universität wien

DIPLOMARBEIT

Titel der Diplomarbeit

ISOLATION, CHARACTERISATION AND STRUCTURE ANALYSIS OF BIOACTIVE NATURAL PRODUCTS FROM RAINFOREST FLORA

angestrebter akademischer Grad

Magister/Magistra der Pharmazie (Mag.pharm.)

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Wien, im 6. Oktober 2008

Abstract

The matter in hand thesis emerged on the collaboration of INBio and the University of Vienna.
INBio tested 30 plants on their bioactivity and as a result of this screenings, two of this plants were decided to be investigated on. Base of the research work done were the two purified extracts of *Amyris pinnata* Kuhnt and *Maianthemum palludicola*.
The author describes general issues on natural products discovery and special points on the bioprospecting work of INBio, Heredia.

Main focuses on the work are:

1. What is the constitution of the plant's chemical fingerprint and how do the chemical structures look like?
2. Which of the compounds is responsible for the bioactivity measured for this plant?

The *Amyris pinnata* extract was pre-separated using Medium pressure liquid chromatography (MPLC), gaining 7 main fractions. Each fraction was examined using Thin layer chromatography (TLC) and Nuclear Magnetic Resonance Spectroscopy (NMR) techniques. The gained fractions were further processed by High Performance Liquid Chromatography (HPLC), preparative Thin Layer Chromatography and recrystallization methods resulting in 3 purified compounds.
This compounds were analyzed using different NMR experiments with the results being discussed.
The author suggests the three Compounds Scopoletin, Luvangetin and the possibility of Ulupterol, with Luvangetin and Ulupterol being new reported for this plant.

The *Maianthemum palludicola* extract was separated using a Sephadex LH-20 Chromatography, gaining 154 fractions with each Fraction monitored using Thin layer Chromatography.
A complex mixture of saponins was gained, which were taken to partial acid hydrolysis in order to make a further separation and monitored on Nuclear Magnetic Resonance Spectroscopy.
Due to time issues, the investigation stops at this point and will be readopted by other persons involved in this project.

Denn die Natur ist so subtil und scharf in ihren Dingen, daß sie nicht ohne große Kunst angewendet werden mag. Denn sie bringt nichts an den Tag, das für sich selbst vollendet wäre, sondern der Mensch muss es vollenden. Diese Vollendung heißt Alchemia. Darum so lerne Alchimiam, die sonst Spagyria heißt, die lehrt zu scheiden das Falsche vom Gerechten.
Paracelsus

ACKNOWLEGDMENT

This work is the result of a cooperation of the University of Vienna with INBio, Costa Rica and the contribution of many people. I could not have done it without them.

First of all, I want to thank Prof. Dr. Dr. Brigitte Kopp for giving me the chance of going to Costa Rica, working at the INBio research centre and sharing her sunstained expert-knowledge with me throughout this project.

I also want to thank Prof. Giselle Tamayo and Master of Scienteae candidate. Victor Vasquez for their support whilst my research in Costa Rica, they have afforded an overwhealming and interesting time. I also want to thank INBio for giving me the possibility of working with their equipment and using their infrastructure.

I dedicate this work to my parents, Silvia and Franz, my grandparents Hilde and Konrad and the rest of my family. They provided me financial and emotional encouragement through the whole time of my education, without them all of this would not have been possible.

Additional thanks go to Mag. Ilkim Erdost, Mag. Elisabeth Granitzer for their help whilst my education.

DECLARATION

Herewith I declare, that I have authored the present Thesis by myself and that no other than the cited auxiliary material was used.

Vienna, 06.10.2008, Signature

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I. GENERAL INTRODUCTION

1.1 Natural Products in Drug Discovery

Ever since the existence of mankind, fauna and flora seemed to play an important role in preventing and treating human diseases. Due to evolutionary selection, natural products provide a wide base of possibly utilizable active ingredients and all around the globe in every culture and at any time of history until present there is evidence, that medicative raw materials of nature have been used for natural product medicine. [1] Today, natural products play a fortiori a highly significant role in the drug discovery and development prozess, particularly in the areas of cancer and infectious diseases. [2] Over 60% and 75% of these drugs, respectively were reported to be of natural origin, based on the number of new drugs approved by regulatory agencies. [3] However, only 5 - 15 % of the world's approximately 250,000 flowering plants have yet been analysed for their possible medicinal uses. [4] Due to this development, biodiversity – the diversity of living forms - has lately attracted a great deal of interest and concern since biological resources constitute an asset with a great deal of immediate as well as potcntial benefit for the quality of life. [5] Ironically, due to the influence of human species, the ascending of industry and the thereby repress and pollution of ecosystems, we realize that there is a current decrease in the number of species and that this may have catastrophic consequences. This loss of species could reduce the availability of natural products used as raw materials for manufacturing and industry. These phenomena pose a serious threat to sustainable development, since species diversity may well be our planet's most important and irreplaceable resource. Once depleted, species regeneration, if at all possible, might take 5 to 10 million years. Its loss would thus have profound negative effects on the overall quality of life on our planet and on our potential development. The consequences of a reduction in biodiversity through loss of species constitute a serious threat to human survival. It could also diminish the future availability of new genetic resources and wild germ plasm essential for breeding crop varieties with higher productivity and with greater resistance to insects, diseases, and adverse climatic conditions. In medical science, the loss of species could reduce the opportunity for treatment of diseases through the loss of medical models and new medicines as a result of reduction of the availability of natural products which have potential medicinal properties. [6] Regarding this trend, biodiversity should be protected and seen as one of the most important treasures that earth endowed us. This is, where bioprospecting begins to play a rising role in order to take advantage of yet available and potentially relevant natural resources.

1.2 The Project

This detailed project is part of the collaboration of the institute of pharmakognosy of the university for life sciences in Vienna, INBio, the CONICIT Program, OEA, the National University of Rosario, Argentina and the University of Panama.

1.3 Costa Rica

The Republic of Costa Rica is a small country located on the central American isthmus. It comprises 51,100 square kilometers and despite of the relatively small extension (0.1% of the world´s land mass), it has 5% of the world´s biodiversity with an enormous plurality in landscapes and ecosystems with many microclimates. [7]

Historically, main export goods of Costa Rica have been agricultural products like pineapples, bananas, coffee and beef, but in present, there is a diversification in economy going on in order to be more independent of the international pricing policy. The country has taken advantage of it´s affluence in flora and fauna and so new categories in economy have been established. One of the most successful sections is ecotourism. This development to a ecotouristic country is the result of a federal nature conservation program. In the last 28 years, 23.7 % of the land´s area have been put under protection. [8] Another reason for the expanding economy is, that the Costa Rican government has implemented a seven year plan of expansion in high tech industry with tax exemptions for companies investing in the country. As a result big companies started developing in the country like intel, Procter & Gamble and Glaxo Smith Kline. [9] An important aspect of Costa Ricans present economy is it´s biodiverity. Since innovation is a motor of economic development of a country, the idea has been born that Costa Rica is in the era of the "economy of knowledge". Knowledge is therefore considered a basic factor of production and research becomes an essential activity to increase this knowledge.
In order to control this development, the government had plans to initiate an instance to gather knowledge of the country´s biodiversity and it´s possible use. Therefore a planing commission was established which advised Costa Rica to initiate an autonomous state National Biodiversity Institute. However because of political difficulties the government failed to implement this proposal, so the comission itself founded this instance. In 1989 INBio got the official status. [10]

1.4 INBio

The National Biodiversity Institute of Costa Rica understands itself as a private, non profit association which conducts research and outreach programs with the aim to increase public awareness towards conservation of biodiversity. [21]
"It is a non-governmental, non-profit, public interest organization of civil society that works in close collaboration with different government institutions, universities, the private sector and other public and private organizations, both within and outside Costa Rica. " [11]

The instituteś five main points of focus are:

Inventory and Monitoring
Conservation
Communications and education
Biodiversity informatics
Bio-prospecting

In the bioprospecting departments of the institute, the samples collected in all parts of the country are chemically analyzed, regenerated and screened biotechnologically. The company´s capacities lie between university and industrial standard, and with its preparative HPLC systems (BioXplore) it has the ability to extract larger quantities of raw materials and to deliver it to interested companies. International companys such as Eli Lilly already engaged the know-how of the INBio scientists. In 1991 INBio and Merck signed a two year agreement. Merck paid INBio a sum of US $1 million, of which $180,000 were spent on equipment, aproximately $60,000 have been spent on training Costa Rican scientists and $100,000 have been invested towards conservation efforts. This agreement was ren-newed three times until 1999. [12] [21]
INBio in return commited to provide plant, insect and environmental samples to Merck for further research. The contract also has a clause which engages Merck to pay a royalty up to 10 % for new products developed by this cooperation. [13]

The Costa Rican government is developing a Wildlife Conservation Law since 1992 that will authorise the right of negotiating agreements with multinational corporations who wish to utilise Costa Rica's natural resources. In addition a Biodiversity Law was decided in 1998. This law would declare all plant and animal species a natural patrimony. Access to Costa Rica's genetic material would be granted to foreign instances. It was implemented to protect Costa Rica's intellectual and biological property. [13] [21]
Enabled by this legal basis for suretyship, INBio made agreements and collaborations with many other international cooperations, universitys and instances in the last years.

1.5 The CONICIT Program

Conicit is an abbreviation for "Consejo Nacional para Investigaciones Científicas y Tecnológicas " which means "National Commission for Scientific and Technological Research". It was founded in 1972 to advance the scientific and technological development of Costa Rica. It belongs to the Network of Specialized Information centers (CIE´s) of the country and focuses on 3 main areas of following activities: Scientific and Technological development, Administrative and Financial matters and the Administration of Scientific and Technological Information. Conicit is the planing, advising, controlling and maintaining institution in the scientific and technological sector. It acts as a coordinating instance to support the scientific and technological activity from any public or private, national or foreign organization, which are linked within the National Program of Development. It wants to create suitable conditions for research programs in areas of national interest and improve the education of science, by co-financing scientific research and educational programs. [14]

1.6 OEA

OEA which stands for „Organización de los Estados Americanos" and is an international organisation with its headquarter in Washington, D.C., United States. The 35 members are the independent nations of the Americans. Costa Rica joined in 1948.
OEA´s major goals are strengthening democracy , working for peace, defending human rights, fostering free trade, fighting drugs trade, promoting sustainable economic development and combating poverty. [15] It also supports regional research programs and was in one of the programs that started the initial screening of the samples discussed in this thesis. [36]

1.7 Bioassay-Guided Fractionation

As part of the INBio – OEA agreement, 30 plants have been tested each year using bioassays from 2003 for their activity, which focuses on 3 main categories of bioactivity:

	2003		***2004***	
	tested	positive	tested	positive
Antifungal	10	1	10	2
Anticancer	10	1	10	2
Antiparasite	10	2	10	2
	30	5	30	6

Tab. 1, bioactivity of plants tested in 2003 and 2004 in context of the INBio – Conicit - OEA agreement

The OEA financed the screening of the 60 plants in 2003 and 2004, while Conicit finaced the isolation and characterization of five selected plants.

1.6.1 Tested Plant Material

Within this detailed project, 2 of the pre tested plants have been investigated, namely *Amyris pinnata* Kunth and *Maianthemum palludicola*.

1.6.2 General considerations

Due to the fact that the two plants had a proven bioactivity, the investigation was concentrated on two focal points for each plant:

3. What is the constitution of the plant's chemical fingerprint and how do the chemical structures look like?
4. Which of the compounds is responsible for the bioactivity measured for this plant?

II Isolation, Characterisation and Structure Analysis of Bioactive Natural Products from Amyris pinnata kunth.

2.1 Introduction

Amyris Pinnata was tested positive of Antifungal activity in 2004 and therefore chosen for further investigation. At the same time there are crossover investigations carried out in Argentina in order to avoid or minimize the risk of errors. It was tested positive in Antifungal bioassays against Microsporum gypseum, *Trichophtyon rubrum, Trichophyton mentagrophytes, Sporothrix schenckii and Fonsecaea pedrosoi*.

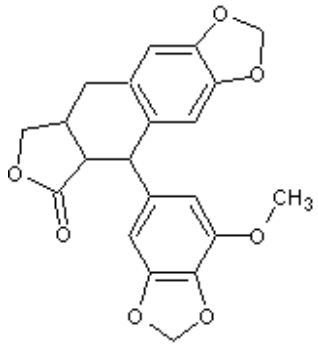
2.1.1 Previous Investigations of *Amyris* species

The alkaloids edulinine and (+/-) isoplatydesmine were isolated from the leaves of *Amyris diatripa*, being the first report of the presence of alkaloids in species of this genus. The presence of coumarins in the plant was also shown by isolation of psoralen, bergapten, marmesin, ulopterol and suberenol. [16]
Essential Amyris oil was tested against 3 mosquito species: *Aedes albopictus, Ae. aegypti*, and *Culex pipiens pallens*. The larvicidal activity was evaluated against 4 instars of each of the 3 mosquito species, and *amyris* oil demonstrated the greatest inhibitory effect of all tested oils with LC50 values in 24 h of 58 microg/ml (LC90 = 72 microg/ml) for *Ae. aegypti*, 78 microg/ml (LC90 = 130 microg/ml) for *Ae. albopictus*, and 77 microg/ml (LC90 = 123 microg/ml) for *Cx. p. pallens*. [17]
Texalin, an oxazole, has been extracted from *Amyris elemifera* and showed antimycobacterial activity, tested against *Mycobacterium tuberculosis, M. avium* and *M. kansasii* using the Middlebrook 7H11 agar medium, the Bactec 460-TB radiometric methodology, and determination of bacterial viable counts. [18]

2.1.2 Previous Investigations of *Amyris Pinnata* Kunth

2.1.2.a Previous chemical Investigations of *Amyris Pinnata Kunth*

In previous reports, the isolation of the coumarins Austrobailignan (**1**), Scopoletin (**2**), Seselin(**3**), Savinin(**4**), Imperatorin (**5**), Phellopterin (**6**), Svetenin (**7**), Heraclenol (**8**) from extracts from the twigs and leaves of *Amyris Pinnata* was described. ß-Sitosterol (**9**) was also reported as a compound of *Amyris pinnata*. [19]



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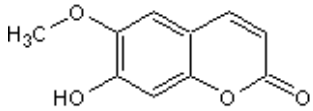

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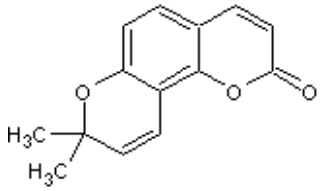

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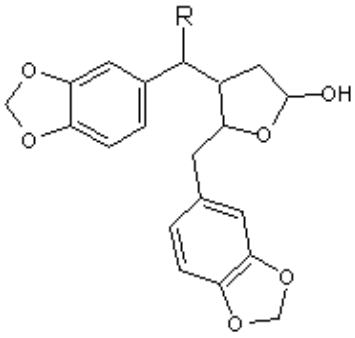
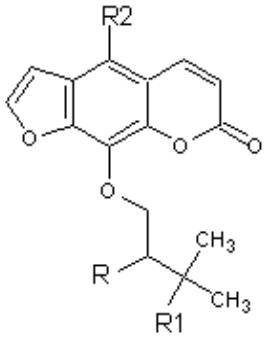
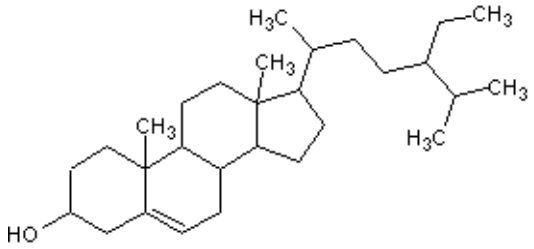




	R	R1	R2
4	**R = H**	**5**	Δ **H**
7	**R = OH**	**6**	Δ **OCH_3**
		8	**HO HO H**



9

pict. 1; reported Compounds of *Amyris pinnata*

2.1.2.b Pre - investigations on bioactive properties of *Amyris Pinnata* Kunth

Antitumor Essays

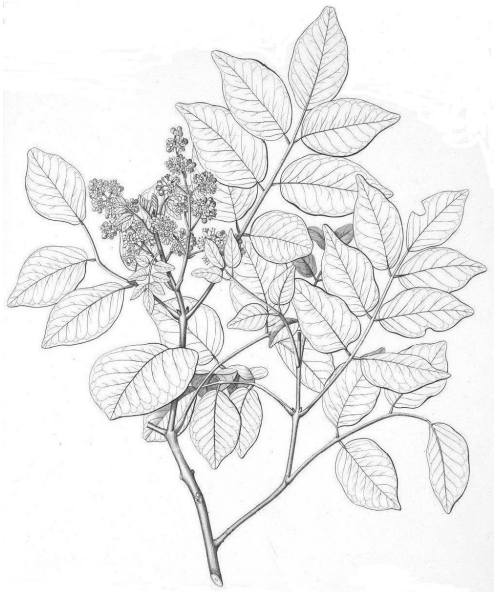
In a previous report, methanolic extracts of the leaves and inflorescences of *Amyris pinnata* were found to possess cytotoxic activity against Eagle`s carcinoma of the Nasopharynx in cell culture (KB). Marginal activity was observed at 100 and 50 mg/kg against the P-388 lymphocytic leukaemia system in mice. [19] An EtOH-H_2O (20:1) extract of flowers, fruit, leaf and stem was tested to have cytotoxic activity for CA-9KB in cell culture. ED50 <20.0 MCG/ML. [20] A methanolic extract of dried inflorescence and leaf had positive antitumor activity against LEUK-P388 in mice. [20]
A methylene chloride extract of dried inflorescence and leave had positive antitumor activity against CA-9KB in cell culture. ED50 0.78 MCG/ML. [20] A MeOH extract of dried inflorescence and leaf had positive antitumor activity against CA-9KB in cell culture. ED50 0.42 MCG/ML. [20]
A petrol ether extract of dried inflorescence and leaf had positive antitumor activity against CA-9KB in cell culture. ED50 2.6 MCG. [20] A MeOH extract of dried twig of Amyris pinnata had positive antitumor activity against LEUK-P388 in mice. [20]

Antifungal Essays

In the context of the OEA and CONICIT collaboration with INBio, the extract of leaves from *Amyris Pinnata* described above in X.X.X was tested positive for inhibiting *Sporothrix Schenkii* (Guatemala). [21] In Argentina it was tested positive for inhibiting *Trichophyton rubrum* and *Trychophyton mentogrophytes*. [21]

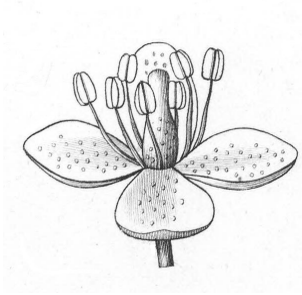
2.2.2 Classification of *Amyris pinnata* Kunth

Appearance

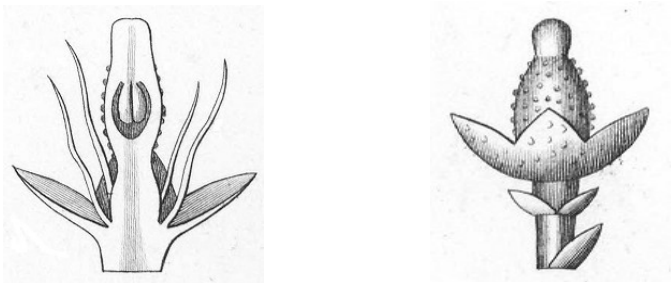


pict. 2; branch of *Amyris pinnata* Kunth; © 1995-2005 Missouri Botanical Garden; http://www.illustratedgarden.org

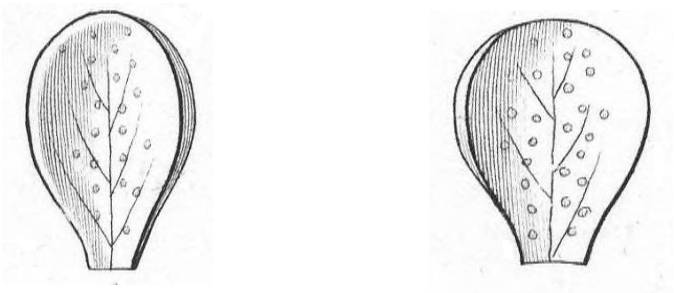
Amyris pinnata is classified a tree, it´s bole is about 25 m x 60 cm.



pict. 3; flower of *Amyris pinnata*; © 1995-2005 Missouri Botanical Garden; http://www.illustratedgarden.org



pict. 4; cross-sectional view of the carpel of *Amyris pinnata* (left), front -view (right); © 1995-2005 Missouri Botanical Garden; http://www.illustratedgarden.org



pict. 5; Petal of the flower of *Amyris pinnata*; © 1995-2005 Missouri Botanical Garden; http://www.illustratedgarden.org

pict. 6; Stamen of *Amyris pinnata*; © 1995-2005 Missouri Botanical Garden; http://www.illustratedgarden.org

Flowers

The flowers have a green calyx, the corolla a creamy color, the stamens are white. The anthers are coffee colored and the pistil is green with an orange colored base. They smell like lemon.
The panicles are terminal and the flowers are open during the morning. The ovarys are spherical and green. [21]

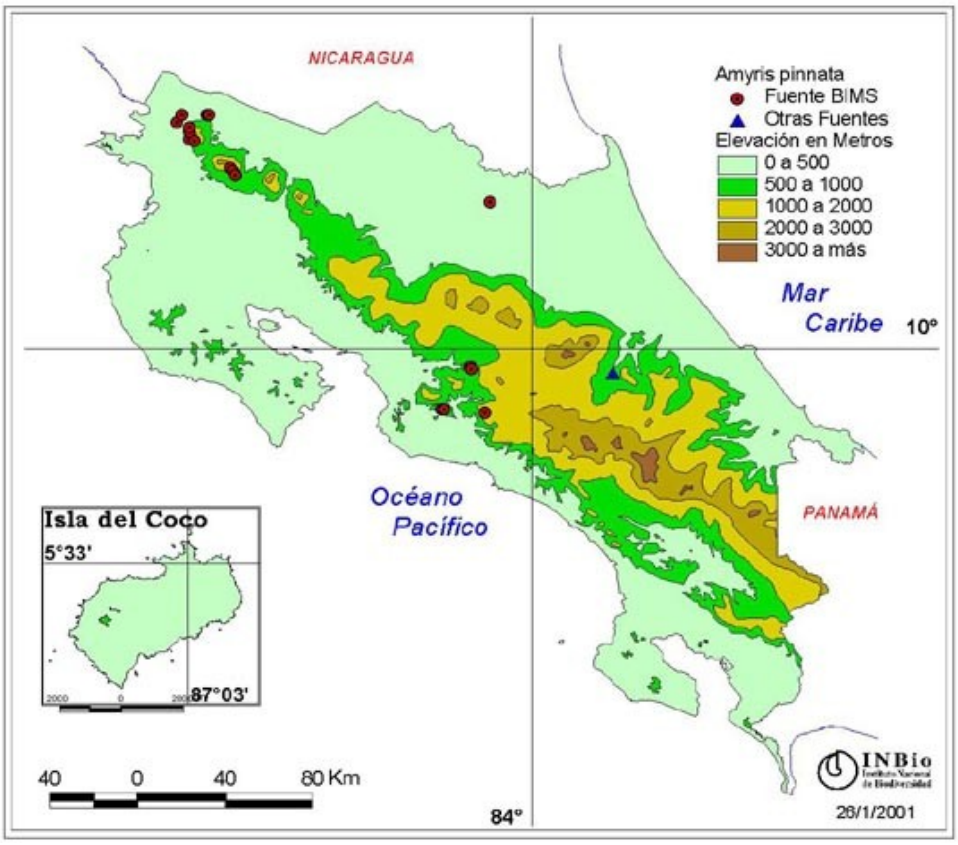
Leaves
The leaves have a length of about 15cm, and consisting of 5 single leaves, which are serrated at the border, have a lancet shape and measure approximately 7-8 cm in length. The upside is brilliant dark green und the underpart is clear green. The stipe is cylindrical and 4-5 cm long and there are translucious points when observed against the light. (see Picture 7) [21]

pict. 7; Leaf of *Amyris pinnata*; © INBio

Cortex and wood
The cortex is clear coffee colored with a fine cross-linking. The wood is cream colored. [21]

Area of distribution

The main area of distribution in Costa Rica is Guanacaste (pict. 8), in an elevation between 400 to 1100 meters. It belongs to the family of Rutaceae. [21]



pict. 8 Evidenced occurrence of *Amyris pinnata* Kunth in rainforests of Costa Rica. © INBio, 2001

Classification data

Kingdom: Plantae
Division: Magnoliophyta
Class: Magnoliopsida
Order: Sapindales
Family: Rutaceae

Synonyms: Torchwood, Manzana,

Rutaceae

The family of Rutaceae consists mainly of trees, shrubs and sometimes herbs. Plants belonging to this family always have aromatic odor, which is originated from various essential oils occuring in glands on the leaves, which are almost visible as pellucid punctations of the leaves.
The plants are mainly pantropical and subtropical and spreads in northern moderate zone. [22]

Amyris

The genus of *Amyris* consists of trees or shrubs, the compound leaves are opposite or imparipinnate. Characteristic of Amrys are the white to yellowish flowers and the indehiscent fruits. [23]

2.2 Results and Discussion

2.2.1 Isolation of Coumarins from *Amyris Pinnata* Kunth

Drying process
3 kg of the leaves of Amyris pinnata were dried at room temperature, yielding 1313 g dry leaves.

Preparation of extracts
The dried plant material was macerated 2 times sequentially with 95% ethanol for 8 hours (at room temperature) to extract the compounds. The resulting extract was filtered and concentrated in vacuum at 40°C in the rotary evaporator and stored at –20 ºC until further use. The 1313 g of the dried leaves were extracted according to the procedure above, resulting in 52.1 g of crude extract.

Preseparation with vacuum liquid chromatography
The preliminary separation was achieved by use of Vacuum liquid chromatography (VLC), using silica for the separation, as referred to in [24]. Therefore a 6.5 x 7 cm column was filled with 50.0 g of silica 70 – 230 µm mesh (Merck), masked with a 6.5 cm diameter round filter paper and washed with 80.0 ml of ethanol, 80.0 ml of methylene chloride and 80.0 ml of n-hexane. The head was prepared with 10.0 g of silica 70 – 230 µm mesh (Merck). 5.0 g of the crude extract of *Amyris pinnata* Kunth were dissolved in 40.0 ml of a mixture of methylene chloride and ethanol in a ratio of 1 : 1. The 10.0 g of the silica 70 – 230 µm mesh (Merck) were added and the mixture was brought up to a rotovapor capable container and dried on the rotovapor in vacuo (550 mm HG), at 35 °C (95 °F) in order to desiccate the silica – extract – mixture. After complete desiccation, the mixture was applicated steadily onto the filter paper covering the silica in the column. In order to have a whole recovery of compounds from apolar to polar, a gradient run was made, using different dissolvents:

Eluation series:

- n-hexane (C_6H_{14})
- n-hexane : methylene chloride (1:1)
- methylene chloride (CH_2Cl_2)
- methylene chloride : ethyl acetate (1:1)
- ethyl acetate ($C_4H_8O_2$)
- ethyl acetate : ethanol (1:1)
- ethanol (C_2H_6O)

Each 300ml.

Taking into consideration that silica is a polar stationary phase, the starting eluent was 300 ml of n-hexane with a relative polarity of 0.009 [25] a dielectric constant of 2.02 and a dipole moment of 0.08. [26]
The polarity was increased in seven steps (300 ml each), including four eluents and three mixtures with

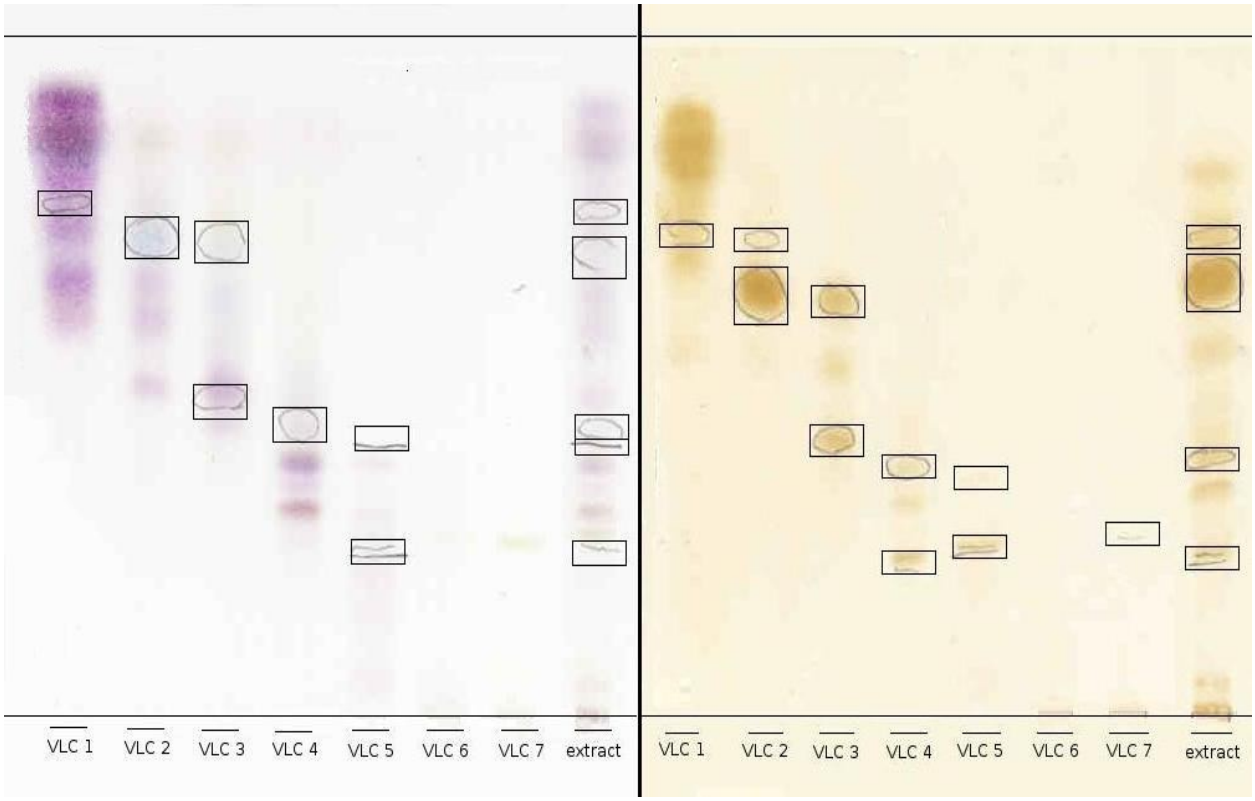
the respectively dilutant having uprising polarity at a 1 : 1 ratio with the last eluent being ethanol with a relative polarity of 0.654 [25]a dielectric constant of 24.3 and a dipole moment of 1.69. [26]
For every eluent and mixture used as mobile phase, one fraction was collected, leading to seven Fractions (VLC_1 – VLC_7). The fractions were dried on the rotorvapor under vacuum to complete desiccation and placed in the cabinet desiccator over night.

fraction	dilutant	weight	color
VLC_1	n-hexane	0.9744 g	dark yellow
VLC_2	n-hexane : methylene chloride (1:1)	1.3839 g	dark green
VLC_3	methylene chloride	0.0364 g	light yellow
VLC_4	methylene chloride : ethyl acetate (1:1)	0.7720 g	dark green
VLC_5	ethyl acetate	0.1713 g	light yellow
VLC_6	ethyl acetate : ethanol (1:1)	1.3061 g	dark green
VLC_7	ethanol	0.635 g	dark green

Tab. 2; data of the primary vacuum liquid chromatography fractions.

Thin Layer Chromatography

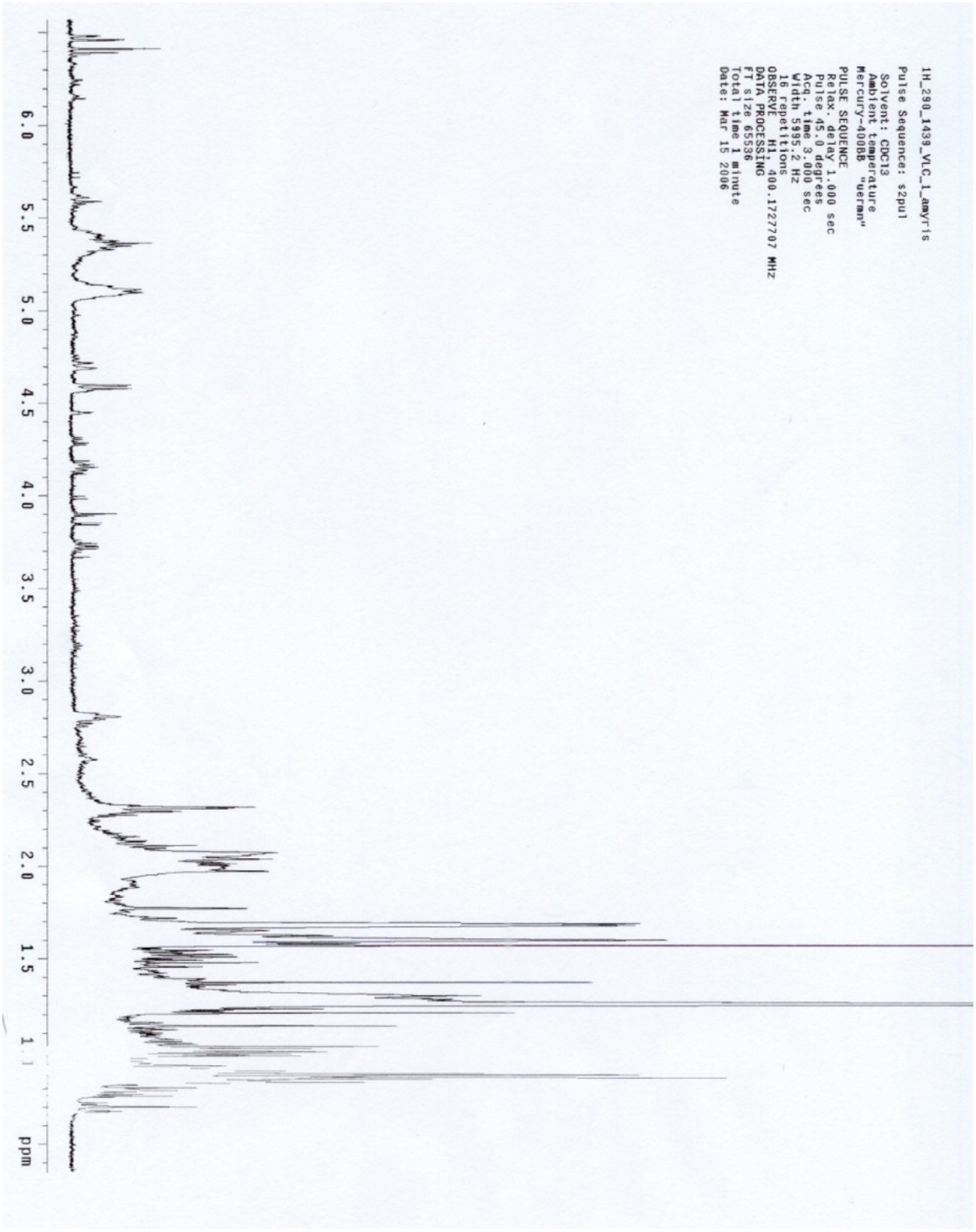
A thin layer chromatography (TLC) of this pre-separated-fractions was made using a 1 mg / ml (1%) dilution in n-hexane of every fraction. The dilution was spotted to a Kieselgel 60 F245 TLC Layer from MERCK using microcapillaries making spots smaller than 0.5 mm in diameter at a starting line of 1.0 cm. In the same way a sample of the crude extract was pointed on the plate for comparison purposes. A specially designed TLC chamber for development was used and filled with 100 ml of a mixture of methylene chloride and ethanol at a ratio of 95 : 5, lined part of the inside with filter paper and sealed for chamber saturation. The plate was developed, to let the solvent-front rise 8.0 cm and was dried afterwards. The plates were observed under UV – light with a wavelenght of λ 254 nm and λ 365 nm and supplementary sprayed with $KMnO_4$ and Vanilla spray agent.



pict. 9; TLC plate of the 7 VLC fractions after spraying with vanilla – spray – agent (left plate) and $KmnO_4$ – spray – agent (right plate). (UV active spots framed.)

A special focus was given to the UV – active compounds. The most obvious physical property of most natural coumarins is the fluorescence they display in UV – light (365nm). This feature has been employed widely for their detection on paper and thin-layer chromatograms since spots can be easily located and, if desired, recovered without the use of a chromogenic reagent. [27]
The plates showed a distribution of compounds in all the fractions of this first pre-separation. Fraction VLC1 (n-hexane) showed spots at a Rf range from 0.55 to 0.95, appearing violet on the vanilla sprayed plate and brown at the $KMnO_4$ treated plate. There was one UV active spot visible at Rf = 0.75. Fration VLC2 (n-hexane : methylene chloride at a ratio of 1:1) showed a distribution of compounds between Rf 0.45 and Rf 0.88, showing 2 relevant UV active spots. The first one was visible on both plates with a blue fluorescence under UV light (365nm) having a Rf of 0.67, the second spot was only visible on the $KMnO_4$ treadted plate, at a Rf of 0.7. Fraction VLC3 (methylene chloride) had a allocation of spots between Rf = 0.42 and Rf = 0.87 showing the same blue fluorescent spot as in Fraction VLC2 at Rf = 0.66 and an additional UV – active spot at Rf = 0.43. Fraction VLC4 (methylene chloride : ethyl acetate at a ratio of 1:1) showed spots in an area between Rf = 0.2 and Rf = 0.45. There were two UV – active spots visible, one on both plates at a Rf of 0.41, the second spot only on the on the $KMnO_4$ treadted plate at Rf = 0.22. The spots of Fraction VLC5 (ethyl acetate) were occuring in a Rf area between 0.0 and 0.44. Two UV – active spots were visible, one at Rf = 0.38, the other spot at Rf = 0.25. Fraction VLC6 (ethyl acetate : ethanol at a ratio of 1:1) had a Rf - range of 0.0 and 2.2, showing no UV activity. Fraction VLC7 showed spots from Rf = 0.0 to Rf = 0.22. There was an UV – active spot visible on the on the $KMnO_4$ treadted plate at Rf = 0.22. (see Picture 9)
In addition a NMR-measurement of each Fraction at the University of Costa Rica was made:

VLC_1



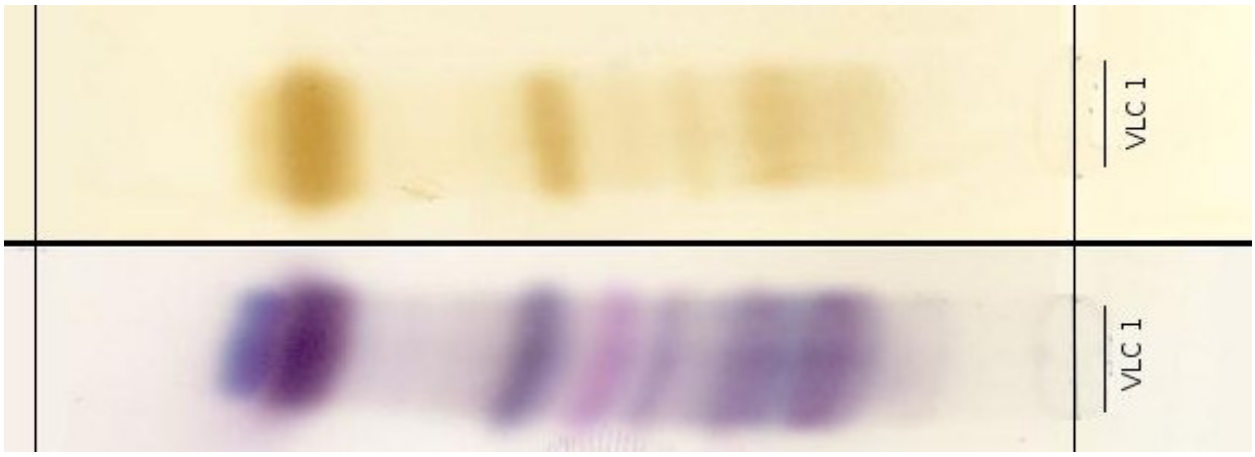
Spextra 1; NMR – spectra of fraction VLC 1. 400 Mhz, $CDCl_3$.

Fraction VLC_1 was brought up ta a a rotovapor capable container and dried on the rotovapor in vacuo at 35 °C (95 °F) in order to desiccate the dilutant, yielding 0.9744 g.

NMR – screening
This fraction showed interesting peaks at ppm values between 0.8 and 2.8 ppm in the ^{1}H screening, giving the sign of aliphatic. (see Spectra 1)

Thin layer chromatography
In order to get a more detailed separation of this fraction, a thin layer chromatography was made using a 1 mg / ml (1%) dilution in n-hexane. Mobile Phase was 100 ml of a mixture of methylene chloride and ethanol at a ratio of 95 : 5 lined part of the inside with filter paper and sealed for chamber saturation. The plate was watched under UV light with a wavelenght of λ 254 nm and λ 365 nm and supplementary sprayed with $KMnO_4$ and Vanilla spray agent. Three mayor zones were determined:
The spot with the strongest retention was only visible after spraying with vanillia spray agent and heating in the oven at 150 °C for 10 minutes. It was neither visible under UV – light, nor after spraying with $KMnO_4$ spray agent. The calculated retention factor (R_f) for this spot was 0.62.
One spot with a R_f of 0.6944 was only visible under UV light with a wave lenght of λ 365 nm having a light yellow fluorescence. The last visible spot had a Rf between 0.777 and 0.888 and consisted of minimum 2 substances which seemed to be very concentrated. They were visible with both, vanilla- (magenta spot), and $KMnO_4$ spray agent but had no fluorescence under UV light.

Due to the fact that this TLC had no well visible separation, the polarity of the dilutant was reduced and another TLC was run with a mixture of n-hexane and chloroform at a ratio of 1 : 1.



pict. 10; TLC spots of fraction VLC 1. Plates sprayed with Vanilla – spray – agent (upper plate), and $KMnO_4$ – spray – agent (lower plate).

This thin layer chromatography provided a much better separation with spots distributed over the whole plate. After spraying with vanilla spray agent, seven concentration zones could be allocated. The plate sprayed with $KMnO_4$ – spray agent showed five of these spots.
The substance with the strongest concentration had a retention factor of 0.73 and a dark violet color after spraying with vanilla spray agent and a brown color with $KMnO_4$ spray agent. It was overlapped

by another substance with a Rf of 0.78. (see Picture 10)

Rf	Vanilla – spray – agent	KmnO4 – spray – agent
0.22	violet	barely visible
0.30	violet	light brown
0.39	violet	light brown
0.43	pink	not visible
0.51	dark violet	brown
0.73	dark violet	brown
0.78	dark blue	barely visible

Tab. 3; Visible spots on the TLC plates of VLC_1 after spraying with KmnO4, vanillia and watching under UV - light.

In consideration of the good separation results of this TLC, a preparative thin layer chromatography was made.

Preparative Thin Layer Chromatography
Three 20 x 20 cm pTLC glassplates were laminated with 1.0 mm of a mixture of silica and water and predried at room temperature for 2 hours. For complete desiccation they were then dried in the cabinet desiccator at 90 °C (194 °F). Starting point was marked 2.0 cm from the lower end with a pencil.
Three specially designed pTLC development chambers were filled with a mixture of n-hexane and chloroform at a ratio of 1 : 1, lined part of the inside with filter paper and sealed for chamber saturation.
3 x 100 mg of fraction VLC_1 were soluted in 3 x 10.0 ml of the same mixture, brought up to the plates and dried to complete desiccation. Then the plates were developed, dried and a zone with a width of 0.5 cm was sprayed with vanilla spray agent and additionally watched under UV254 and UV365 to locate the spots. Six different zones were distinguished and the plate was fragmented in six fractions by cutting the silica layer with a razor blade, paring the zones and collecting the silica in vessels.
To each of this fractions 50.0 ml of a mixture of n-hexane and chloroform at a ratio of 1 : 1 were added. The vessels were brought up to the ultrasonic bath to solute the substances and filtered through cellulose filters. The filtered fractions were collected in containers and dried on the rotovapor.

Rf - start	Rf - stop	weight
0	0.27	0.0171 g
0.27	0.38	0.0548 g
0.38	0.5	0.0434 g
0.5	0.66	0.0373 g
0.66	0.86	0.0776 g
0.86	1	0.0395 g

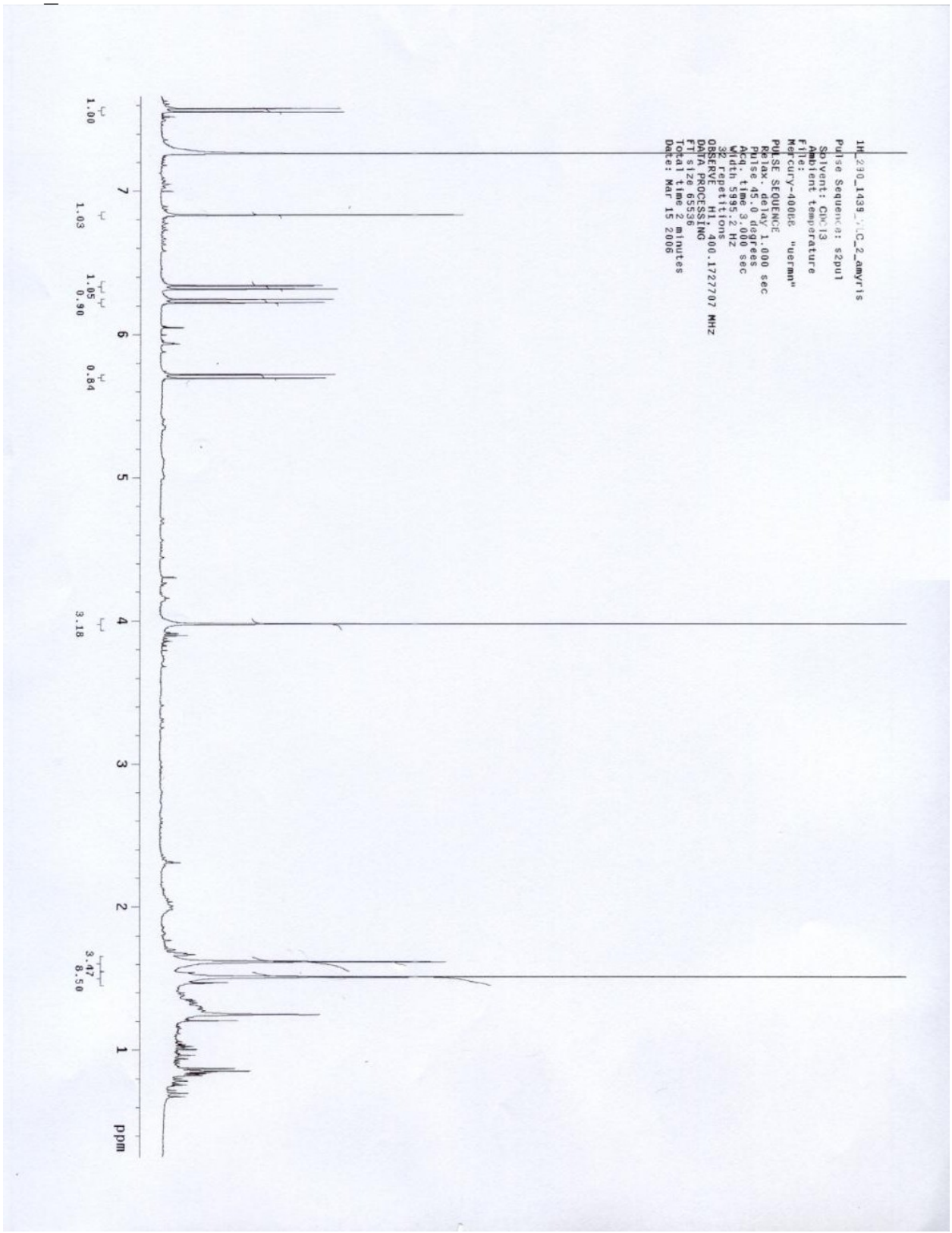
Tab. 4; Fractions of the pTLC separation of VLC_1 (Solvent Gradient: n-hexane and chloroforme 1:1)

A TLC of this fractions was made in order to control the separation results of this pTLC.
Dilutant was a mixture of n-hexane and chloroform (1:1), the plates were sprayed with vanilla -, and $KMnO_4$ – spray – agents and additionally watched under UV – light.

pict. 11; TLC fractions of the preparative thin layer chromatography separation. (UV active spots framed.) Plates sprayed with Vanilla spray agent (left plate), and $KMnO_4$ spray agent (right plate).

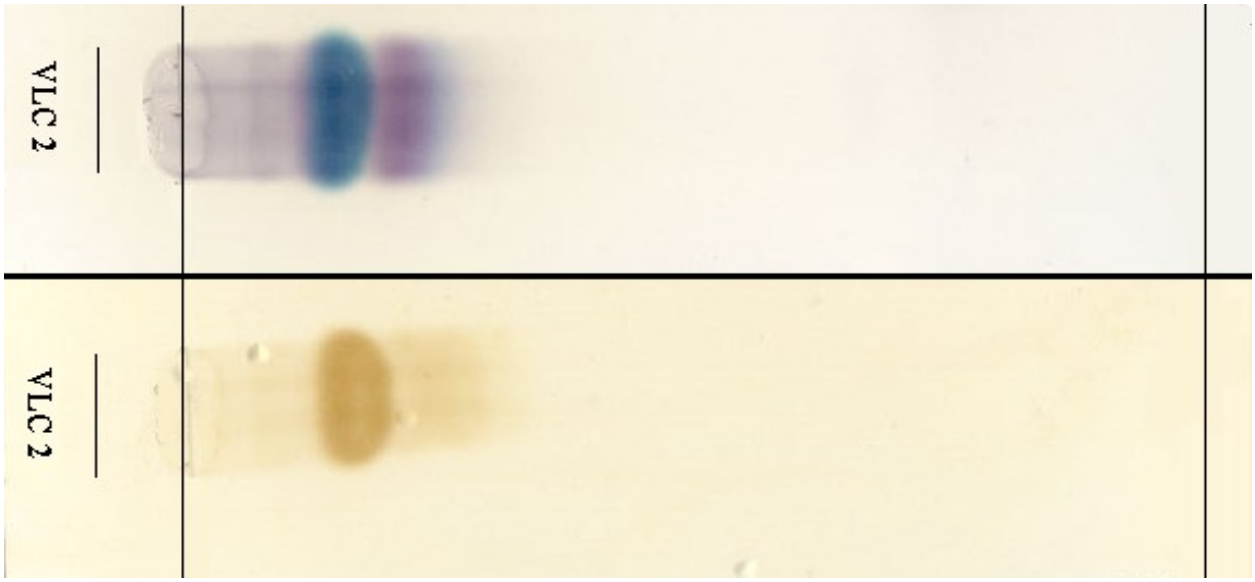
Fraction 1, 2 and 3 showed the same 2 spots visible only in UV λ 365 nm with a green fluorescence. The first spot had a Rf of 0.26 and a light-green fluorescence, the second spot had a Rf of 0.31 with a light blue fluorescence. Fraction four and five had a spot with a Rf of 0.57 visible after spraying with $KMnO_4$ agent. Fraction 5 and 6 showed both the same spot at Rf : 0.8 visible after spraying with vanilla – agent. (see Picture 11)
These fractions were sent to NMR for further structure analysis, solvent for the NMR-measurement was methanol-d4.

VLC_2



Spectra 2; NMR spectra of fraction VLC 2. 400 Mhz, $CDCl_3$.

A thin layer chromatography of this fraction was made in order to determine the number of existing compounds. Solvent was a mixture of n-hexane and chloroform at a ratio of 1:1, concentration of applied fraction was 1 mg / ml in the same dilutant.



pict. 12; TLC spots of fraction VLC 2. Plates sprayed with Vanilla spray agent (upper plate), and $KMnO_4$ spray agent (lower plate).

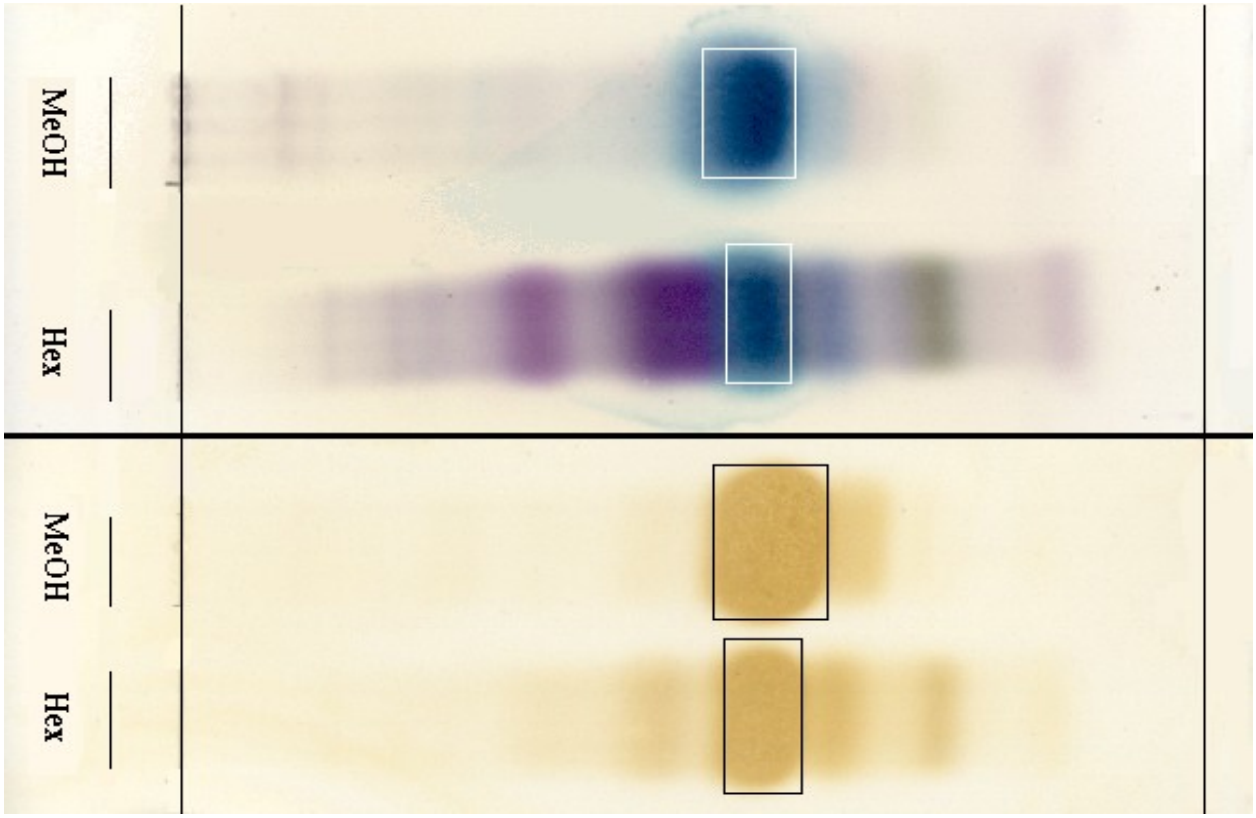
This fraction showed 2 spots visible after spraying with vanilla – spray – agent. The first one had a Rf of 0.13 and was visible with both spray agents showing a blue fluorescence. The second spot was only visible with vanilla spray agent and had a Rf of 0.22 and showed no fluorescence.

Liquid – liquid – separation

This fraction was then divided by liquid-liquid separation. The fraction was diluted in 90.0 ml of n-hexane then 10.0 ml of methanol were added. The mixture was brought up to a 150 ml separating funnel. The mixture was agitated for 4 minutes and after phase separation, the methanol phase was discharged and collected in another vessel. This separation procedure was repeated two times by adding each 10.0 ml of methanol, agitating and separating the methanol – phase. The three discharged methanol phases were collected, united and washed with 30.0 ml of n-hexane in a new separation funnel. The n-hexane phases were united and dried on the rotorvapor. After complete desiccation, the n-hexane phase had an amount of 0.5527 g and a green, semi – liquid - consistency.
The methanolic phase was dried on the rotrovapor resulting in 0.6873 g of a green colored residue.

TLC

These two phases were compared by thin layer chromatography by using a mixture of dichloromethane and methanol at a ratio of 98 : 2 as dilutant. The concentration of applied fraction was 1 mg / ml diluted in the same mixture. (seee Picture 13)



pict. 13; TLC spots of the liquid – liquid separation of fraction VLC 2. Plates sprayed with Vanilla spray agent (upper plate), and $KMnO_4$ spray agent (lower plate).

n-hexane fraction

After developing the TLC plates, watching them under UV light and spraying them with vanilla, and $KMnO_4$ spray agent, it could be stated, that the n-hexane fraction consisted of at least four compounds. The compound with the strongest retention had a Rf of 0.35 and showed a violet spot on the vanilla sprayed plate. Going down with retention, the next spot had a Rf of 0.48 with a violet spot with vanilla spray agent and a lightbrown spot on the $KMnO_4$ plate. The next spot was visible in both, the n-hexane , and the methanolic fraction. It had a Rf of 0.56 and showed a big azur spot on the vanilla plate and a brown spot on the $KMnO_4$ plate. The last spot with a Rf of 0.78 was dark green on the vanilla, and light brown on the KMnO4 plate. (see Picture 13)

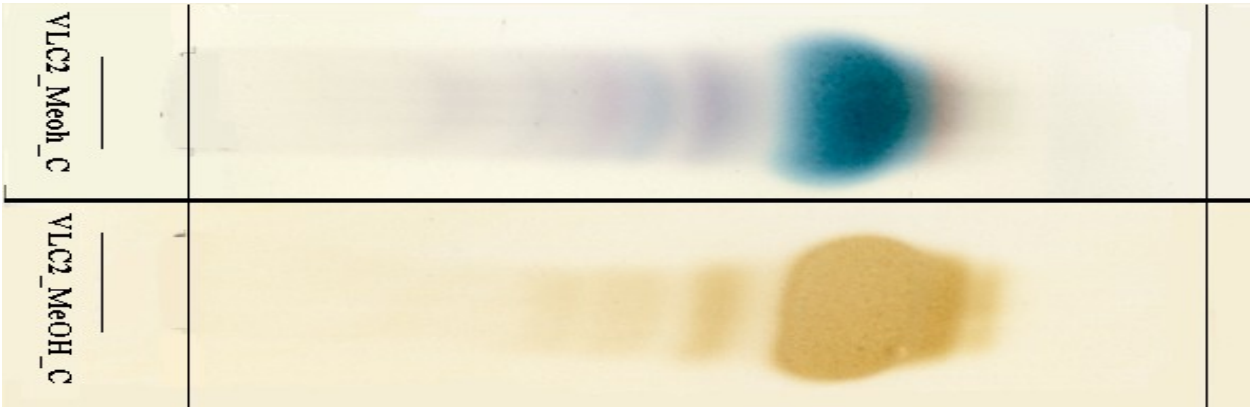
methanolic – fraction

The methanolic fraction produced 2 spots on the TLC-plates. One big spot, which was also visible in the n-hexane phase with a Rf of 0.56 and a second spot which was barely visible with vanilla spray agent having a lightbrown color on the $KMnO_4$ plate. The Rf of this spot was 0.65 and the spots of this two compounds were not completely separated from each other. (see picture 13)

The methanolic fraction was soluted in 6.0 ml of ethanol, 2.0 g of activated carbon were added and after 5 minutes to repose, it was filtered through a cellulose filter. After concentration in the rotovapor and final desiccation in the cabinet desiccator, 0.6405g of material was gained in the filtered methanolic fraction consisting of lucent prisms and a green semi – liquid part.

Thin layer chromatography

A TLC of this cleaned fraction was made, using a mixture of dichloromethane and methanol at a ratio of 98 : 2 as dilutant. The TLC - plates had a lenght of 10.0 cm.



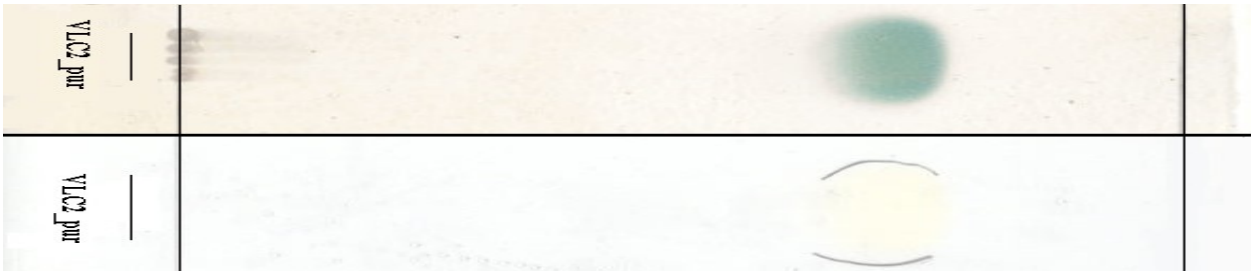
pict. 14; TLC spots of the methanolic fraction of VLC 2 after tratment with activated carbon. Plates sprayed with vanilla spray agent (upper plate), and $KMnO_4$ spray agent (lower plate).

Recrystallization

In order to separate the two compounds, the 0.6405g of this fraction were soluted in warm ethyl acetate using the Ultrasonic Bath. After complete solution of the fraction, it was allowed to cool slowly down at room temperature and was afterwards frozen slowly in the freezer. White Crystals dropped out, were filtered in cellulose filters and washed with cold ethyl acetate.
This afforded 0.1381 g of the white prisms of **Compound 1** (**x**).

Thin layer chromatography

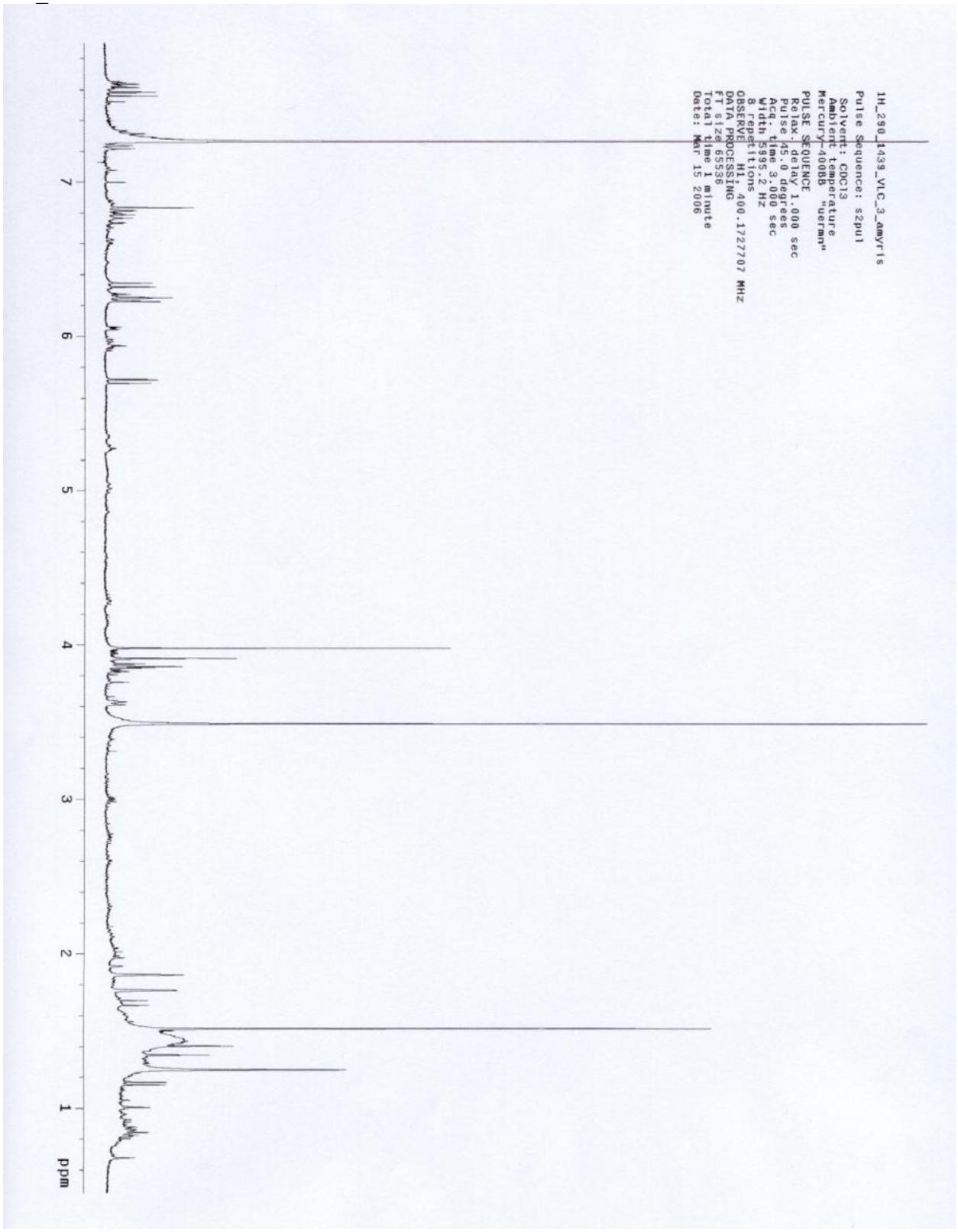
A TLC was made by using a mixture of methylene chloride and methanol at a ratio of 95 :5.



pict. 15; TLC spots of the depurated compound of VLC 2. Plates sprayed with Vanilla spray agent (upper plate), and $KMnO_4$ spray agent (lower plate).

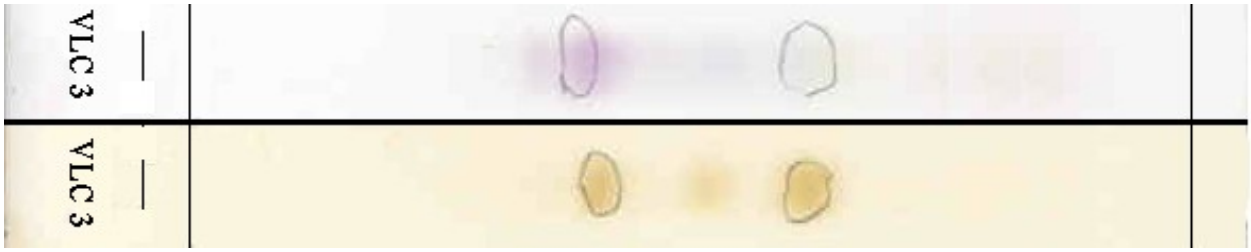
The ethyl acetate phase was dried at the rotorvapor and yielded 0.5490 g. Hence this phase was containing compound 1 too, it was diluted in 5.0 ml of ethylacetate and united with the hexane phase for quantification purposes. The united fraction was dried on the rotorvapor and weighting then 1.1017 g.

VLC_3



Spectra 3; NMR spectra of fraction VLC 3. 400 Mhz, $CDCl_3$.

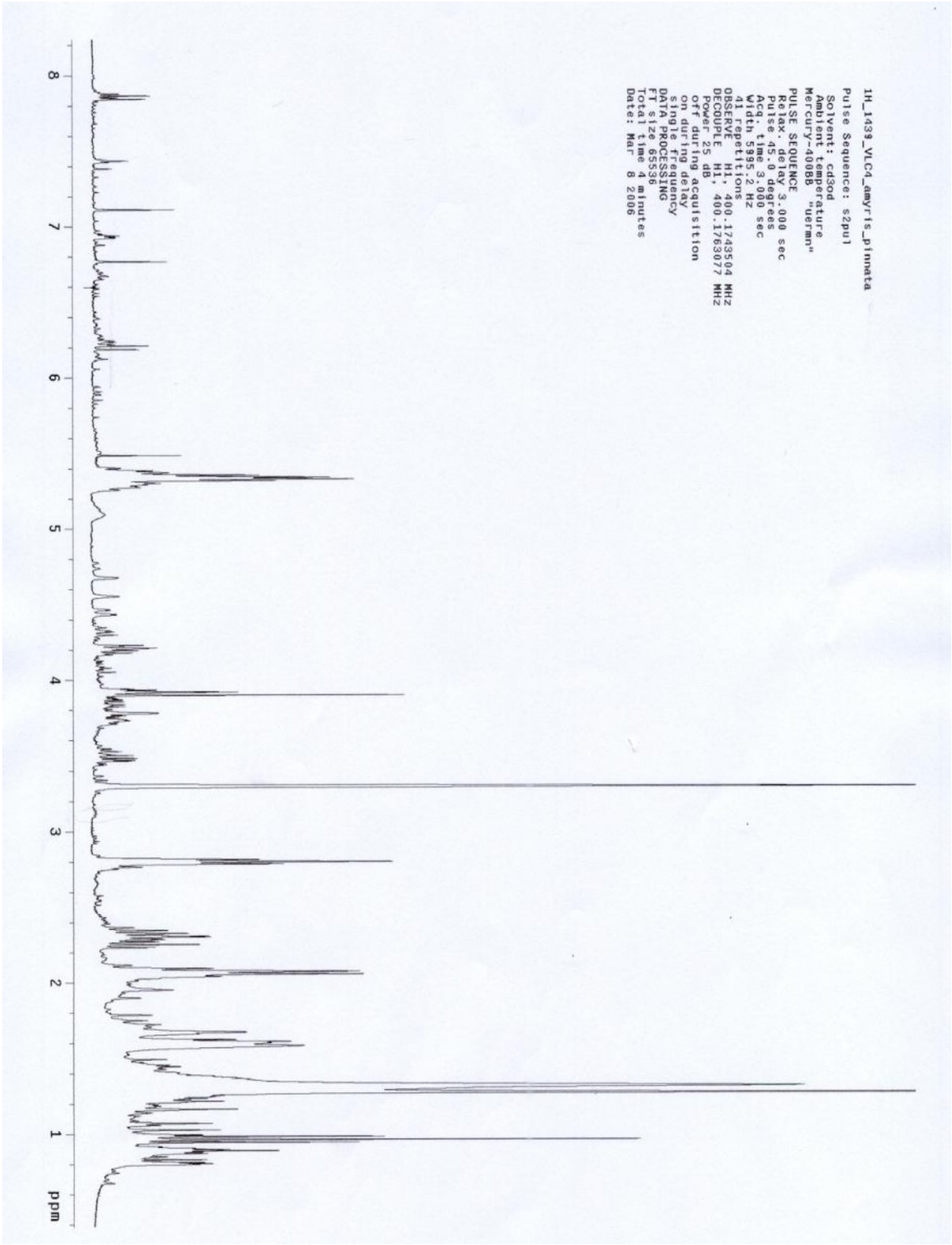
A thin layer chromatography of this fraction was made by using a mixture of methylene chloride and methanol at a ratio of 95 : 5.



pict. 16; TLC spots of VLC 3. Plates sprayed with vanilla spray agent (upper plate), and $KMnO_4$ spray agent (lower plate).

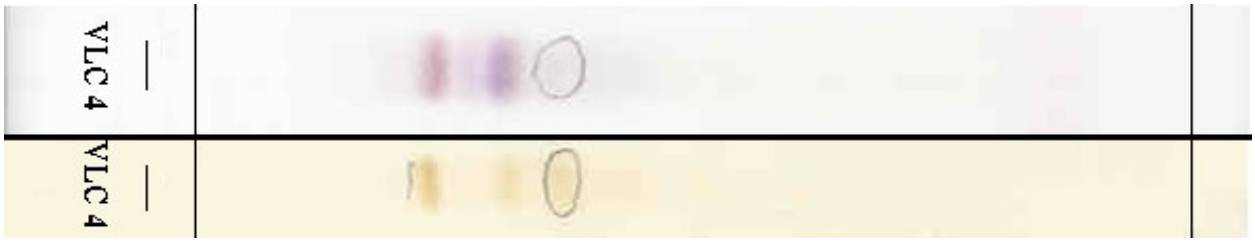
After developing the plates three spots could be located. (see picture 16)The spot having the smallest retention with a Rf of 0.416 was visible under UV light (yellow flourescence) and after spraying with $KMnO_4$ (resulting a brown spot).Next compound with a Rf of 0.52 was only visible on the $KMnO_4$ plate, resulting a brown spot. The last spot had a Rf of 0.62 and was visible under UV light (yellow to green fluorescence). After spraying with $KMnO_4$ agent (producing a brown color), it was visible in both fractions, VLC_2 and VLC_3.
Due to the low yield of this fraction (0.0364g) no further steps of separation were made.

VLC_4



Spectra 4; NMR spectra of fraction VLC 4. 400 Mhz, $CDCl_3$

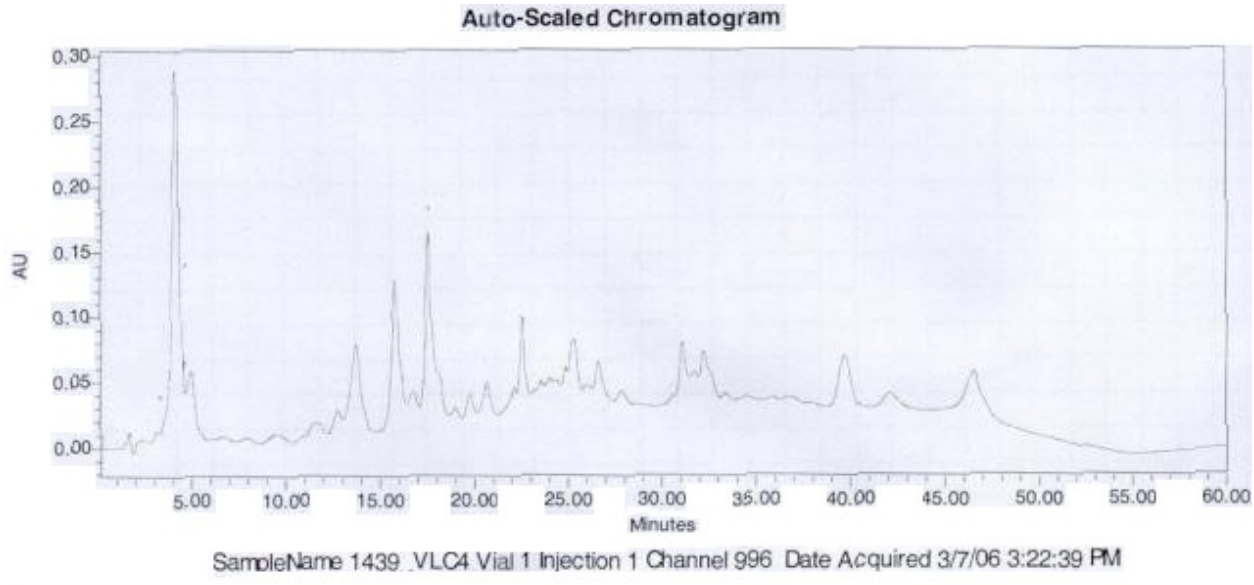
Thin layer chromatography
For the thin layer chromatography a mixture of methylene chloride and methanol at a ratio of 95 : 5 were used as dilutant. The plates were watched under UV – light with a wave lenght of λ 254 nm and λ 365 nm and supplementary sprayed with vanilla , and $KMnO_4$ spray agent.

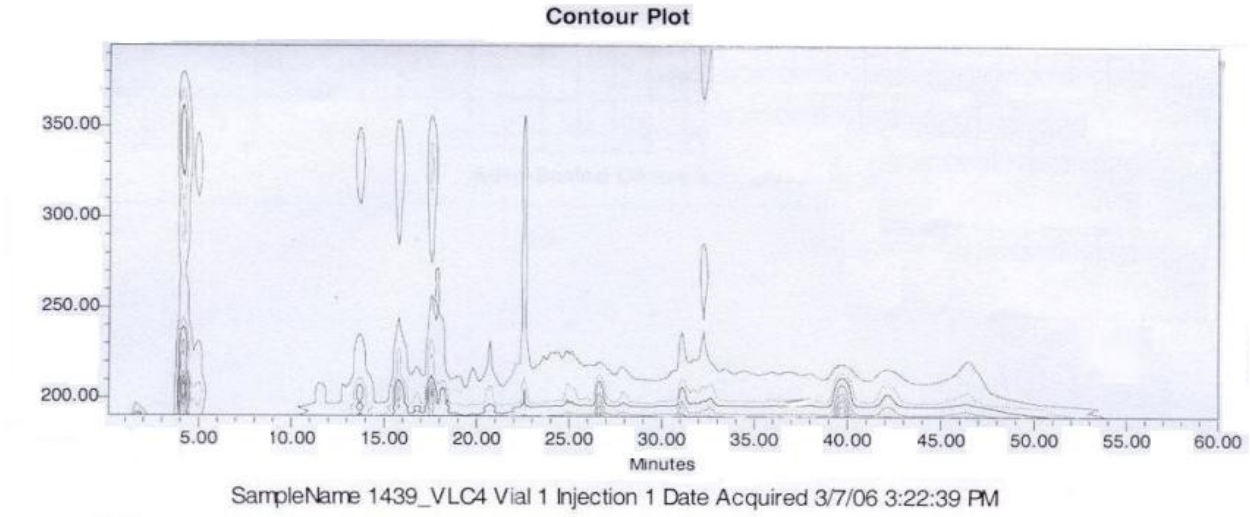


pict. 17; TLC spots of VLC 4. Plates sprayed with vanilla spray agent (upper plate), and $KMnO_4$ spray agent (lower plate).

Three spots could be located, the first one showed a fluorescence decrease in UV – light with a wave lenght of λ 254 nm resulting in a brown spot after spraying with $KMnO_4$ spray agent, having a Rf was 0.229. Next spot with a Rf of 0.313 was only visible on the $KMnO_4$ sprayed plate. The last spot showed a yellow fluorescence under UV light with a wavelenght of λ 365 nm and had a brown spot with $KMnO_4$ agent. The Rf for this compound was 0.375. None of these spots of this fraction was visible after spraying with vanilla spray agent. (see Picture 17)

High performance liquid chromatography
In order to determine the quantity of UV active compounds at a high resolution a HPLC separation combined with a λ – absobance measurement was made. Injection volume was 20 µg of the fraction in 20 µl 60% methanol. Column was a Waters Prep-Nova-Pak HR-C18 (60 Å, 6 µm, 7.8 x 300 mm), dilutant a mixture of methanol in H_2O at a ratio of 9:1 and running time 60 minutes.
The separation was detected by using a Waters 2487 Photo Diode Array detector. (see Spectra 5)





Spectra 5; UV activity (auto scaled chromatogram of the different components of VLC_4 during HPLC separation.

Reversed phase medium pressure liquid chromatography

Fraction VLC_4 was separated by reversed phase medum pressure liquid chromatography, using a 14 x 3cm column. A cotton filter was brought up to the column as bottom layer and it was filled to a height of 11cm with LiChromprep RP-18 (25-40 µm mesh) from MERCK. On top of the silica, a round filter paper was attached in order to mask it. The RP 18 was washed with 200.0 ml of acetonitrile then with 200.0 ml of a mixture of acetonitrile and methylene chloride at a ratio of 1 : 1.
For the head of the column the 0.7720 g of this fraction were soluted in 20.0 ml of methylene chloride and 2.0 g of of LiChromprep RP-18 (25-40 µm mesh) (MERCK) were added. This mixture was then dried on the rotorvapor. After complete desiccation the mixture was applicated steadily onto the filter paper covering the silica in the column. To get a quantitative run of all the compounds, a eluation gradient was used starting with the high relative polarity of 10 % acetonitrile in 90 % water going down to medium relative polarity of acetonitrile.

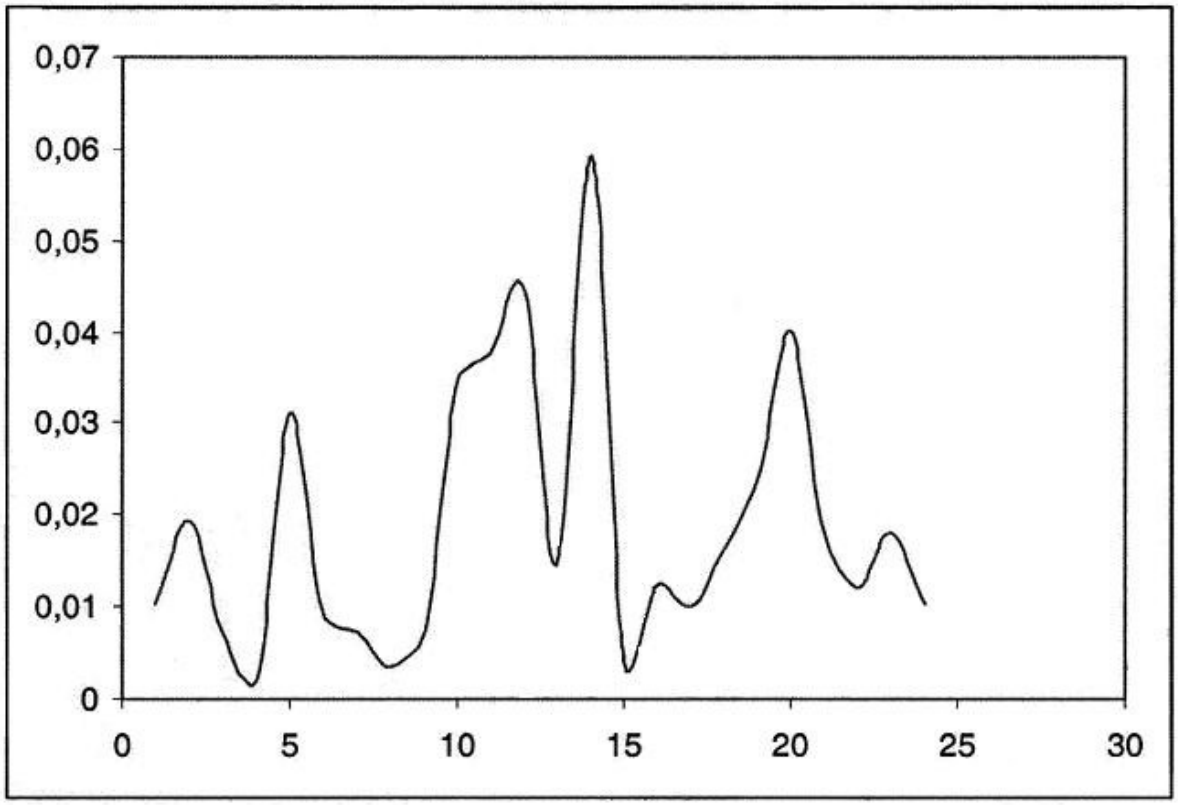
Eluation gradient:

- 90 % H_20 + 10% Acetonitrile 150ml
- 80 % H_20 + 20% Acetonitrile 150ml
- 70 % H_20 + 30% Acetonitrile 150ml
- 50 % H_20 + 50% Acetonitrile 150ml
- 40 % H_20 + 60% Acetonitrile 150ml
- 20 % H_20 + 80% Acetonitrile 150ml
- 100% Acetonitrile 150ml

This was carried out to afford 24 fractions with every fraction having a quantity 43,75ml.
Due to the fact that the RP – 18 had a lightgreen color after the run and in order to get a quantitative separation the column was washed first with 200.0 ml of methanol and second with 200.0 ml of acetone gaining 2 additional fractions. The fractions were dried on the rotorvapor under vacuum to complete desiccation and were placed in the cabinet desiccator over night.

fraction	weight	color
MPLC_1	0.0104 g	colorless
MPLC_2	0.0193 g	colorless
MPLC_3	0.0072 g	colorless
MPLC_4	0.0023 g	nearly colorless
MPLC_5	0.0311 g	light yellow
MPLC_6	0.0096 g	light yellow
MPLC_7	0.0074 g	nearly colorless
MPLC_8	0.0035 g	colorless
MPLC_9	0.0072 g	colorless
MPLC_10	0.0346 g	yellow
MPLC_11	0.0377 g	yellow
MPLC_12	0.0123 g	yellow
MPLC_13	0.0144 g	yellow to gold
MPLC_14	0.0592 g	yellow
MPLC_15	0.004 g	yellow
MPLC_16	0.0124 g	yellow to green
MPLC_17	0.01 g	yellow
MPLC_18	0.0161 g	yellow
MPLC_19	0.0242 g	dark yellow
MPLC_20	0.0401 g	dark yellow
MPLC_21	0.0185 g	dark yellow
MPLC_22	0.012 g	yellow to orange
MPLC_23	0.0181 g	yellow
MPLC_24	0.0102 g	light yellow
MPLC_methanol	0.9626 g	green
MPLC_acetone	0.2103 g	dark green

Tab. 5; Distribution of weights and colors of the MPLC fractions.

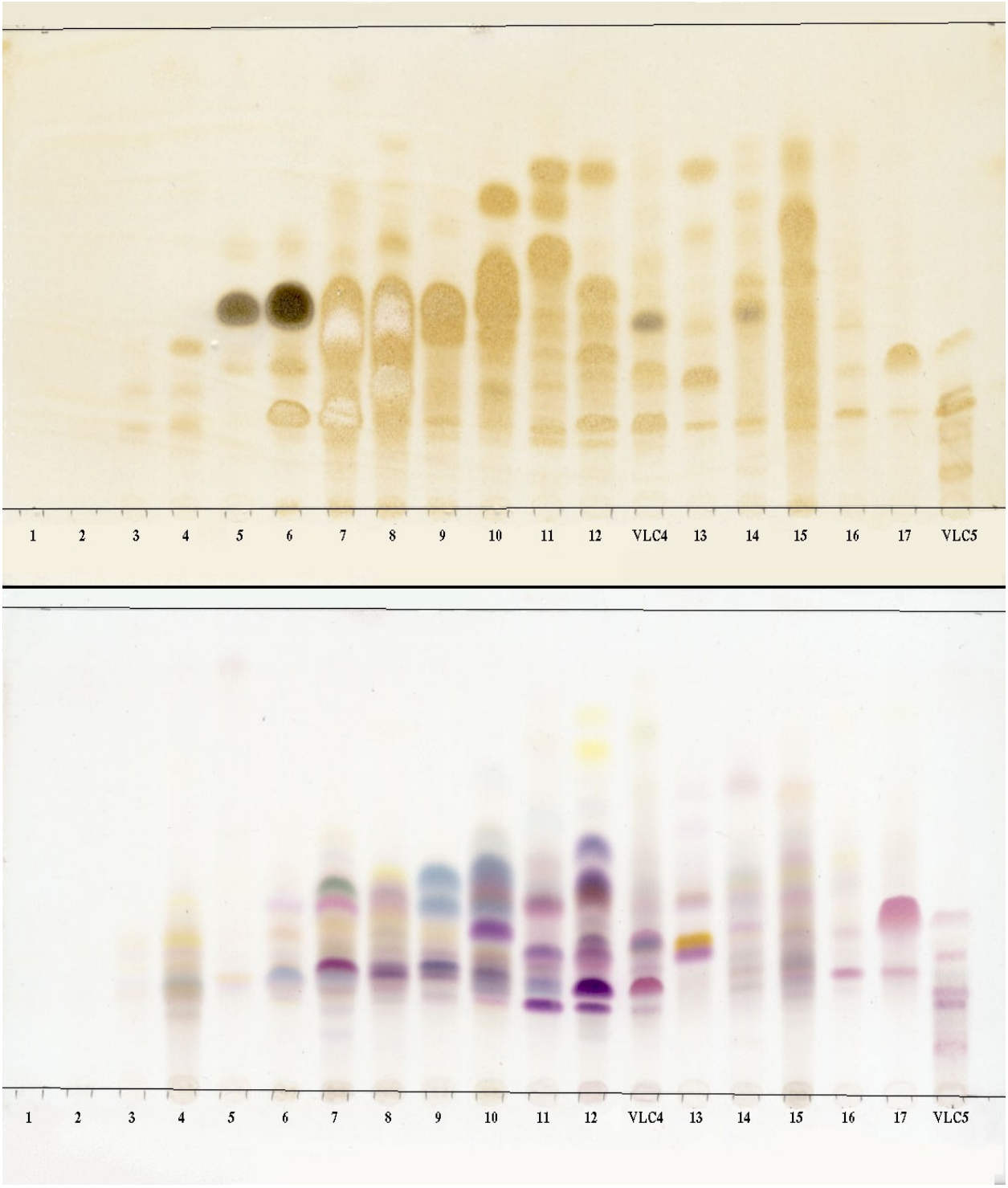


Picture 18; Weight distribution of the 24 MPLC fractions of VLC_4, excluding the two washing fractions. Weight in gram plotted on y - axis, fraction number plotted on x - axis.

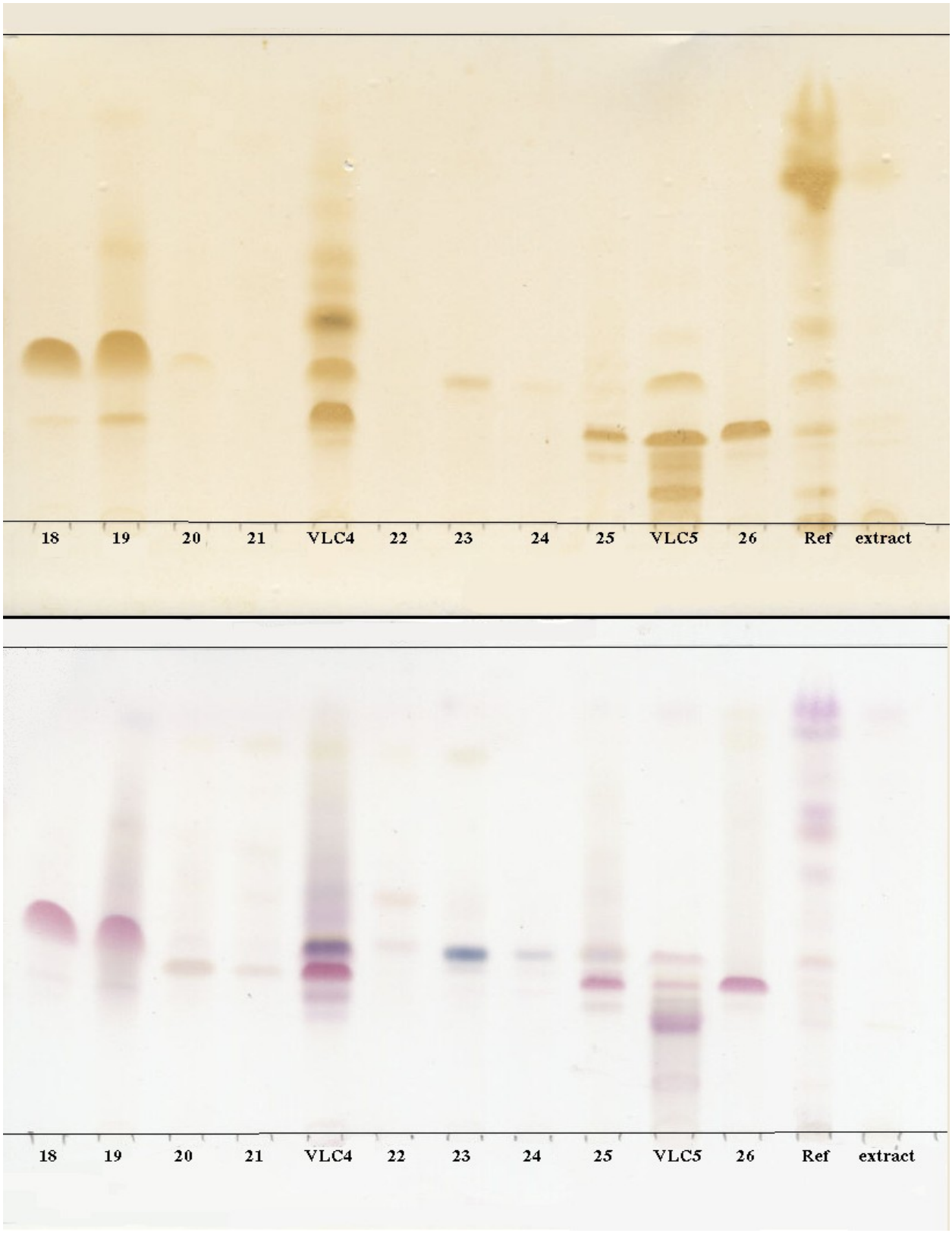
NMR measurements of each fraction were made at the University of Costa Rica in order to determine the presence of conjugated/aromatic systems.

Thin layer chromatography

A thin layer chromatography was made to compare all this fractions, using a 1 mg / ml (1%) dilution of every fraction in a mixture of dichloromethan and methanol at a ratio of 95 : 5.
Dilutant was the same mixture of dichloromethan and methanol.
After watching the TLC plates under UV light and spraying them with both vanilla, and $KMnO_4$ spray agent some of the fractions showed the same composition. So they were united, by soluting them in respectively 10.0 ml of a mixture of dichloromethan and methanol at a ratio of 95 : 5, and drying them on the rotorvapor.



pict. 19; TLC spots of the MPLC fractions 1 to 17 compared with VLC4 and VLC5. Plates sprayed with vanilla spray agent (lower plate), and $KMnO_4$ spray agent (upper plate).



Pict. 20; TLC spots of the MPLC fractions 18 to 26 compared with VLC4, VLC5, an internal reference and the extract. Plates sprayed with vanilla spray agent (lower plate) and $KMnO_4$ spray agent (upper plate).

fraction	united fractions	spots visible on TLC - plate	weight
1	1 + 2	0	0.151 g
3	3 + 4	0	0.0076 g
5	5 + 6	7	0.0314 g
7	7 + 8 + 9	12	0.0122 g
10	10	10	0.0321 g
11	11	6	0.0362 g
12	12 + 13	9	0.0267 g
14	14	9	0.0074 g
15	15	∞	0.0038 g
16	16	5	0.0121 g
17	17 + 18 +19	2	0.0456 g
20	20 + 21	2 - 3	0.1819 g
22	22	2	0.0117 g
23	23 + 24	2	0.03 g
25	25 + 26	3	0.276 g

Tab. 6; Properties of united fractions of the MPLC - separation.

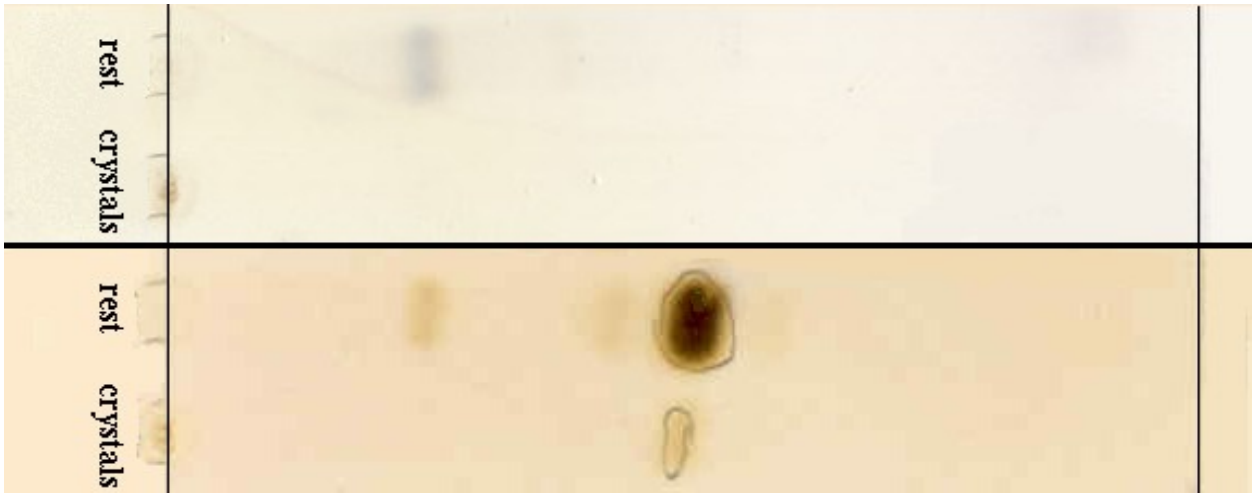
Fraction MPLC_5

This fraction showed a light blue fluorescence at daylight when soluted in methanol.
The NMR measurements evidenced the presence of relevant conjugated double bounds.
Under consideration that this fraction consisted of beige to yellow crystals a recrystallization was accomplished:

Recrystallization

Therefore, the fraction was soluted in a 5.0 ml of hot water (90 °C) allowing it to cool down slowly at room temperature and frozen to 5 °C for a quantitative precipitation of substance. Water and crystals were separated by filtering through a cellulose filter. The crystals were washed with cold water, dried on the rotorvapor and put in the cabinet desiccator over night which resulted 0.0161 g of white needles with a sweet, honey – like smell.

Thin layer chromatography
A TLC was made by using a mixture of methylene chloride and methanol at a ratio of 95 : 5.



pict. 21; TLC spots of the recrystalisation of MPLC5. Plates sprayed with vanilla spray agent (upper plate) and $KMnO_4$ spray agent (lower plate).

This fraction showed only one spot resulting in an intense black spot after spraying with $KMnO_4$ spray agent. The vanilla reaction showed a light azul color and had a blue flourescence when irridated under UV light with a wavelength of λ 365 nm. Rf was 0.413.

NMR measurement
A part of the crystals was soluted in Methanol-d4, filtered through cotton in a NMR tube and sent to the University of Costa Rica to accomplish a ^1H – NMR measurement.
This was resulting **Compound 2**.

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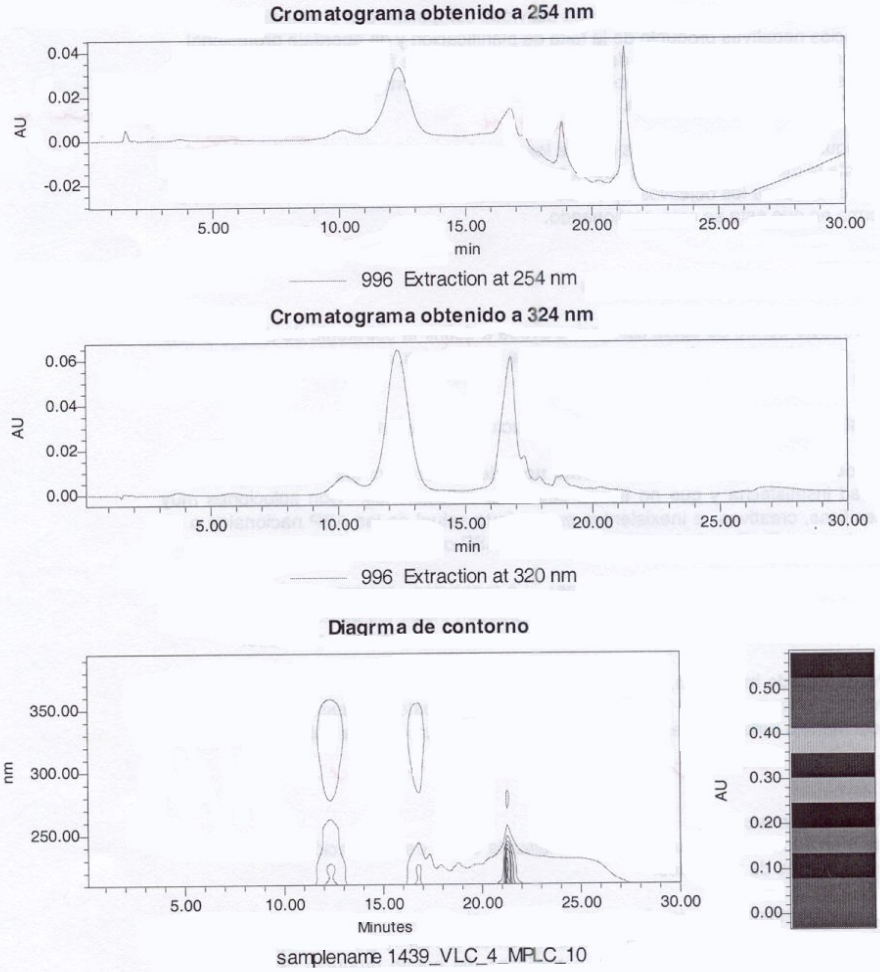
REPORTE DE MONITOREO DE EXTRACTOS EN HPLC

Project Name CONICIT_OEA
samplename 1439_VLC_4_MPLC_10
CODIGO_INBIO
COD_LQ

Acq Method Set Gradiente_amyris
Injection Volume 20.00 ul
TIPODECOLUMNA
System Name SistemaPDA



Pict 22, HPLC data of MPLC 10.

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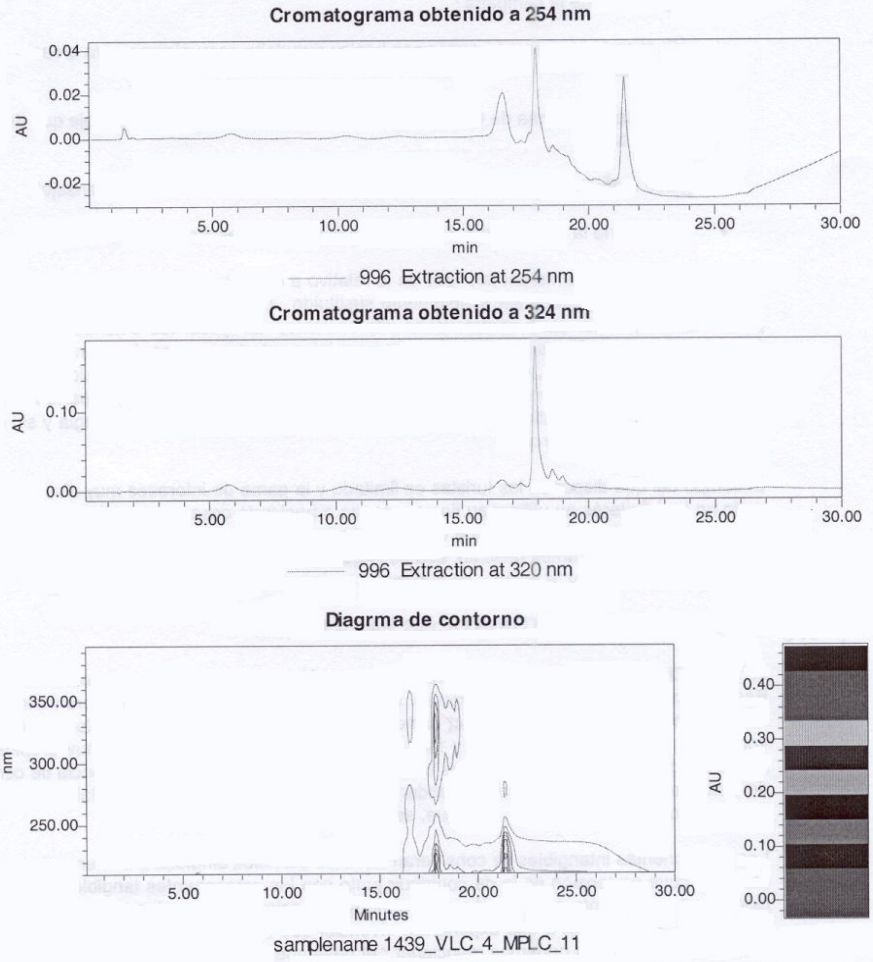
de Biodiversidad

REPORTE DE MONITOREO DE EXTRACTOS EN HPLC

Project Name CONICIT_OEA
samplename 1439_VLC_4_MPLC_11
CODIGO_INBIO
COD_LQ

Acq Method Set Gradiente_amyris
Injection Volume 20.00 ul
TIPODECOLUMNA
System Name SistemaPDA



Pict 23, HPLC data of MPLC 11.

Thin layer chromatography
The TLC using a mixture of methylene chloride and methanol at a ratio of 95 : 5 as dilutant showed nine spots and due to the proportion of nine substances in a relative small amount of substance (0.0161 g) no further steps have been taken at this point. (Picture 19)

UV activity

MPLC 10
This fraction showed two peaks at 324nm in the HPLC. The first peak had an average retention of 13 minutes, the second one at 17 minutes. Another absorption maximum could be determined at 254nm wave length which had a retention of 21.5 minutes. (see picture 22) so it seemed that this fractions contained at least 3 interesting uv – active compounds.

MPLC 11
This fraction showed a absorption maximum at 324 nm wave lenght with a retention of 18 minutes. Measured at 254 nm wave length, this fraction showed the same absorption maximum as MPLC 10 at a retention time of 21.5 minutes. (see picture 23) So this fraction seemed to contain at least one more uv – active compound.

MPLC 12
Fraction MPLC 12 had an absorption maximum, measured at 324n nm wave length, with a retention of 19,2 minutes. Measured at 254 nm wave length two peaks were visible. One with a retention of 19.2 minutes, which seems to be uv – active also at w wavelenght of 324 nm. The other smaller peak had a retention time of 21.3 minutes. (see picture 24)

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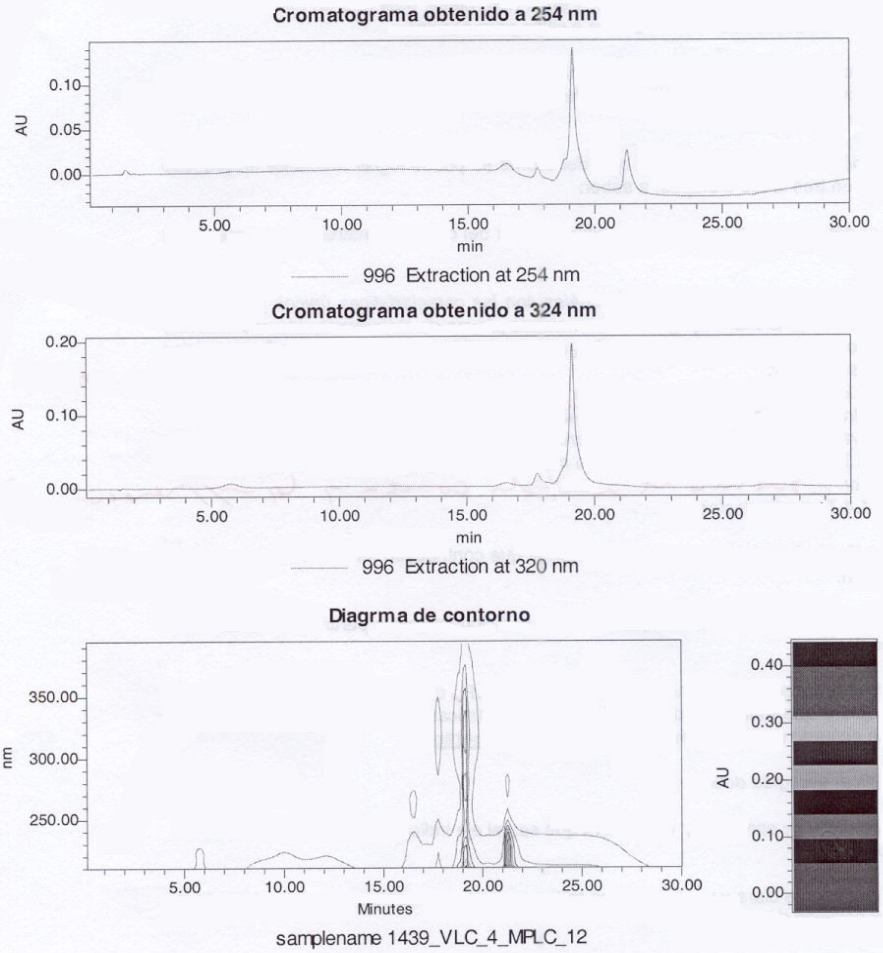
REPORTE DE MONITOREO DE EXTRACTOS EN HPLC

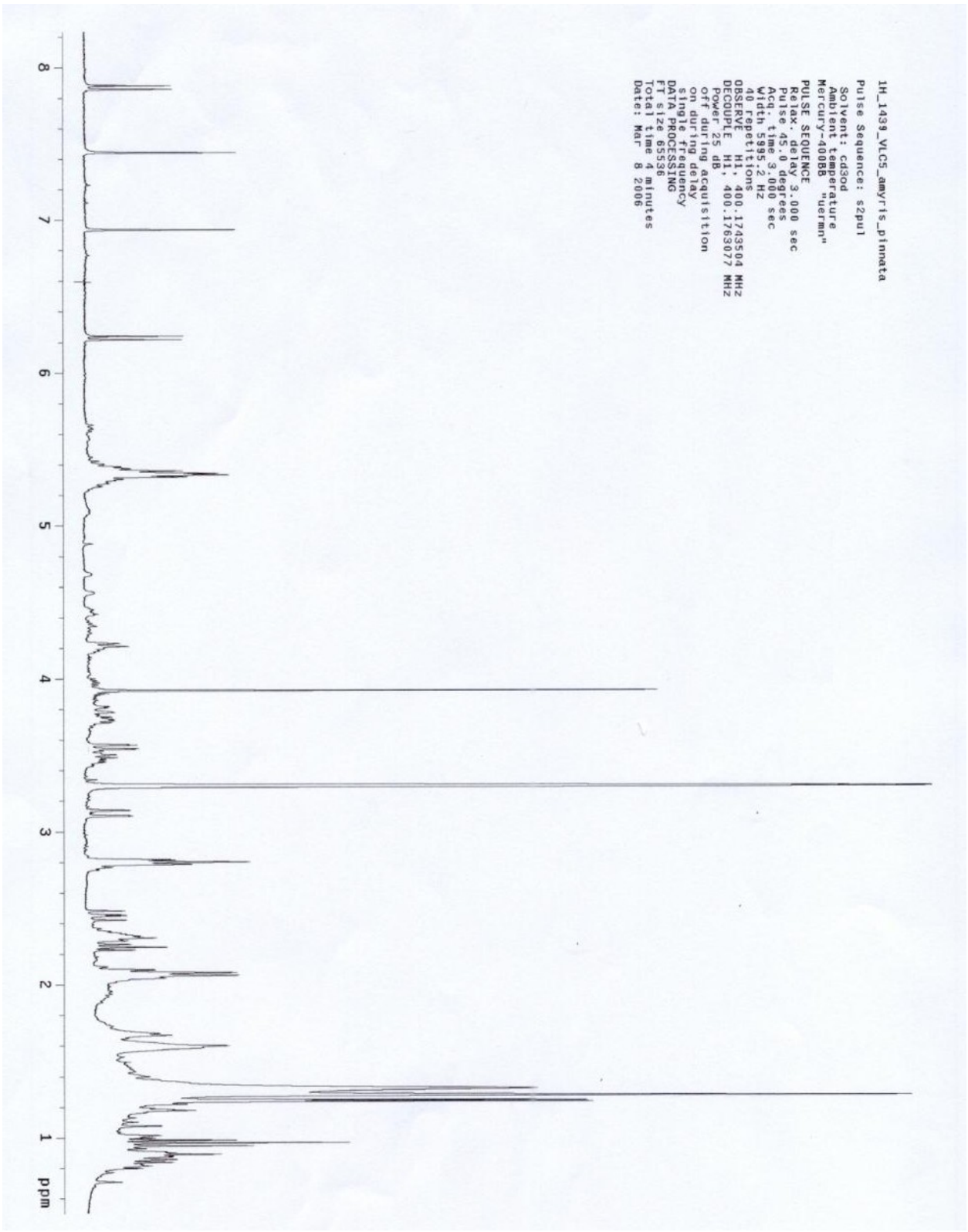
Project Name CONICIT_OEA
samplename 1439_VLC_4_MPLC_12
CODIGO_INBIO
COD_LQ

Acq Method Set Gradiente_amyris
Injection Volume 20.00 ul
TIPODECOLUMNA
System Name SistemaPDA



Pict 24, HPLC data of MPLC 12.

VLC_5



Spectra 6; NMR spectra of fraction VLC 5. 400 Mhz, $CDCl_3$

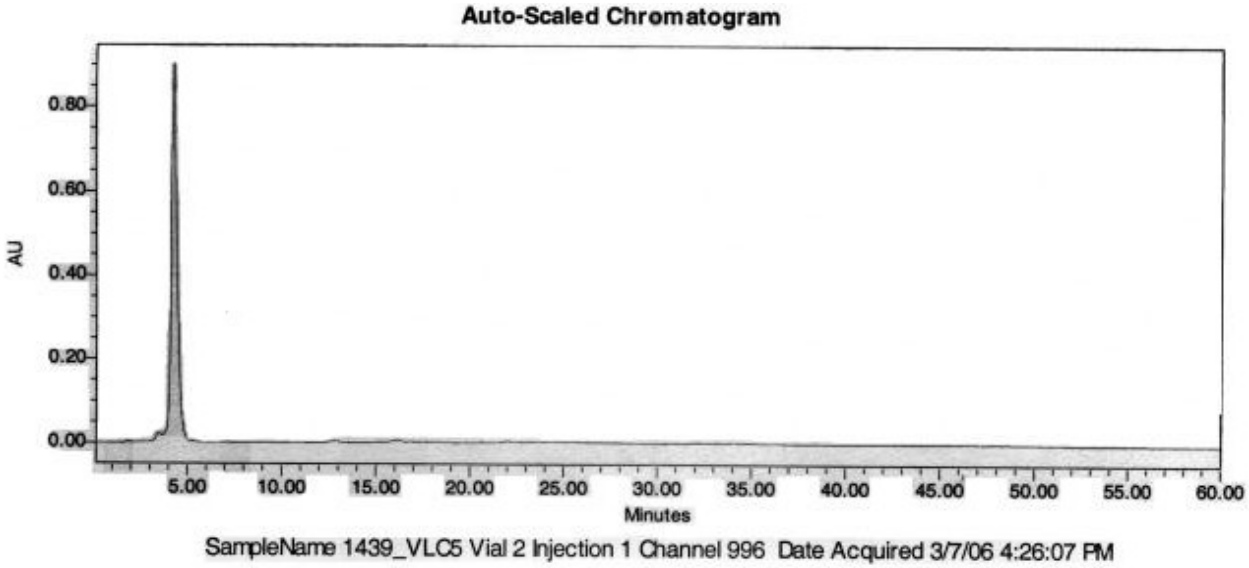
This fraction yielded 0.1713g and had a light yellow color.

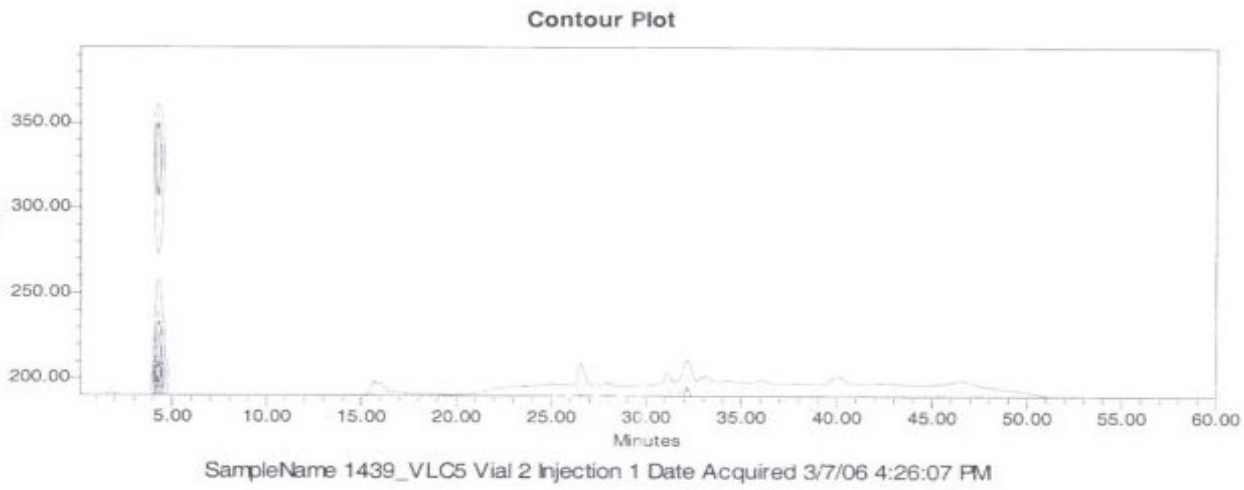
Thin layer chromatography
The thin layer chromatography for this fraction was made by using a mixture of methylene chloride and methanol at a ratio of 95 : 5.

pict. 25; TLC spots of VLC5. Plates sprayed with vanilla spray agent (upper plate) and $KMnO_4$ spray agent (lower plate).

The fraction showed two spots with UV irridation (λ 365 nm), one with a Rf of 0.253 that was also visible as a brown spot after spraying with $KMnO_4$ spray agent, the other one with a Rf of 0.318 that was neither visible with $KMnO_4$, nor with vanilla spray agent. (see picture 25)

High performance liquid chromatography
In order to determine the number of UV active compounds at a high resolution a HPLC separation combined with a λ – absorbance measurement was made. Injection volume was 20 µg of the fraction (VLC_5) in 20 µl 60% methanol. Column was a Waters Prep-Nova-Pak HR-C18 (60 Å, 6 µm, 7.8 x 300 mm), dilutant a mixture of methanol in H_2O at a ratio of 9:1 and running time 60 minutes.
The separation was detected by using a Waters 2487 dual λ absorbance detector.





pict.26; UV activity (auto scaled chromatogram of the different components of VLC_5 during HPLC separation.

Solid phase separation

To obtain a further separation of this fraction a reversed phase column chromatography was applied by using two pre packed Supelco RP-18 Columns. (Half of the sample per column.) The whole 0.1713 g of fraction VLC_5 were soluted in 20.0 ml of 10% MeOH in H_2O and apportioned into two parts by 10.0 ml per part. These two parts were brought up respectively to one of the two Supelco RP-18 Columns. The columns were put into a vacuum chamber and a vacuum was used in order to get a faster run.

Eluation gradient was:

- H_2O
- H_2O/MeOH 1:1
- MeOH

20.0 ml each fraction, 10.0 ml per column;

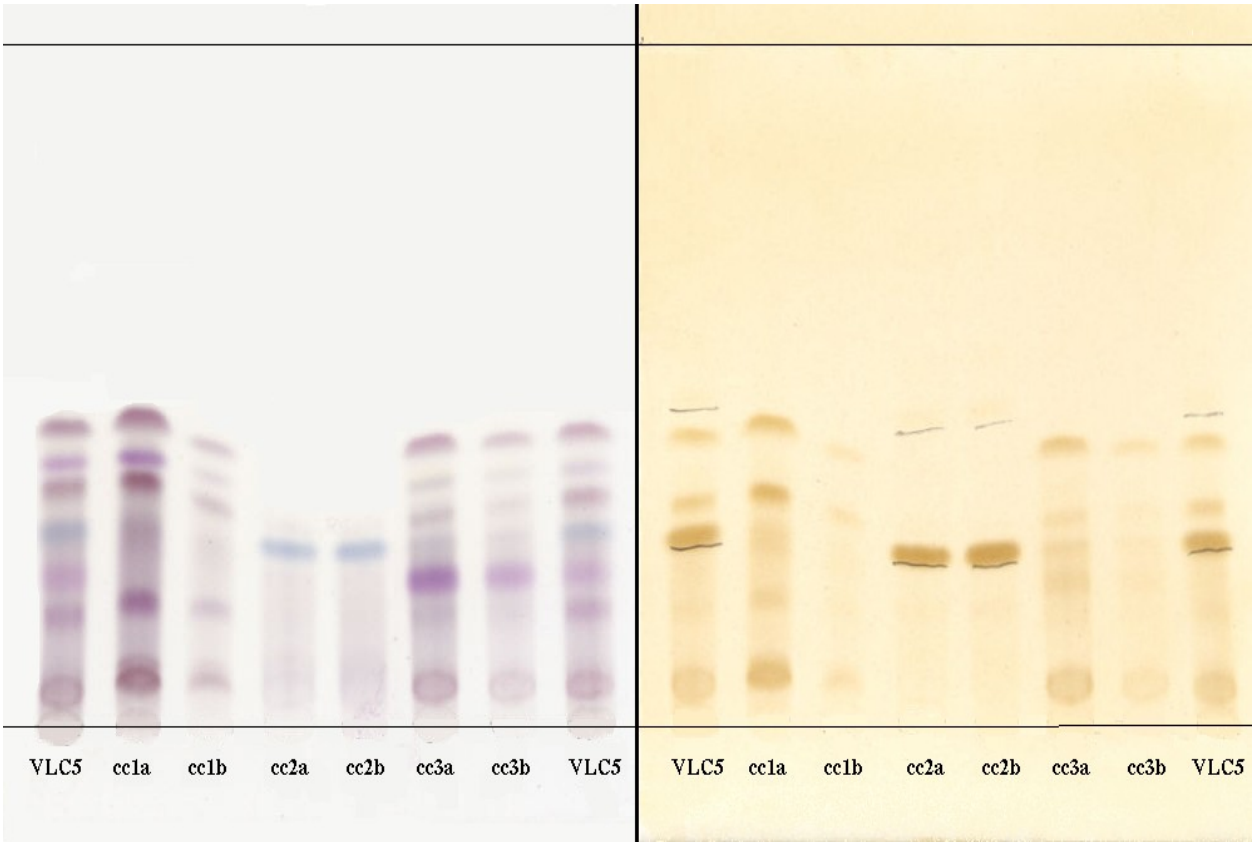
This resulted in six fractions, three fractions (10.0 ml each) per column. The fractions were dried on the rotorvapor and weighed:

fraction	weight	dilutant
CC_1a	0.0251 g	H_2O
CC_1b	0.0262 g	H_2O
CC_2a	0.0173 g	H_2O/MeOH (1:1)
CC_2b	0.0190 g	H_2O/MeOH (1:1)
CC_3a	0.0201 g	MeOH
CC_3b	0.0198 g	MeOH

Tab. 7; Properties of the reversed phase column chromatography fractions of VLC_5.

Thin layer chromatography

To analyze the results of this separation a thin layer chromatography was made by using a mixture of methylene chloride and methanol at a ratio of 95 : 5 as eluent.



pict. 27; TLC spots of the column chromatography fractions of VLC5. Plates sprayed with Vanilla spray agent (left plate) and $KMnO_4$ spray agent (right plate).

CC_1a and CC_1b

The first two parallel aquired fractions with H_2O as dilutant showed six spots on the TLC plate:

Rf	$KMnO_4$	vanillia	UV
0.061	brown	grey	-
0.184	brown	grey	-
0.255	brown	blue	+
0.327	brown	grey to brown	-
0.408	-	light grey	-
0.449	brown	grey	-

tab. 8; properties of the TLC spots of fraction CC_1 and CC_3

Taken in consideration that this two fractions had the same spots they were soluted in respectively 20.0 ml H_2O, united and dried on the rotorvapor. After drying this fraction (CC_1) had an amount of 0.0513 g.

CC_3a and CC_3b
These two fractions appeared to have the same spots as fractions CC_1a and CC_1b. The fractions were soluted in each 20.0 ml of warm methanol, united and dried on the rotorvapor. It yielded 0.0399 g.

CC_2a and CC_2b
The next two fractions only had two spots on the TLC plates. One with a Rf of 0.245 and beeing visible on the $KmnO_4$ sprayed plate as a brown spot and under irridation of UV – light with a wave lenght of λ 365 nm having a yellow flourescence. The second spot had a Rf of 0.429 and was only visible with UV irridation (λ 365 nm) with a yellow flourescence. These two fractions were soluted in each 20.0 ml of a mixture of H_2O and methanol at a ratio of 1 : 1. They were united as they had the same composition and dried on the rotorvapor. The united fraction yielded 0.0363 g.

Recrystallization
The 0.0363 g of this fraction were soluted in warm ethyl acetate on the ultrasonic bath and allowed to cool down slowly at room temperature and afterwards frozen to 5 °C. White to beige crytals precipitated from the solvent. They were filtered through a cellulose filter and the crystals were washed with cold ethylacetate. This led to 0.0232 g of crystals after drying in the cabinet desiccator over night. The solvent was evaporated on the rotorvapor yielded 0.0029 g of material.
A part of this fraction was soluted in Methanol-3D, filtered with cotton and a NMR – measurement was accomplished. This measurement abandoned that the fraction was not pure so further separation had to be made.

High performance liquid chromatography
The 0.0029 g were soluted in 0.5 ml of 30 % methanol in H_2O and injected in the HPLC. Column was a Waters Prep-Nova-Pak HR-C18 (60 Å, 6 µm, 7.8 x 300 mm), dilutant a mixture of methanol in H_2O at a ratio of 3:10 and running time 30 minutes.

fraction	weight	minutes
HPLC_1	0.0027 g	1
HPLC_2	0.0006 g	5
HPLC_3	0.0054 g	9
HPLC_4	0.0018 g	10.2
HPLC_5	0.001 g	15
HPLC_6	0.001 g	30

Tab. 9; Preferences of the HPLC fractions of CC_2.

Thin layer chromatography
To control this separation a thin layer chromatography was made by using a mixture of dichloromethan and methanol at a ratio of 95 : 5 as eluent.
Only fraction HPLC_3 (0.0054 g) had the relevant spot at Rf = 0.245 visible under UV irridation

consisting of **Compound 3** (**10**). The other fractions showed no UV active compounds.
In order to obtain a bigger amount of Compound 3 the washing phase of the recrystalisation of CC_2 (0.0029 g) and the 2 remaining Fractions of the reversed phase supelco – column – chromatography (CC_1 and CC_3) were soluted in respectively 10.0 ml of a mixture of methanol and H_2O at a ratio of 1 : 1, united and dried on the rotorvapor.
This added up to 0.0940 g of material.

High performance liquid chromatography
The 0.0940 g were soluted in 0.5 ml of 50 % methanol in H_2O and injected in the HPLC.
Column was a X-Terra RP-18 column (5 µm, 3.9x50mm).

Eluation Gradient was:

- 60 ml of 40% MeOH in H_2O
- 40 ml of 100% MeOH in H_2O
- 20 ml of 40% MeOH in H_2O

time	flow	100 % MeOH	40% MeOH
0	4		100
5	4		100
15	4	100	
20	4	100	
25	4		100
30	4		100

Tab. 10; Gradient data of the HPLC separation of the 3 united fractions containing Compound 3.

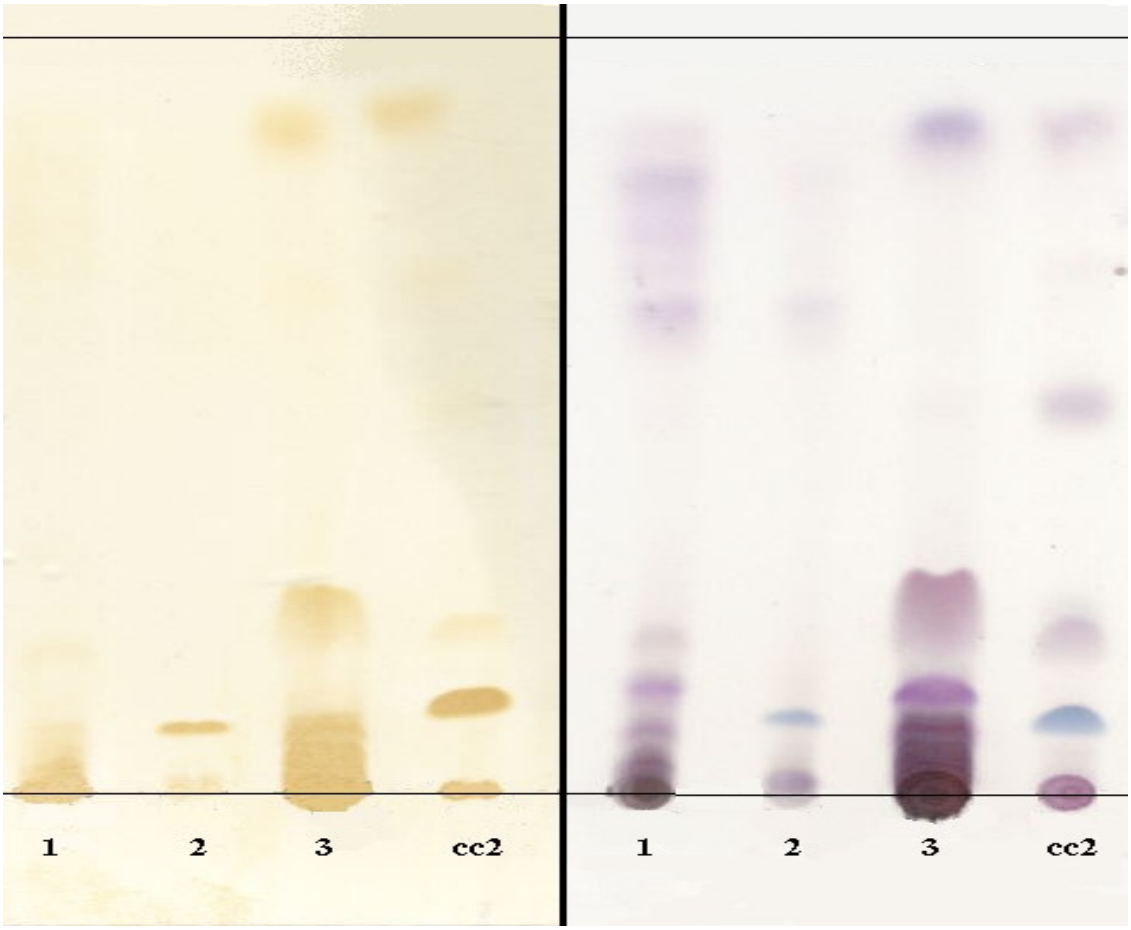
Controller was a Waters 600, HPLC pumps were Waters 600 and Detector was a Knauer differential refractometer.

This separation resulted 3 fractions:

fraction	weight	time	UV active spot at Rf ~ 0.245
HPLC_1	0.0069 g	0 – 17.8	-
HPLC_2	0.0015 g	17.81 - 19.98	+
HPLC_3	0.061 g	20.0 - 30	-

Tab. 11; Data of the three fractions of the HPLC separation of the 3 united fractions, containing Compound 3.

Thin layer chromatography
A thin layer chromatography was made by using a mixture of methylene chloride and methanol at a ratio of 95 : 5 as eluent.

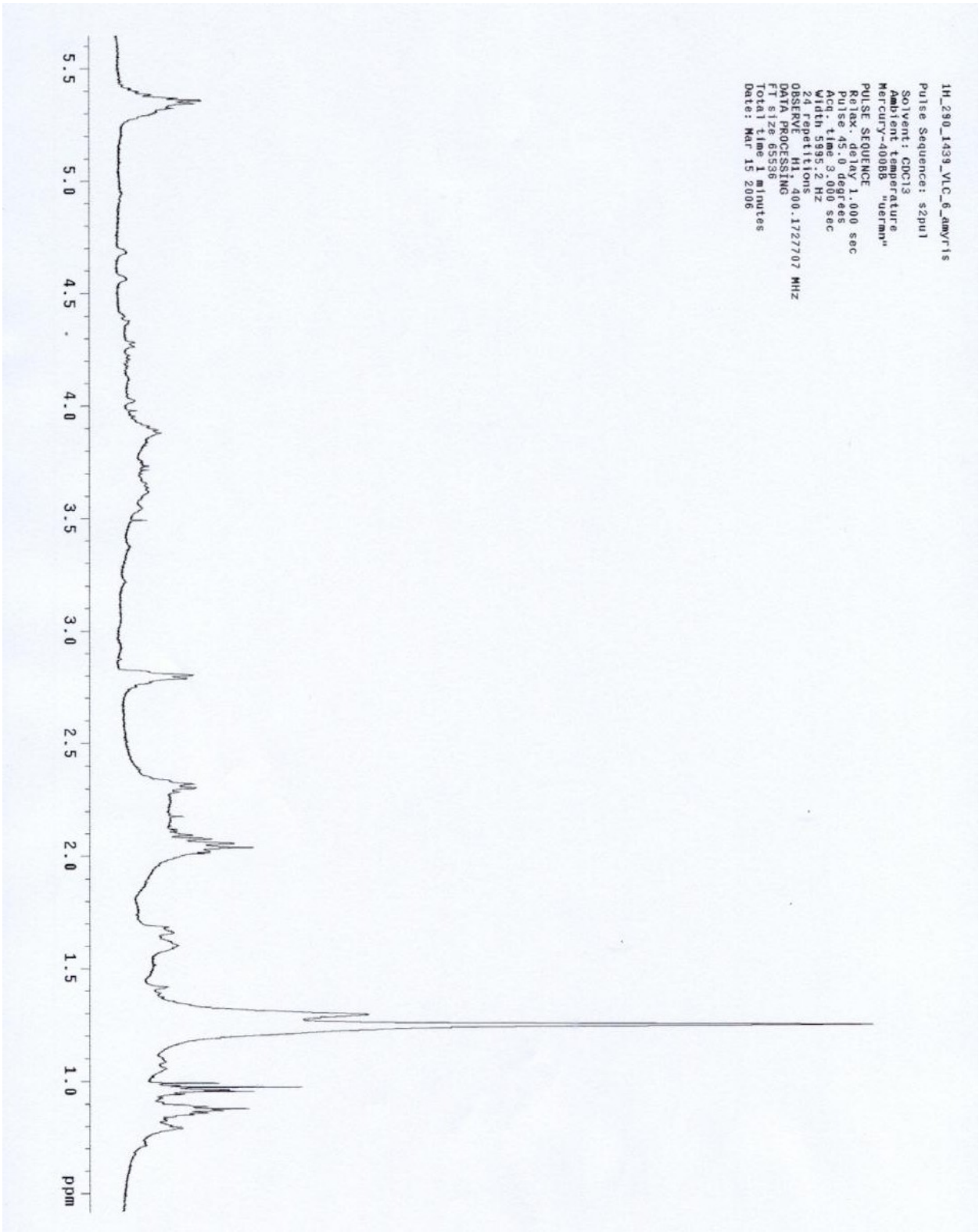


pict. 28; TLC – spots of the HPLC fractions of VLC5. Plates sprayed with vanilla spray agent (left plate) and $KMnO_4$ spray agent (right plate).

Only fraction HPLC_2 (0.0015 g) had the relevant spot at Rf = 0.248 having a UV activity when irridated with a wavelenght of λ 365 nm, consisting of **Compound 3** (**10**). The other fractions showed no UV active compounds. (see Picture 28)

This purified fraction was soluted in 2.0 ml of 50% methanol in H_2O. Additionally the other fraction containing Compound 3 (0.0054 g) was soluted in 4.0 ml of the same mixture. The two fractions were united and dried on the rotorvapor and added up to to 0.0069g of **Compound 3** (**10**).

VLC_6



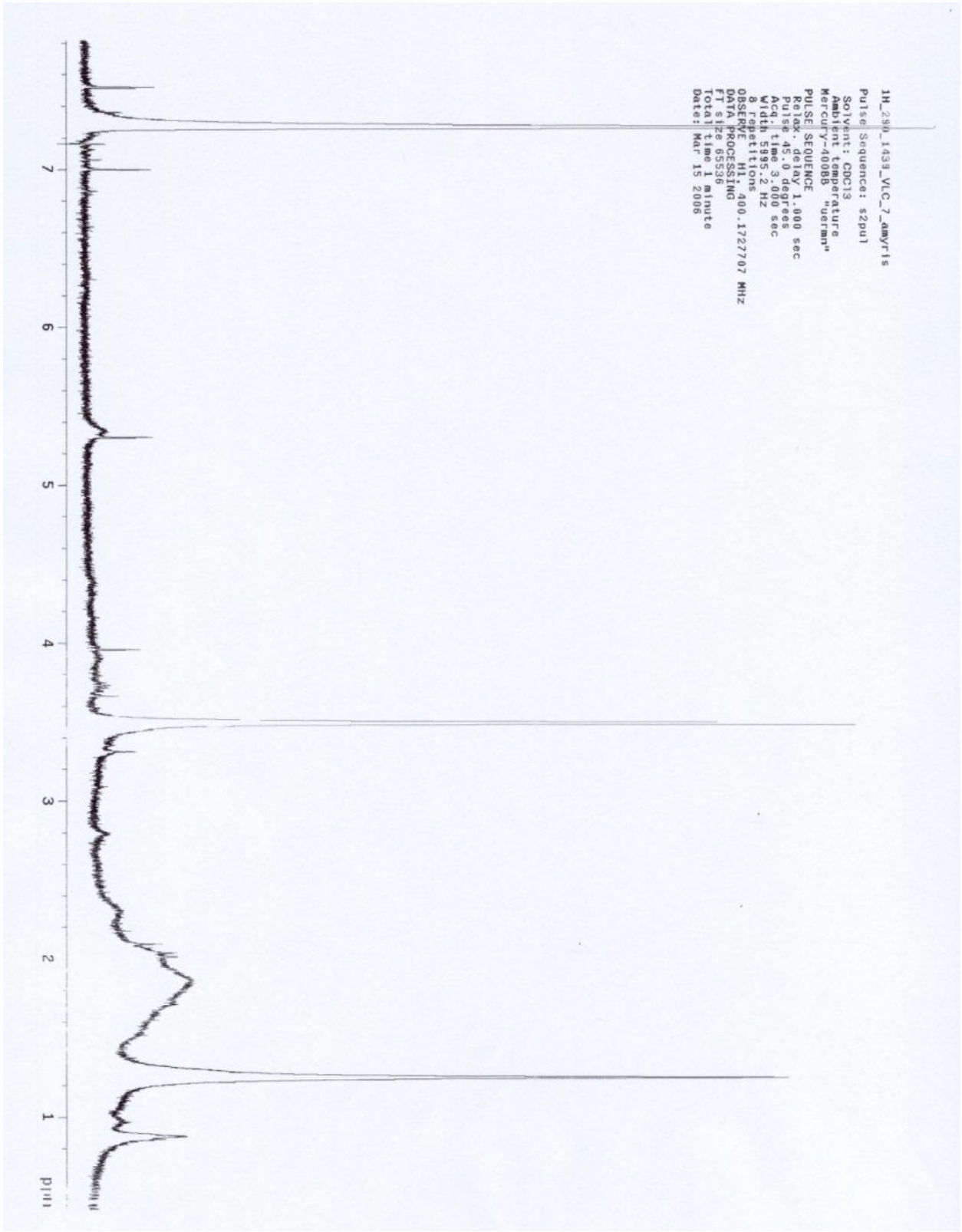
Spectra 7; NMR spectra of fraction VLC 6. 400 Mhz, $CDCl_3$

This fraction yielded 1.3061 g with ethyl acetate : ethanol (1:1) as dilutant in the vacuum liquid chromatography and had a dark green color with a viscous texture.

Thin layer chromatography
A thin layer chromatography was made by using a mixture of methylene chloride and ethanol at a ratio of 95 : 5.
This fraction showed neither spots after spraying with $KmnO_4$ – , or vanilla – spray – agent nor when watched under UV light (λ 254 nm and λ 365 nm).
Additionally, a ^1H - NMR – measurement was made showing no relevant conjugated bonds.

According to this facts, no further steps were taken for this fraction.

VLC_7



Spectra 8; NMR spectra of fraction VLC 6. 400 Mhz, $CDCl_3$

Fraction VLC_6 had a weight of 0.635 g, a darkgreen color and a semi - solid texture.

Thin layer chromatography
The thin layer chromatography was made by using a mixture of methylene chloride and ethanol at a ratio of 95 : 5.
This fraction showed only one spot that appeared brown after spraying with $KmnO_4$ spray agent, and beige after treated with vanilla spray agent. There was no UV - activity. (λ 254 nm or λ 365 nm). The Rf was 0.259. (see picture 28)

pict. 29; TLC spot of VLC7. Plates sprayed with vanilla spray agent (upper plate), and $KMnO_4$ spray agent (lower plate).

The ^1H - NMR measurement showed no relevant conjugated bonds.

Due to the absence of relevant compounds no further separation steps have been taken for this fraction.

2.2.2 Characterisation of Coumarins from *Amyris Pinnata* Kunth

Results

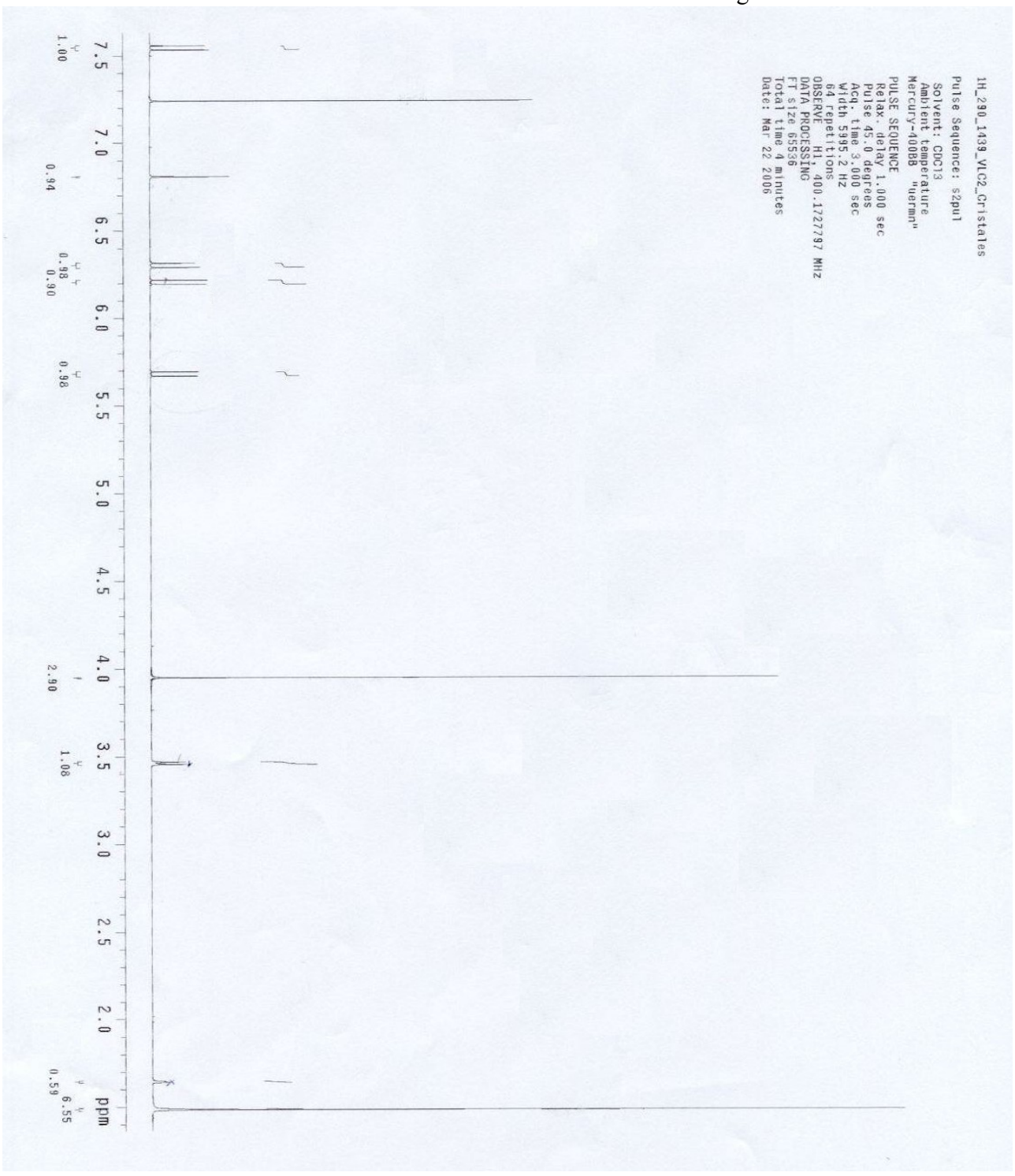
Identification of isolated Compounds

Compound 1			Compound 2		Compound 3		
Position	**δ - C**	**δ - H**	**Position**	**δ - H**	**Position**	**δ - C**	**δ - H**
2	160.80 S		H3	6.23, (d)	1	163.77 S	
3	113.35 D	6.23 d	H4	7.90 (d)	2	113.00 D	6.25 (d)
4	143.77 D	7.56 d	H5	7.14, (s)	3	146.06 D	7.89 (d)
5	119.24 D	6.81 s	H8	6.80 (s)	4	113.30 S	
6	119.22 S		-OCH3	3.78 (s)	5	131.18 D	7.48 (s)
7	149.43 S				6	162.73 S	
8	135.74 S				7	127.60 S	
9	148.40 S				8	99.48 D	6.98 (s)
10	113.11 S				9	156.00 S	
1′	121.20 D	6.31 d			10	33.05 T	2.50 (t) / 2.18 (d)
2′	131.37 D	5.69 d			11	78.50 D	3.60 (d)
3′	77.93 S				12	73.89 S	
4′	38.32 Q	1.51 s			13	25.66 Q	1.31
5′	38.32 Q	1.51 s			14	25.18 Q	1.31
-OMe	61.55 Q	3.97 s			15	56.55 Q	3.98 (s)

Table 12: $^{1}H + ^{13}C$ NMR Data (δ in Hz) for Compounds 1, 2 and 3. 400 Mhz.

2.2.2.a Compound 1 (Luvangetin)

The basis of structure analysis of this compound were NMR experiments. The first experiments made were $^{1}H + ^{13}C$ NMR measurements in order to determine the aromatic configurations.



Spectra 9. ^{1}H – NMR Signals of Compound 1. 400 Mhz, Solvent : $CDCL_3$

^{1}H – NMR Signals

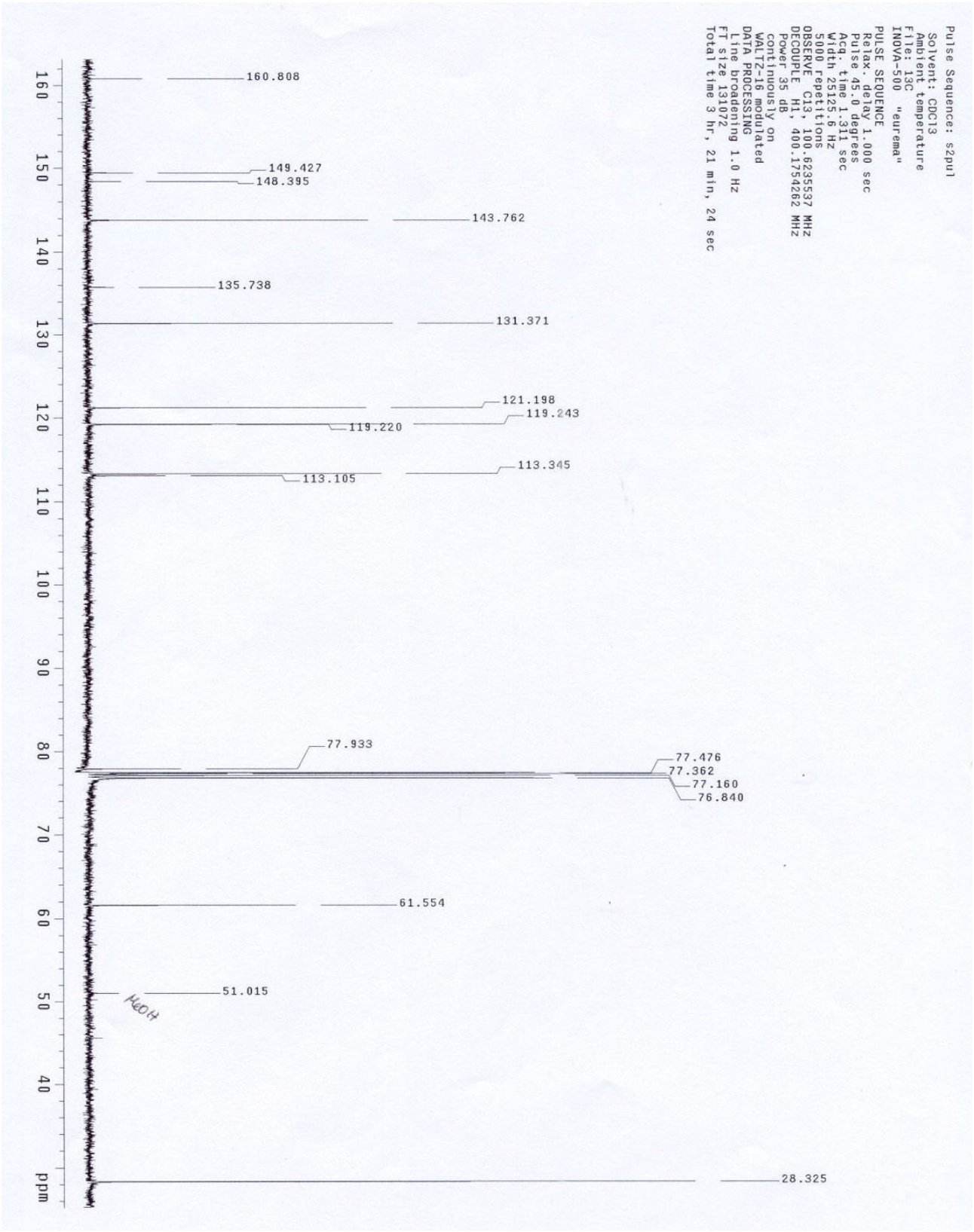
The ^{1}H - NMR experiment resulted in eight peaks with the following chemical shifts: δ 7.56 (doublet), δ 6.81 (singlet), δ 6.31 (doublet), δ 6.23 (doublet), δ 5.69 (doublet), δ 3.97 (singlet), δ 1.51 (singlet) and δ 1.51 (singlet). (see spectra 9)

According to [28] the peak with the following chemical shifts could indicate following groups:

δ 1.51 (singlet) : H3C-C-Hal, a H3C-C=C or a H3C-CC group.
δ 3.97 (singlet) : H3C-SO2- , H3C-N- , H3C-O-Alkyl , H3C-O-Aryl or a -O-CO- group.
δ 5.69 (doublet) : -O-CH2-CO- , -O-CH2-O-, -Aryl , CH-Hal , CH-Aryl,-NR-,-O- , CH=C- , Aryl-OH- , Aryl-NH2- or a Aryl2-NH- group.
δ 6.23 (doublet) : CH-Aryl, -NR- , -O- , CH=C- , Ar-H , Aryl-OH or a R-CO-NH- .
δ 6.31 (doublet) : CH-Aryl, -NR- , -O- , CH=C- , Ar-H , Aryl-OH or a R-CO-NH- .
δ 6.81 (singlet) : CH-Aryl, -NR- , -O- , CH=C- , Ar-H , Aryl-OH or a R-CO-NH- .
δ 7.56 (doublet) : CH=C- , Ar-H , Aryl-OH and R-CO-NH- .

^{13}C – NMR Signals

The ^{13}C experiment resulted in 15 peaks consisting of seven singlets, five doublets and 3 quadruplets. The chemical shifts were: δ 160.80 (singlet), δ 149.43 (singlet), δ 148.40 (singlet), δ 143.77 (doublet), δ 135.74 (singlet), δ 131.37 (doublet), δ 121.20 (doublet), δ 119.24 (doublet), δ 119.22 (singlet), δ 113.35 (doublet), δ 113.11 (singlet), δ 77.93 (singlet), δ 61.55 (quadruplet), δ 38.32 (quadruplet) and δ 38.32 (quadruplet). (see spectra 10)



Spectra 10; ^{13}C – NMR Signals of Compound 1. 400 Mhz, Solvent : $CDCL_3$

According to [29] ^{13}C – NMR Signals with the following chemical shifts can allude to the following groups:

δ 160.80 (singlet):
>C=N- : >C=N-OH , >C=NH , >C=N- ;
>C=C< : =CH-O- , =CH< -R, -O- , =C< ;
Aromatic: Furan C_2 , Pyridin C_2 ;
Benzol : C_1 -F , C_1 -O- , C_1 -N< ;
X ... >C=O : R-, >N- >C=O , $(>N-)_2C=O$, >N-, -O- >C=O , R-, J- >C=O ;
C ... >C=O : $(-O-)_2C=O$.

δ 149.43 (singlet):
Benzol : C_1 -C , C_1 -Cl , C_1 -N< , C_1 -S- , C_1 -P< ;
Aromatic : Pyridin C_3 , Furan C_2 ;
>C=C< : =C< , =CH- , =CH-O- , **=C<** -R, -S- ;
>C=N- : >C=N- , S=C=N- ;
-C≡N : $-N^+\equiv C^-$;

δ 148.40 (singlet):
Benzol : C_1 -C , C_1 -Cl , C_1 -N< , C_1 -S- , C_1 -P< ;
Aromatic : Pyridin C_3 , Furan C_2 ;
>C=C< : =C< , =CH- , =CH-O- , **=C<** -R, -S- ;
>C=N- : >C=N- , S=C=N- ;
-C≡N : $-N^+\equiv C^-$;

δ 143.77 (doublet):
Benzol : C-H , C_1 -C , C_1 -Cl , C_1 -N< , C_1 -S- , C_1 -P< , C_1 -P(O)< ;
Aromatic : Pyridin C_3 ;
>C=C< : =C< , =CH- , =CH-O- , **=C<** -R, -S- , =C< -R, $-SO_2-$, >C=C<-R $-SO_2-$;
>C=N- : O=C=N- , S=C=N- ;
-C≡N : $-N^+\equiv C^-$;

δ 135.74 (singlet):
Benzol : C-H , C_1 -Br , C_1 -P(O)< ;
Aromatic : Pyridin C_4 , Thiofuran $C_{2,3}$;
>C=C< : =CH- , =CH< -R, -Cl , =CH-N< , **>C=C<**-R $-SO_2-$;
>C=N- : O=C=N- , S=C=N- ;
>C=N- : -C≡N , $-N^+\equiv C^-$;
C ... Hal : $-CF_3$;

δ 131.37 (doublet):
Benzol : C-H , C_1 -Br ;
Aromatic : Pyridin C_4 , Thiofuran $C_{2,3}$;
>C=C< : =CH- , =CH< -R, -Cl , =CH-N< , >C=C<-R $-SO_2-$;
>C=N- : =C-C=N- , O=C=N- , S=C=N- ;
>C=N- : -C≡N ;

C ... Hal : -CF3 ;

δ 121.20 (doublet):
Benzol : C-H , C1 -Br ;
Aromatic : Furan C3 ;
>C=C< : =CH2 , =CH< -R, -Cl , **>C=C<** -R, -N< ;
>C=N- : =C-C=N- , O=C=N- ;
-C≡N : -O-C≡N , -S-C≡N ;
C ... O : CH(-O-)3 ;

δ 119.24 (doublet):
Benzol : C1 -Br , C1 -J ;
Aromatic : Furan C3 ;
>C=C< : =CH2 , **>C=C<** -R, -N< ;
>C=N- : =C-C=N- ;
-C≡N : -O-C≡N , -S-C≡N ;
C ... O : CH(-O-)3 , >C(-O-)2 ;

δ 119.22 (singlet):
Benzol : C1 -Br , C1 -J ;
Aromatic : Furan C3 ;
>C=C< : =CH2 , **>C=C<** -R, -N< ;
>C=N- : =C-C=N- ;
-C≡N : -O-C≡N , -S-C≡N ;
C ... O : CH(-O-)3 , >C(-O-)2 ;

δ 113.35 (doublet):
Benzol : C1 -Br , C1 -J ;
Aromatic : Furan C3 ;
>C=C< : =CH2 , **>C=C**< -R, -O- , >C=C< -R, -N< ;
>C=N- : =C-C=N- ;
-C≡N : -O-C≡N ;
C ... O : >C(-O-)2 ;
C ... Hal : >CH-F , -CCl3 ;

δ 113.11 (singlet):
Benzol : C1 -Br , C1 -J ;
Aromatic : Furan C3 ;
>C=C< : =CH2 , **>C=C**< -R, -O- , >C=C< -R, -N< ;
>C=N- : =C-C=N- ;
-C≡N : -O-C≡N ;
C ... O : >C(-O-)2 ;
C ... Hal : >CH-F , -CCl3 ;

δ 77.93 (singlet):
-C≡C- : ≡C-H , ≡C-S- ;
C ... O : (R3-)C-O- , >CH-O- , -CH2-O- ;
C ... N : R3C-N< , -CH2-NO2 , -CH2-O-N=O ;

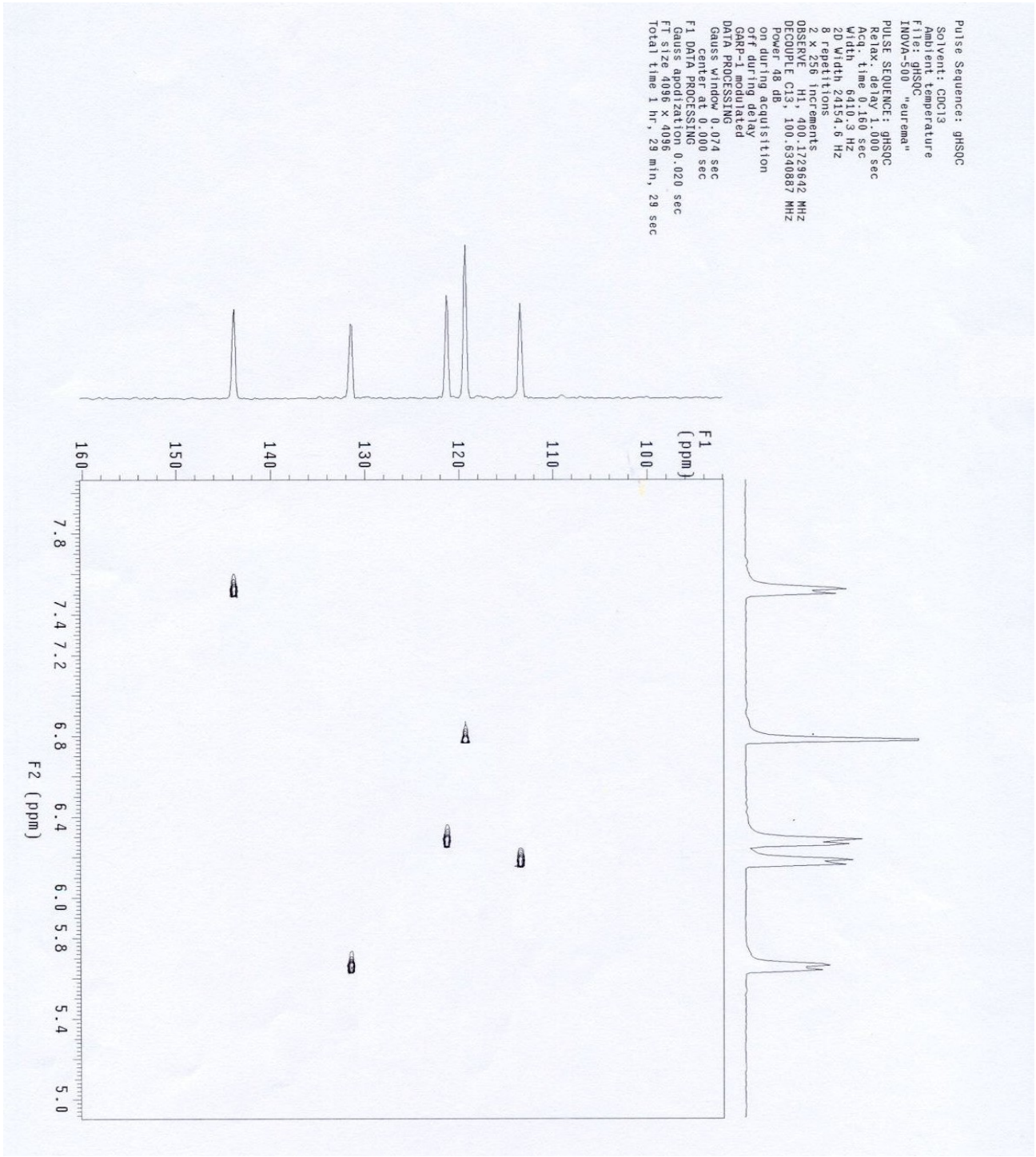
C ... S : R_3C-S- ;
C ... Hal : R_3C-Cl , R_3C-Br ;
C ... P : (R_3C)-P=O ;

δ 61.55 (quadruplet):
-CHn : -CH_2- , -CH< , C_q ;
C ... O : -CH_2-O- , **CH_3-O-** ;
C ... N : R_3C-N< , R_2CH-N< , R-CH_2-N< , -CH< -COO, -N< ;
C ... S : R_2CH-S- , R_3C-SO- , R-CH_2-SO_2- ;
C ... Hal : >CH-Cl , -CH_2-Cl , >CH-Br ;
C ... P : (R_3C-O)$_3$P ;

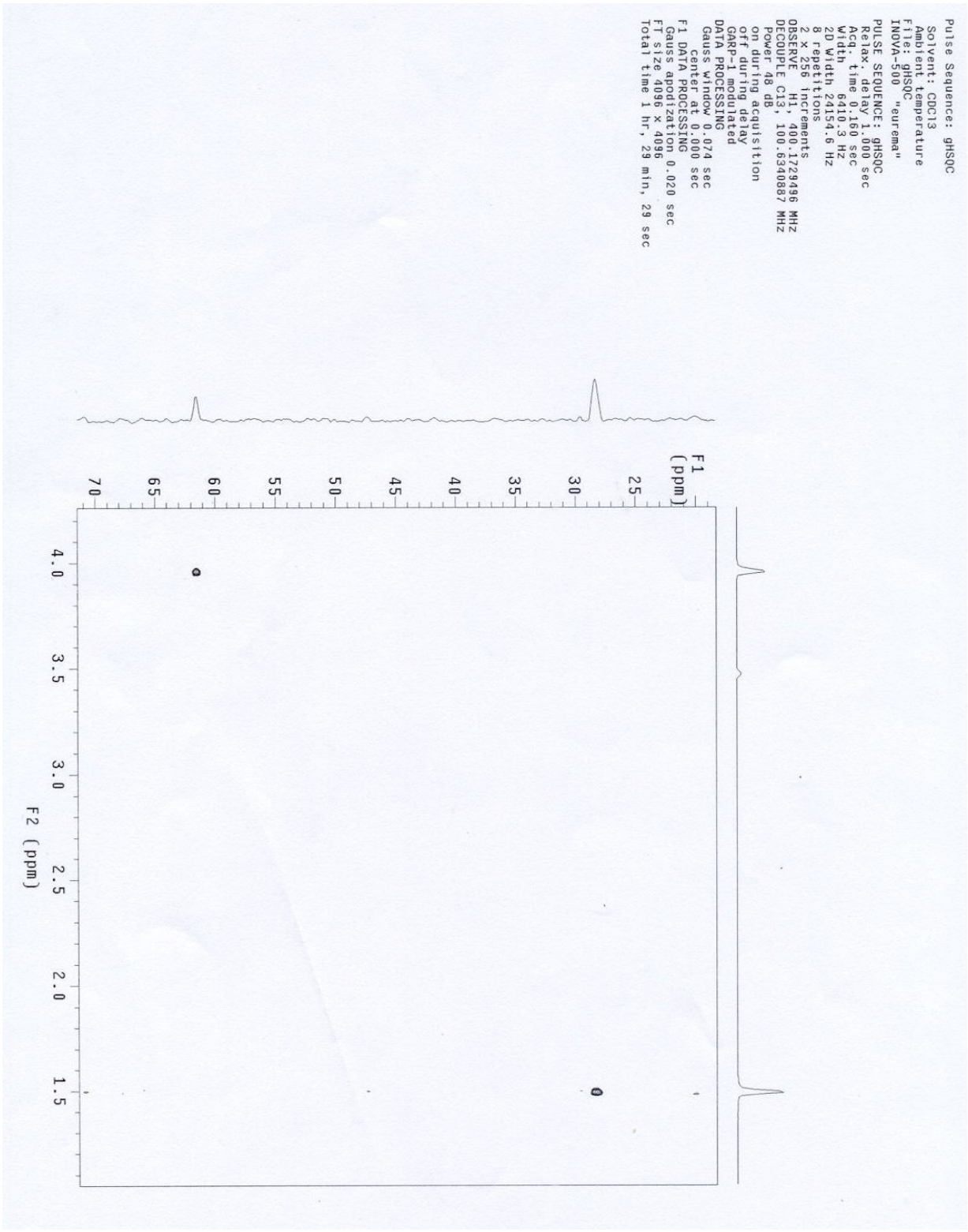
δ 38.32 (quadruplet):
-C≡C- : -C≡C-O- ;
-CHn : -CH_3 , -CH_2- , -CH< ;
C ... O : R-CH_2-C=O , CH_3-C=O ;
C ... N : CH_3-N< ;
C ... S : R-CH_2-S- , CH_3-S- ;
C ... Hal : -CH_2-Br ;
C ... P : -CH_2-P< ;

gHSQC

To associate the ^{13}C – peaks with the correlating ^{1}H - peaks a 2D – gHSQC - NMR experiment was made.



Spectra 11. GHSQC Spectra of Compound 1. (160 – 80 ppm y - Axis). 400 Mhz Solvent : $CDCL_3$



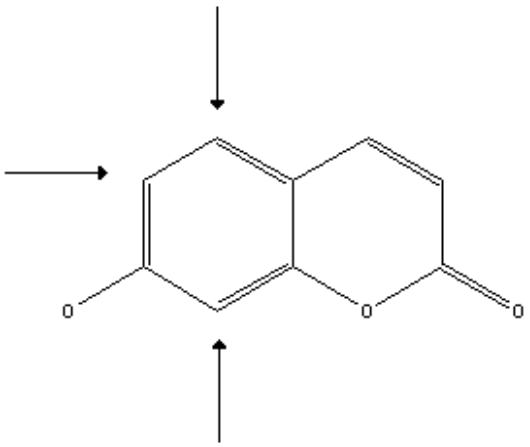
Spectra 12. GHSQC Spectra of Compound 1. (72 – 20 ppm y - Axis). 400 Mhz, Solvent : $CDCL_3$

This enabled the conclusion that the following shifts were
belonging together: δ 38.32 (Q, H: 1.51 (s) 6H), 38.32 (Q, H: 1.51 (s) 6H), 61.55 (Q, H: 3.97 (s), 3H), 119.24 (D, H: 6.81 (s) 1H), 121.20 (D, H: 6.31 (d) 1H), 131.37 (D, H: 5.69 (d) 1H) and 143.77 (D, H: 7.56 (d) 1H). (see spectra 11 & 12)

If the correlating $^{1}H + ^{13}C$ Peaks are coordinated together the following argumentation can be made:

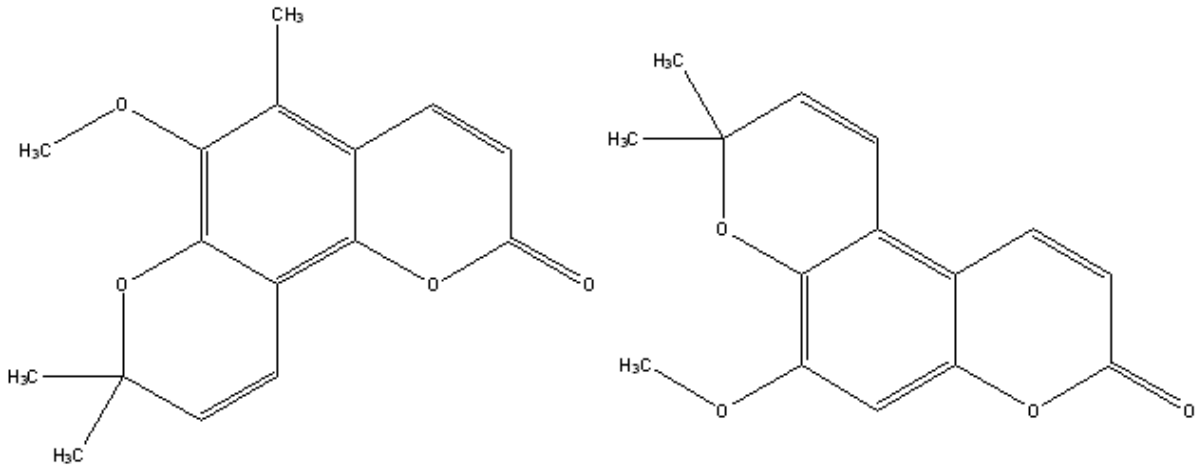
- ^{13}C – NMR peak: δ 38.32 (Q) plus corresponding ^{1}H – NMR peak: δ 1.51 (s) --> **-CH3**
- ^{13}C – NMR peak: δ 61.55 (Q) plus corresponding ^{1}H – NMR peak: δ 3.97 (s) --> **-O- CH3**
- ^{13}C – NMR peak: δ 131.37 (D) plus corresponding ^{1}H – NMR peak: δ 5.69 (d) --> **CH=C-**
- ^{13}C – NMR peak: δ 113.3 (D) plus corresponding ^{1}H – NMR peak: δ 6.23 (d) --> **CH=C-**
- ^{13}C – NMR peak: δ 121.2 (D) plus corresponding ^{1}H – NMR peak: δ 6.31 (d) --> **CH=C-**
- ^{13}C – NMR peak: δ 119.2 (D) plus corresponding ^{1}H – NMR peak: δ 6.81 (s) --> **CH=C-**
- ^{13}C – NMR peak: δ 143.8 (D) plus corresponding ^{1}H – NMR peak: δ 7.56 (d) --> **CH=C-**

Taking the basic biogenetic structures in consideration a logic skeletal structure can be assumed, the oxidation of C7 is precursory.



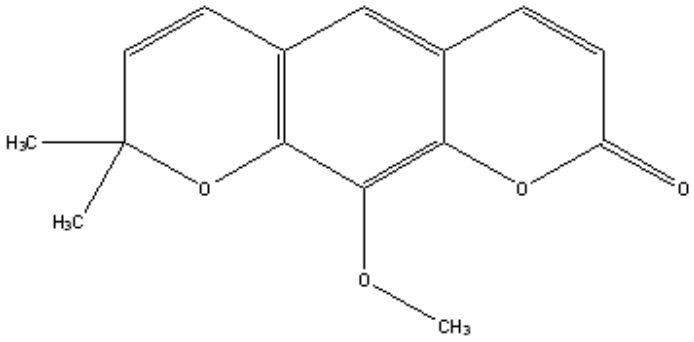
pict. 30; logical skeletal structure and possible positions of oxidation of Cumarin 1

There are two possible structures when the molecule is oxidized in C-6:



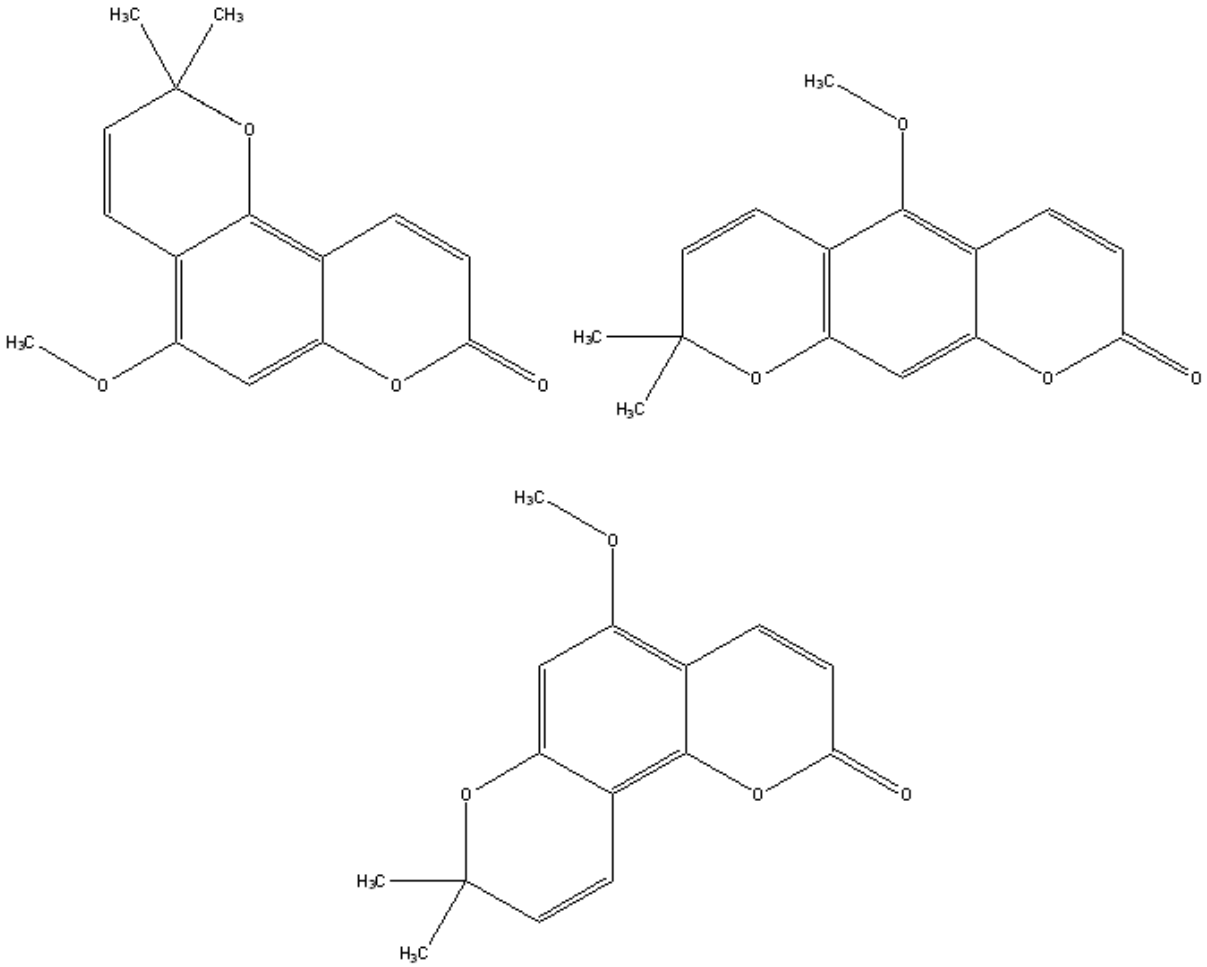
pict. 31; possible structures of Cumarin 1 if C-6 is oxidized

There is another possibility when the molecule is oxidized in C – 8:



pict. 32; possible structure of cumarin 1 if C-8 is oxidized.

There are three possible structures when the molecule is oxidized in C - 5:

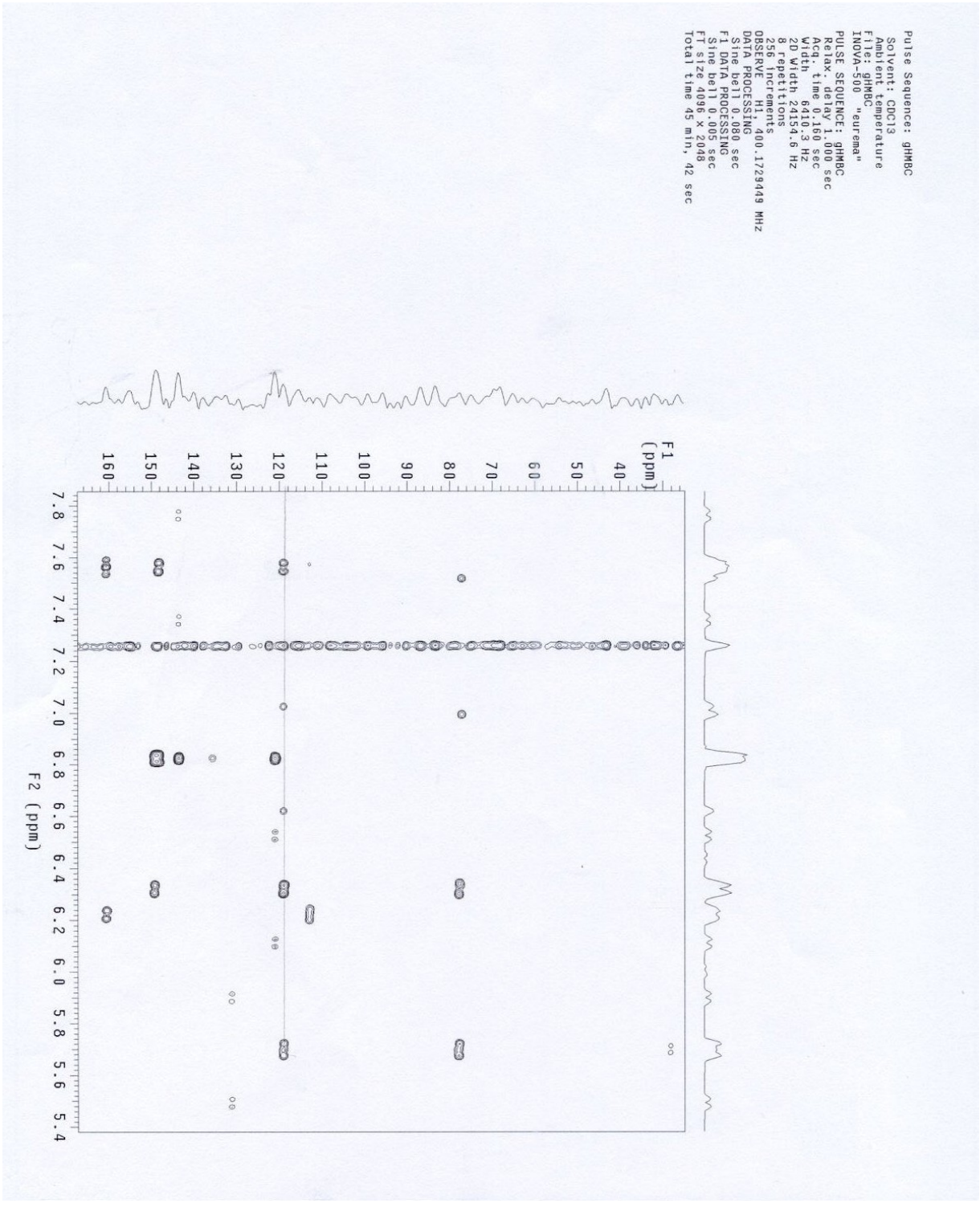


pict. 33; possible structure of Cumarin 1 if C-5 is oxidized.

To determine which of these structures is Cumarin 1, additional gHMBC and gCOSY experiments were made.

gHMBC

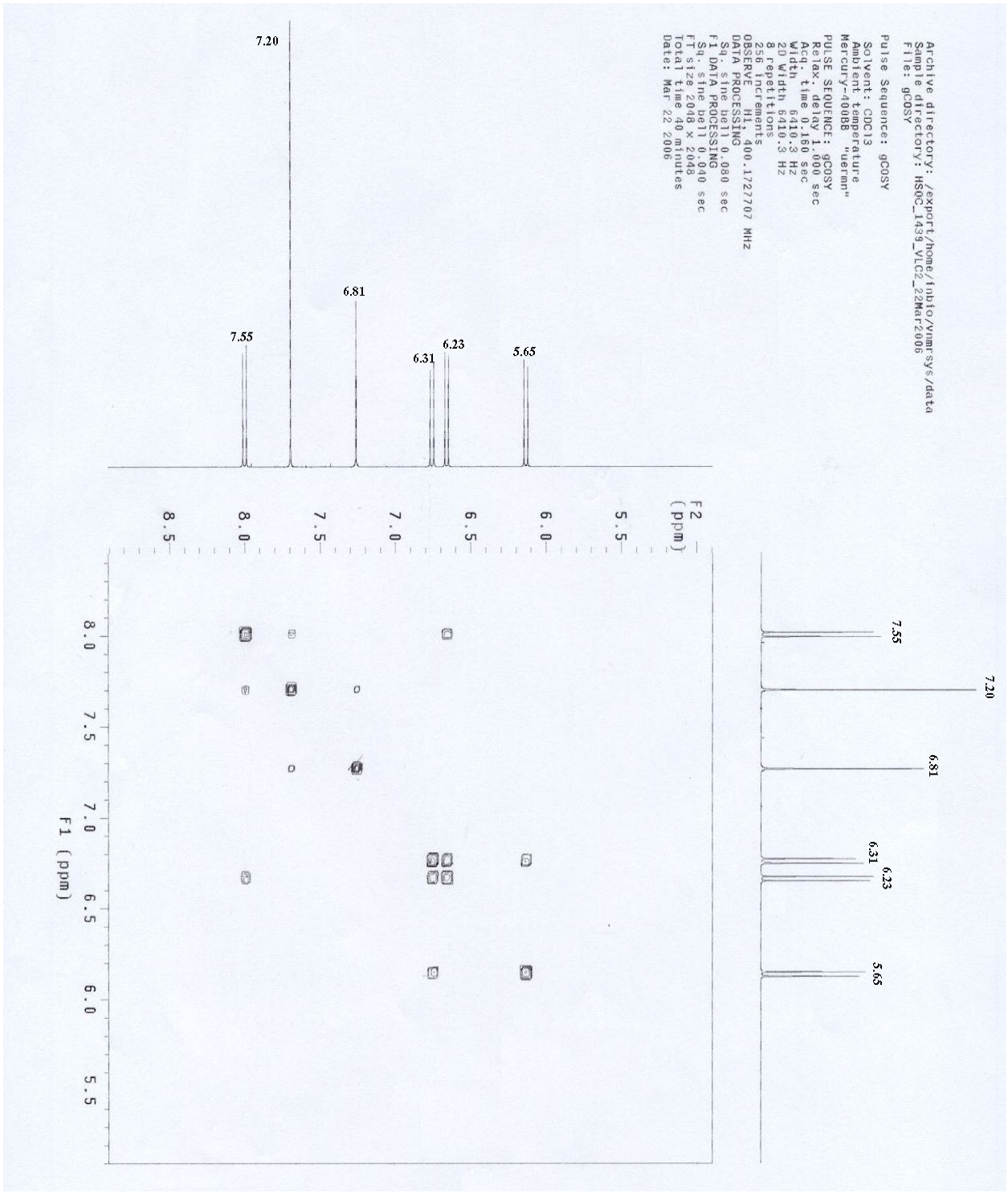
To detect the longer range 2-3 bond couplings a Gradient Heteronuclear Multiple Bond Coherence experiment was made.



Spectra 13. gHMBC Spectra of Compound . 400 MHz, Solvent : $CDCL_3$

gCOSY

This Gradient Selected Correlation Spectroscopy experiment gives correlation between J – coupled signals.

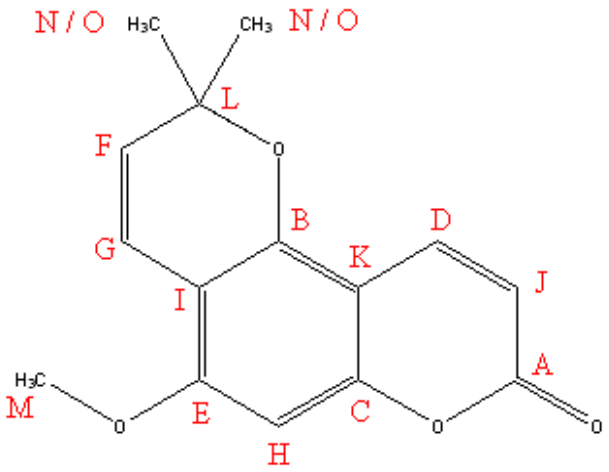


Spectra 14. gCOSY Spectra of Compound 1. 400 Mhz, Solvent : $CDCL_3$.

Position	δ -^{13}C	multiplicity	HSQC / DEPT	multiplicity	gCOSY	gHMBC
A	160.8	S				
B	149.4	S				
C	148.4	S				
D	143.8	D	7.56	d	j	A, C, H/I
E	135.7	S				
F	131.4	D	5.69	d		L, N/O, H, I
G	121.2	D	6.31	d		B, L, H/I
H	119.2	D	6.81	s		B/C, D, G
I	119.2	S				
J	113.3	D	6.23	d	d	A, H/I, K
K	113.1	S				
L	77.9	S				
M	61.6	Q	3.97 (3H)	s		E
N/O	38.3	Q	1.51 (6H)	s		F, L, N/O

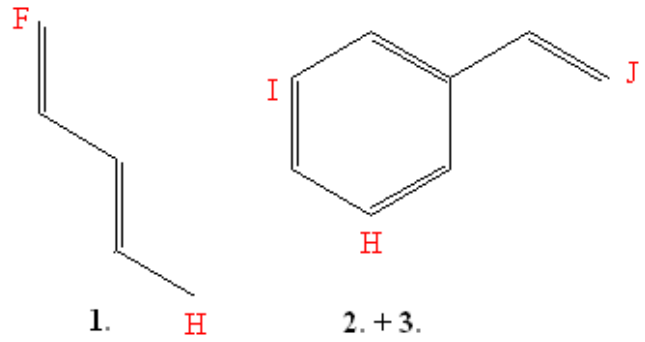
Table 13: ^{1}H + ^{13}C NMR Data (δ in Hz), gHMBC, gHSQC and gCOSY Data for Compound 1. 400 MHz.

Posibilities for the molecule when oxidized in the C – 5 position:



pict. 34; Long distance correlations of possibility 1 for the C – 5 oxidized molecule.

With this molecule three of the longer range bond couplings measured in the gHMBC experiment are not found:

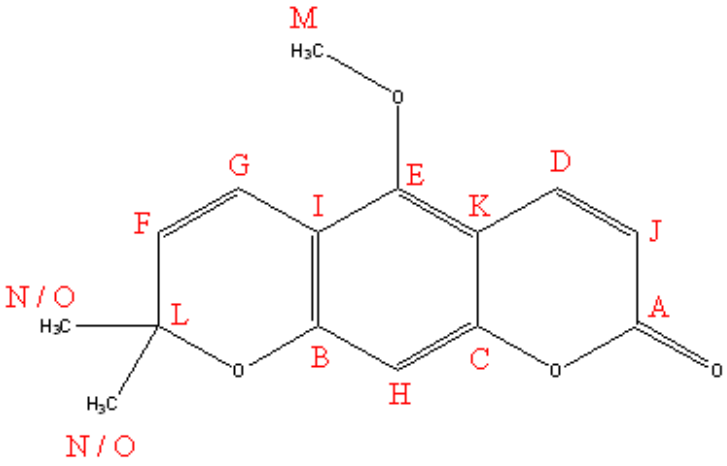


pict. 35; Unmet longer range bond couplings of possibility 1 for the C – 5 oxidized molecule.

- The correlation between F and H is not applicable because the coupling would imply 4 bindings.

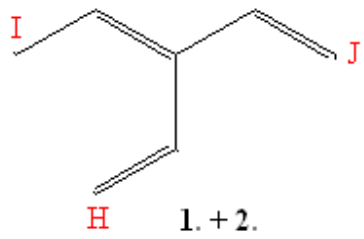
2.+3. The correlation between J and H and acordingly the correlation between J and I is not applicable because the coupling would imply 4 bindings.

Due to this three unmet dependencies, possibility one for a C – 5 oxidized molecule has to be precluded.



pict. 36; Long distance correlations of possibility 2 for the C – 5 oxidized molecule.

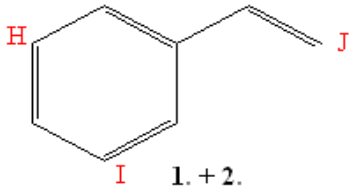
According to the measurements, this possibility has 2 unmet long distance correlations:



pict. 37; Unmet longer range bond couplings of possibility 2 for the C – 5 oxidized molecule.

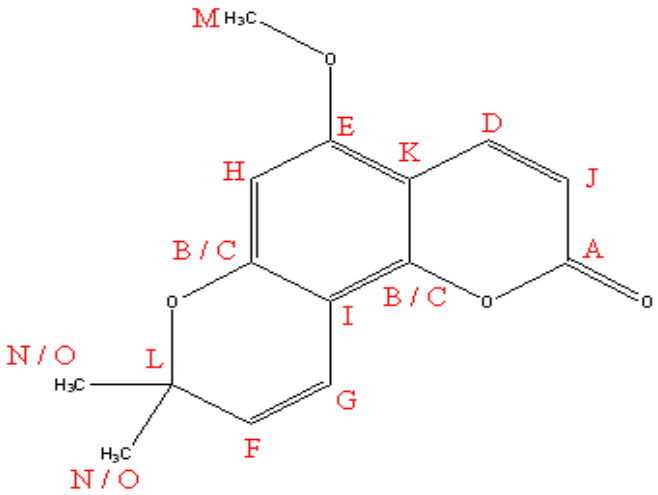
- The correlation between J and H is not applicable because the coupling would imply 4 bindings.
- The correlation between J and I is not applicable because the coupling would imply 4 bindings.

This molecule would have 2 unmet dependencies, so it is abolished.



pict. 38; Long distance correlations of possibility 3 for the C – 5 oxidized molecule.

This possibility has 2 unmet dependencies:

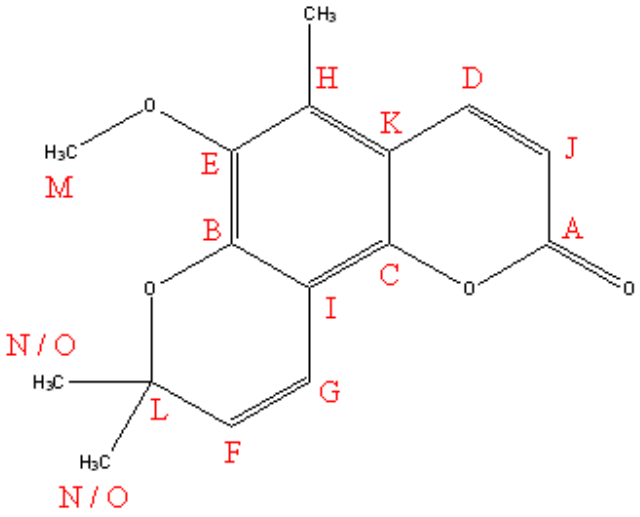


pict. 39; Unmet longer range bond couplings of possibility 3 for the C – 5 oxidized molecule.

- The correlation between J and H is not applicable because the coupling implies 4 bindings.
- The correlation between J and I is not applicable because the coupling implies 4 bindings.

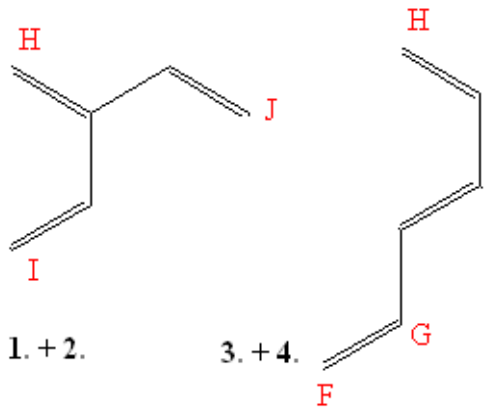
This molecule has 2 unmet dependencies, so it is overruled. None of this three possibilities with an oxidized C – 5 is the tested molecule.

Posibilities for the molecule when oxidized in the C – 6 position:



pict. 40; Long distance correlations of possibility 1 for the C – 6 oxidized molecule.

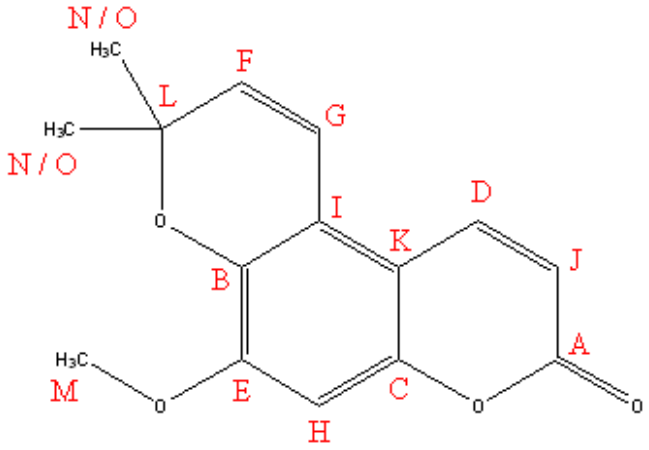
In this case not all of the longer range bond couplings measured in the gHMBC – experiment are complied with:



pict. 41; Unmet longer range bond couplings of possibility 1 for the C – 6 oxidized molecule.

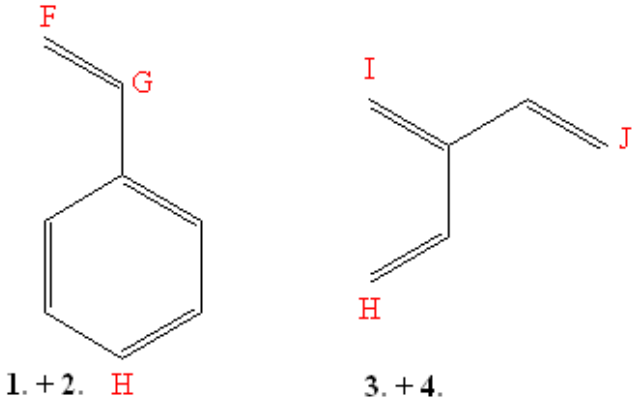
1. The correlation between I and J is not applicable because the coupling implies 4 bindings, but:
2. The correlation between H and J is applicable, because it implies 3 bindings.
3. The correlation between H and G is not applicable because the coupling implies 4 bindings.
4. The correlation between H and F is not applicable because the coupling implies 5 bindings.

This unmet dependencies implement possibility 1 for a C – 6 oxidized molecule can be discarded.



pict. 42; Long distance correlations of possibility 2 for the C – 6 oxidized molecule.

With this molecule 2 of the longer range bond couplings measured in the gHMBC – experiment are unmatched:

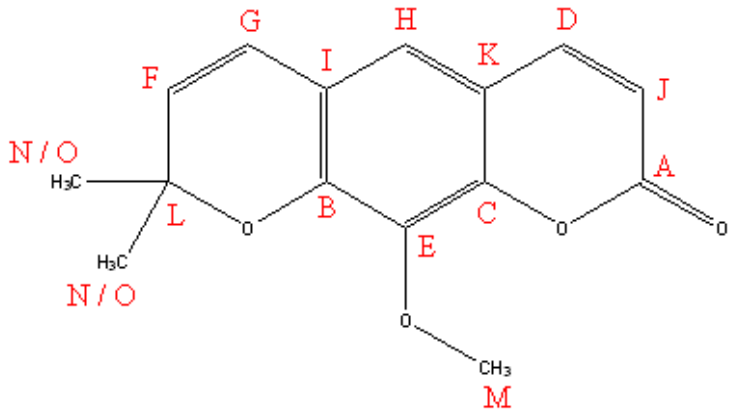


pict. 43; Unmet longer range bond couplings of possibility 2 for the C – 6 oxidized molecule.

1. The correlation between F and H is not applicable because the coupling implies 5 bindings.
2. The correlation between G and H is not applicable because the coupling implies 4 bindings.
3. The correlation between J and H is not applicable because the coupling implies 4 bindings, but:
4. The correlation between J and I is applicable because the coupling implies 3 bindings.

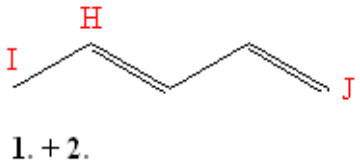
Posibility 2 can be discarded too, so non of the C – 6 oxidized possibilities is the tested molecule.

Possibilities for the molecule when oxidized in the C – 8 position:



pict. 44; Long distance correlations of the possibility with an oxidized C – 8.

This molecule is the only option where all of the long distance correlations are matching the measured correlations:



pict. 45; No unmet longer range bond couplings for the C – 8 oxidized molecule.

1. The correlation between J and I is not applicable because the coupling implies 4 bindings, but:
2. The correlation between J and H is applicable because the coupling implies 3 bindings.

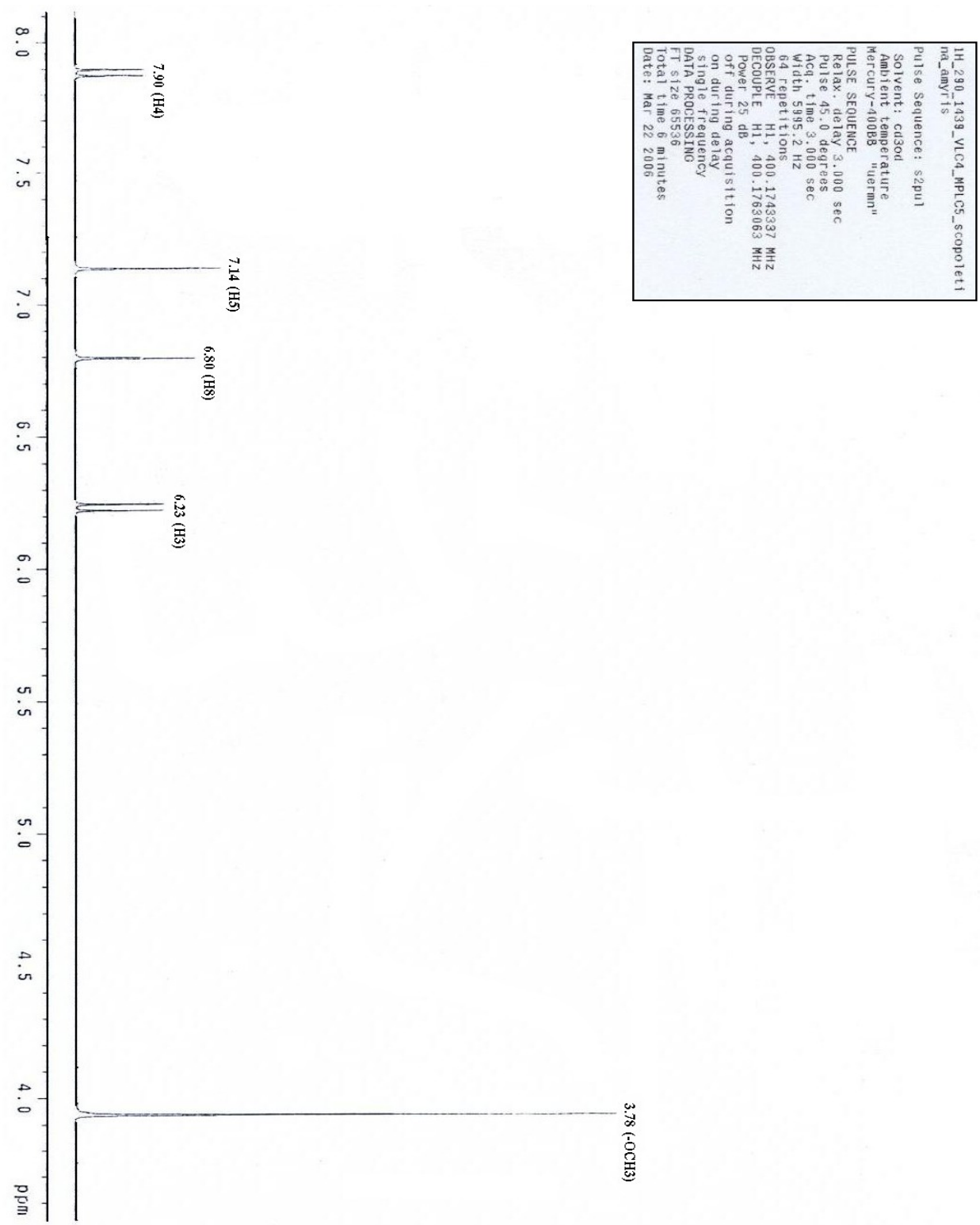
According to the longer range 2-3 bond couplings, the other possible structures that are oxidized in C - 5 and C - 6 can be excluded. Only the molecule oxidized in C – 8 gives the right long range 2 – 3 bond couplings. This Coumarin can be identified as Luvangetin.

Luvangetin
White prisms (MeOH). ^{13}C + ^{1}H NMR (CDCl3, 400 Mhz) δ **38.32** (Q, C5′ – H: 1.51 (s) 6H), **38.32** (Q, C4′ – H: 1.51 (s) 6H), **61.55** (Q, -Ome – H: 3.97 (s), 3H), **77.9** (S, C3′), **113.11** (S, C10), **119.22** (S, C6), **119.24** (D, C5 – H: **6.81** (s) 1H), **121.20** (D, C1′- H: 6.31 (d) 1H), **131.37** (D, C2′ - H: 5.69 (d) 1H), **135.74** (S, C8), **143.77** (D, C4 – H: 7.56 (d) 1H), **148.40** (S, C9), **149.43** (S, C7), **160.80** (S, C2). Synonyms: `10-Methoxy-8,8-dimethyl-2H,8H-benzo[1,2-b:5,4-b']dipyran-2-one.`

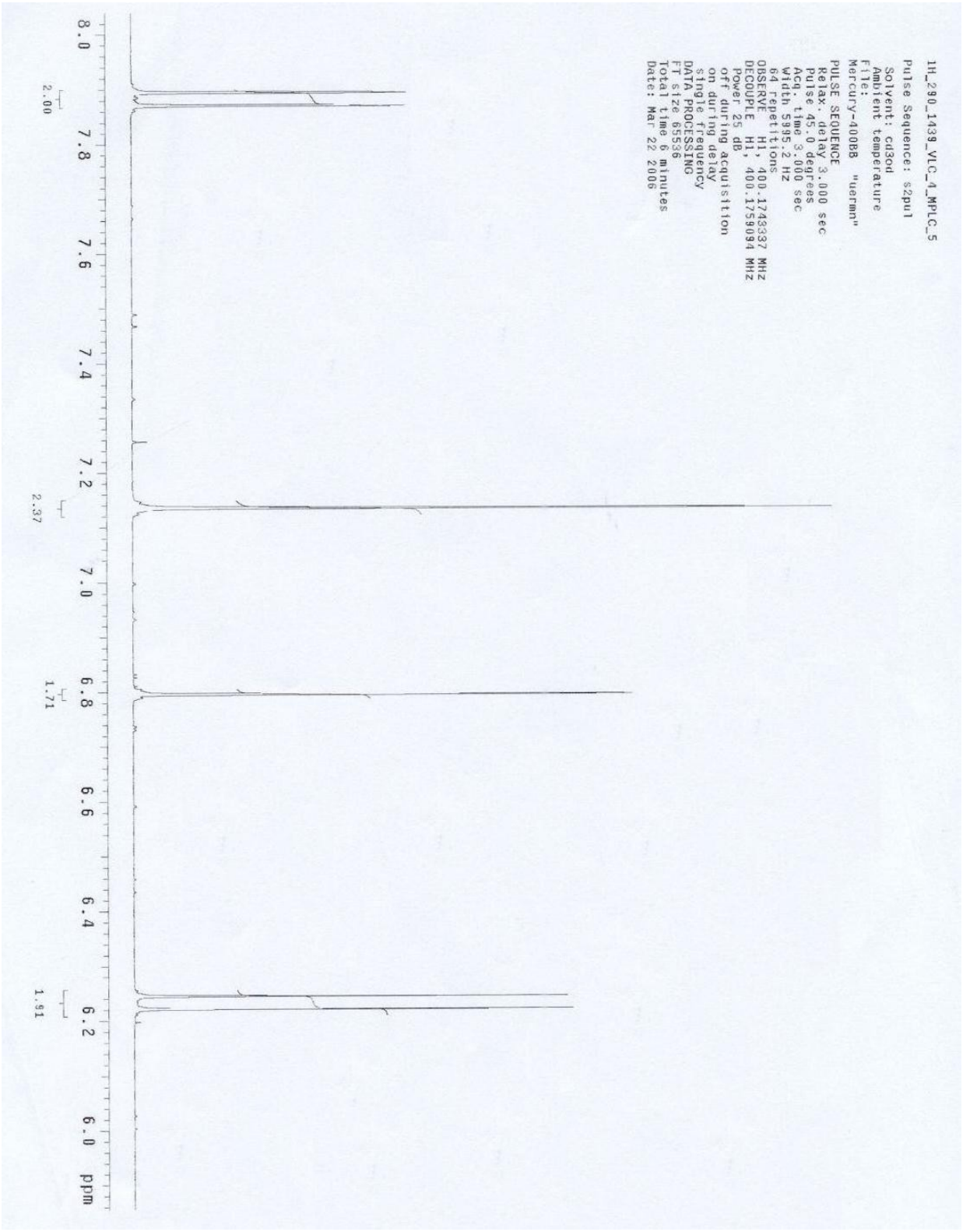
Reported bioacticity of Luvangetin

The pyranocoumarin Luvangetin, which also has been isolated from the seeds of *Aegle marmelos Correa*, showed significant protection against pylorus-ligated and aspirin-induced gastric ulcers in rats and cold restraint stress-induced gastric ulcers in rats and guinea pigs. In difference to bergenin and norbergenin, which have been also a part of the study, luvangetin (1-10 micrograms/ml) did not produce any stimulatory effect on prostaglandins release by human colonic mucosal incubates and therefore some other mucosal defensive factors may be involved. [30]

2.2.2.b Compound 2 (Scopoletin)



Spectra 15. ^{1}H – NMR Signals of Compound 2. 400 Mhz, Solvent : MeOD

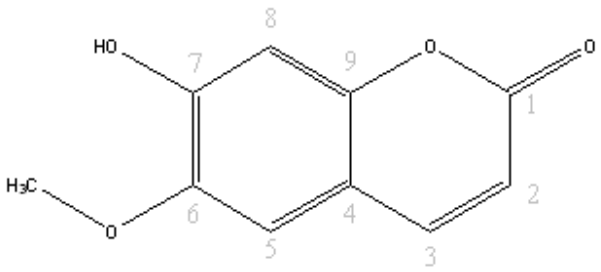


Spectra 16. ^{1}H – NMR Signals of Compound 2. (8.0 – 6.0 ppm, invluding coupling constants.) 400 Mhz, Solvent : MeOD

This compound crystallized in yellow to beige needles. A ^{1}H – NMR measurement was made and resulted in 5 peaks.
The chemical shifts of this substance were compared with literature spectras [31]
and identified as Scopoletin.

Position	δ - H	multiplication
H3	6.23	d
H4	7.90	d
H5	7.14	s
H8	6.80	s
-OCH3	3.78	s

Tab. 14; NMR Data and correlating positions of Compound 2



pict. 46; Correlating positions to NMR Data of Compound 2

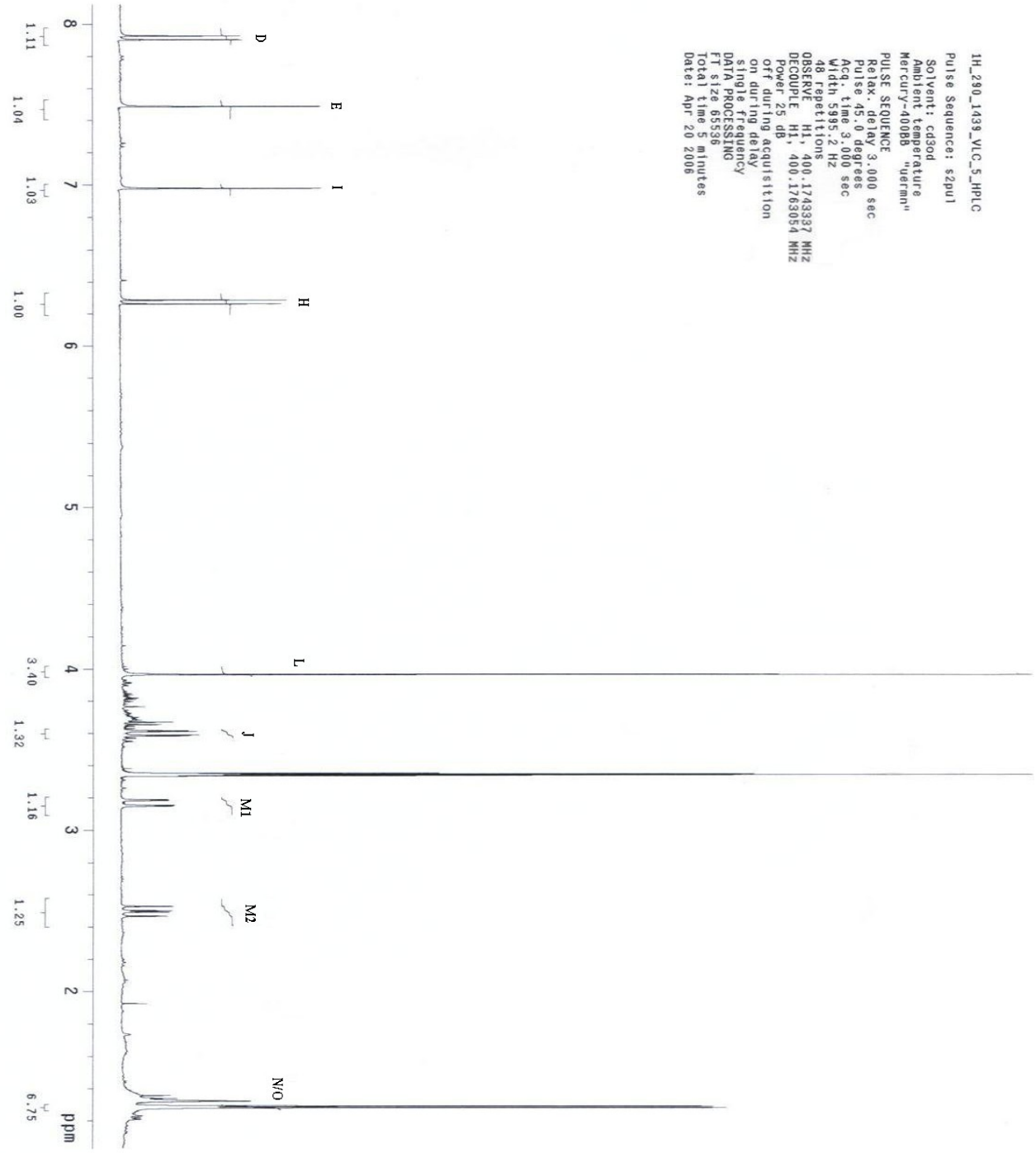
Scopoletin
Yellow to beige crystalline needles (MeOH-H20). ^{1}H-NMR (CDCl3, 400 Mhz) δ **3.78**, (s, 3H, C6-OCH3), **6.23** (d, J=9.52 Hz, 1H, H3), **6.80** (s, 1H, H8), **7.14** (s, 1H, H5), **7.90** (d, J=9.55 Hz, 1H, H4)
Synonyms: 7-Hydroxy-6-methoxycoumarin, gelseminic acid, chrysatropic acid, esculetin-6-methyl ether. MW: 192.17112, Formula: $C_{10}H_8O_4$, mp (°C): 200 - 207;

2.2.2.c Compound 3

Compound 3 consisted of white to beige crystals.

^{1}H – NMR Signals

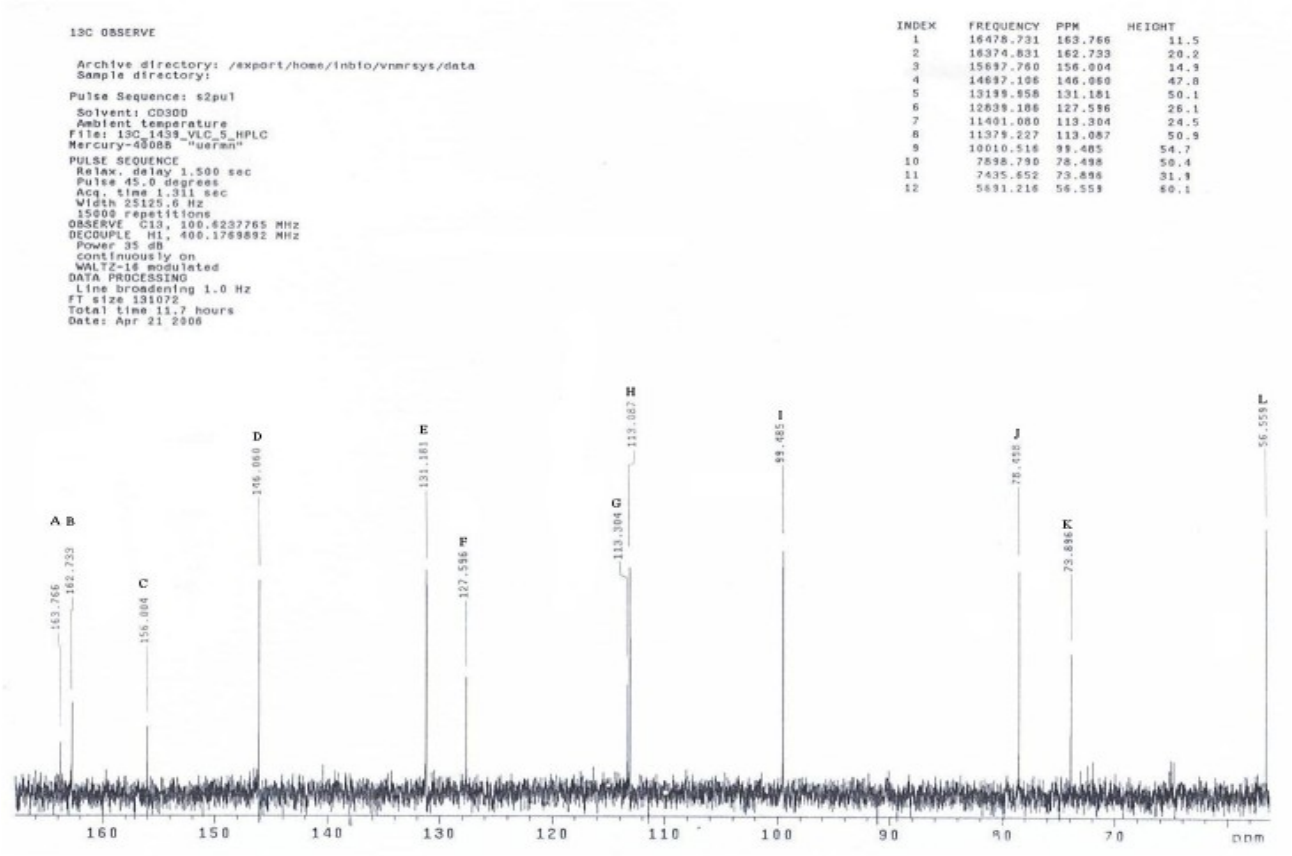
To identify the aromaticity, a ^{1}H – NMR measurement was made giving peaks with the following chemical shifts: 7.89 (d), 7.48 (s), 6.98 (s), 6.25 (d), 3.98 (s), 3.60 (d), 2.50 (t), 2.18 (d), 1.31



Spectra 17. ^{1}H – NMR Signals of Compound 3. (8.0 – 6.0 ppm, invluding coupling constants.) 400 Mhz, Solvent: $CDCL_3$.

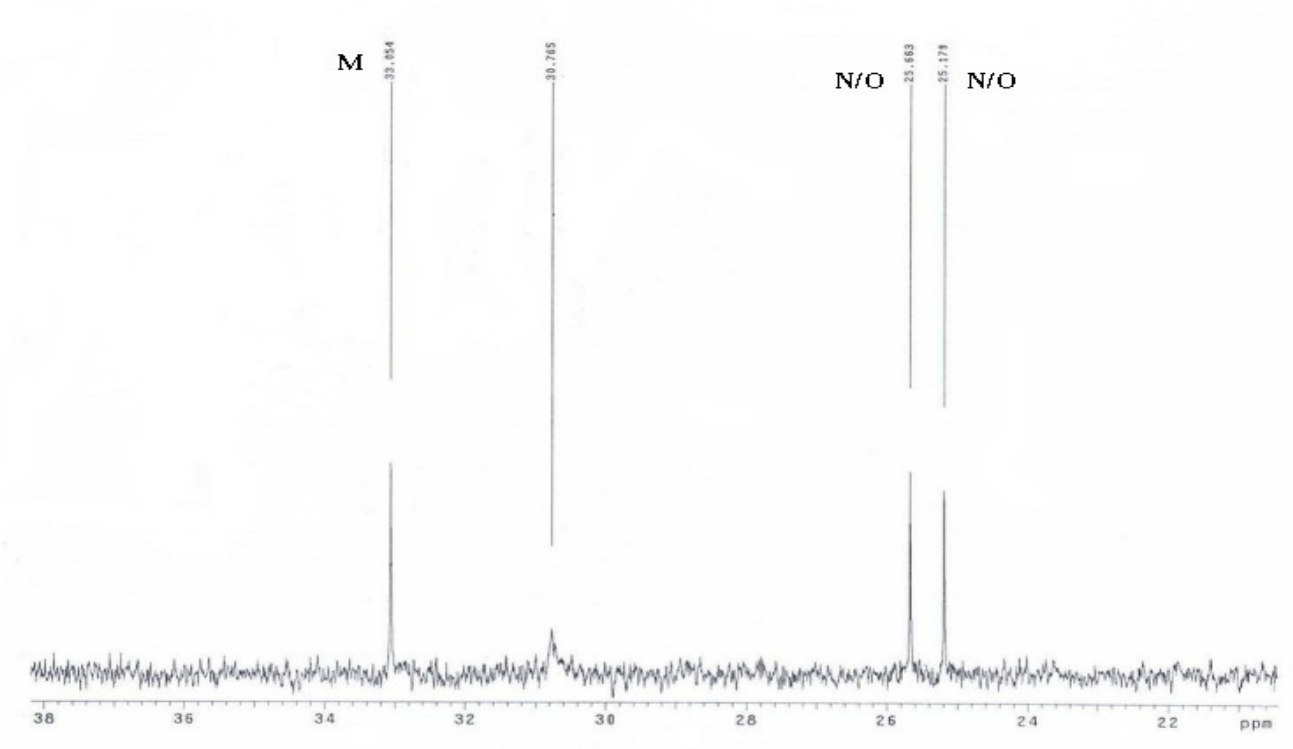
According to [28] the peak with the following chemical shifts could indicate following groups:

δ 1.31 (singlet) : H3C-C-Hal ; H3C-C=C ; H3C-CC ; H3C-CO ; C-CH2-Alkyl ; C-CH2-CO ; CH-Alkyl ; Alkyl-OH ; Alkyl-SH ; Alkyl-NH2 ; Alkyl2-NH
δ 2.50 (triplet) : H3C-Aryl,-Heteroaryl ; H3C-CO ; H3C-S- ; H3C-SO2- ; H3C-N ; C-CH2-CO ; C-CH2-N ; C=C-CH2-C=C ; N-CH2-CO- ; C-CH-N ; Alkyl-OH ; Alkyl-SH ; Alkyl-NH2 ; Alkyl2-NH;
δ 3.18 (doublet) : H3C-SO2- ; H3C-N ; H3C-O-Alkyl ; C-CH2-N ;
δ 3.60 (doublet) : H3C-SO2- ; H3C-N ; H3C-O-Alkyl ; H3C-O-Aryl ; -O-CO- ; C-CH2-O- ; C-CH2-S ; C-CH2-N ; N-CH2-CO- ; C-CH-O- ; C-CH-N ; CO-CH-C=C ; CH-Aryl,-NR-,-O- ; -CCH, Alkine ; Alkyl-OH ; Aryl-OH ; Alkyl-SH ; Aryl-SH Aryl-NH2 ; Aryl2-NH ;
δ 3.98 (singlet) : H3C-O-Aryl ; -O-CO- ; C-CH2-O- ; C-CH2-S ; N-CH2-CO- ; C-CH-O- ; C-CH-N ; CO-CH-C=C ; CH-Aryl,-NR-,-O- ; -CCH Alkine ; Alkyl-OH ; Aryl-OH ; Alkyl-SH ; Aryl-SH ; Aryl-NH2, Aryl2-NH ;
δ 6.25 (doublet) : CH-Aryl,-NR-,-O- ; CH=C- ; Ar-H ; Aryl-OH ; R-CO-NH- ;
δ 6.98 (singlet) : CH=C- ; Ar-H ; Aryl-OH ; R-CO-NH- ;
δ 7.48 (singlet) : CH=C- ; Ar-H ; Aryl-OH ; R-CO-NH- ;
δ 7.98 (doublet) : Ar-H ; Aryl-OH ; R-CO-NH- ;



^{13}C – NMR Signals

Spectra 18;^{13}C – NMR signals of Compound 3. (165 – 65 ppm) 400 Mhz, Solvent : MeOD



Spectra 19; ^{13}C – NMR signals of Compound 3. (28 – 21 ppm) 400 Mhz, Solvent : MeOD

In order to determine the number of C – Atoms a ^{13}C – NMR measurement was made. This resulted in 15 peaks with six singlets, five doublets, one triplet and three quadruplets for C – Atoms: δ 163,77 (singlet), δ 162.73 (singlet), δ 156.00 (singlet), δ 146.06 (doublet), δ 131.18 (doublet), δ 127.60 (singlet), δ 113.30 (singlet), δ 113.00 (doublet), δ 99.48 (doublet), δ 78.50 (doublet), δ 73.89 (singlet), δ 56.55 (quadruplet), δ 33.05 (triplet), δ 25.66 (quadruplet), δ 25.18 (quadruplet**).**

According to [29] ^{13}C – NMR signals with the following chemical shifts can be alluded to the following groups:

δ 163,77 (singlet)
C ... >C=O : =C-COOR **;** $(-O-)_2C=O$ **;**
X ... >C=O : $-N<(C=O)_2$ **;** R-, >N- >C=O **;** $(>N-)_2C=O$ **;** R-, J- >C=O **;**
benzole : C_1 -F **;** C_1 -O- **;**
>C=N- : >C=N- **;** >C=NH **;** >C=N-OH **;**

δ 162.73 (singlet)
C ... >C=O : =C-COOR **;** $(-O-)_2C=O$ **;**
X ... >C=O : $-N<(C=O)_2$ **;** R-, >N- >C=O **;** $(>N-)_2C=O$ **;** R-, J- >C=O **;**
benzole : C_1 -F **;** C_1 -O- **;**
>C=N- : >C=N- **;** >C=NH **;** >C=N-OH **;**

δ 156.00 (singlet)

C ... >C=O : (-O-)$_2$C=O ;
X ... >C=O : R-, >N- >C=O ; (>N-)$_2$C=O ; >N-, -O- >C=O ; R-, J- >C=O ;
benzole : C$_1$ -F ; C$_1$ -O- ; C$_1$ -N< ;
aromatic: Pyridin C$_2$;
>C=C< : =CH< -R, -O- ;
>C=N- : >C=N- ; >C=NH ; >C=N-OH ;

δ 146.06 (doublet)
benzol : C$_1$ -C ; C$_1$ -F ; C$_1$ -N< ; C$_1$ -S- ;
aromatic : Pyridin C$_2$; Furan C$_2$;
>C=C< : =C< ; =CH- ; =CH< -R, -O- ; =CH-O- ; =C< -R, -S- ;
>C=N- : >C=N- ;
-C≡N : -N^+≡C^- ;

δ 131.18 (doublet)
benzole : C-H ; C$_1$ -C ; C$_1$ -Cl ; C$_1$ -S- ; C$_1$ -P< ; C$_1$ -P(O)< ;
aromatic : Pyridin C$_3$; Thiofuran C$_{2,3}$;
>C=C< : =C< ; =CH- ; =C< -R, -S- ; =CH-N< ; =C< -R, -SO$_2$- ; >C=C<-R -SO$_2$- ;
>C=N- : O=C=N- ; S=C=N- ;
-C≡N : -N^+≡C^-

δ 127.60 (singlet)
benzole : C-H ; C$_1$ -Br ; C$_1$ -P(O)< ;
aromatic : Pyridin C$_3$; Thiofuran C$_{2,3}$;
>C=C< : =CH- ; =CH$_2$; =CH< -R, -Cl ; =CH-N< ; =C< -R, -SO$_2$- ; >C=C<-R -SO$_2$- ;
>C=N- : O=C=N- ; S=C=N- ;
-C≡N : -C≡N ; -N^+≡C^- ;
C ... Hal : -CF$_3$;

δ 113.30 (singlet)
benzole : C-H ; C$_1$ -Br ;
aromatic : Pyridin C$_4$; Furan C$_3$;
>C=C< : =CH- ; =CH$_2$; =CH< -R, -Cl ;
>C=N- : =C-C=N- ; O=C=N- ;
-C≡N : -C≡N ; -O-C≡N ; -S-C≡N ;
C ... O : CH(-O-)$_3$;
C ... Hal : -CF$_3$;

δ 113.00 (doublet)
benzole : C-H ; C$_1$ -Br ;
aromatic : Pyridin C$_4$; Furan C$_3$;
>C=C< : =CH- ; =CH$_2$; =CH< -R, -Cl ;
>C=N- : =C-C=N- ; O=C=N- ;
-C≡N : -C≡N ; -O-C≡N ; -S-C≡N ;
C ... O : CH(-O-)$_3$;
C ... Hal : -CF3 ;

δ 99.48 (doublet)
benzole : C_1 -J ;
>C=C< : >C=C< -R, -O- ; >C=C< -R, -N< ; H_2C=C< -R, -N< ;
C ... O : >C(-O-)$_2$; -CH(-O-)$_2$;
C ... Hal : >CH-F ; -CCl_3 ;

δ 78.50 (doublet)
benzole : C_1 -J ;
>C=C< : =C=C< ; =C=CH_2 ;
-C≡C- : ≡C-C ; ≡C-H ; ≡C-S- ;
C ... O : (R_3-)C-O- ; >CH-O- ;
C ... N : -CH_2-NO_2 ;
C ... Hal : -CH_2-F ; R_3C-Cl ;

δ 73.89 (singlet)
-C≡C- : ≡C-C ; ≡C-H ; ≡C-S- ;
C ... O : (R_3-)C-O- ; >CH-O- ;
C ... N : -CH_2-NO_2 ; -CH_2-O-N=O ;
C ... Hal : -CH_2-F ; R_3C-Cl ;
C ... P : (R_3C)-P=O ;

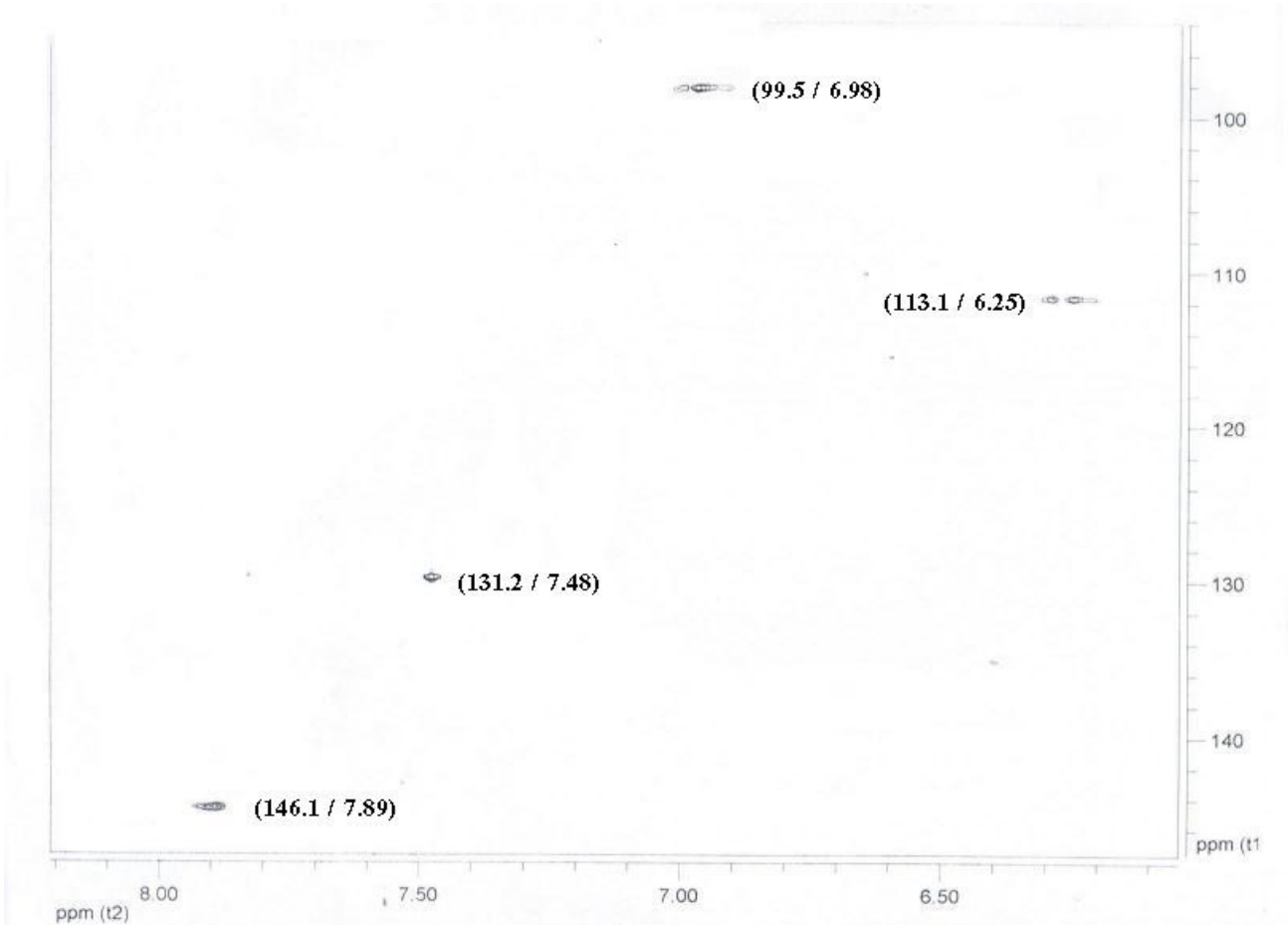
δ 56.55 (quadruplet)
-CHn : -CH_2- ; -CH< ; C_q ;
C ... O : >CH-O- ; -CH_2-O- ; CH_3-O- ;
C ... N : R_3C-N< ; R_2CH-N< ; R-CH_2-N< ; -CH_2-O-N=O ; -CH< -COO, -N< ;
C ... S : R_3C-S- ; R_2CH-S- ;
C ... Hal : R_3C-Br ;
C ... P : (R_3C-O)$_3$P ;

δ 33.05 (triplet)
-C≡C- : -C≡C-O- ;
-CHn : -CH_2- ; -CH< ;
C ... O : R-$_2$CH-C=O ; R-CH_2-C=O ;
C ... N : CH_3-N< ; -CH_2-N< -N=O ;
C ... S : R_2CH-S- ; R-CH_2-S- ;
C ... Hal : -CH_2-Br ; R_3C-J ;
C ... P : -CH_2-P< ;

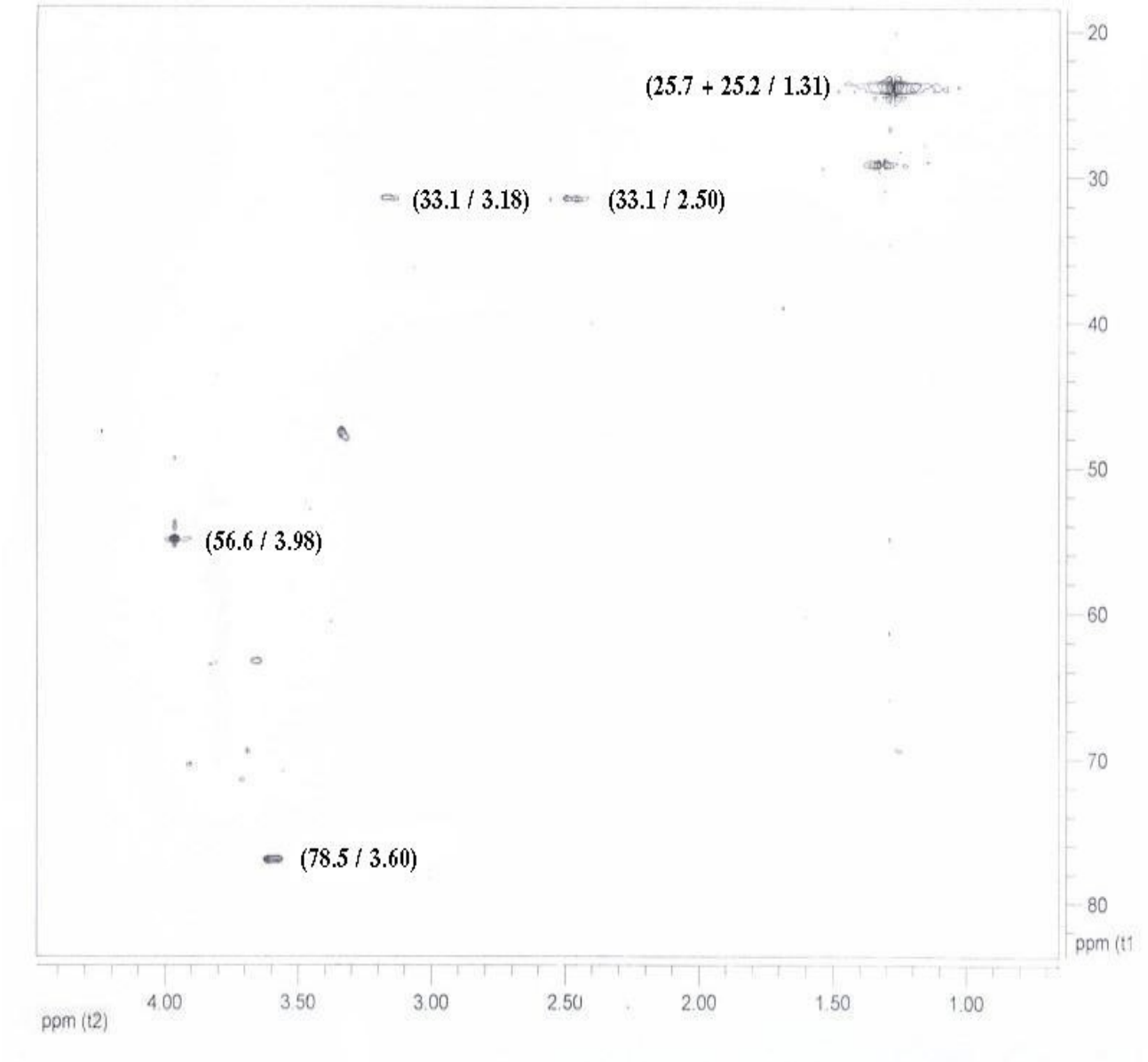
δ 25.66 (quadruplet)
-C≡C- : -C≡C-O- ;
-CHn : -CH_3 ; -CH_2- ; -CH< ;
C ... O : R-CH_2-C=O ; CH_3-C=O ;
C ... S : R-CH_2-S- ; CH_3-S- ;
C ... Hal : -CH_2-Br ; >CH-J ;
C ... P : -CH_2-P< ; R_3C-P(O) (OR)$_2$;
δ 25.18 (quadruplet)
-C≡C- : -C≡C-O- ;
-CHn : -CH_3 ; -CH_2- ; -CH< ;

C ... O : R-CH_2-C=O **;** CH_3-C=O **;**
C ... S : R-CH_2-S- **;** CH_3-S- **;**
C ... Hal : -CH_2-Br **;** >CH-J **;**
C ... P : -CH2-P< ; R3C-P(O) (OR)2 ;

gHSQC



Spectra 20. GHSQC Spectra of Compound 3. (150 – 100 ppm y - Axis). 400 Mhz Solvent : MeOD



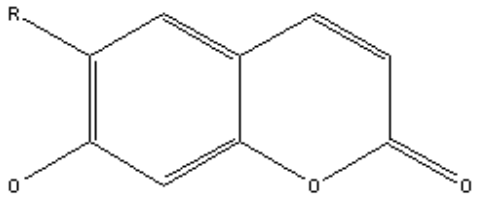
Spectra 21. GHSQC Spectra of Compound 3. (80 – 20 ppm y - Axis). 400 Mhz Solvent : MeOD

An additional gHSQC experiment was made in order to coordinate this two experiments.
The experiment allows the statement that the following ^{1}H – peaks are linked with the following ^{13}C – peaks: 146.06 (D, 7.89 (d)), 131.18 (D, 7.48 (s)), 113.00 (D, 6.25 (d)), 99.48 (D, 6.98 (s)), 78.50 (D, 3.60 (d)), 56.55 (Q, 3.98 (s)), 33.05 (T, 2,50 (t) / 3.18 (d)), 25.66 (Q, 1.31 (t)), 25.18 (Q, 1.31 (t)).

If the correlating $^{1}H + ^{13}C$ Peaks are coordinated together the following argumentation can be made:

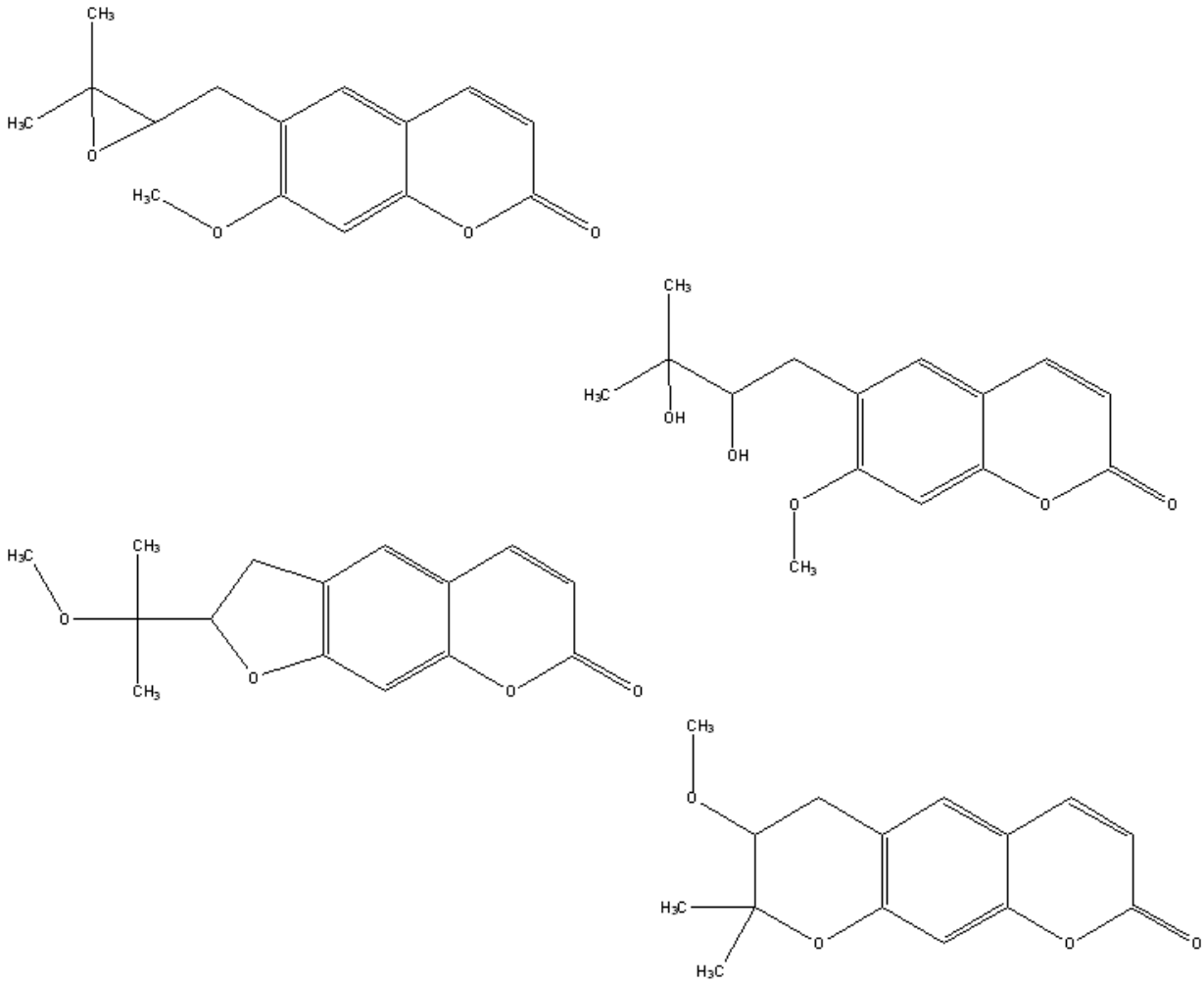
- ^{13}C – NMR peak: δ 25.66 / 25.18 (Q / Q) plus corresponding ^{1}H – NMR peak: δ 1.31 (s) --> **-CH3**
- ^{13}C – NMR peak: δ 33.05 (T) plus corresponding ^{1}H – NMR peak: δ 2.50 (t) , 3.18 (d)--> **-CH2-**
- ^{13}C – NMR peak: δ 78.50 (D) plus corresponding ^{1}H – NMR peak: δ 3.60 (d) --> **>CH-O-**
- ^{13}C – NMR peak: δ 56.55 (Q) plus corresponding ^{1}H – NMR peak: δ 3.98 (s) --> **CH3-O-**
- ^{13}C – NMR peak: δ 113.0 (D) plus corresponding ^{1}H – NMR peak: δ 6.25 (d) --> **CH=C-**
- ^{13}C – NMR peak: δ 99.48 (D) plus corresponding ^{1}H – NMR peak: δ 6.98 (s) --> **CH=C-**
- ^{13}C – NMR peak: δ 131.2 (D) plus corresponding ^{1}H – NMR peak: δ 7.48 (s) --> **CH=C-**
- ^{13}C – NMR peak: δ 146.1 (D) plus corresponding ^{1}H – NMR peak: δ 7.98 (d) --> **=CH-**

According to the biogenetic scheme, starting with of umbeliferone [27] this molecule seems to be prenilated in position C-6; there are subsequent matching products with this spectra:



pict. 47; Skeletal structure of Cumarin 3

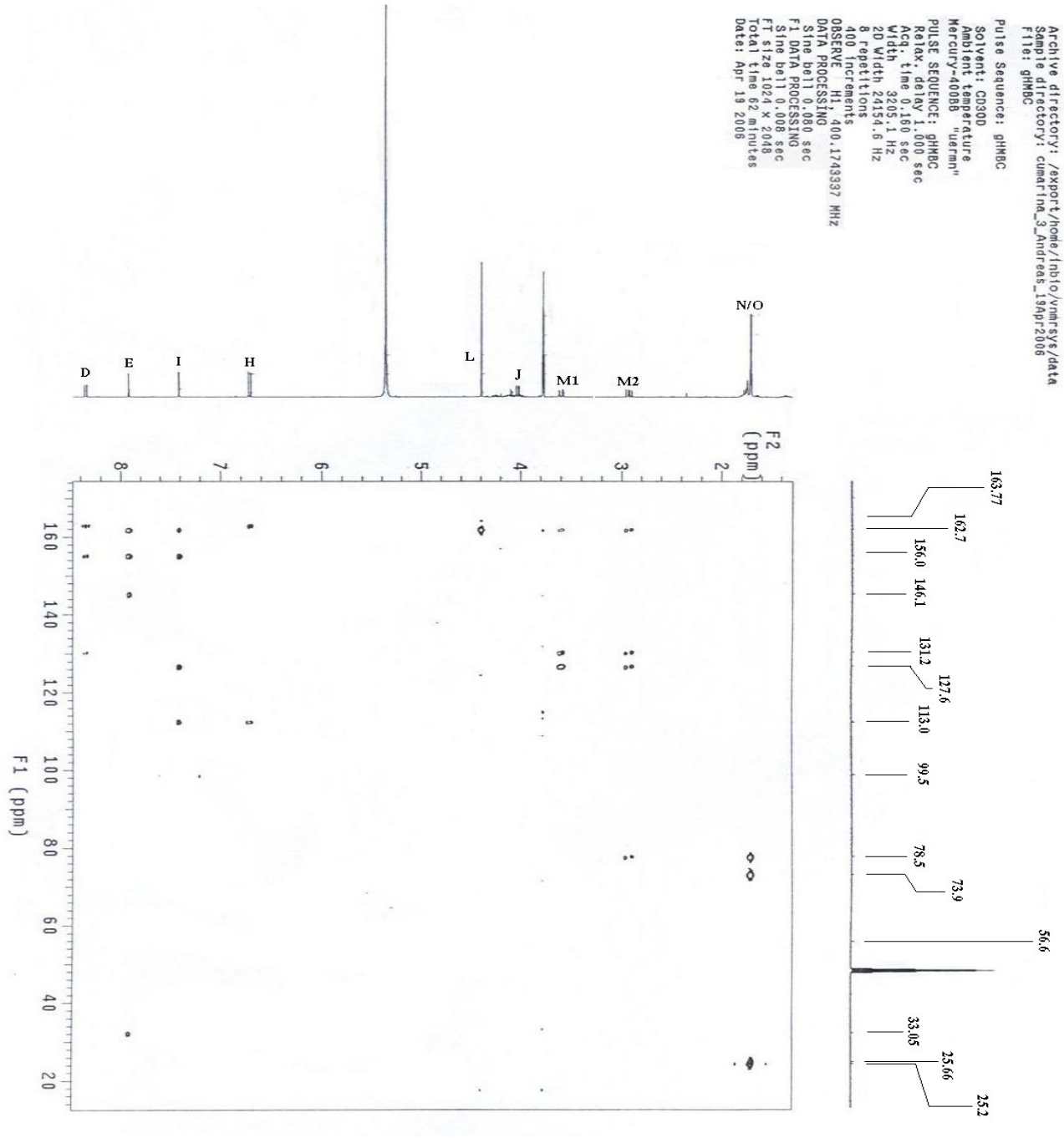
If this skeletal structure is combined with the other peaks of the ^{1}H, ^{13}C and the gHSQC experiment, four posibilities arise:



pict. 48; Possible structures of Cumarin 3 after $^{1}H + ^{13}C$ and gHSQC measurement.

To exclude the other possibilities an additional gHMBC experiment was made.

gHMBC



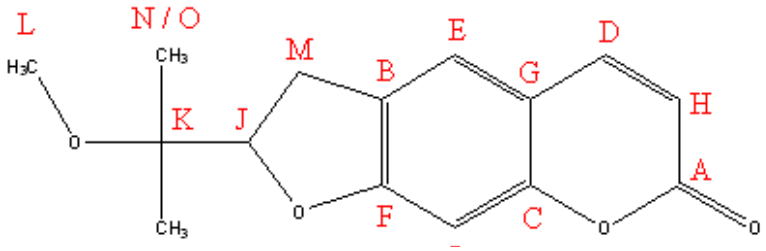
Spectra 22. gHMBC spectra of Compound . 400 MHz, Solvent : MeOD

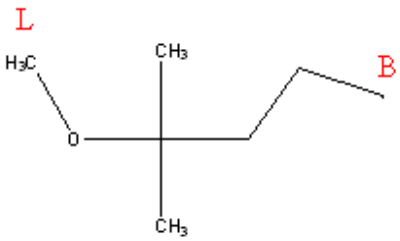
Position	δ -^{13}C	multiplicity	Phase sensitive HSQC	gHMBC
A	163.77	S		D, H
B	162.73	S		E, I, L M
C	156.00	S		D, E, I
D	146.06	D	7.89 (d)	A, C, E
E	131.18	D	7.48 (s)	B, C, D, J, M
F	127.60	S		I, J, M
G	113.30	S		
H	113.00	D	6.25 (d)	A, E
I	99.48	D	6.98 (s)	B, C, F
J	78.50	D	3.60 (d)	N, O
K	73.89	S		N, O
L	56.55	Q	3.98 (s)	**B**
M	33.05	T	2.50 (t); 3.18 (d)	B, E, F, J
N / O	25.66	Q	1.31	K, N, O, J
N / O	25.18	Q	1.31	K, N, O, J

Table 15: ^{1}H + ^{13}C NMR Data (δ in Hz), gHMBC, gHSQC Data for Compound 3. 400 Mhz.

The first of the four possible structures posesses a dihydrofuran partial structure:

pict. 49; Long distance correlations of the possibility with a dihydrofuran partial structure.

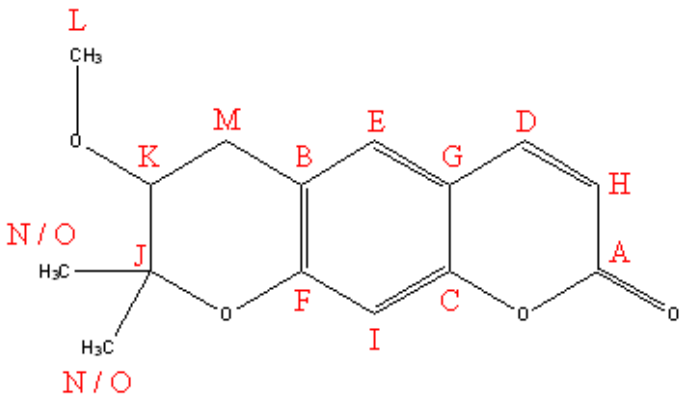




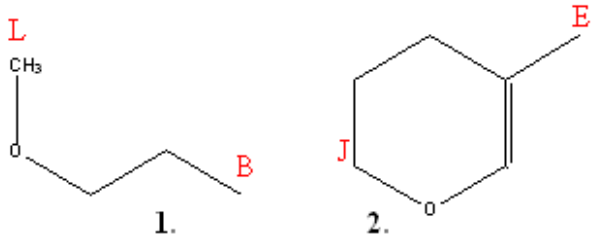
pict. 50; Unmet longer range bond couplings of the molecule containing a dihydrofuran partial structure.

In the HMBC – measurement a long distance correlation between the H - atom at position L and the C – atom at position B and vice versa was measured. But with this molecule no such correlation would be possible because the two positions are separated by 5 bindings.
This structure option can therefore be discarded.

The second possibility has an Oxacylohexane (Tetrahydropyran) partial structure:



pict. 51; Long distance correlations of the possibility with a tetrahydropyran partial structure.

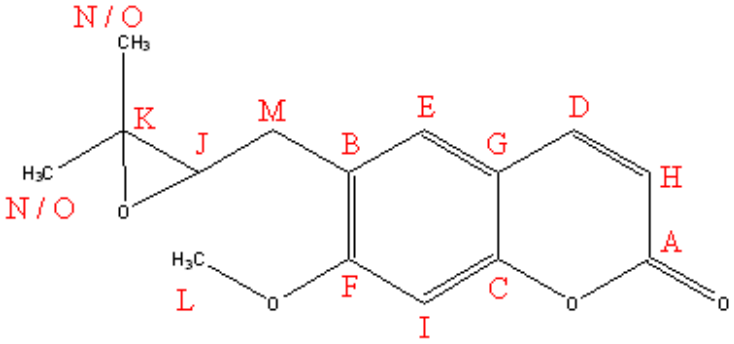


pict. 52; Unmet longer range bond couplings of the molecule containing a tetrahydropyran partial structure.

1. The correlation between L and B is not applicable because the coupling implies 4 bindings.
2. The correlation between J and B is not applicable because the coupling implies 4 bindings.

By means of this two unmet dependencies this structure option with a tetrahydropyran partial structure can be discarded.

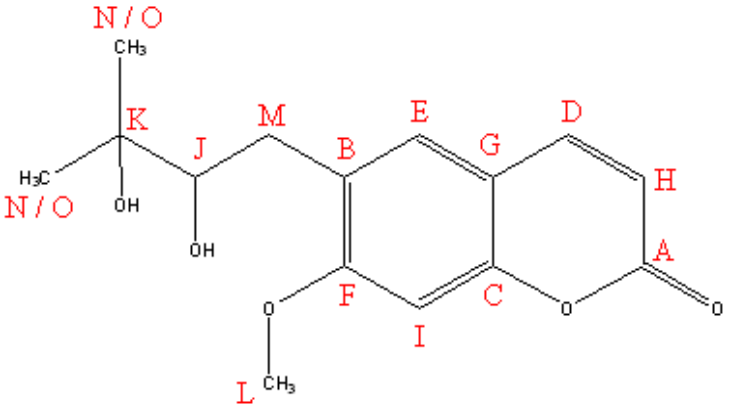
The third possibility for this structure is subersine epoxide. This molecule has an oxirane partial structure:



pict. 53; Long distance correlations of subersine epoxide.

The structure of Suberosine epoxide has no unmet dependencies to the peaks measured at the HMBC experiment for Cumarin 3.

The last option for this molecule is Pubesinole. It has two hydroxy groups instead of the oxirane partial structure of suberosine epoxide:



pict. 54; Long distance correlations of pubesinole.

The structure of Pubesinole has no unmet dependencies to the peaks measured at the HMBC experiment for Cumarin 3.
According to $^{1}H + ^{13}C$ NMR measurements and the HSQC / DEPT and gHMBC two dimensional experiments these two options (Suberosine epoxide and Pubesinole) are the contingent structures of Cumarin 3. Suberosin epoxide has a melting point of 120 – 121 °C, Pubesinole has a melting point of 135 – 140 °C. Due to the loss of substance while HPLC separation no further analysis has been carried out.

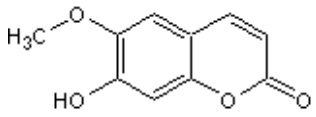
Cumarin 3
White to beige Crystals. (MeOH). ^{13}C + ^{1}H NMR (MeOD, 400 Mhz) δ 163,77 (S, C-1), δ 162.73 (S, C-6), δ 156.00 (S, C-9), δ 146.06 (D, C-3 – H: 7.89 (d) 1H), δ 131.18 (D, C-5 – H: 7.48 (s) 1H), δ 127.60 (S, C-7), δ 113.30 (S, C-4), δ 113.00 (D. C-2 – H: 6.25 (d) 1H), δ 99.48 (D, C-8, H: 6.98 (s) 1H), δ 78.50 (D, C-11 – H: 3.60 (d) 1H), δ 73.89 (S, C-12), δ 56.55 (Q, C-15 – H: 3.98 (s) 3H), δ 33.05 (T, C-10 – H: 2,50 (t) 2H / 3.18 (d) 1H), δ 25.66 (Q, C-13 – H: 1.31 (t) 3H), δ 25.18 (Q, C-14 – H: 1.31 (t) 3H).

2.2.3 Quantity of isolated Coumarins

Substance	*Weight in Fraction*	*% in ethanolic extract*	*% in dried leaves*	*% in fresh leaves*
Scopoletin	0.0116 g	0.232 %	0.009205619 %	0.004029067 %
Luvangetine	0.51234749 g	10.2469 %	0.406592723 %	0.177955362 %
Coumarin 3	0.0069 g	0.138 %	0.00547577556 %	0.0023966 %

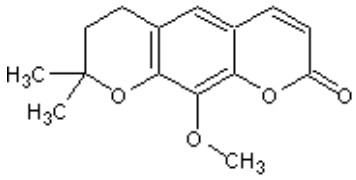
Table 16; Weight distribution of the 3 measured Compounds.

2.2.4 The Structure of Scopoletin



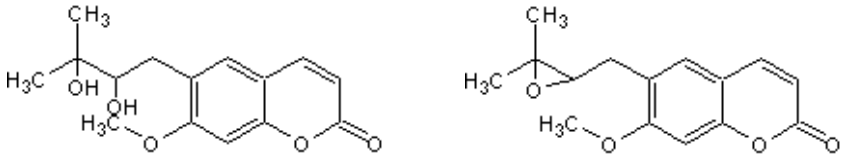
pict. 55, structure of Scopoletine

2.2.5 The Structure of Luvangetin



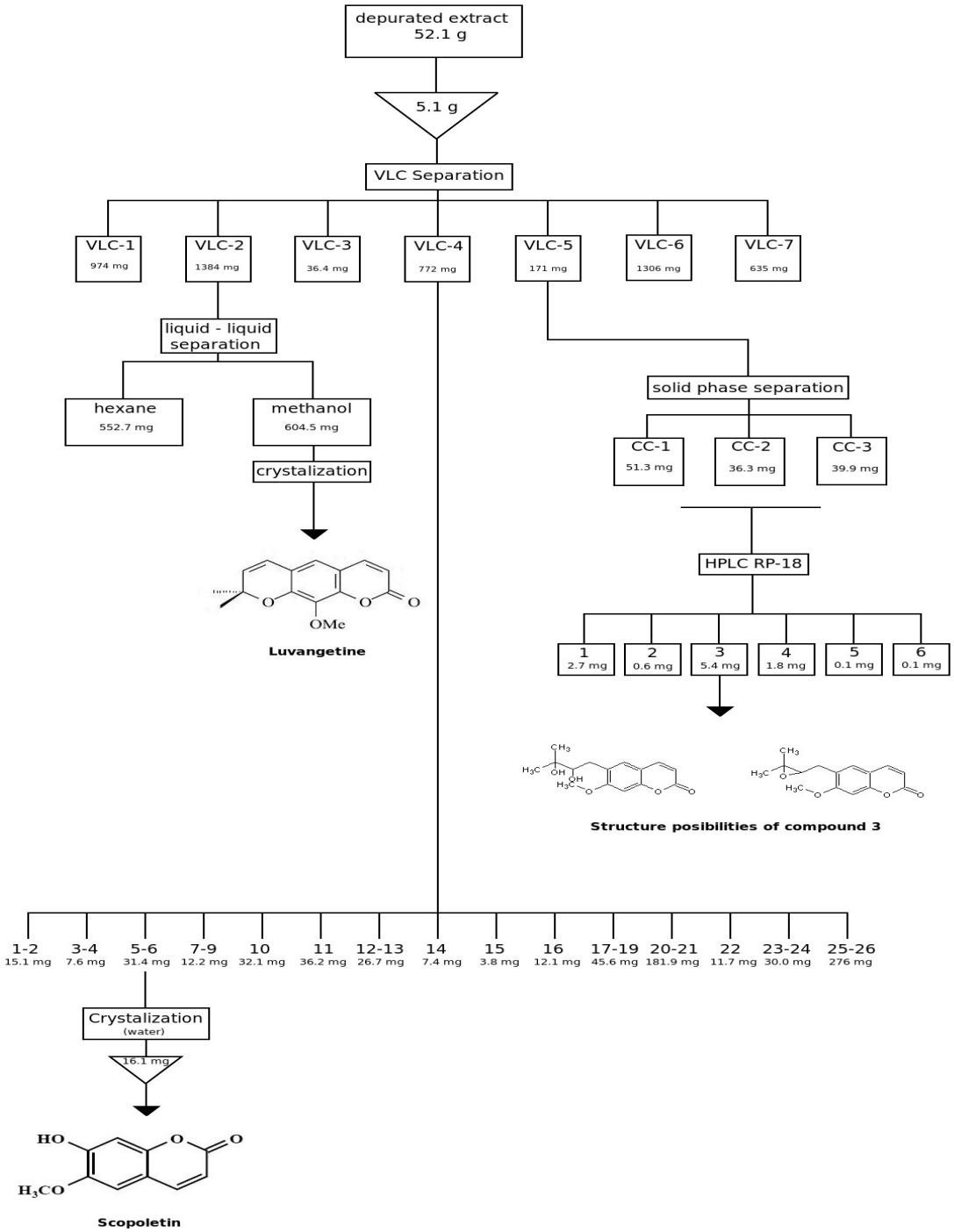
pict. 56, structure of Luvangetine

2.2.6 Structure posibilities of Compound 3



pict. 57, structure possibilities of Compound 3

2.2.7 Flow chart of the separation



Picture 58; Flow chart of the separation of *Amyris pinnata* Compounds.

2.2.8 Bioactive details

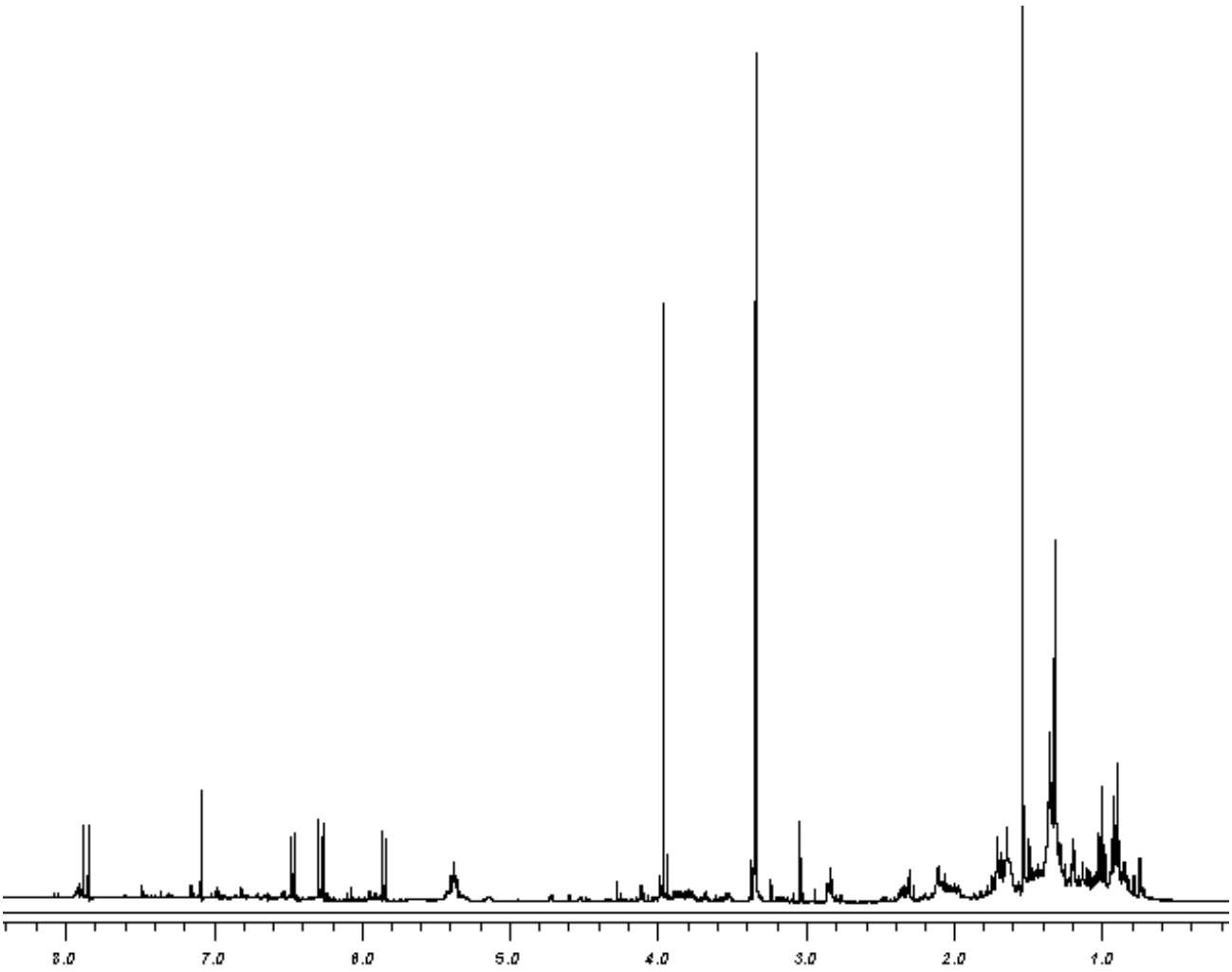
2.2.8.a Bioactive details of the purified extract

The purified extract of this species showed a antifungal activity against the following dermatophytic strains: *Trichophyton rubrum* (C 110), *T. mentagrophytes* (ATCC 9972) and *M. gypseum* (C 115). [32]

M. gypseum	**T. rubrum**	**T. mentagrophytes**
MIC in g/ml	MIC in g/ml	MIC in g/ml
62.5	62.5	62.5

Tab. 17; Reported antifungal activity – Argentinia OEA 2004

Due to the promising outcome of antifungal activity reported by this species *Amyris pinnata* showed a great potential for further investigations.



Spectra 23; NMR measurement of the purified *Amyris pinnata* extract.

The spectrum clearly shows a large proportion of fatty acids and waxes as well as tannins and chlorophylls. Obviously the presence of aromatic systems and the presence of a compound majority.

2.2.7.b Bioactive details of the isolated compounds

The isolated compounds were sent for determination of their biological activity while in parallel still structural elucidation is in progress. The following bioactivities were associated with the structures of the compounds which had been sent for evaluation.

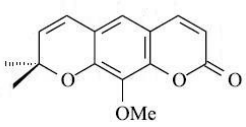
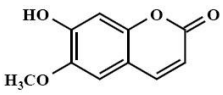
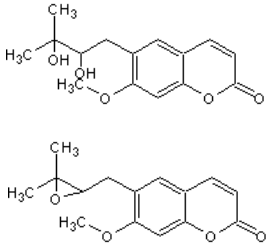
Name	Structure						
		M. Gypseum	T. Rubrum	T. Mentagrophytes	Afu	Afl	Ani
Luvangetin		250	250	125	31.2	62.5	31.2
Scopoletin		>250	>250	>250	>250	>250	>250
Ulupterol		>250	>250	>250	>250	>250	>250
Amyris pinnata depurated extract		62.5	62.5	62.5	>1000	>1000	>1000

Table 18; Bioactivity details for the purified extract and the 3 Compounds.

As shown in the table above the initially refined crude extract shows an activity for the group of household dermatophytes but applied as isolated pure compound Luvangetina (X) loses its activity in this group and shows increased activity in the group of hialohifomicetos (*Aspergillus fumigatus, flavus* and *niger*). This could be due to the synergy and antagonism of compounds in the mix of the extract in each of these groups evaluated. [33]

3.1 Characterization of *Maianthemum paludicola*

Domain: eukaryotes
Subkingdom: Viridaeplantae
Phylum: tracheophyta Sinnot (auct.)
Subphylum: Spermatophytina
Infraphylum: Angiospermae auct.
Class: Liliopsida Scopoli
Subclass: Liliidae Takhtajan
Superorder: Lilianae Takhtajan
Order: Asparagales Perleb
Family: Liliaceae
Genus: Maianthemum
Specific epithet: paludicola LaFrankie

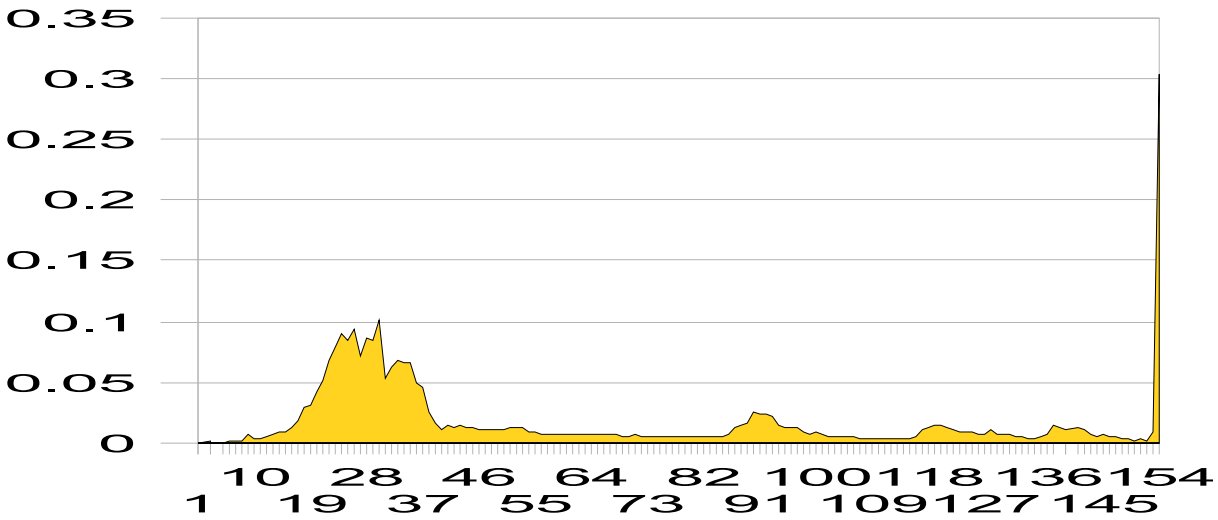
Maianthemum paludicola is a terrestrial plant which stems achieve at least 1.8 m altitude. Petals have about 2-5 mm, leaves 9.6-15.7 cm × 2.1-4.9 cm with lance-ovate to elliptic or lanceolate form. Inflorescences have an extension of 8-14 cm, clusters 16-22, 2-7.5 cm., Pedicels 3-10 mm.. The tepals are white with purple anthers with a diameter of 7-9 mm. [34] The plant grows in rainforests, peatbogs and boggish zones at a height of 2500-3100 m. and blooms from April to November. The straight and linear rhizomes are the only reliable character to determine M. paludicola. According to LaFrankie (1986) lateral veins of the leaves of this spezies look all the same. But regarding INBioś notations there are more distinctive veins alternating with veins less distinctive. Most collections of this species have been made next to Highway Interamericana. [35]

3.2 Separation of Compounds

Sephadex Chromatography

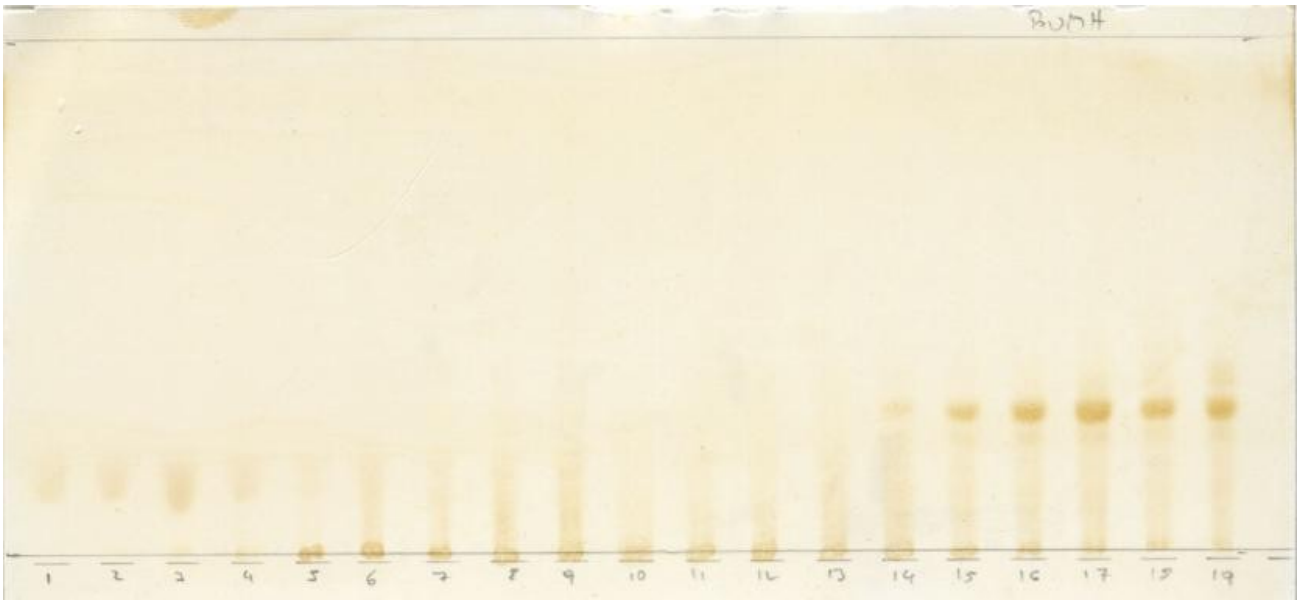
2.6 g of the depurated extract, which was formerly freed of tannins, were brought to a 89x3cm column filled to a height of 75 cm with lipophilic Sephadex LH-20. Mobile Phase was 90% Methanol, then Methanol/$CHCl_3$ 1:1.
The quantity of each collected fraction was 10,0 ml at a flow of 1,75 ml per minute and concentrated to complete dryness on the rotorvapor.

Resulting quantity distribution Sephadex LH-20

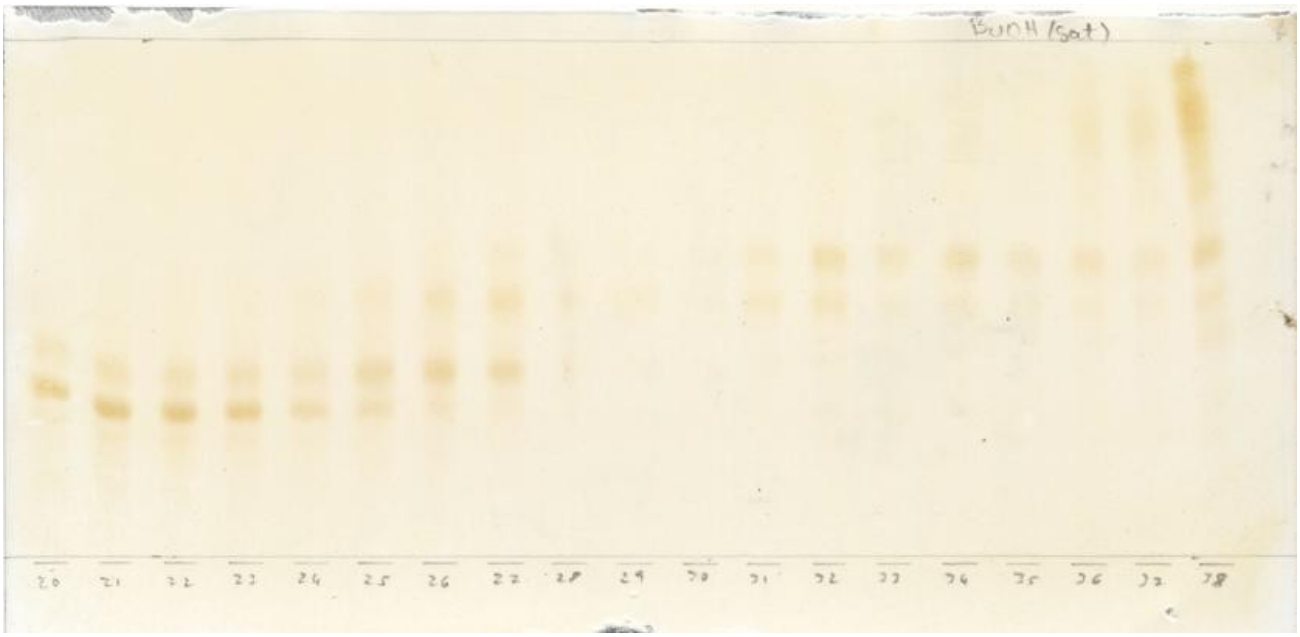


Tab. 19; Weight distribution of the Sephadex fractions of Maianthemum paludicola LaFrankie.

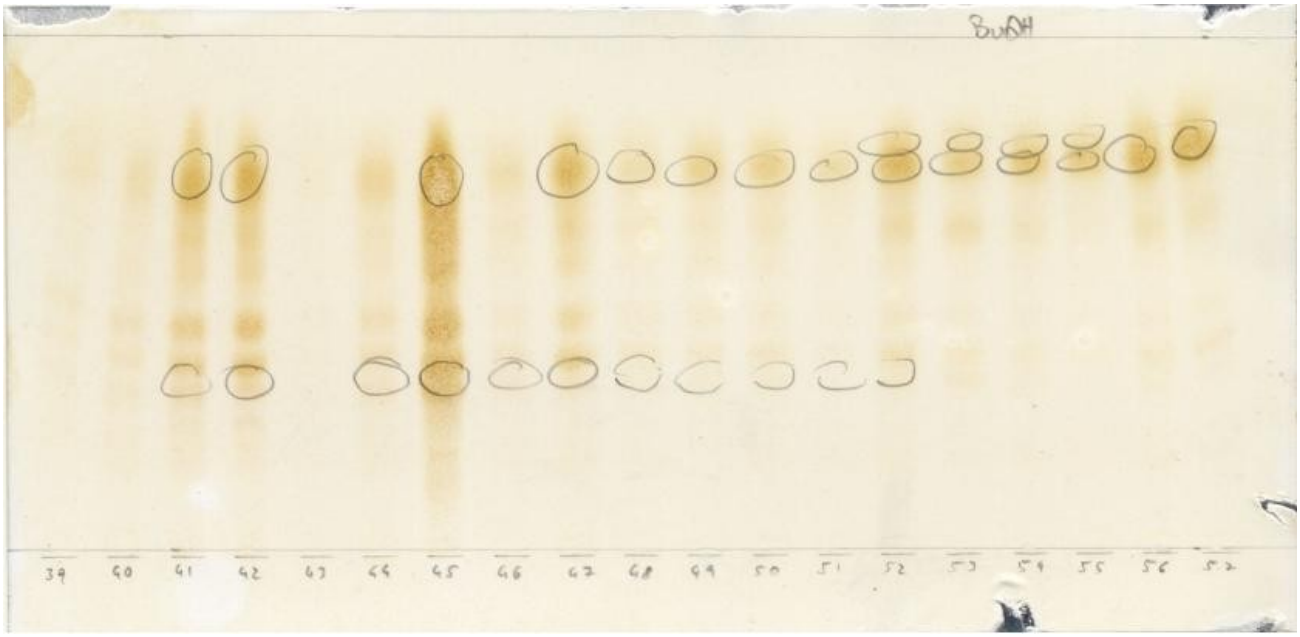
In order to get an overview of the distribution of constituents a comparing thin layer chromatography was made by using CHCl3:Methanol (9:1) as mobile phase.
KmnO4 was used as spraying agent.



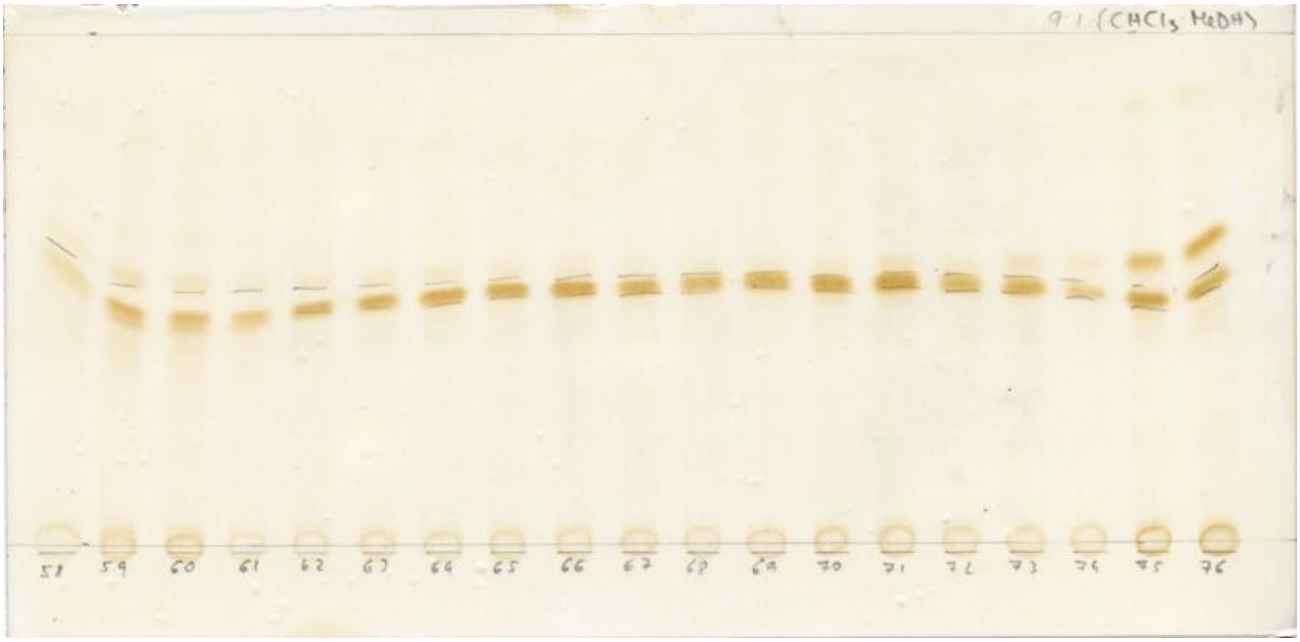
Pict 59; Fractions cc-1 to cc-19 of *Maianthemum palludicola*



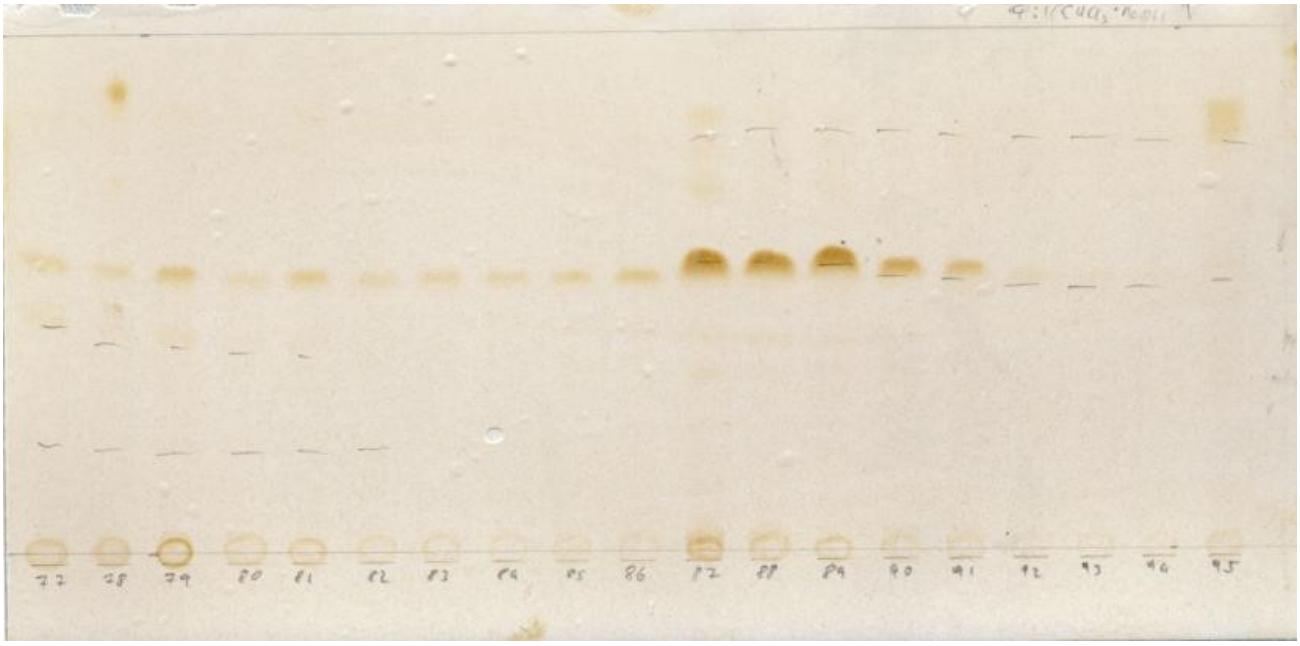
Pict 60; Fractions cc-20 to cc-38 of *Maianthemum palludicola*.



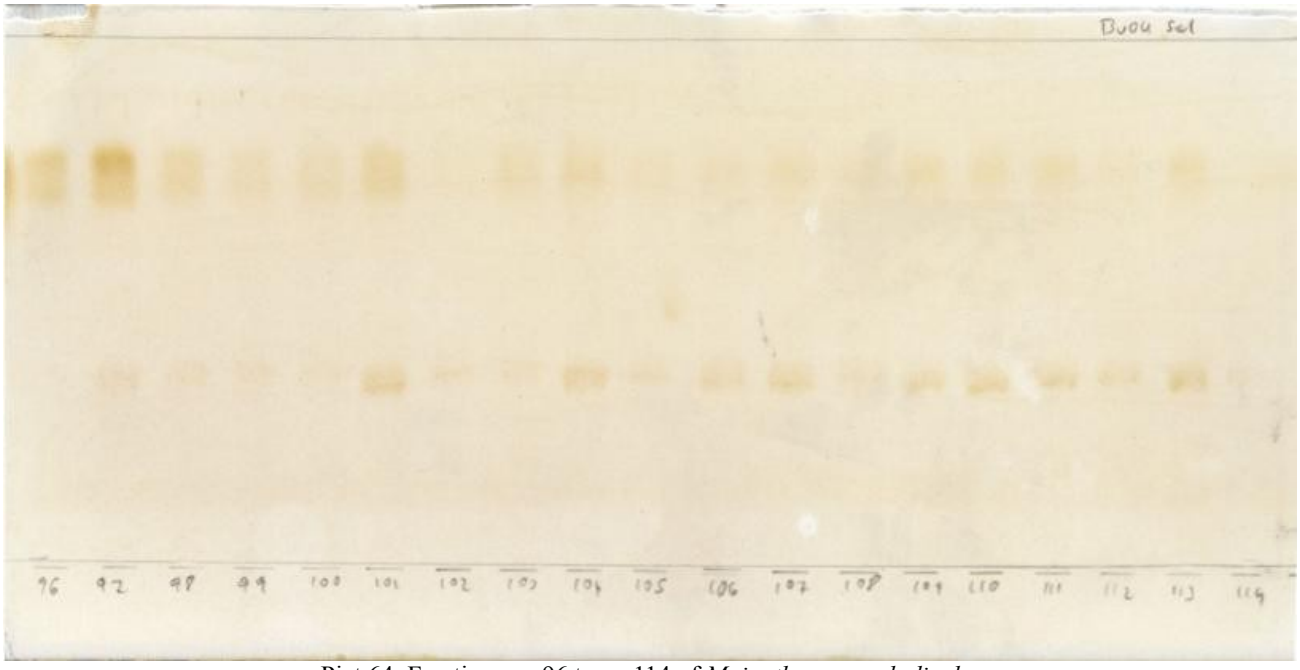
Pict 61; Fractions cc-39 to cc-57 of *Maianthemum paludicola*.(UV active spots marked)



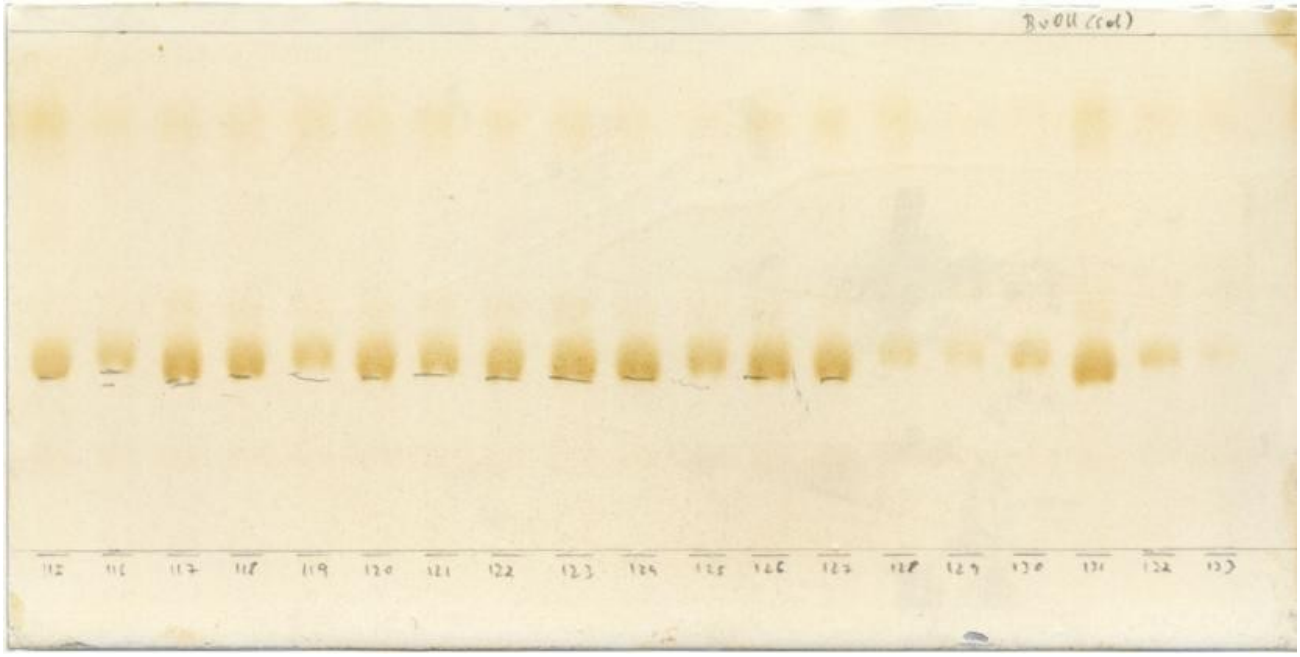
Pict 62; Fractions cc-58 to cc-76 of *Maianthemum palludicola*. (UV active spots marked)



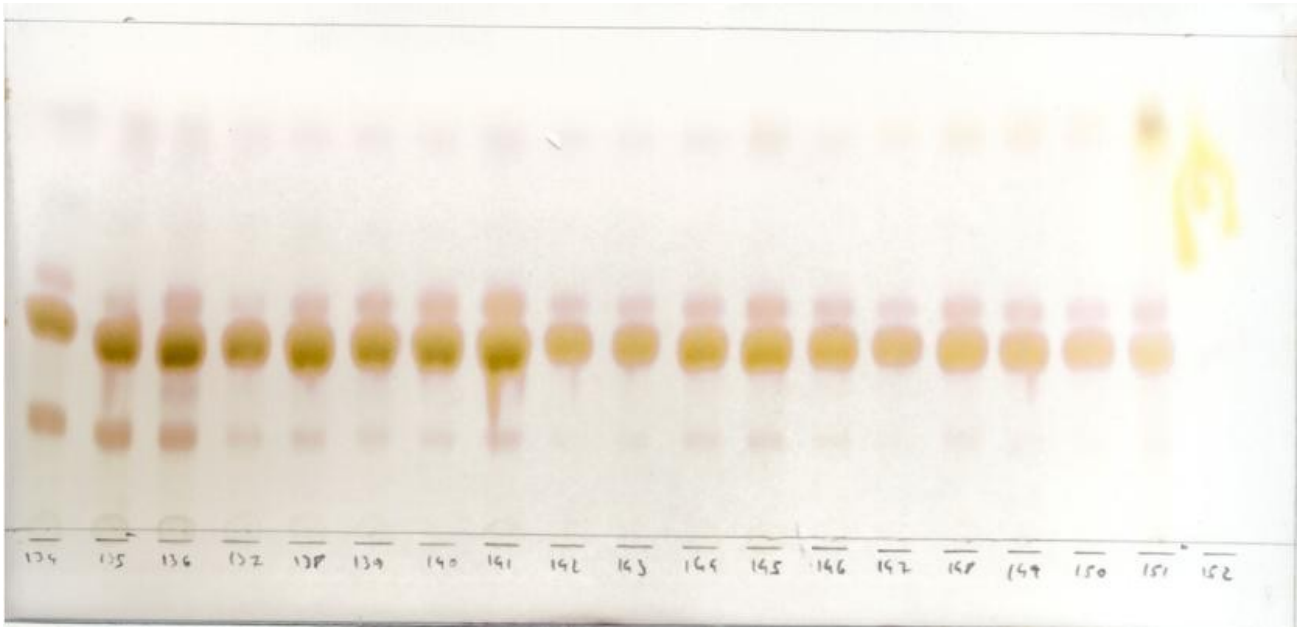
Pict 63; Fractions cc-77 to cc-95 of *Maianthemum paludicola*. (UV active spots marked)



Pict 64; Fractions cc-96 to cc-114 of *Maianthemum paludicola*.



Pict 65; Fractions cc-115 to cc-133 of *Maianthemum paludicla*. (UV active spots marked)

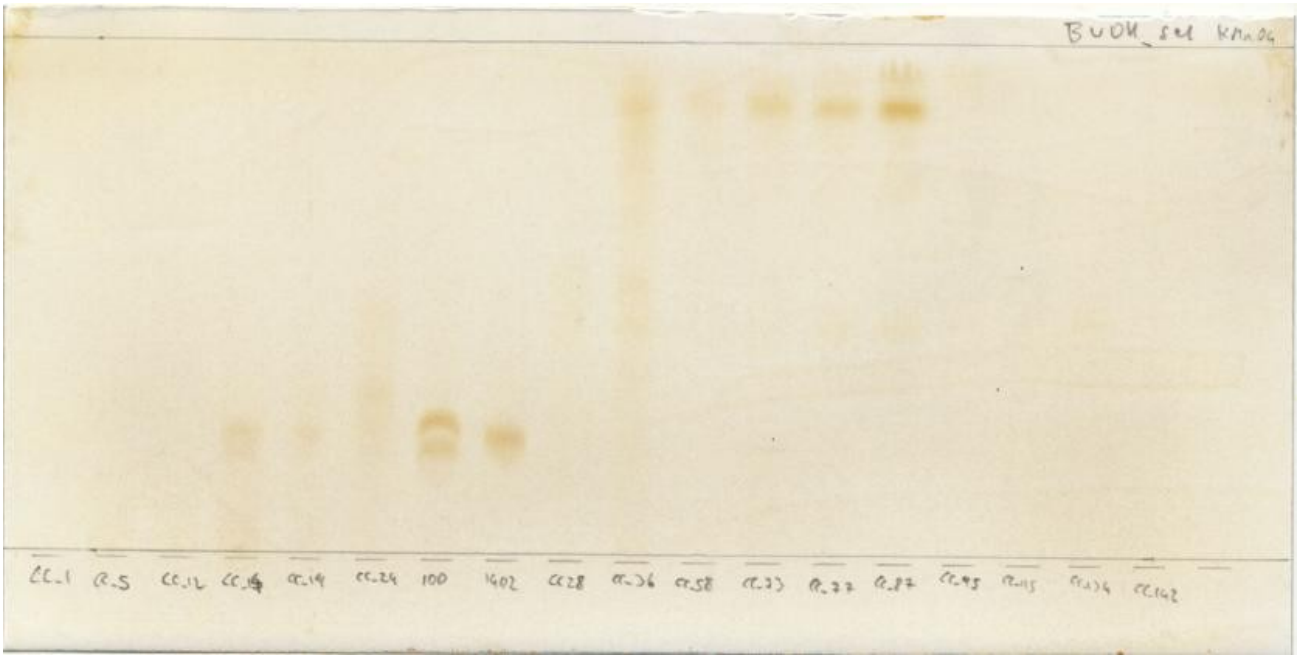


Pict 66 Fractions cc-134 to cc-152 of *Maianthemum paludicola*. (UV active spots marked)

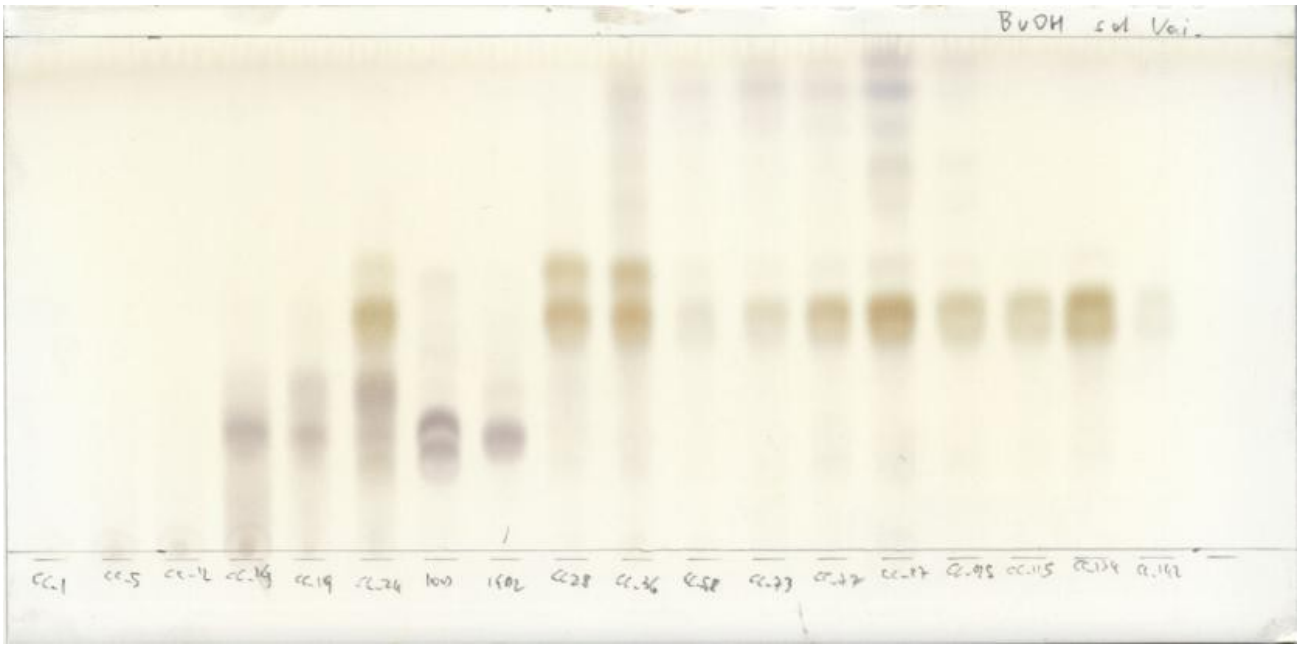
United fractions	name	Weight (g)
cc-1 - cc-4	cc-1	0.0033
cc-5 - cc-11	cc-5	0.0230
cc-14 - cc18	cc-14	0.1268
cc-19 - cc-23	cc-19	0.3031
cc-24 - cc-27	cc-24	0.2521
cc-28 - cc-35	cc-28	0.38
cc-36 - cc-57	cc-36	0.2051
cc-58 - cc-72	cc-58	0.0842
cc-73 - cc-76	cc-73	0.0147
cc-77 - cc-86	cc-77	0.056
cc-87 - cc94	cc-87	0.0718
cc-95 - cc-114	cc-95	0.1086
cc-115 - cc-133	cc-115	0.1472
cc-134 - cc141	cc-134	0.0668
cc-142 - cc-150	cc-142	0.026

Tab 20; United fractions and corresponding weights

The fractions were eluted using thin layer chromatography using saturated Butanol as mobile phase. Spraying agents were vanilla spray agent and KmnO4.



Pict 67; United fractions (BuOH, KmnO4 as spray agent)



Pict 68; United fractions (BuOH, vanilla as spray agent)

cc-19

Partial acid hydrolysis

50 mg of fraction cc-19 were taken to partial acid hydrolysis using 10.0 ml of a mixture of 1.0 HCl concentrated and 9.0 ml of destillated water. The mixture was heated and stirred for 4 hours for complete hydrolysis. During this process it changed the colour to black. After cooling to room

temperature the ingredients were divided by using 3 x 20.0 ml CHCl3 for liquid-dispersion. The CHCl3-Phase was washed with 50.0 ml H2O and after that with a 50.0 ml of a 2% solution of sodiumbicarbonate in H2O. After drying the CHCl3-Phase on the rotorvapor it yielded 0.0122 g, while the H2O-Phase yielded 0.0420 g.

cc-28

Fraction cc-28 was separated into two ways of processing:

Crystallization/Hydrolysis
330.0 mg of cc-28 were solluted in a warm mixture of 90% Methanol and 10% H2O and allowed to cool down slowly with the result of the crystallization of white needles. This procedure was repeated 2 times resulting in 44.1 mg of the crystals. The rest was dried on the rotorvapor yielding 217.0 mg substance.
The 44.1 mg of crystals were solluted in 9.0 ml of H2O and 1.0 ml of concentrated HCl were added. This mixture was heated to 60.0 °C and stirred for 6 hours in order to get a quantitative acid-hydrolysis. After cooling down to room-temperature the ingredients were divided using 3 x 20.0 ml CHCl3 for liquid-dispersion. The CHCl3 phase was washed with 3 x 30.0 ml H2O and after that with a 3 x 30.0 ml of a 2% solution of sodiumbicarbonate in H2O. After drying the CHCl3 phase on the rotorvapor it yielded 0.0212 g while the H2O-Phase yielded 0.0289 g of substance.

The 0.0289 g of the H2O-Phase were extracted with 3 x 20.0 ml Buthanol using liquid-liquid-separation and the Buthanol phase was washed with 3 x 20.0 ml H2O and after that with a 3 x 20.0 ml of a 2% solution of sodiumbicarbonate in H2O.
As a result of this the Buthanol-phase resulted in 0.0091 g substance and the H2O-phase in 0.0394 g.

Hydrolysis/PTLC-separation
50 mg of fraction cc-28 were also taken to partial acid hydrolysis using 10.0 ml of a mixture of 1.0 HCl concentrated and 9.0 ml of destillated water. The mixture was heated and stirred for 4 hours for complete hydrolysis. After cooling to room temperature the ingredients were divided using 3 x 20.0 ml CHCl3 for liquid dispersion. The CHCl3 phase was washed with 50.0 ml H2O and after that with a 50.0 ml of a 2% solution of sodiumbicarbonate in H2O. After drying the CHCl3 phase on the rotorvapor it yielded 0.0207 g while the H2O-Phase yielded 0.0275 g.

The 20.7 mg of the CHCl3-Fraction were separated using 20.0x20.0 TLC plates of 0.5mm silica. A mixture of CHCl3 and Methanol at a ratio of 9:1 were used as mobile phase. After running the plate, it was divided into 5 zones:

Area on plate	Weight (g)
0 – 2.5 cm	0.0036
2.5 – 7.0 cm	0.0018
7.0 – 8.5 cm	0.0018
8.5 – 13.5 cm	0.0071
13.5 – 15 cm	0.0027

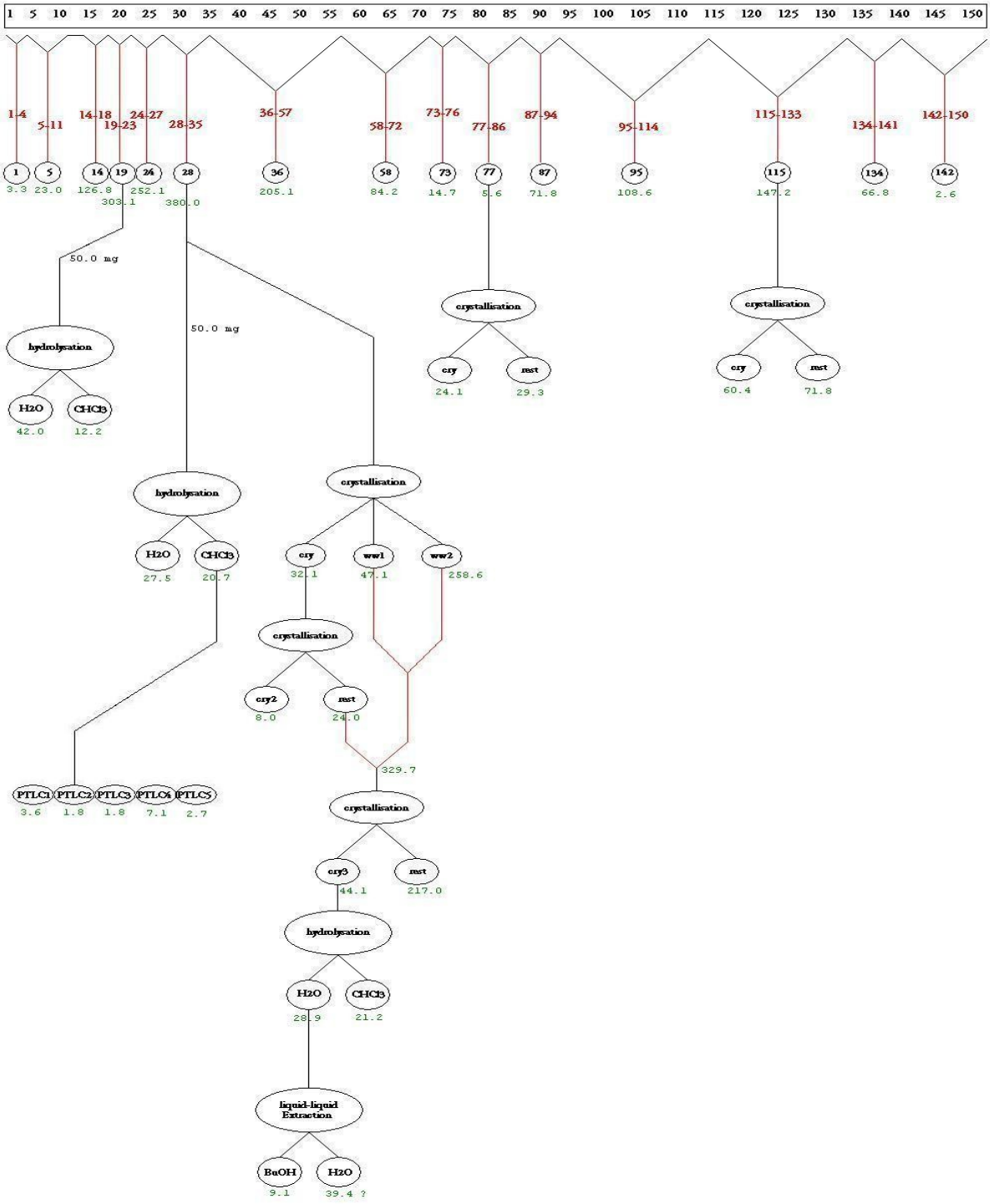
Table 21; Preparative thin layer chromatography zones of cc-28

The zones were separated, cut into 0.5 cm2 pieces and dissolved in a mixture of CHCl3 and Methanol at a ratio of 9:1. In order to remove silica in the fractions they were filtered with a Waters 0.45 µm Filter and dried on the rotorvapor.

cc-77
The 0.056 g of fraction cc-77 were soluted in warm Ethanol and allowed to cool down slowly. This resulted in 0.0241 g of crystals and the rest of 0.0293.

cc-115
Fraction cc-115 was recrystallised in warm Methanol which resulted in 0.064 g of a colloidal precipitate and 0.0718 of the rest of this fraction.

3.3 process chart of the separation



Picture 67; Process chart of the separation for *Maianthemum paludicola* LaFrankie.

3.4 Results

Due to time issues and the malfunction of the HPLC device, investigations on this species have been stopped at this point and will be readoptet by other persons being involved in this project.

IV Experimental

4.1 Material

4.1.a Plant Material

The fresh leaves from *Amyris pinnata* Kunth were collected from tropical forests in the pacific side of Costa Rica in the "Sector Laguna Meandrica. Bosque secundario." in Puntarenas, Pacífico Central. The plant was steril, the location was 9:47 Latitude and, -84:35 Longitude and 70 Altitude. It was collected on 30th of March, 2000 and identified by Luis Guillermo Acosta from INBio, Heredia and the voucher specimen was deposited at the National Biodiversity Institute, Heredria, Costa Rica.

4.2.a Solvents

Solvents used were science-quality solvents from Merck.

4.3 Bioassays Employed for evaluation

For the antifungal evaluation strains of the American Type Culture Collection (ATCC, Rockville, MD, USA) and CEREMIC (C, Centro de Referencia en Micología, Facultad de Ciencias Bioquímicas y Farmacéuticas, Universidad Nacional de Rosario, Rosario, Argentina) had been used. The plants had been tested against the following strains: *Candida albicans* (ATCC 1023), *Candida tropicalis* (C 131), *Saccharomyces cerevisiae* (ATTC 9763), *Cryptococcus neoformans* (ATCC 32264), *Aspergillus flavus* (ATTCC 9170), *Aspergillus niger* (ATCC 9029), *Aspergillus fumigatus* (ATCC 26934), *Microsporum gypseum* (C 115), *Microsporum canis*, *Trichophtyon rubrum* (C 110), *Trichophyton mentagrophytes* (ATCC 9972), *Epidermophyton floccosum*, S*porothrix schenckii* and *Fonsecaea pedrosoi*.

4.4 Chromatography

Vacuum liquid chromatography
The head was prepared with 10 g of Silica 70 – 230 µm mesh from Merck for the vacuum liquid chromatography.

thin layer chromatography
For thin layer chromatography, Kieselgel 60 F245 TLC Layers from MERCK both with and without fluorescence indicator were used.

preparative thin layer chromatography
20x20cm pTLC glassplates were used laminated with 1mm of silica for preparative thin layer chromatography (Merck).

Medium Pressure Liquid Chormatography
We used a 14x3cm column, packed with LiChromprep RP-18 (25-40 μm mesh) from MERCK. The head was prepared with 2.0 g of LiChromprep RP-18 (25-40 μm mesh).

Supelco column chromatography
Two Supelco RP-18 Columns were used.

High pressure liquid chromatography
2 columns were used to divide fraction VLC_5:
- X-Terra RP-18 column 5 μm, 3.9x50mm.
- Waters Prep-Nova-Pak HR-C18 column, 60A, 6 , 7.8x300mm.

Controller: Waters 600
Pump: Waters 600
Detector: Waters 2487 dual λ absorbance detector, Waters 996 Photodiode array detector;

V Lists of afterimages

5.1 Table of pictures

5.2 List of tables

5.3 List of spectras

VI Literature

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[36] Prof. Giselle Tamayo

VII Assessment of INBio

INBio
Instituto Nacional
de Biodiversidad

06 de Octubre del 2008
BP-138-08

Dear sir,

Mr. Andreas Vallant worked on 2006 as a trainee in our department, concentrating his efforts on the isolation and characterization of active components of the plants *Amyris pinnata* Kunth and *Maianthemum palludicola*.

During his stay, he learned chromatographic techniques, such as vacuum liquid chromatography (VLC), thin layer chromatography (TLC), HPLC (high performance liquid chromatography) and SPE (solid phase extraction). He showed also abilities to perform crystallizations, and liquid-liquid partitioning.

From these separations, he obtained fractions which should be further purified up to pure compounds. He was able to obtain the three main components of *A. pinnata* Kunth and a preliminary separation on *M. palludicola*. The latter could not be accomplished up to its entire separation, due to time constraints

We are fairly satisfied with the performance and commitment showed by Mr. Vallant, and thereby we grade his performance with A (9.5).

With kind regards,

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Bioprospecting-INBio
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VII Abstract (german)

Diese Arbeit entstand durch die Zusammenarbeit von INBio und dem Department für Pharmakognosie der Universität Wien. 30 Pflanzen wurden auf ihre biologische Aktivität getestet und anhand dieser Kriterien wurden 2 Pflanzen für weiterführende Untersuchungen ausgesucht.
Ausgangspunkt der durchgeführten Untersuchungen waren gereinigte Extrakte von *Amyris pinnata* Kuhnt und *Maianthemum palludicola***.**
Es werden generelle Themen der Gewinnung natürlicher Produkte besprochen mit besonderem Augenmerk der Tätigkeiten von INBio, Heredia.

Schwerpunkte der Arbeit sind:

5. Wie sieht der chemische Fingerabdruck der Pflanzen aus und welche chemischen Strukturen verbergen sich dahinter?
6. Welcher der chemischen Inhaltsstoffe ist für die biologische Aktivität verantwortlich?

Der Extrakt von *Amyris pinnata* wurde mittels MPLC Chromatographie aufgetrennt, wobei jede dieser 7 Fraktionen durch Dünnschichtchromatographie und Kernspinresonanz überprüft wurde. Weiters wurde mit Hilfe von Hochleistungsflüssigkeitschromatographie, präparativer Dünnschichtchromatographie und Umkristallisation weitere Reinigungsschritte durchgeführt, was zu 3 reinen Verbindungen führte.
Die gereinigten Verbindungen wurden mittels verschiedener Kernspinresonanztechniken gemessen und die Ergebnisse diskutiert. Der Autor schlägt Scopoletin, Luvangetin und die Möglichkeit von Ulupterol als Hauptinhaltsstoffe vor, wobei Luvangetin und Ulupterol noch nicht in dieser Pflanze gefunden wurde.

Der *Maianthemum palludicola* Extrakt wurde mittels Sephadex LH-20 Chromatographie aufgetrennt, 154 Fraktionen gesammelt und jede dieser Fraktionen wurde mittels Dünnschichtchromatographie überprüft. Der Autor erhielt eine komplexe Mischung von Saponinen, die durch partielle saure Hydrolyse gespalten und mittels Kernspinresonanz gemessen wurde.
Auf Grund von Zeitproblemen wurden die Untersuchungen an diesem Punkt beendet und werden durch andere Mitarbeiter des Projekts wiederaufgenommen.

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mother	Lisbeth Silvia Vallant, teacher
nationality	austrian

education

1986 –1990	Elementary shool, Villach
1990 - 1998	Bundesrealgymnasium Perau, Villach
1998 - 1999	community service at the red cross, Villach
seit 1999	pharmacy academic studies at the University of Vienna

employment:

2000-2004	2 months of full-time employment every year, Lind Apotheke, Villach
2004 - 2006	part-time employment, Allerheiligenapotheke, Wien
Feb. 2006 – Juli 2006	laboratory research, INBio National Biodiversity Institute, Costa Rica
Nov. 2006 -	part-time employment, Apotheke zum Papst, Wien

Knowledge:

languages	german, english, spanish (basics)
computer - applications	MS Windows 98/ME/2000/XP and Office MS network administration Linux: Debian, Redhat, Suse, Ubuntu Open Office pharmacy applications: Apotronic, Ritter, Optipharm Datapharm