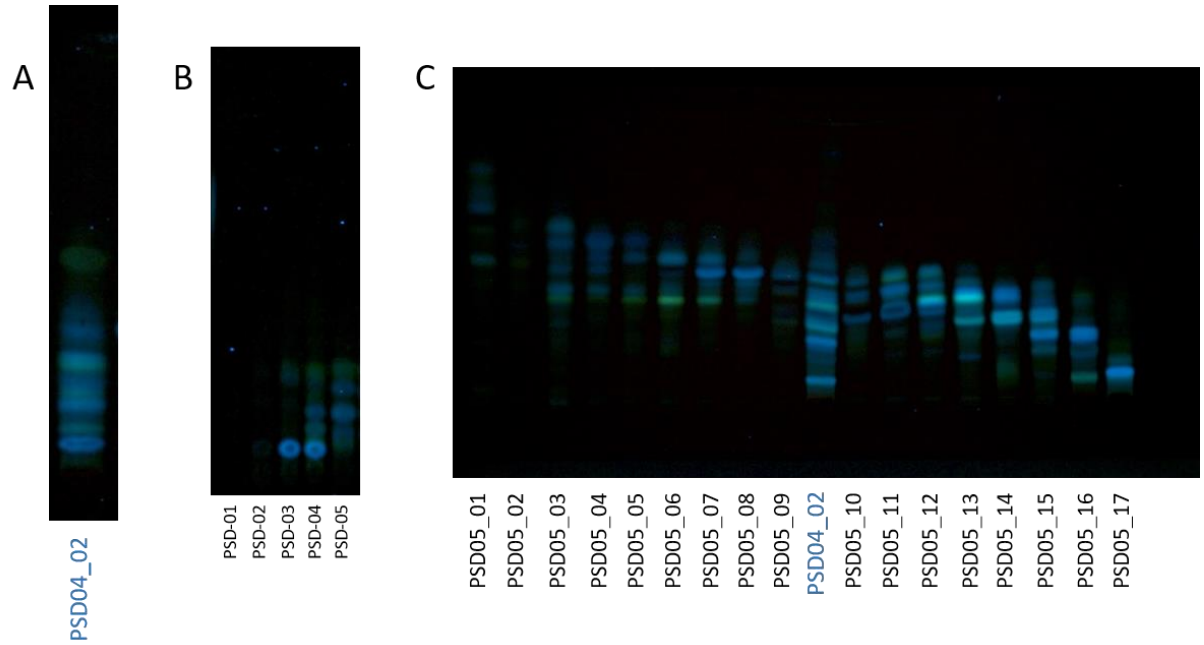


Figure 27 - TLC comparison of package 1 with the sub-fractions of PSD01_03, detected at 366 nm; A: PSD04_02, B: package 1 + PSD-05, C: InterHPCCC fractions of PSD04_02; TLC parameters are given in 7.1.3

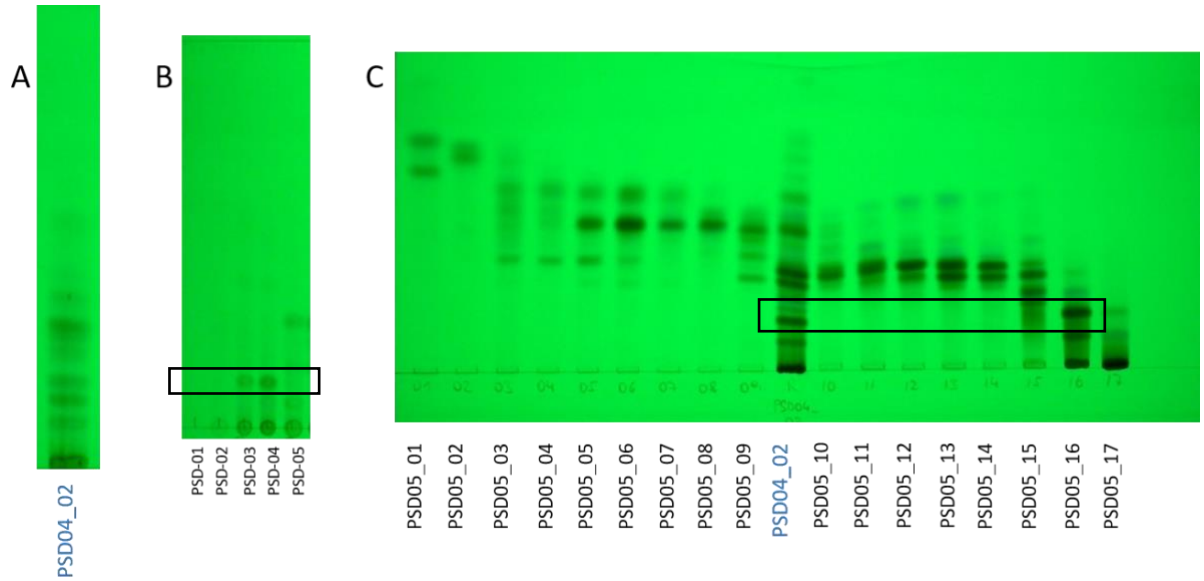


Figure 28 - TLC comparison of package 1 with sub-fractions of PSD01_03, detected at 254 nm; A: PSD04_02, B: package 1 + PSD-05, C: InterHPCCC fractions of PSD04_02; TLC parameters are given in 7.1.3

Because of the significant bioactivity results of PSD04_02 in the *C. elegans* lifespan assay (see chapter 5.1.2), this fraction was already fractionated with InterHPCCC into 17 sub-fractions, i.e., PSD05_01 to PSD05_17. A comparison of both, the TLC and UPLC chromatograms, revealed that the more polar sub-fractions of PSD04_02, namely PSD05_13 to PSD05_16 were pointed out to be the fraction(s) that could contain the ELINA predicted active compound(s) in package 1. A mass dereplication of PSD05_13 to PSD05_17 and the MFs of package 1 was planned, to get more information in which fraction(s) the active compound(s) are contained. Therefore, all MFs of package 1 were chromatographed with the same method as the sub-fractions of PSD04_02, i.e., PSD05_01 to PSD05_17.

5.3.2.1.2 Dereplication of the selected sub-fractions of PSD04_02

For a better separation of the compounds and in order to facilitate a comparison with the sub-fractions of PSD04_02, the UPLC method that was employed for all MFs (see Table 18) was optimized. With this optimized method, another UPLC run was performed with the MFs PSD-03, PSD-04 and PSD-05. These chromatograms were used for a MS-based dereplication, as it can be seen in Figure 29. In “A” the MFs chromatographed with the old method, and in “B” the MFs chromatographed with the new method are shown. The mass analysis for the selected peak at 2.02 min (concerning to the old method) and at 5.60 min (concerning to the new method) revealed a *m/z* value of 330.00.

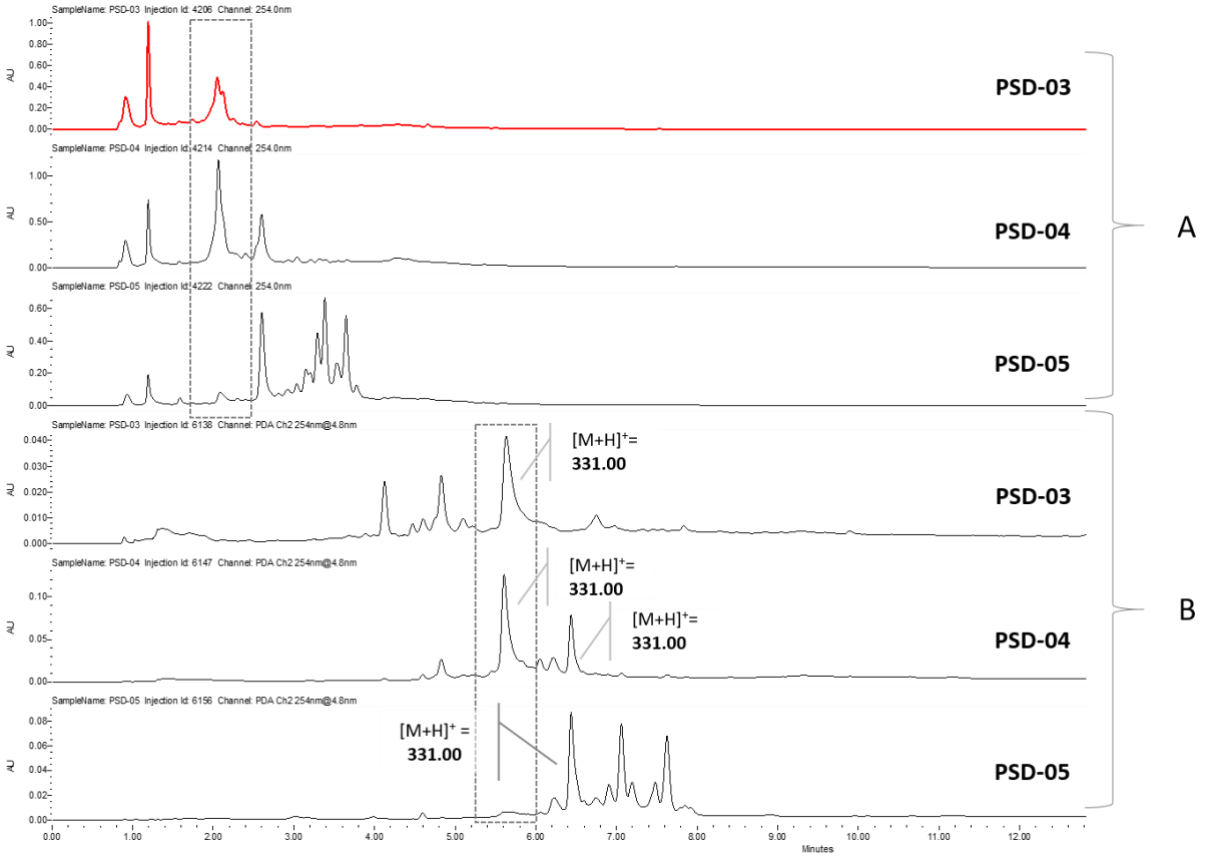


Figure 29 - UPLC - PDA (254 nm) analyses of PSD-03 - PSD-05 with two different methods (A: old method; B: new method) and m/z value in positive mode of the selected peak; UPLC parameters are given in 7.1.6

The selected peak with a *m/z* value of 330.00 was found in one InterHPCCC fraction of PSD04_02, namely PSD05_16. To isolate the selected compound, a further purification of PSD05_16 with InterHPCCC was the method of choice. The parameters we used is shown in Table 30 and in Table 31 in the chapter 7.2. 3. Since the total yield of this fraction was only 37.81 mg, a fractionation in an analytical, normal phase mode was performed. 86 fractions were collected and 3 μL of each fraction was applied on a TLC plate for monitoring. Compounds that appeared similar on the plate were united, 3 fractions were pooled (namely PSD10_01, PSD10_02 and PSD10_02) and yields were determined (see Table 25). A collective TLC of the active MF PSD-04, the fraction PSD04_02, its sub-fraction PSD05_16 together with the generated InterHPCCC fractions PSD10_01 to PSD10_03 is shown in Figure 30.

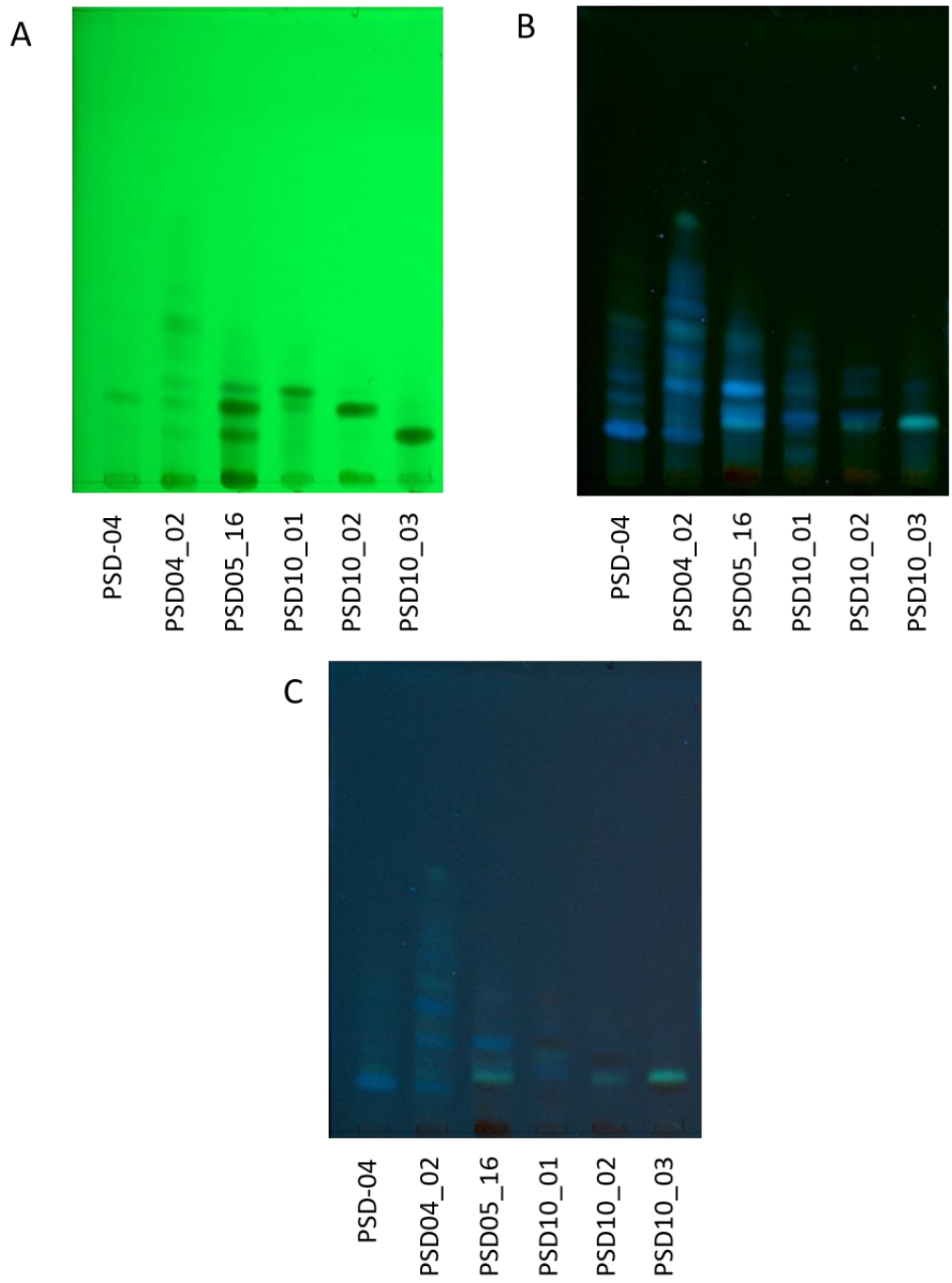


Figure 30 - Collective TLC of PSD-04, PSD04_02 and derived by InterHPCCC fractions; A: detection at 254 nm, B: detection at 366 nm, C: detection at 366 nm after using spraying reagent Vanillin/H_2SO_4; TLC parameters are given in 7.1.3

The obtained fractions were further chromatographed over UPLC at a concentration of 1 mg/mL with an injection volume of 1 μL. In Figure 31 a comparison of the UPLC-PDA analyses of PSD-04, PSD05_16 and the thereof obtained InterHPCCC fractions (PSD10_01 to PSD10_03) is shown. It became obvious, that the selected peak appears in PSD10_02, since the peak had

the same retention time and molecular mass, respectively. Anyway, the peak with a *m/z* value of 330.00 and a retention time of 6.50 that appears in PSD10_03 is also given in the MF PSD-04 and not given in PSD-03 (see Figure 29). This compound could be involved as an additive in the activity of this MF. Both molecules were forwarded to 2D NMR structure elucidation and bioactivity testing in HUVECs.

To prove the anti-inflammatory activity of the isolated molecules, further testing in a cell-based assay quantifying mRNA expression of CX3CL1 or other equivalent assays would be necessary and could be very interesting for future research. The bioactivity results of the isolated compounds are going to be tested by the collaboration partner (February 2021).

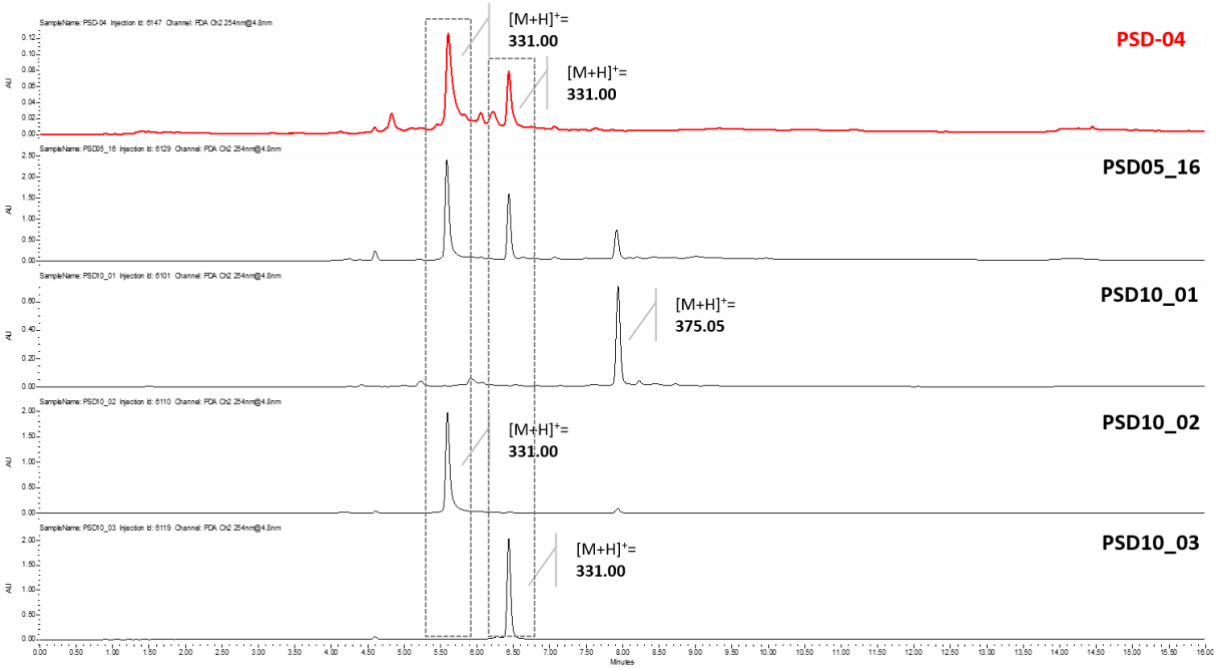


Figure 31 - Comparison of UPLC - PDA chromatograms of PSD-04, PSD05_16 and its InterHPCCC fractions with MS-dereplication, detection at 254 nm; UPLC parameters are given in 7.1.6

6 CONCLUSION

In previous studies a lead-like enhanced heartwood extract (PtesanXDM) from *P. santalinus,* the DCM fraction of this extract (PtesanXM_DCM) and one of its sub-fraction (PSD01_03) showed a significant lifespan-prolonging effect in *C. elegans,* and promising anti-inflammatory activities in an *in vitro* cell-based assay quantifying mRNA expression of the chemokine CX3CL1. Within the scope of this diploma thesis, the active fraction PSD01_03 was fractionated via Puriflash Interchim to ten sub-fractions (PSD04_01 – PSD04_10) and 35 MFs (PSD-01 – PSD-35), respectively. Based on these bioactivity results several phytochemical procedures (e.g., size exclusion/column chromatography, HPCCC, TLC, LC-MS and 1D and 2D NMR experiments) were performed in order to isolate and identify the bioactive compounds from the complex fraction PSD01_03. The work was thematically split into two sections:

The phytochemical processing of PSD04_01 – PSD04_10 affecting the life span of *C. elegans* and the biochemometric approach ELINA employed with the 35 MFs in an anti-inflammatory assay. These two effects of the active sub-fraction, PSD01_03, were examined in more detail in this thesis.

6.1 Phytochemical Investigation of a Bioactive Fraction from *Pterocarpus santalinus* Affecting the Health Span of *Caenorhabditis elegans*

With ~ 4000 mg of the bioactive fraction PSD01_03 a large-scale fractionation was performed using a Puriflash Interchim column chromatography. The collected fractions were combined to ten sub-fractions, namely, PSD04_01 – PSD04_10. These were forwarded to bioactivity testing in a *C. elegans* lifespan assay (tested at 25 μg/mL) and compared with the positive control reserpine (at 30 μM) used as positive control. Six out of ten sub-fractions increased the lifespan of the nematodes significantly in comparison to vehicle control (DMSO 1%). In the course of this diploma thesis the most active fractions were selected for further phytochemical investigations. PSD04_02 resulted in a DT_{50} extension of 20.63% ($p < 0.01$), PSD04_04 led to a DT_{50} extension of 17.97% ($p < 0.01$) and worms treated with PSD04_07 showed a DT_{50} extension of 15.87% ($p < 0.01$) when compared to the vehicle control. The further purification of these three sub-fractions was performed with an optimized chromatographic system that

combines two techniques, the High-Performance Counter Current Chromatography (HPCCC), and an automatic chromatography system for preparative fractionations, the Puriflash Interchim. The optimization of the chromatographic separation was very successful in terms of:

(i) separation of the disruptive red pigments from putative bioactive compounds and
(ii) purification of complex fractions with compounds of a wide range of polarity via gradient elution of the InterHPCCC

From the sub-fraction PSD04_07, 3 compounds were isolated with the InterHPCCC performance (i.e., PSD06_01, PSD06_02, PSD06_04) and after a subsequent separation of PSD06_03 with Sephadex CC two further compounds were isolated, namely PSD07_01 and PSD07_02. Fraction PSD04_04 was purified in the same manner with InterHPCCC and successive Sephadex CC. Three metabolites were isolated (i.e., PSD09_01, PSD09_02, PSD09_03) and forwarded to 2D NMR structure elucidation for an unambiguous identification of the isolates. Separation of the most complex fraction, namely PSD04_02, via InterHPCCC resulted in 16 sub-fractions (i.e., PSD05_01 – PSD05_16). One of those fractions, i.e., PSD05_16, was part of a targeted isolation predicted by the biochemometric ELINA approach (see next chapter). Testing of the generated fractions and isolated compounds in the *C. elegans* lifespan assay will be necessary to identify those compounds with lifespan prolonging activity.

6.2 A Biochemometric Approach for the Targeted Isolation of *in vitro* Anti-Inflammatory Compounds in *Ptercarpus santalinus*

For the ELINA approach 35 MFs (PSD-01 - PSD-35) were generated from ~1000 mg PSD01_03 via Purifalsh Interchim. These MFs were further chromatographed over UPLC detected with ELSD and PDA and forwarded to ^{1}H NMR measurement, each at a concentration of 3 mg/mL. For bioactivity testing they were assayed for CX3XL1 expression in HUVECs at a concentration of 25 μg/mL. Based on the bioactivity results, two packages were selected with a continuous alteration in activity: Package 1 (PSD-01 - PSD-04) with an increasing activity over the course of the selected MFs and Package 2 (PSD-06 - PSD-08) with a decreasing activity over the course of the package. Due to time limitations, a targeted isolation of ELINA predicted active

compound(s) was only performed for Package 1. Unfortunately, the correlation of ^{1}H NMR data and biological data did not work for the selected package, as all NMR signals were positively correlated with bioactivity. Therefore, the semi-quantitative ELSD was used to compare the AUC of the contained peaks and detect molecules with potential activity. Two compounds, namely PSD10_02 and PSD10_03, present in the MF with the highest bioactivity (PSD-04), were successfully isolated from a sub-fraction of PSD04_02, namely PSD05_16 via InterHPCCC, and were forwarded to NMR structure elucidation and bioactivity testing in HUVECs.

In the scope of this diploma thesis, two different phytochemical approaches were performed with a selected bioactive fraction of *P. santalinus* (i.e., PSD01_03): (i) a traditional bioactivity-guided fractionation/isolation procedure with lifespan-prolonging fractions (PSD04_01 – PSD04_10) in the nematode *C. elegans* and (ii) the advanced biochemometric approach ELINA targeting the anti-inflammatory potential of 35 generated MFs (PSD-01 – PSD-35) in HUVECs. The final bioactivity in both test systems has to be confirmed by (i) testing the generated sub-fractions PSD05_01 – PSD05_16, PSD06_01 – PSD06_07, PSD08_01 – PSD08_11 (obtained from the fractions PSD04_02, PSD04_07 and PSD04_04) as well as the isolates (PSD09_01 – PSD09_03 and PSD07_01 – PSD07_02) in the *C. elegans* lifespan assay and (ii) the compounds obtained via targeted isolation using the ELINA approach, i.e. PSD10_02 and PSD10_03 to prove the predictability of ELINA in the anti-inflammatory assay addressing the chemokine CX3CL1 via qPCR analysis. These experiments have to be conducted at both, the Division of Pharmacognosy, Department of Pharmaceutical Sciences, University of Vienna and the cooperation partner Prof. Dr. Rainer de Martin from the Medical University of Vienna. It can be concluded that in comparison to the bioactivity-guided fractionation/isolation approach, the ELINA approach is more straight-forward regarding identification and isolation of putative bioactives. Since only a hand-full of isolates have to be tested for their anti-inflammatory potential (i.e., PSD10_02 and PSD10_03) in order to proof the strength and predictability of ELINA, this biochemometric approach saves time, resources and has the ability to accelerate the NPs drug discovery process.

7 MATERIALS AND METHODS

This chapter contains detailed information about the phytochemical methods and all used parameters described in Chapter 4.

7.1 Phytochemical Investigation of a Bioactive Fraction from *Pterocarpus santalinus* Affecting the Health Span of *Caenorhabditis elegans*

In past work a large-scale extract of *P. santalinus* was generated using dichloromethane and methanol as extraction solvent (Thrakl, 2019). In a continuing diploma thesis, this extract was separated through a liquid – liquid separation with dichloromethane and methanol water (1:1). The dichloromethane extract was further fractionated with the Puriflash Interchim to 4 fractions in normal phase mode (PSD01_01 to PSD01_04). The third fraction, PSD01_03, showed a significant activity in a *C. elegans* life span assay, as well as in the assay of Dr. Rainer de Martin. Therefore, this fraction was selected for further purification. For more details regarding the process of extraction and fractionation see chapter 5.1.4

7.1.1 Chromatographic Methods

7.1.1.1 PuriFlash Interchim

The PuriFlash Interchim is an automatic chromatographic system for preparative fractionations. By implementation of a so called dry-load, it is possible to load a greater amount of the sample, than for instance with methods like HPLC or HPCCC. With a suitable column size the amounts can range from 100 mg to more than 10 g for dry-load. This system is equipped with an evaporative light scattering detector (ELSD), a photodiode array (PDA; possible ranges from 200 – 400 nm), and an automated fraction manager with a closed fraction collector, which is controlled by the Interchim software. Also, the Flash has a solvent manager with a robust pump technology that allows you to mix 4 channels with 1 % accuracy (interchim.com, 2021).

7.1.1.2 High-Performance Counter-Current Chromatography

The Counter-Current Chromatography (CCC) or HPCCC is a liquid chromatography (LC) technique using a biphasic liquid system. During the separation process the analytes migrate between the two immiscible liquids according to their partition coefficient (K_D), while the support-free stationary phase is held in place by centrifugal force and the mobile phase is pumped through the column. The basic requirement is that the extract/fraction to be separated has affinity to both phases. The composition of the solvents is determined by screening HEMWat systems and can be specially selected for each complex mixture (Berthod et al., 2009).

7.1.1.3 „InterHPCCC“: A Flash-HPCCC-Hybrid

As descriped in Chapter 5.2.1.2 we optimized the chromatographic separation by combination of two techniques, the PuriFlash Interchim and the HPCCC.

7.1.1.4 Ultra-Performance Liquid Chromatography

The UPLC is an advanced LC technology with high resolution due to instrumentation and column technology. The adsorptive stationary phase has a smaller particle size (1.7 – 1.8 μm) than in the conventional HPLC technology. The mobile phase is pumped through the column at pressures up to 1030 bar (15.000 psi). Thus, provides a higher sensitivity and resolution of the chromatographic performance, also it shortens the time of the analysis (waters.com, 2021).

7.1.1.5 Sephadex

Sephadex (i.e.: separation Pharmacia dextran) is a LC – chromatography which separates the molecules by sizing. The column is filled with a dextran gel, which, in conjunction with aqueous solutions (e.g.: MeOH), forms a gel with uniform pore size (sigmaaldrich.com, 2021).

7.1.2 Labeling of Extracts and Fractions

In previous diploma theses, the heartwood of P*. santalinus* was extracted with both, DCM and MeOH (i.e., PtesanXDM). After a liquid-liquid fractionation between DCM (PtesanXDM_DCM) and aqueous MeOH (PtesanXDM_MW), PteanXDM_DCM was further fractionated with Puriflash Interchim. The fraction PSD01_03, which was the starting point in this work, was labeled by using the first letter of genus and species, the D is for the extraction solvent DCM. The number comes from the consecutively fractionation, in this case it was the first fractionation of PtesanXM_DCM and the third fraction yielded. The ten sub-fractions of PSD01_03 were labeled in an equal way, e.g., PSD04_01 for the first fraction and PSD04_10 for the tenth fraction. This type of designation was continued for all the deriving fractions. The MFs were also labeled by using the first letters of genus, species and the extraction solvent, namely PSD-01 to PSD-35.

7.1.3 TLC – System

The parameters for TLC are given in Table 3, this system was used in the whole work. The TLC was carried out after the chamber has reached saturation. The compounds were visualized using visible light and ultraviolet light (254 nm and 366 nm) before and after derivatization with vanillin/sulfuric acid and heating it up to approximately 100°C for ~ 1 minute.

Table 3 - TLC system for P. santalinus

Stationary phase:	TLC silica gel 60 F_{254}
Mobile phase:	Chloroform : formic acid : ethyl acetate (8:1:1)
Spraying reagent:	Vanillin 1% in MeOH and sulfuric acid 5% in MeOH
Detection:	Vis, UV_{254}, UV_{366} and after derivatization

7.1.4 Method for the Fractionation of PSD01_03 with PuriFlash Interchim

The third fraction of the dichloromethane extract PtesanXM_DCM was fractionated using a Puriflash Interchim in a reversed phase mode, using a gradient elution. The used method is shown in Table 4 and the composition of the solvents for gradient elution is shown in Table 5. After pooling out the fractions, yields were determined, these are shown in Table 22. Except the fraction volume, the same method was used for the microfractioantion, which was performed one time with 1000 mg. The parameters for the fraction manager are shown in Table 6.

Table 4 - Method for Fractionation of PSD01_03 with Puriflash Interchim; reveresd phase mode

Column	PuriFlash Column 15 C18 HQ 35G
Amount of sample	1000 mg (4x)
Flow rate	15 mL/min
Mobile phase	Solvent A: H_2O Solvent B: Acetonitrile

Table 5 - Composition of Gradient Elution for Fractionation with Puriflash Interchim

Time	% A	% B
00:00	95	5
10:00	95	5
20:00	70	30
52:41	70	30
01:17:41	50	50
01:35:40	50	50
01:46:20	02	98
02:15:40	02	98

Table 6 - Parameters for Fraction Manager, Fractionation with Puriflash Interchim

	Ten Sub-fractions	35 Micro-fractions
Collection volume	20 mL each fraction	5 mL each fraction
Collected fractions	90 – 100 per run	350

7.1.5 Methods for the Purification of Selected Bioactive Fractions with InterHPCCC and Sephadex

The prioritized sub-fractions PSD04_02, PSD04_04 and PSD04_07 were further purified. As described in Chapter 5.2.1.2, the chromatographic separation with a self-made hybrid of the Puriflash Interchim and the HPCCC, designated InterHPCCC, was the method of choice for the purpose of this diploma thesis.

Before each InterHPCCC performance in a semi-preparative mode, an analytical run (i.e., monitoring with a smaller amount of sample and solvents, respectively) was performed for monitoring and optimizing the used parameters such as appropriate HEMWat systems.

PSD04_02:

Table 7 - Tested HEMWat Systems PSD04_02

No.	n-hexane	ethyl acetate	methanol	water
13	2	5	2	5
14	1	2	1	2
15	2	3	2	3
16	5	6	5	6
17	1	1	1	1

Table 8 - Method for Fractionation with InterHPCCC in normal phase mode, PSD04_02

Mode	Semi-preparative
Amount of sample	~ 250 mg
Stationary phase	HEMWat 17 lower layer (solvent A)
Mobile phase	HEMWat 17 upper layer (solvent B) HEMWat 14 upper layer (solvent C)
Flow rate	6 mL/min
Collection volume	3 mL each fraction
Collected fractions	323

Table 9 - Composition of Gradient Elution for Fractionation with InterHPCCC, PSD04_02

Time	Flow rate	%A	%B	%C
00:00	10 mL/min	**100**	0	0
30:00	10 mL/min	**100**	0	0
32:00	6 mL/min	**100**	0	0
32:03	6 mL/min	0	**100**	0
01:30:00	6 mL/min	0	**100**	0
02:15:00	6 mL/min	0	0	**100**
03:30:00	6 mL/min	0	0	**100**

PSD04_04:

Table 10 - Tested HEMWat Systems PSD04_04

No.	n-hexane	ethyl acetate	methanol	water
14	1	2	1	2
15	2	3	2	3
16	5	6	5	6
17	1	1	1	1
18	6	5	6	5
19	3	2	3	2

Table 11 - Method for Fractionation with InterHPCCC in normal phase mode, PSD04_04

Mode	Semi-preparative
Amount of sample	~ 250 mg
Stationary phase	HEMWat 17 lower layer (solvent A)
Mobile phase	HEMWat 17 upper layer (solvent B) HEMWat 16 upper layer (solvent C) HEMWat 15 upper layer (solvent D) HEMWat 14 upper layer (solvent E)
Flow rate	6 mL/min
Collection volume	6 mL each fraction
Collected fractions	249

Table 12 - Composition of Gradient Elution for Fractionation with InterHPCCC, PSD04_04

Time	Flow rate	%A	%B	%C	%D	%E
00:00	10 mL/min	**100**	0	0	0	0
30:00	10 mL/min	**100**	0	0	0	0
32:00	6 mL/min	**100**	0	0	0	0
32:03	6 mL/min	0	**100**	0	0	0
01:20:00	6 mL/min	0	**100**	0	0	0
02:05:00	6 mL/min	0	0	**100**	0	0
02:50:00	6 mL/min	0	0	**100**	0	0
03:35:00	6 mL/min	0	0	0	**100**	0
04:20:00	6 mL/min	0	0	0	**100**	0
04:36:00	6 mL/min	0	0	0	0	**100**
04:56:00	6 mL/min	0	0	0	0	**100**

PSD04_07:

Table 13 - Tested HEMWat Systems PSD04_07

No.	n-hexane	ethyl acetate	methanol	water
19	3	2	3	2
20	2	1	2	1
21	5	2	5	2
22	3	1	3	1

Table 14 - Method for Fractionation with InterHPCCC in normal phase mode, PSD04_07, two runs performed

Mode	Semi-preparative
Amount of sample	~ 270 mg + ~ 250 mg
Stationary phase	HEMWat 20 lower layer (solvent A)
Mobile phase	HEMWat 20 upper layer (solvent B) HEMWat 18 upper layer (solvent C) HEMWat 16 upper layer (solvent D)
Flow rate	6 mL/min
Collection volume	6 mL each fraction
Collected fractions	404

Table 15 - Composition of Gradient Elution for Fractionation with InterHPCCC, PSD04_07

Time	Flow rate	%A	%B	%C	%D
00:00	10 mL/min	**100**	0	0	0
30:00	10 mL/min	**100**	0	0	0
32:00	6 mL/min	**100**	0	0	0
32:03	6 mL/min	0	**100**	0	0
01:15:00	6 mL/min	0	**100**	0	0
01:30:00	6 mL/min	0	0	**100**	**0**
02:00:00	6 mL/min	0	0	**100**	**0**
03:00:00	6 mL/min	0	0	0	**100**
04:10:00	6 mL/min	0	0	0	**100**

PSD06_03:

Table 16 - Method for Fractionation with Sephadex, PSD06_03

Stationary phase	LH-20 in MeOH
Mobile Phase	MeOH
Applied amount	8.47 mg
Pre run	12 Fractions in 30 minutes
Collected fractions	108 in 2 minutes

PSD08_05:

Table 17 - Method for Fractionation with Sephadex, PSD08_05

Stationary phase	LH-20 in MeOH
Mobile Phase	MeOH
Applied amount	20.56 mg
Pre run	12 Fractions in 30 minutes
Collected fractions	110 in 2 minutes

7.1.6 Methods for UPLC analysis

Table 18 - Used UPLC Method for PSD01_03, Sub-fractions and Micro-fractions, reversed phase mode

PtesanXDM_PDA_ELSD_col2				**PtesanXDM_PDA_QDa_col2**				
Instrument	Col.	Temp. [°C] Col.	Temp. [°C] Sample	Flow rate [mL/min]	Inj vol [μL]	Config.		Det. Wavel. [nm]
UHPLC Waters	HSS T3 100 mm	40 °C	8 °C	0.250	1	PDA ELSD	PDA QDa	210

Time [min]	%A Water	%B Acetonitrile
00:00	70	30
00.50	70	30
02:00	55	45
03:50	50	50
12:00	45	55
12:10	2	98
18:00	2	98
18:10	70	30
19:00	70	30

Table 19 - Used UPLC Method for PSD04_02 and deriving Sub-fractions, reversed phase mode

PSD04_02_PDA_ELSD_col2_16min								**PSD04_02_PDA_QDa_col2_16min**
Instrument	Col.	Temp. [°C] Col.	Temp. [°C] Sample	Flow rate [mL/min]	Inj. Vol. [μL]	Config.		Det. Wavel. [nm]
UHPLC Waters	HSS T3 100 mm	40 °C	8 °C	0.250	1	PDA ELSD	PDA QDa	210

Time [min]	%A Water	%B Acetonitrile
00:00	85	15
00:50	85	15
04:00	70	30
12:00	48	52
12:10	2	98
15:50	2	98
15:60	85	15
16:00	85	15

Table 20 - Used UPLC Method for PSD04_07 and deriving Sub-fractions, reversed phase mode

PSD04_07_PDA_ELSD_col2_16min						**PSD04_07_PDA_QDa_col2_16min**		
Instrument	Col.	Temp. [°C] Col.	Temp. [°C] Sample	Flow rate [mL/min]	Inj vol [μL]	Config.		Det. Wavel. [nm]
UHPLC Waters	HSS T3 100 mm	40 °C	8 °C	0.250	1	PDA ELSD	PDA Qda	210

Time [min]	%A Water	%B Acetonitrile
00:00	60	40
00:50	60	40
00:60	55	45
09:00	45	55
10:00	2	98
10:10	2	98
15:00	2	98
15:10	60	40
16:00	60	40

Table 21 - Used ISM Method for UPLC analysis with QDa

Isocratic Solvent Manager with splitter kit		
Solvent	Splitter	Flow rate [mL/min]
Ammonium formate 10 mM, dissolved in H_2O:MeOH (90:10)	1:10	0.150

7.1.7 Fraction Yields

Table 22 - Fraction Yields PSD04_01 to PSD04_10

Sub-fractions PSD01_03	Yield [mg]
PSD04_01	165.5
PSD04_02	315.9
PSD04_03	519.14
PSD04_04	431.4
PSD04_05	248.6
PSD04_06	513.3
PSD04_07	538.72
PSD04_08	311.78
PSD04_09	263.39
PSD04_10	502.19

Table 23 – Micro-fraction Yields PSD-01 to PSD-35

Micro-fractions PSD01_03	Vial weight [g]	Fraction yield [mg]
PSD-01	4.93278	38.27
PSD-02	4.97883	4.18
PSD-03	4.87628	6.67
PSD-04	4.84214	15.95
PSD-05	4.93869	13.34
PSD-06	4.87614	17.04
PSD-07	4.90237	11.93
PSD-08	4.83780	15.36
PSD-09	4.80429	14.06
PSD-10	4.93357	35.54
PSD-11	4.95521	25.18
PSD-12	4.91620	30.80
PSD-13	4.81823	24.92

PSD-14	4.91654	41.25
PSD-15	4.88351	27.12
PSD-16	4.95459	33.13
PSD-17	4.95175	20.75
PSD-18	4.90051	24.71
PSD-19	4.93532	45.83
PSD-20	4.92249	44.04
PSD-21	4.92842	59.34
PSD-22	4.93349	48.67
PSD-23	4.79884	34.74
PSD-24	4.92491	24.75
PSD-25	4.82579	14.98
PSD-26	4.86156	24.69
PSD-27	4.81952	8.89
PSD-28	4.87456	10.49
PSD-29	4.90572	14.22
PSD-30	4.88566	8.15
PSD-31	4.87794	5.89
PSD-32	4.93913	4.30
PSD-33	4.90740	3.21
PSD-34	4.82617	8.58
PSD-35	4.92400	9.14

Table 24 - Fraction Yields PSD05_01 – PSD05_17; PSD05_07+_08 and PSD05_13+_14 were combined to one Fraction

Fractions PSD04_02	Vial Weight [g]	Fraction Yield [mg]
PSD05_01	4.89935	1.98
PSD05_02	4.80235	1.15
PSD05_03	4.78012	3.64
PSD05_04	4.92341	2.09

PSD05_05	4.79679	3.98
PSD05_06	4.91021	7.13
PSD05_07+08	4.81641	6.22
PSD05_08		
PSD05_09	4.96132	10.62
PSD05_10	4.95642	6.20
PSD05_11	4.91132	7.55
PSD05_12	4.81970	9.55
PSD05_13+14	4.82021	24.72
PSD05_14		
PSD05_15	4.85149	26.00
PSD05_16	4.84425	37.81
PSD05_17	4.84030	61.73

Table 25 - Fraction Yields PSD10_01 - PSD10_03

Fractions PSD05_16	Vial Weight [g]	Fraction Yield [mg]
PSD10_01	4.92673	2.46
PSD10_02	5.00419	3.51
PSD10_03	5.01662	2.08

Table 26 - Fractions Yields PSD06_01 - PSD06_07

Fractions PSD04_07	Vial Weight [g]	Fraction Yield [mg]
PSD06_01	4.91188	5.37
PSD06_02	4.90923	11.12
PSD06_03	4.83954	8.47
PSD06_04	4.79714	17.59
PSD06_05	4.85379	19.15
PSD06_06	4.89809	30.31
PSD06_07	4.99657	168.20

Table 27 - Fraction Yields PSD07_01 & PSD07_02

Fractions PSD06_03	Vial Weight [g]	Fraction YIeld [mg]
PSD07_01	4.90506	2.54
PSD07_02	4.84953	0.80

Table 28 - Fraction Yields PSD08_01 - PSD08_11

Fractions PSD04_04	Vial Weight [g]	Fraction Yield [mg]
PSD08_01	5.05272	19.33
PSD08_02	4.89747	5.57
PSD08_03	4.92321	43.29
PSD08_04	4.84767	33.80
PSD08_05	5.00293	20.56
PSD08_06	5.00790	3.71
PSD08_07	4.83448	10.29
PSD08_08	4.90099	8.71
PSD08_09	4.97660	21.48
PSD08_10	5.01012	23.09
PSD08_11	4.97607	25.62

Table 29 - Fraction Yields PSD09_01 - PSD09_03

Fractions PSD08_05	Vial Weight [g]	Fraction Yield [mg]
PSD09_01	4.92275	0.64
PSD09_02	4.97517	7.73
PSD09_03	4.95644	4.79

7.1.8 Instruments Solvents and Reagents

7.1.8.1 Miscellaneous Instruments

Analytical Balance	BP210D
Centrfigue	Zentrifuge Heraeus Labofuge 4
Chiller (HPCCC)	Thermo Scientific Accel 500 f
Chiller (rotary evaporator)	Umlaufkühler F-314, Büchi Lab
Concentrator	Sample concentrator FSC400D, Techne
HPCCC	Dynamic extractions Spectrum
Laminar Air Flow	Steril Werkbank B10
NMR	Bruker NMR 500 MHz with cryo probe
Pipette	Eppendorf Research (1000 μL, 100 μL, 10 μL)
Pipette Tips	Eppendorf; Sarstedt; StarLab
Puriflash Interchim	Puriflash 4250, Interchim
Rotary Evaporator	Rotavapor RII mit Heizbad, Büchi
TLC Visualizer	CAMAG®
Ultrasonic Bath	Transsonic T 460, Elma
UPLC	ACQUITY UPLC H-Class System, Waters
UV Lamp	CAMAG®
Vacuum Pump	V-710, Büchi
Vortex	Genius 3, IKA

7.1.8.2 Solvents and Reagents

Acetone	Rectapur; ÖAB, distilled
Acetonitrile for HPLC	Chromaorm Super Gradient VWR BDH 83639.320
Ammonium Formiate 10 mM	Dissolved in 90:10 Water:MeOH
DCM	Normapur, VWR BDH 23366.327

ddH_2O	Double-distilled water
DMSO	Emsure, Merck 1.02952.1000
Ethanol 96% p.a.	ÖAB, distilled, Brenntag CEE
Ethyl acetate	Rectapur ÖAB, distilled, VWR BDH23880.461
H_2O for HPLC	Purified by ion exchanger
Methanol	Chromanorm LC-MS Grade, VWR BDH 83638.320
n-Hexane p.a.	Normapur; VWR BDH 24577.460
Reserpine	Sigma-Aldrich; 83580-1G
Sulphuric Acid	ACS Reagent, 95.0-98.0%, Sigma-Aldrich, S/No: 320501
Vanillin	ReagentPlus, 99%, Sigma-Aldrich, S/No: V1104

7.1.8.3 UPLC Columns

High Strength Silica Trifunctional Alkyl-C_{18} (HSS T3)

7.1.8.4 Spraying Reagent for TLC Analysis

1% Vanillin in MeOH; spraying solvent

5% Methanol. H_2SO_4; spraying solvent

7.1.8.5 TLC Plates

Merck TLC Silica Gel 60 F_{254} 20 x 20 cm TLC Plates

7.1.8.6 Sephadex Columns

Sephadex LH-20 column

7.2 A Biochemometric Approach for the Targeted Isolation of *in vitro* Anti-Inflammatory Compounds in *Ptercarpus santalinus*

7.2.1 Method for the Targeted Isolation of ELINA Predicted Active Constituents with InterHPCCC

PSD05_16:

The choice of the used HEMWat Systems is based on the screening of PSD04_02 shown in Figure 13.

Table 30 - Method for Targeted Isolation with InterHPCCC in normal phase mode, PSD05_16

Mode	Analytical
Amount of sample	~ 35 mg
Stationary phase	HEMWat 13 lower layer (solvent A)
Mobile phase	HEMWat 14 upper layer (solvent B) HEMWat 13 upper layer (solvent C)
Flow rate	1 mL/min
Collection volume	2 mL each fraction
Collected fractions	86

Table 31 - Composition of Gradient Elution for Targeted Isolation with InterHPCCC, PSD05_16

Time	Flow rate	%A	%B	%C
00:00	2 mL/min	**100**	0	0
25:00	2 mL/min	**100**	0	0
27:00	1 mL/min	**100**	0	0
27:03	1 mL/min	0	**100**	0
01:25:00	1 mL/min	0	**100**	0
01:55:00	1 mL/min	0	0	**100**
03:30:00	1 mL/min	0	0	**100**

8 APPENDIX

8.1 UHPLC Chromatograms of *Pterocarpus santalinus* Fractions after Fractionation with PuriFlash Interchim

8.1.1 UPLC Chromatograms of PSD04_01 – PSD04_10 and deriving InterHPCCC fractions

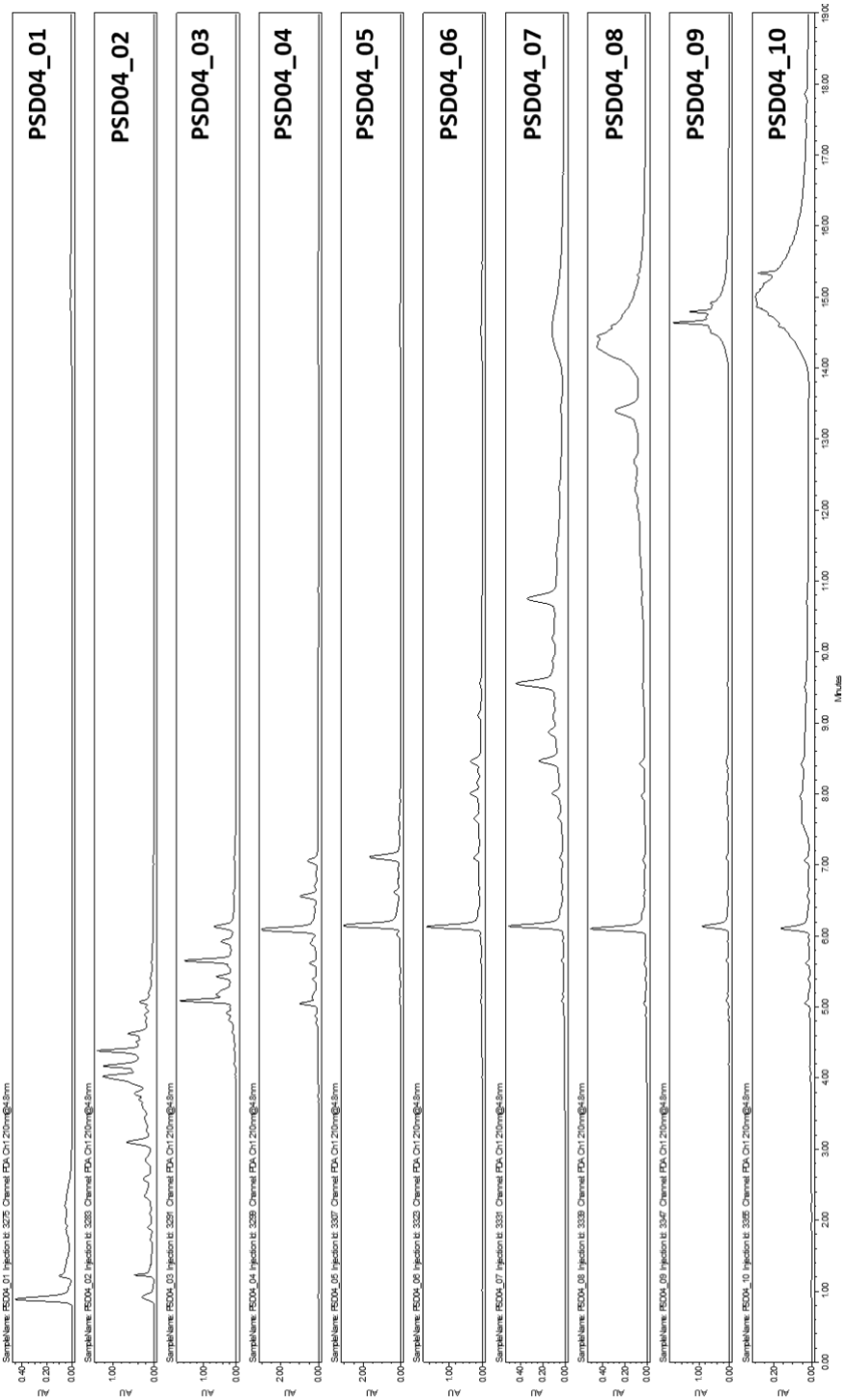


Figure 32 - PSD01_03 deriving Interchim Fractions PSD04_01 – PSD04_10 PDA at 210 nm

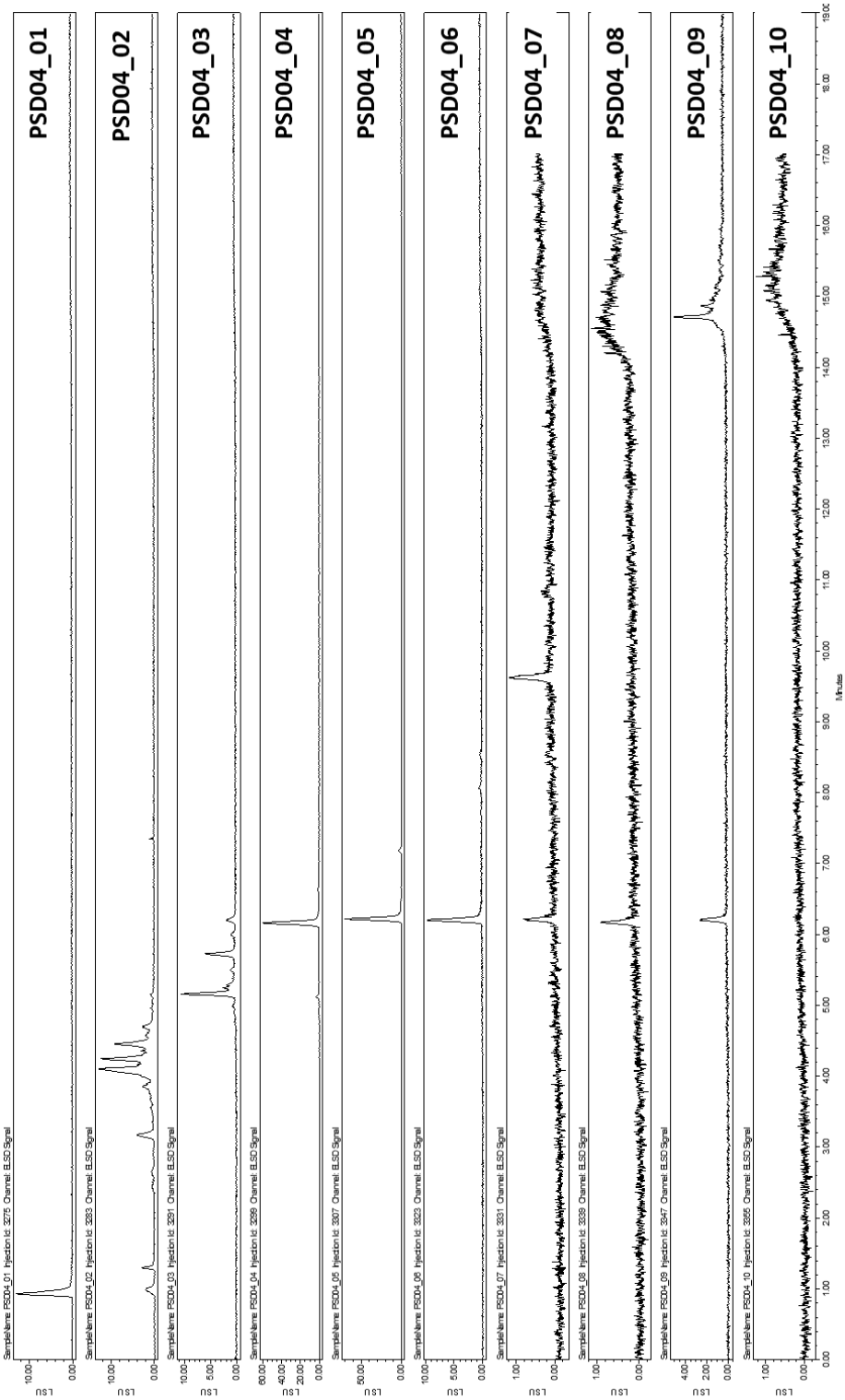


Figure 33 - PSD01_03 deriving Interchim Fractions PSD04_01 – PSD04_10 with ELSD

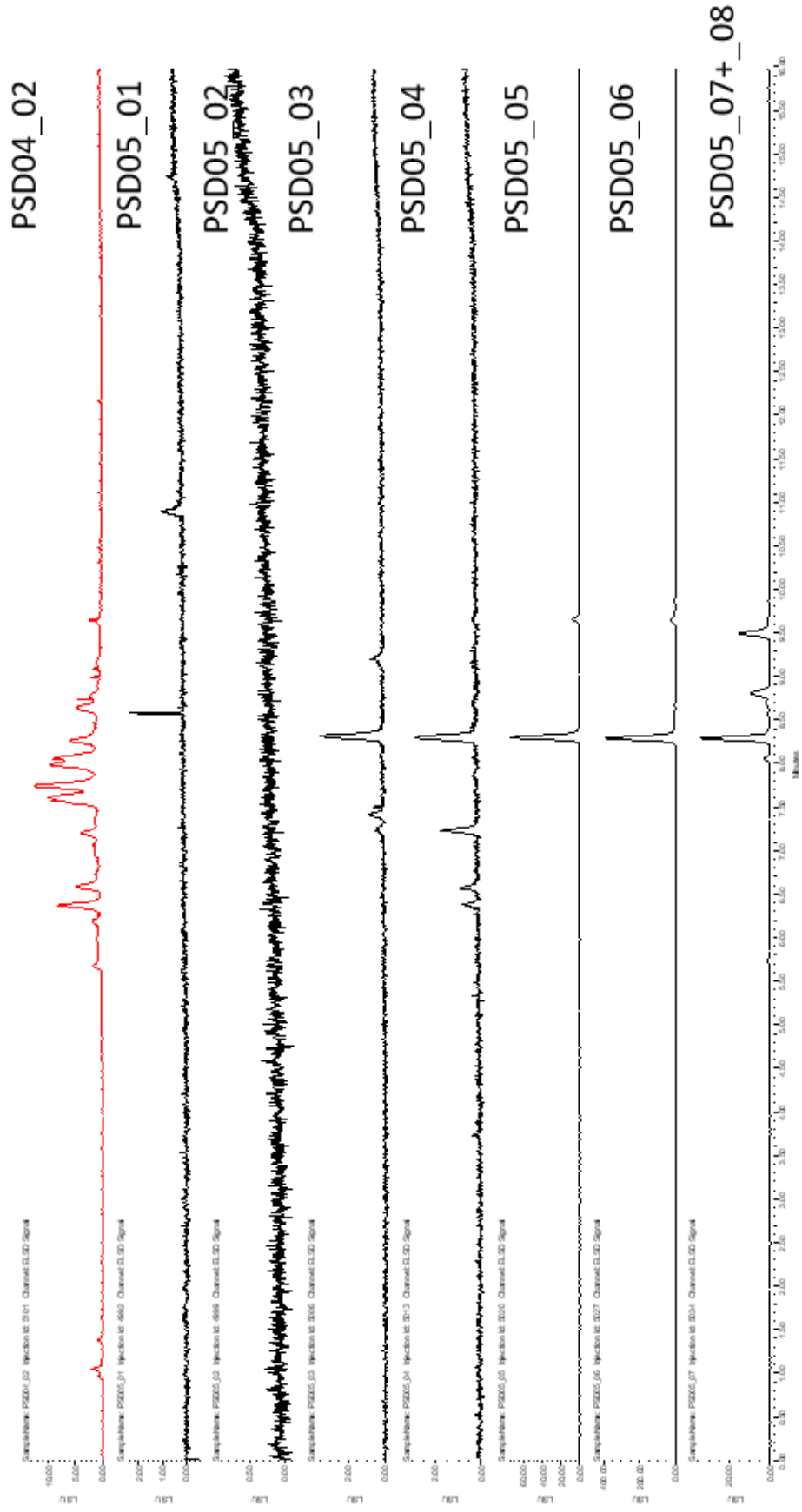


Figure 34 - UPLC chromatogram of PSD04_02 and deriving InterHPCCC fractions PSD05_01 – PSD05_07 with ELSD

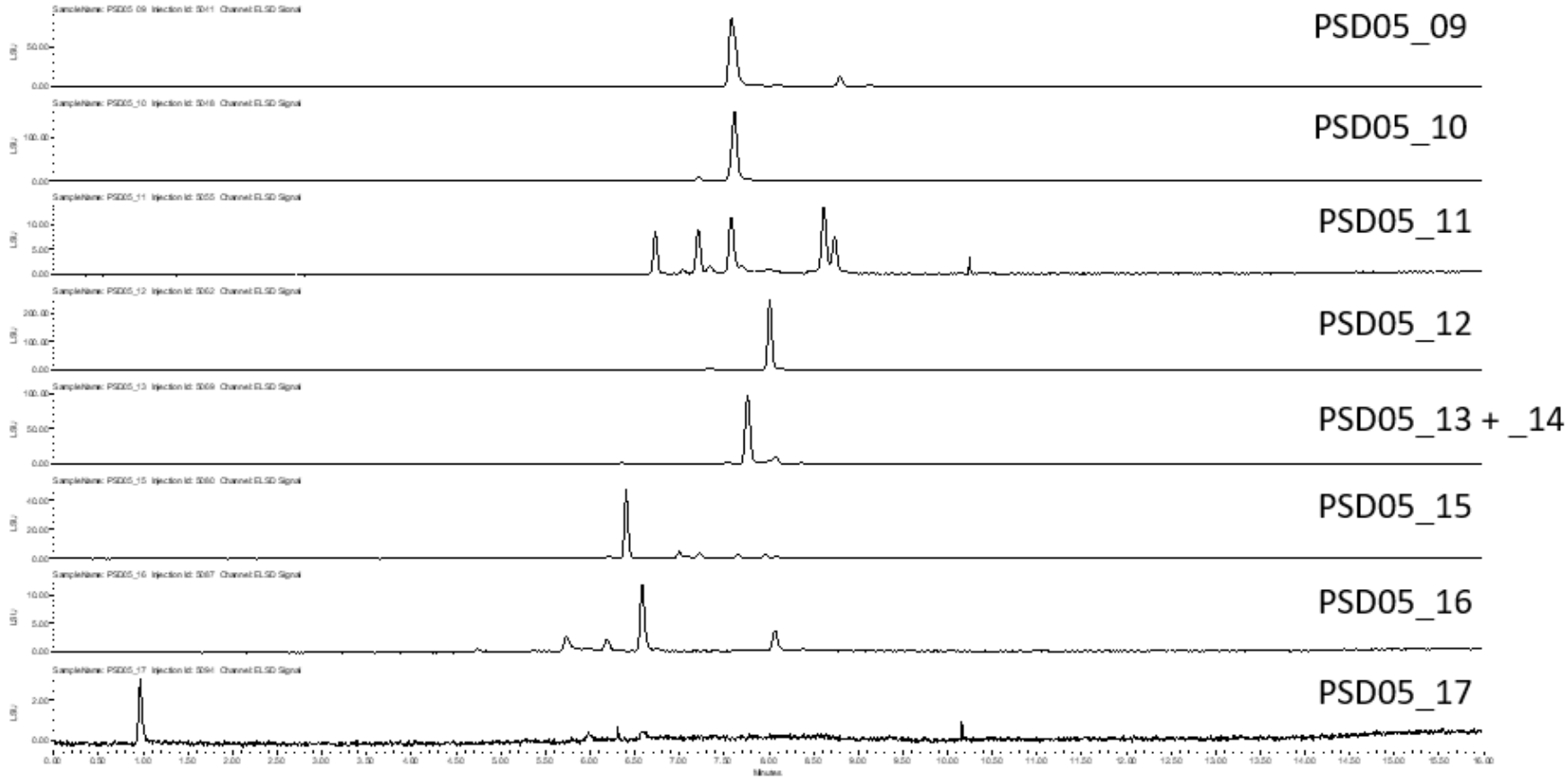

PSD05_09
PSD05_10
PSD05_11
PSD05_12
PSD05_13 + _14
PSD05_15
PSD05_16
PSD05_17

Figure 35 - UPLC chromatogram of PSD04_02 and deriving InterHPCCC fractions PSD05_09 – PSD05_17 with ELSD

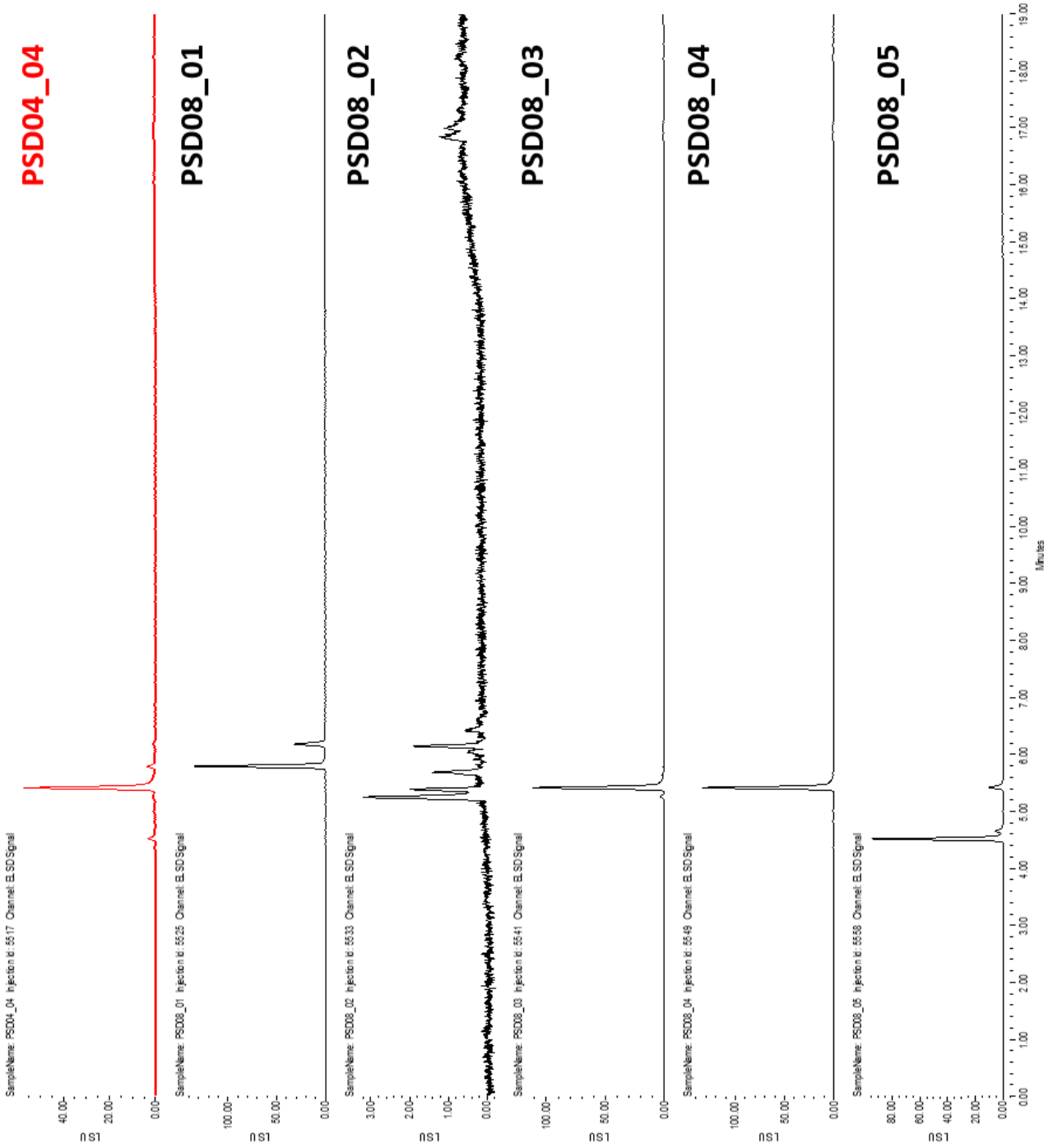


Figure 36 - UPLC chrmatogram of PSD04_04 and deriving InterHPCCC fractions PSD08_01 - PSD08_05 with ELSD

Figure 37 - UPLC chrmatogram of PSD04_04 and deriving InterHPCCC fractions PSD08_06 - PSD08_11 with ELSD

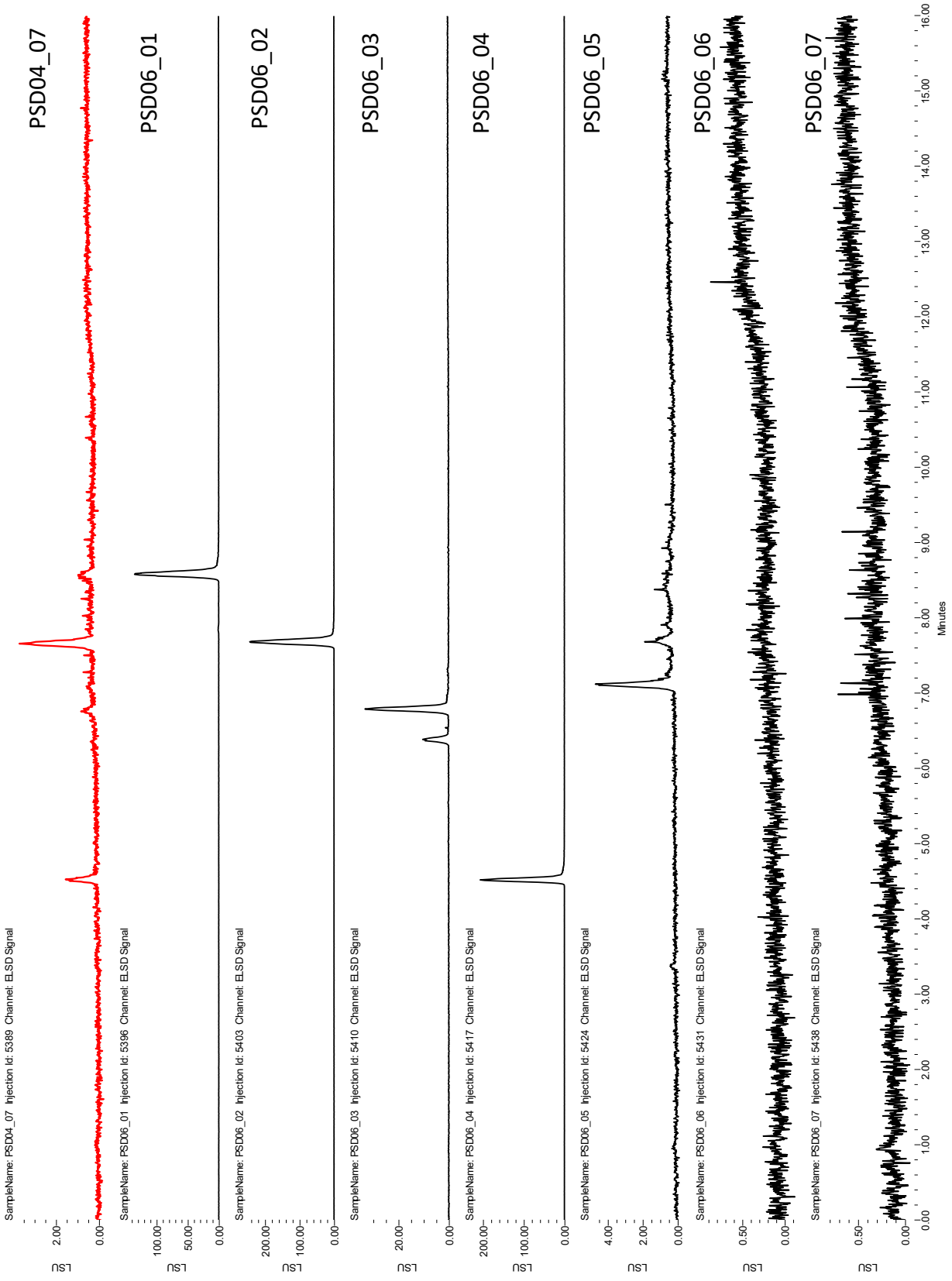


Figure 38 - UPLC chromatogram of PSD04_07 and deriving InterHPCCC fractions PSD06_01 - PSD06_07 with ELSD

8.1.2 UPLC Chromatograms of PSD-01 – PSD-35

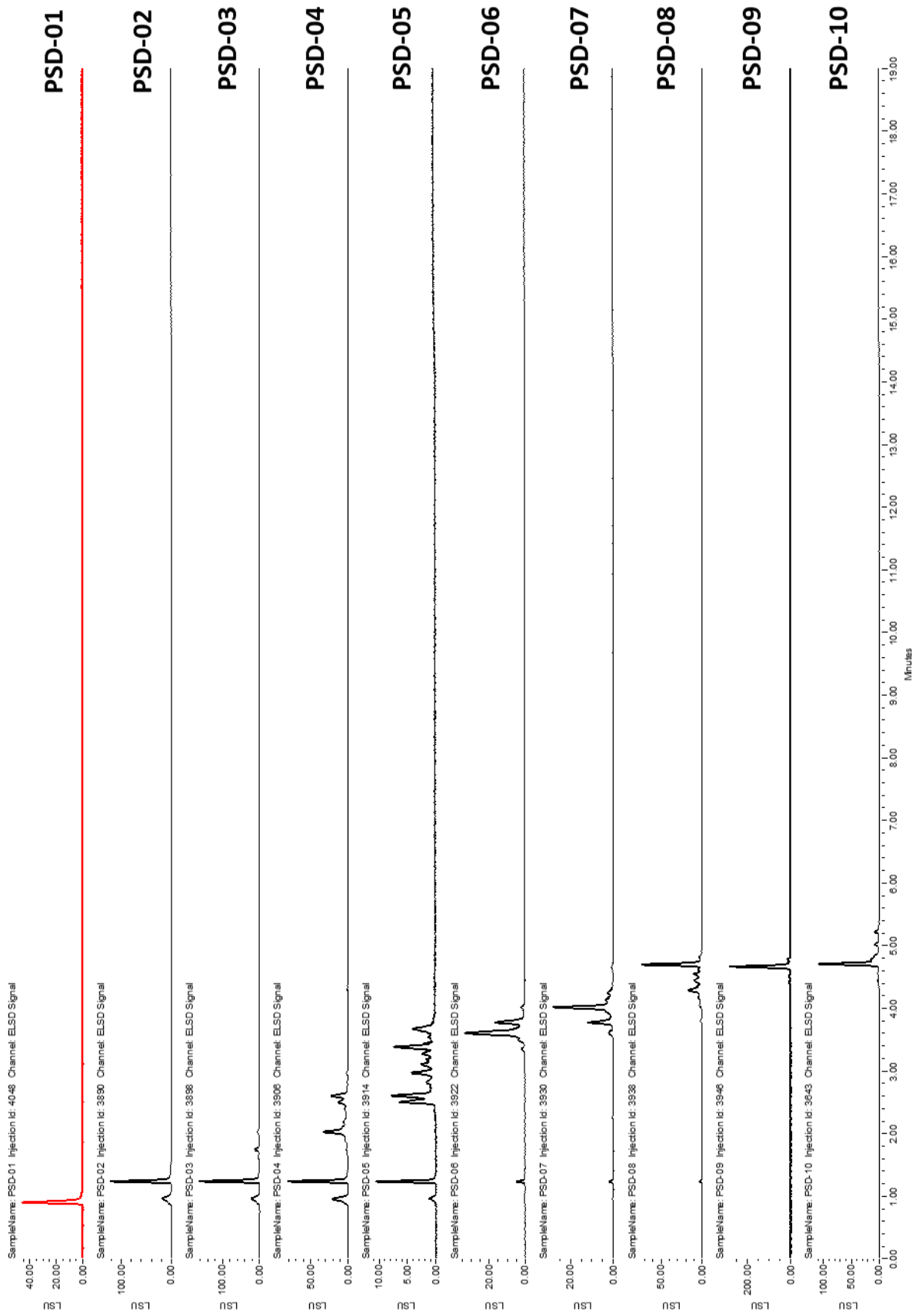


Figure 39 - UPLC chromatogram of the microfractions PSD-01 – PSD-10 with ELSD

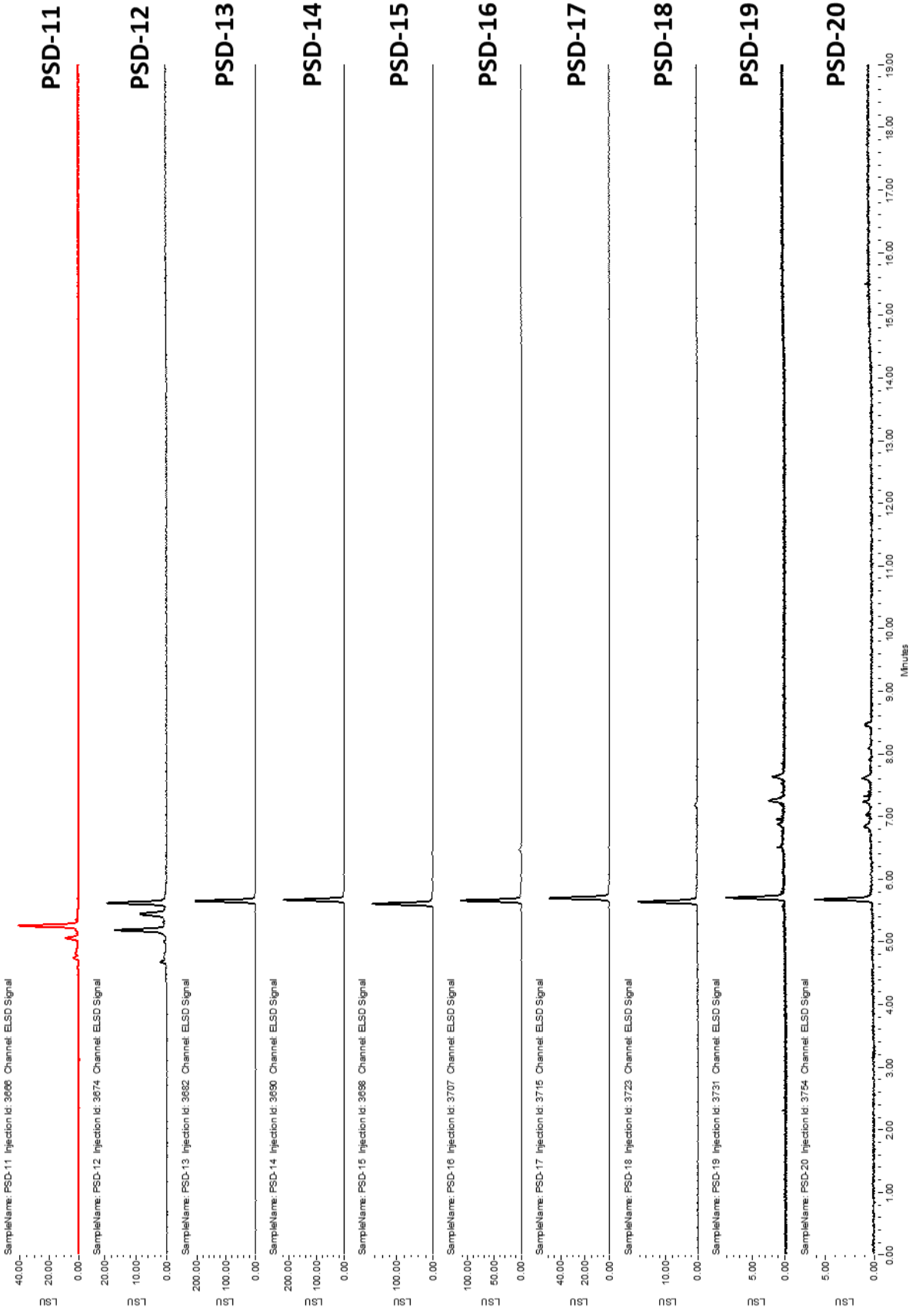


Figure 40 - UPLC chromatogram of the microfractions PSD-11 – PSD-20 with ELSD

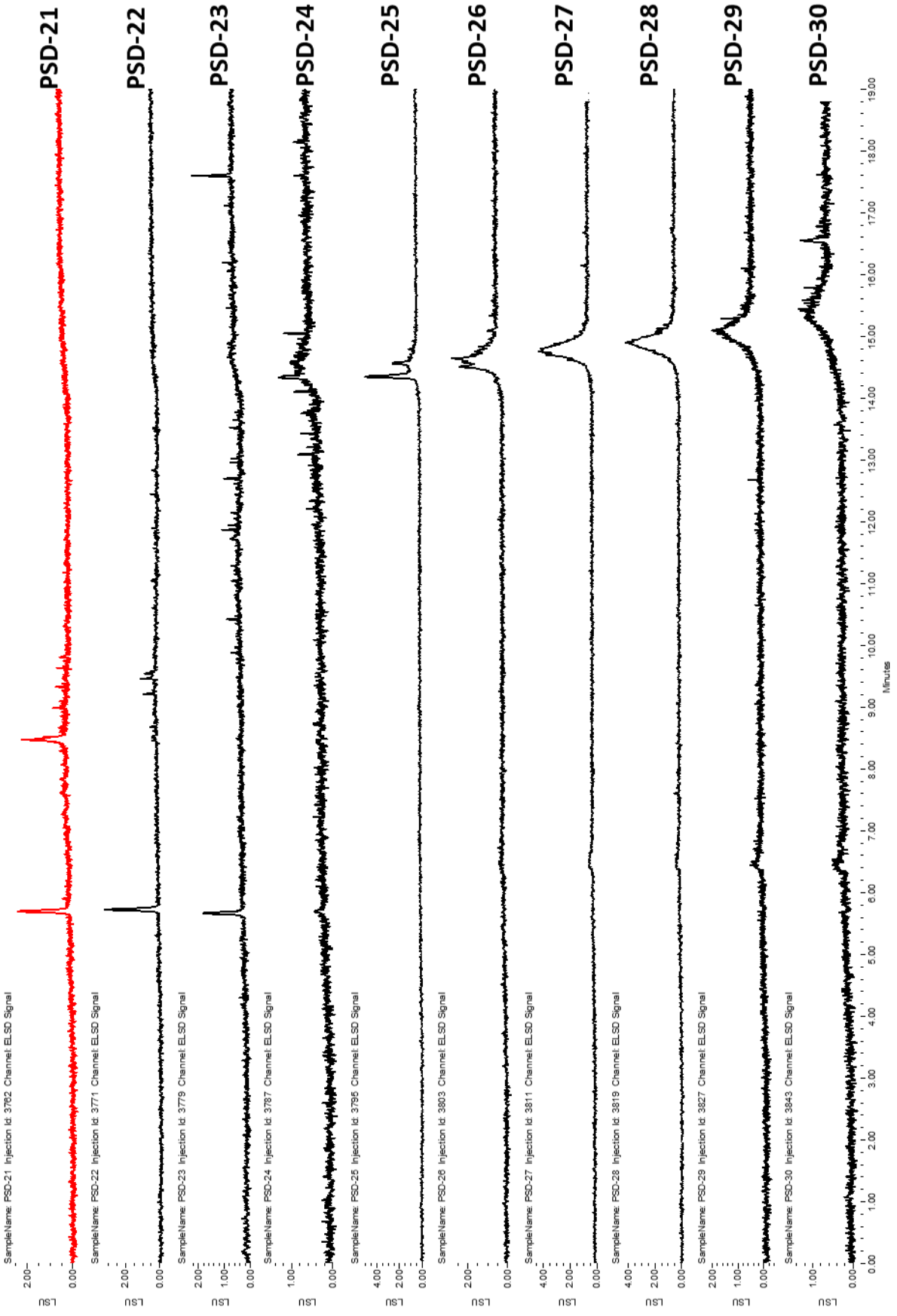


Figure 41 - UPLC chromatogram of the microfractions PSD-21 – PSD-30 with ELSD

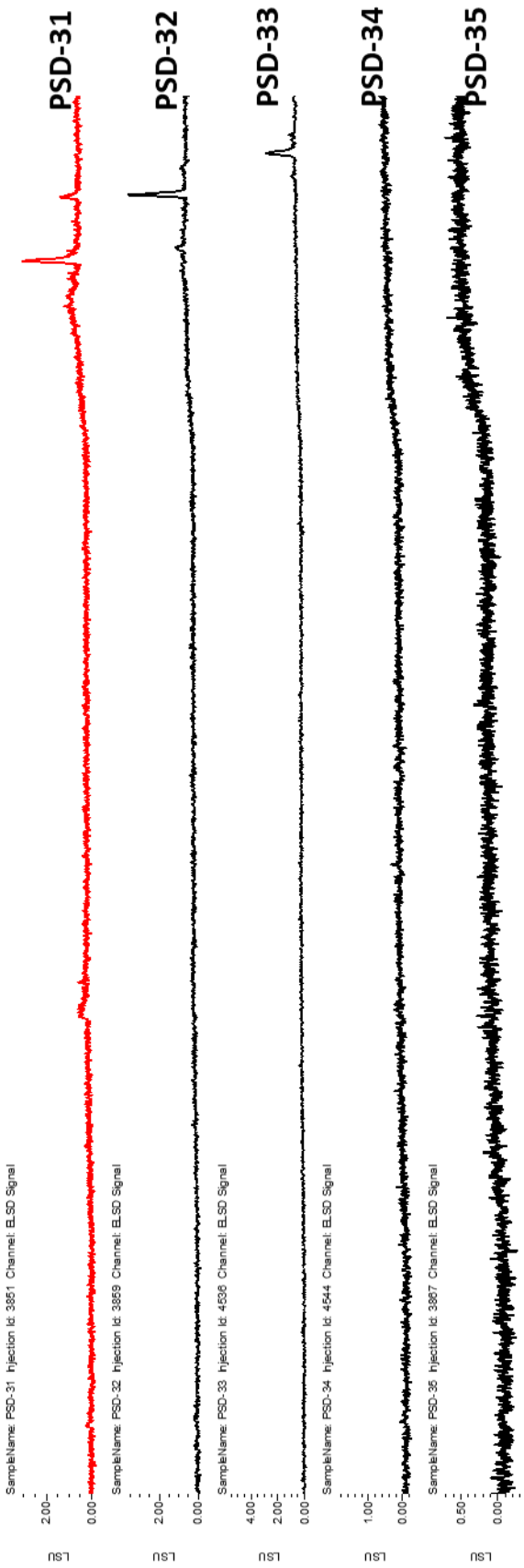


Figure 42 - UPLC chromatogram of the microfractions PSD-31 – PSD-35 with ELSD

8.2 Table Directory

8.3 List of Figures

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