Characterization of Medicinal Properties of *Cannabis sativa* L. Roots

Prepared for

Lou Riesenberg, Professor and Department Head
Agricultural and Extension Education, College of Agricultural and Life Sciences
University of Idaho
Moscow, ID 83844

Prepared by

Blair Van Pelt
Ag 404

Work performed at

Department of Plant Metabolomics, Institute Biology Leiden
Leiden University, The Netherlands

December 15, 2008

Characterization of Medicinal Properties of *Cannabis sativa* L. Roots

Table of Contents

Characterization of Medicinal Properties of *Cannabis sativa* L. Roots

Abstract

A quick and easy test for the detection of Acetylcholine Esterase inhibitors was used for the bioassay guided fractionation of cannabis root extract. The roots of 9 *Cannabis sativa* plants (variety Bedrocan) were collected from Bedrocan BV, the Netherlands, and analyzed at the department of plant metabolomics at Leiden University. They were extracted by a range of solvents in order to extract the maximum range of compounds with different polarity. Although alkaloids were expected to be present, they were not found in this study. Instead, mainly lipids and sugars were detected. The compound finally isolated appeared to be a lipid but could not be uniquely identified. More sensitive and selective analytical methods and a wider range of bioassays are needed to further explore the medicinal potential of cannabis root.

Botanical drawing of *Cannabis sativa* L.

Introduction

Cannabis is known for its multi-purpose nature. It can be used to make food, fuel, fiber, and medicine. The root is remembered by some as an old folk remedy for arthritis or joint pain (Bott 2008). References were also found stating the roots emetic and cathartic properties (Indian Hemp 2008). Only in the last 30 years have we begun to understand why. Over 10,000 scientific papers about cannabis have been published. Still the root of *Cannabis sativa* L. is the least studied part.

Characterization of Medicinal Properties of *Cannabis sativa* L. Roots

Historical uses
Cannabis root has been used medicinally for centuries from different parts of the world and to treat a wide range of maladies. The earliest report is from medieval times when the root was said to relieve the agonies of gout and other painful diseases (Nature's Pharmacy 2007).

In1696 Georg Eberhard Rumpf (Rumphius), a German physician at the service of the Dutch Crown reported on the use of cannabis root in Indonesia to treat gonorrhea (Rumpf and Beekman 1981, Russo 2002). By 1763 The New English Dictionary said cannabis root applied to skin eases inflammation (Marijuana as Medicine 2005). In the Chinese Materia Medica "juice of the root is thought to have a beneficial action in retained placenta and post partum hemorrhage, (Stuart 1928).

In Argentina cannabis is considered a real panacea and is used to treat tetanus, colic, gastralgia, swelling of the liver, gonorrhea, sterility, impotency, abortion, tuberculosis of the lungs and asthma even the root-bark has been collected in spring, and employed as a febrifuge, tonic, for treatment of dysentery and gastralgia, either pulverized or in form of decoctions. The root when ground and applied to burns is said to relieve pain. Oil from the seeds has been frequently used even in treatment of cancer (Kabelik, 1960).

Historical preparations
Historically cannabis roots have been prepared in several ways. Usually the roots are dried and ground into a powder or boiled. The powder is then used to make a poultice or an oil salve to be used topically, and sometimes the boiled root juice is drunk. The decoction of the root is said help remedy hard tumors and knots in the joints (Duke 1983).

The roots can be ground to form a paste and applied to relieve the pain of surgery and broken bones (Frank 1978). Mixed with oil and butter, the root was used as a salve it is said to treat burns from the newly introduced gunpowder (Natures' Pharmacy 2008).

Identified components of roots
The roots are the least studied part of the cannabis plant; still, several components and compounds were identified since the 1970's. Although glandular hairs are where the majority of cannabinoids are produced they have also been detected in the roots by immunoassays (Tanaka and Shoyama 1999) and chemical analysis (Potter 2004).

Terpenes have been detected and isolated from essential oil from flowers, leaves and roots (Slatkin et al. 1971). The terpenes are responsible for the flavor of different varieties of cannabis and determine the preference of the cannabis users (Flores-Sanchez 2008).

Alkaloids are another class of chemical constituents that have been found in cannabis. Both piperidine and pyrrolidine were identified and isolated from the roots, leaves, stems, pollen, and seeds (Paris et al. 1975; El-Feraly and Turner 1975; Elsohly at al. 1978). The biosynthesis of choline and atropine by hairy root cultures have also been reported (Wahby et al. 2006)

"Cannabis fruits and roots (Sakaibara at al. 1995) have yielded 11 compounds identified as phenolic amides and lignanamides." A review done by Flores-Sanchez found phenolic amides to

have cytotoxic, anti-inflammatory, antineoplastic, cardiovascular and mild analgesic activity and the lignans to have insecticidal effects.

Work done in this study
Based on literature reviewed and recent studies about cannabis root (Wahby 2006) it is expected that alkaloids be present. The roots of a medicinal variety of cannabis (Bedrocan) were extracted and compounds were fractionated in order to detect biologically active components by bioassay guided fractionation. The Acetylcholine Esterase assay was chosen as bioactivity assay because of availability and limited time (6 weeks).

Materials and Methods

Everything described below is schematically shown in figure 1 of the figure appendix.

Solvents and chemicals
All organic solvents were analytical grade and obtained from Merck Biosolve Ltd. Valkenswaard, The Netherlands. Deuteriated chloroform ($CDCl_3$, 99.8%), water (D_20) and methanol (MeOD) were obtained from Eurisotop (Gif-sur-Yvette, France).

General equipment used

- RotaVapor: RotarVap R-200, Büchi
- Speed Vac: SPD 121P, ThermoElectron Corporation
- Centrifuge: Varifuge 3.0 R, Heraeus Sepatech
- Freeze Dryer: Modulyo, Edwards
- Sonicator: 5510, Branson
- Blender: Blend-o-matic, Waring
- UV Spectrophotometer: Reprostar II, Camag

Plant material and extraction
Fresh root material (see figure 6) was collected from Bedrocan BV, the Netherlands, and stored at -20ºC until used. The roots were air dried and then pulverized in liquid nitrogen. The resulting powder was freeze-dried and weighed to determine yield.

Powder (13 g) was extracted sequentially with four different solvents of increasing polarity: hexane, chloroform, acetone, and methanol (extracts: A, B, C, D respectively). Each extraction was preformed twice with 200 mL of solvent, while being sonicated for five minutes. For each solvent, both extracts were combined and filtered over paper.

The same root powder was then extracted with boiling water for thirty minutes. The water extract was filtered over paper (extract E). Extracts A-D were concentrated to 10 mL under vacuum in a RotaVapor while extract E was concentrated in a freeze-dryer. All obtained extracts were analyzed by TLC. Extracts B, C, and D were combined due to similar composition.

Characterization of Medicinal Properties of *Cannabis sativa* L. Roots

Thin Layer Chromatography (TLC)
For efficient and rapid analysis of large numbers of fractions TLC was used (Hazekamp 2007). Samples (20µL) were manually spotted on 10x20 cm normal phase silica gel plates F254 No. 105554 (Merck, Darmstadt, Germany) and developed in saturated normal chambers (saturation time 15 minutes). Eluent was hexane/diethylether/formic acid, 50:50:1 (v/v/v). After development, visual inspection was done under UV 254 and 366 nm. Subsequently, general visualization of compounds was done by spraying with modified anisaldehyde-sulphuric acid spray reagent (see figure 8), followed by heating with a heat gun (see figure 9).

In some experiments also the following spray reagents were used:

Fast blue b: 0.5% fast blue B salt (*o*-dianisidine-*bis*-(diazotized)-zinc double salt) (Sigma) in water

Dragendorf: Solution a) 0.6gr bismuth subnitrate in 2 ml conc. HCl and 10 ml water. Solution b) 6 gr KI in 10 ml water. Mix together with 7 ml conc. HCl and 15 ml water. Dilute to 400 ml with water.

Preparative HPLC
Combined fractions B-D were further fractionated by affinity chromatography using a preparative HPLC system (Shimadzu) under the following conditions:

Column: Phenomenex Luna C_{18}, 5µm, 100Å
Eluent: 50% methanol + 50% water
Flow: 4 ml/min
Fraction size: 31 fractions of 10 ml
UV detection: 254 nm

All obtained fractions were analyzed by TLC and fractions 0, 14, 18, and 20 were selected for further analysis by AchE assay.

Acetylcholine Esterase Inhibition Assay (AchE)
This test was performed according to Rhee et al. (2001). With this assay it is possible to detect compounds that inhibit Acetylcholine Esterase in a simply and rapid manner. The advantage of this TLC assay method is that there is no disturbance from sample dissolving solvents as in the microplate assay.

To perform the assay the following solutions were freshly prepared:

- Buffer A: 50 mM Tris-HCL, pH 8
- Substrate/dye solution: 1mM Acetylthicholine Iodide (ATCI) + 1mM 5.5'-dithiobis-(2-nitrobenzoic acid) (DTNB) in buffer A
- Enzyme solution: 3U/mL of Acetylcholine Esterase (AchE) from electric eel (type VI-s Sigma chemical Co.) in buffer A.

Investigated fractions were spotted on TLC plate and were developed as described above. After thorough drying, the plate was sprayed with the substrate/dye solution. The plate was allowed to dry for 3-5 minutes and then sprayed with the enzyme solution. Active spots were identified as

white spots against a yellow background. The alkaloid galanthamine was used as a positive control for this assay.

Acid hydrolysis
Fraction 0 obtained from prep HPLC was hydrolyzed by heating for 1 hour at 85°C in 1 mL methanol, 1 mL water and 0.2 mL hydrochloric acid. The methanol was evaporated under vacuum so that only the acidic water remained. Hydrolyzed compound was then extracted from the water by partitioning with 1 mL of ethyl-acetate, followed by 1 mL of hexane. Both organic extracts were combined and evaporated. Residue was re-dissolved in 1 mL of ethanol and analyzed by AchE assay.

Preparative TLC
The aglycons obtained after acid hydrolysis were further fractionated by preparative TLC. Sample (500 µL) was spotted manually using a capillary on 10x20 cm normal phase silica gel plate F254 No. 105554 (Merck, Darmstadt, Germany) and developed in saturated normal chambers (saturation time 15 minutes). Eluent was hexane/ethyl-acetate 1:1 (v/v).

After development, visual inspection was done under UV 254nm. One inch of each side of plate was cut off for general visualization of bands by spraying with modified anisaldehyde-sulphuric acid spray reagent. The most intense band was scraped off the plate, and the obtained silica was extracted with chloroform. The extract was then evaporated and re-dissolved in $CDCL_3$ for NMR analysis.

NMR analysis
NMR analysis was used several times during the study either to determine relative purity of fractions or to identify major components present in fractions. ^{1}H-NMR spectra were recorded in $CDCl_3$, D_2O, and MeOD using a Bruker DPX 300 spectrometer, equipped with an Indy Silicon Graphics computer. For each sample, 64 scans were recorded with the following parameters: 32K data points, pulse width of 4.0 µs and relaxation delay of 1 second. FID's were Fourier transformed with LB of 0.5 Hz. For quantitative analysis, peak area was used after baseline correction.

Results

Cannabis root was extracted and fractionated in order to isolate and identify potential compounds with an inhibitory effect on Acetylcholine Esterase.

After the roots were washed, pulverized, and dried; the yield was 26.06 grams. The TLC analysis done with the first extractions showed similar compounds in fractions B-D and these were therefore combined. From that point the fractions were renamed as: fraction 1 (non-polar, fraction A), fraction 2 (medium polar, fraction B-D) and fraction 3 (polar, fraction E). NMR analysis showed that fraction 1 was composed mostly of lipids, fraction 2 contained mixed compounds, and fraction 3 contained mostly sugars.

TLC plates were sprayed for cannabinoids with fast blue b spray reagent, but none were detected. Another set of TLC plates was sprayed with dragendorf spray reagent to specifically detect alkaloids and results indicated possible presence of alkaloids in fraction 2.

Initial fractionation of the compounds present in fraction 2 was performed with Prep HPLC. AchE assay was done on all 31 fractions to detect possible bioactivity, but only false positive results were obtained. Also, no alkaloids could be detected after spraying with dragendorf. At this point, the trail of bioactivity was unfortunately lost. It was therefore decided to continue based on the fractions with the most visible compounds and the purest composition (based on TLC).

Preparative HPLC fractions 0, 14, 18, 20 were selected for NMR analysis, and the main components were tentatively identified as follows:
Fraction 0: glycoside of unknown structure
Fraction 14: saturated fatty acid
Fraction 18: fatty acid
Fraction 20: unsaturated fatty acid

Fraction 0 was the most interesting because glycosides are often biologically active. Because glycosides are hard to analyze as such, the fraction was hydrolyzed to remove sugar groups and yield the free aglycons. AchE assay was done on the organic phase, containing the aglycons of fraction 0 but the results were inconclusive. After the same organic phase was separated by using preparative TLC, the most intense band (Rf value: 0.49; see figure 10) was successfully scraped off and extracted. Finally the isolated compound was identified by NMR analysis as a type of lipid (see figure 11). However the exact identity could not be determined due to time constraints.

Discussion

The root of Cannabis, as an underground part of the plant, has been scientifically overlooked. Although the stem, flowers, seeds and leaves have received an overwhelming amount of attention, almost nothing has so far been published on the roots.

In this study I tried to find compounds in root extracts with an inhibitory effect on Acetylcholine Esterase. Often compounds which display this effect belong to the chemical class of alkaloids (e.g.: galanthamine). Alkaloids have been detected in cannabis roots, albeit at a very low level. The concentration of choline and neurine from dried roots is only 0.01% (Turner and Mole 1973). This could be the reason no alkaloids were found.

Roots are the primary storage organs of plants. The high content of lipids and sugars present in the used cannabis roots may therefore have complicated the isolation, analysis, and identification of minor compounds. In the final step of the bioassay guided fractionation, a lipid was identified. However, it is unclear whether this is really a biologically active compound or not, because the results on the second AchE assay were inconclusive. It may therefore be concluded that the glycoside (fr.0 from prep HPC) is the active compound and its activity was lost as a result of

hydrolysis. However, the inconclusive result of the final AchE test may also be due to residual acid from the hydrolysis still present in the sample.

In conclusion it was not possible to clearly identify compounds with an inhibitory effect on Acetylcholine Esterase in cannabis root. More sensitive and selective analytical methods and a wider range of bioassays are needed to further explore the medicinal potential of cannabis root.

My Personal Learning Experiences

Two years ago I started working for a doctor who employed cannabis therapeutics in her practice. We saw positive results on patients using cannabis root to treat staph infections. This experience sparked the initial idea for this project. In April 2008, I went to the Cannabis Therapeutics Conference and met several cannabis researchers. One in particular (Arno Hazekamp) has been amazing at helping me manifest this project as well facilitating my learning and development every step of the way.

Going to Bedrocan BV in Groningen, the Netherlands was a complete dream come true. I was blown away by the legality and quality of the entire facility. I was fortunate enough to get to meet several of the key people involved in Dutch cannabis policy: Tjalling Erkelens, Director of Bedrocan BV; John Jespers, Director of the cannabis crimes division; Peter van Dijk from the Drug information and monitoring system of the Trimbos Institute and all the members of The Dutch Association for Legal Cannabis and its Constituents as Medicine.

When I finally got to the lab at Leiden University I was struck with the hard reality that I had never had any experience in a chemistry lab. I was overwhelmed at first but quickly dove right in absorbed as much as I possibly could. Justin Fishedick and Andrea Lubbe (PhD students working with cannabis) were very helpful in the practical lab learning. I went to Isvett Flores-Sanchez's PhD thesis defense on cannabis and her work proved helpful for the literature review of this project.

I learned more and was challenged more in the past 3 months on this project than I ever have on any other school project. Even though my results were not what I expected or wanted my experiences in the lab taught me patience, humility and the importance of a good lab journal. I loved every minute of it. I am clearer about the direction of my studies and I look forward to doing more research in the near future. As much as I liked being in the lab, I also realized that I want to do more hands on practical field research (on industrial hemp).

I am profoundly grateful to have had this learning experience.

Characterization of Medicinal Properties of *Cannabis sativa* L. Roots

Literature Cited

Bott, C., Bishop, D. "Frequently Asked Questions About Cannabis**."** The Eldorado County Chapter of The American Alliance for Medical Cannabis. 29 September, 2008 <http://www.eldoradocountyaamc.com/faq.htm>

Duke, James A. *Cannabis sativa* L. 1983. Handbook of Energy Crops. unpublished Purdue University. 29 September 2008 <http://www.hort.purdue.edu/newcrop/duke_energy/cannabis_sativa.html>

El-Feraly, F.S. and Turner, C.E. "Alkaloids of *Cannabis sativa* leaves." Phytochemistry 14 (1975): 2304.

Elsohly, M.A. Turner, C.E., Pheobe, C.H., et al. "Anhydrocannabisativine, a New Alkaloid from *Cannabis sativa."* Journal of Pharmacy and Pharmaceutical Sciences 67 (1978): 124.

Flores-Sanchez, I.J. and Verpoorte, R. "Secondary Metabolism in Cannabis." Phytochemistry Reviews 7 (2008): 615-639.

Frank, Mel and Rosenthal, Ed. Marijuana Growers Guide. Berkeley: And/Or Press, 1978.

Hazekamp, PhD, Arno. Cannabis: Extracting the Medicine. Print Partners Ipskamp, 2007.

Indian Hemp. Dictonary. 29 September 2008 <http://dictionary.die.net/indian%20hemp>

Kabelik, J. Marijuana: History of Marijuana. A1B2B3. 29 September 2008 < http://www.a1b2c3.com/drugs/mj002b.htm>

Marijuana as Medicine: A Short History. June 2005. MedGadget. 29 September 2008 <http://medgadget.com/archives/2005/06/marijuana_as_me.html>

Nature's Pharmacy: Ancient Knowledge, Modern Medicine; Marijuana (Cannabis sativa). July 2007. U of Iowa Medical Museum. 29 September 2008 <http://www.uihealthcare.com/depts/medmuseum/galleryexhibits/naturespharmacy/marijuanaplant/marijuana.html>

Paris, M., Boucher, F., Cosson, L. "The Constituents of *Cannabis sativa* Pollen." Economic Botany 29 (1975): 245-253.

Potter, D., "Growth and Morphology of Medicinal Cannabis." P. 17-54 The Medicinal Uses of Cannabis and Cannabinoids. Guy, G.W., Whittle, B.A., Robson, P.J. (eds), London , UK, Pharmaceutical Press London, 2004.

Rhee IK, van de Meent M, Ingkaninan K, Verpoorte R. "Screening for acetylcholinesterase inhibitors from Amaryllidaceae using silica gel thin-layer chromatography in combination with bioactivity staining" Journal of Chromatography A 915(1-2) (2001): 217-23

Rumpf, G.E., and E.M. Beekman. 1981 The Poison Tree: Selected Writings of Rumpfius on the Natural History of the Indies. Library of the Indies. Amherst: University of Massachusetts.

Russo, Ethan. "Cannabis treatments in Obstetrics and Gynecology: A Historical Review." Journal of Cannabis Therapeutics Vol.2 No ¾ (2002): 5-35.

Sakaibara, I., Ikeya, Y., Hayashi, et al. "Three acyclic *bis*-phenylpropane lignanamides from fruits of *Cannabis sativa."* Phytochemistry 38 (1995): 1003-1007.

Slatkin, D.J., Doorenbos, N.J., Harris, L.S., et al. "Chemical Constituents of *Cannabis sativa* L. root." Journal of Pharmacy and Pharmeceutical Sciences 60 (1971): 1891-1892.

Stuart, G.A. Chinese Materia Medica. Shanghai: Presbyterian Mission 1928.

Tanaka, H. and Shoyama, Y. " Monoclonal Antibody Against Tetrahydracannabinolic Acid Distinguishes *Cannabis sativa* Samples from Other Plant Species." Forensic Science International 106 (1999): 135-146.

Turner, C.E., Mole, M.L. "Chemical Components of *Cannabis sativa."* Journal of the American Medical Association. 225 (1973): 639.

Wahby, I., Arrare-Roman, D., Segura-Carretero, A., et al. "Analysis of Choline and Atropine in Hairy Root Cultures of *Cannabis sativa* L. by Capillary Electrophoresis-Electrospray Mass Spectrometry." Electrophoresis 27 (2006): 2208-2215.

Other References

Conrad, Chris. Hemp for Health: The Medicinal and Nutritional Uses of Cannabis Sativa. Healing Arts Press, 1997.

Grotenhermen, MD, Franjo, Russo, MD, Ethan. Cannabis and Cannabinoids: Pharmacology, Toxicology, and Therapeutic Potential. The Hayworth Integrative Healing Press, 2002.

Russo, MD, Ethan. Cannabis: From Pariah to Prescription. The Hayworth Integrative Healing Press, 2003.

Characterization of Medicinal Properties of *Cannabis sativa* L. Roots

Figure Appendix

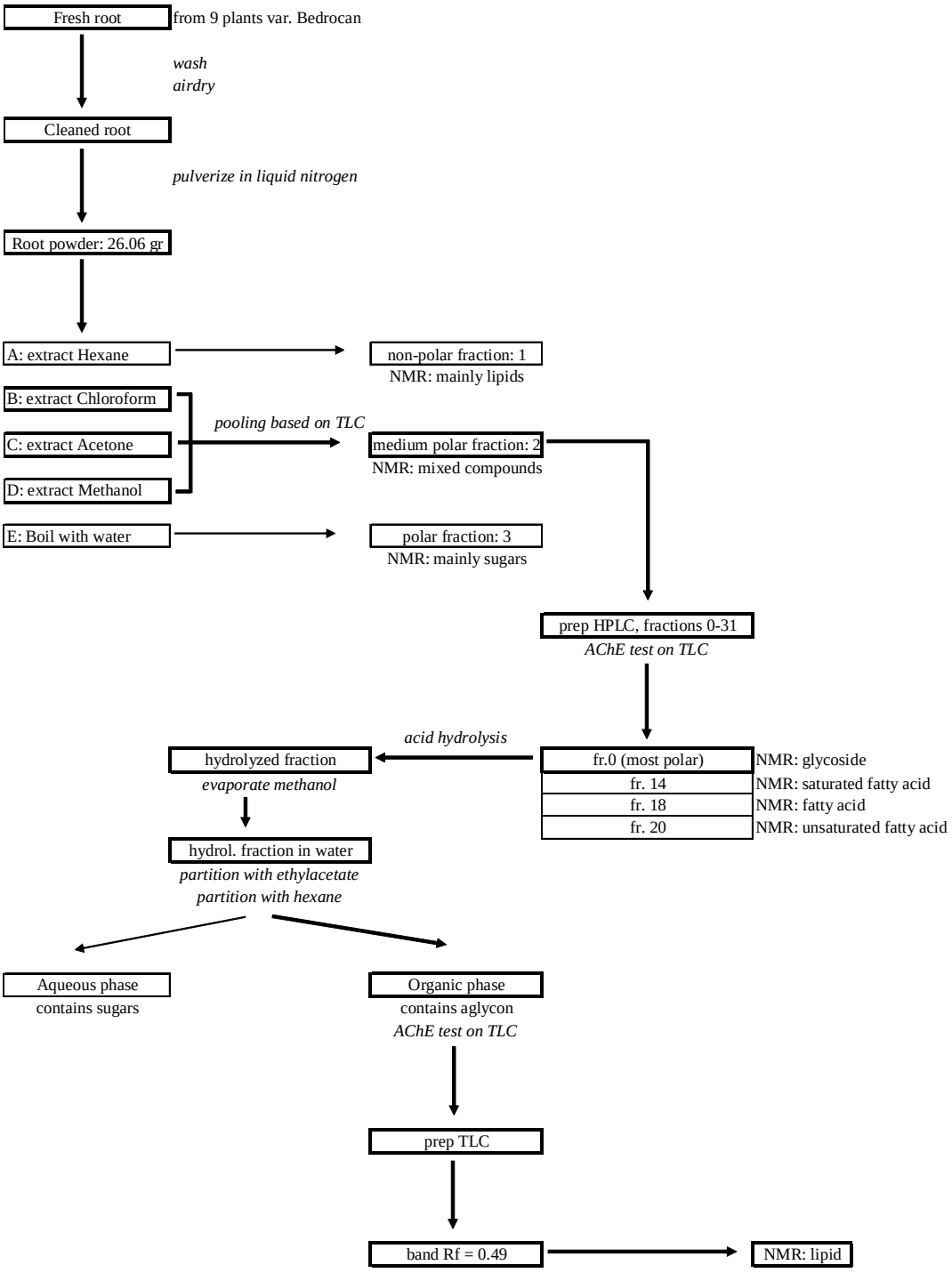

Figure 1. Lab-tree showing the flow of experiments performed in this study. Bold arrows indicate followed trail of activity.

Figure 2. Blair in the clean grow-room on harvest day.

Figure 3. Bedrocan grow-room during the harvest, root samples were taken from 9 of the pots visible here.

Figure 4. Blair in experimental cannabis grow-room, where new varieties are developed.

Figure 5. Fresh hemp root system. (not used in experiment).

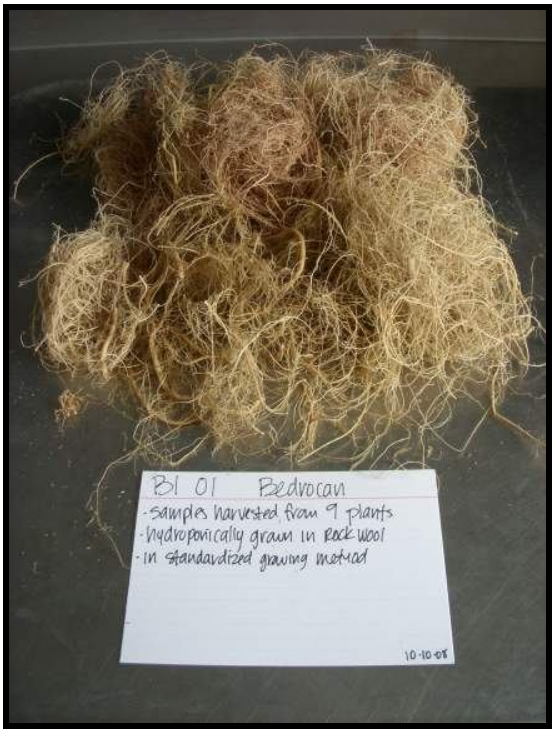

Figure 6. Root samples collected from Bedrocan for analysis, after washing and drying.

Figure 7. Blair pipeting cannabis extracts in the fume hood.

Figure 8. Blair spraying anisaldehyde on TLC plates.

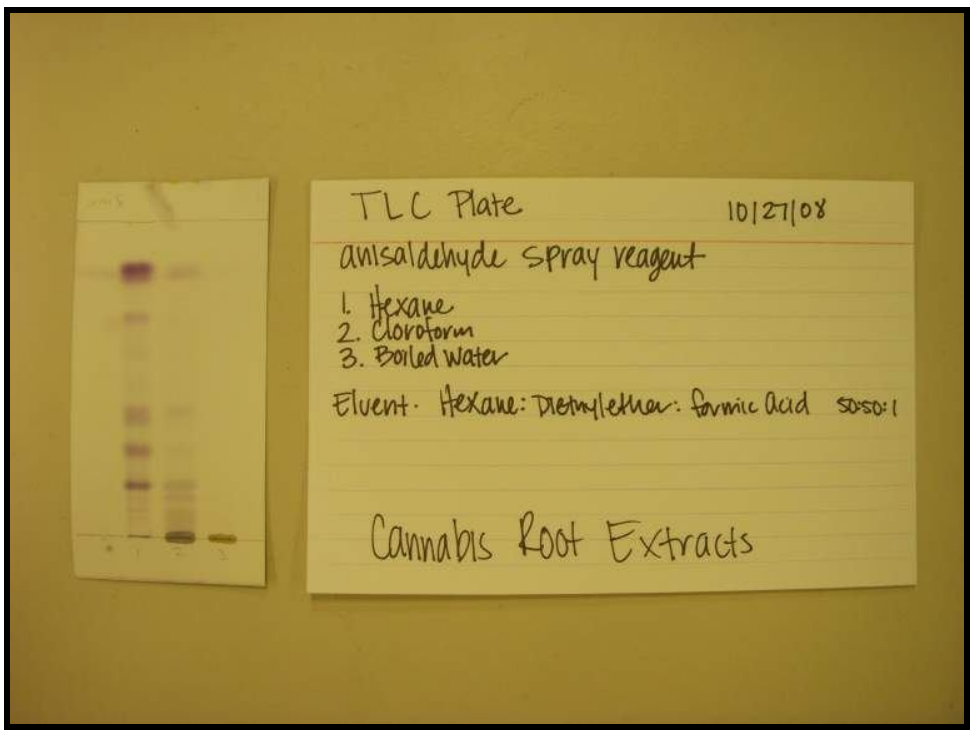

Figure 9. TLC plate after development.

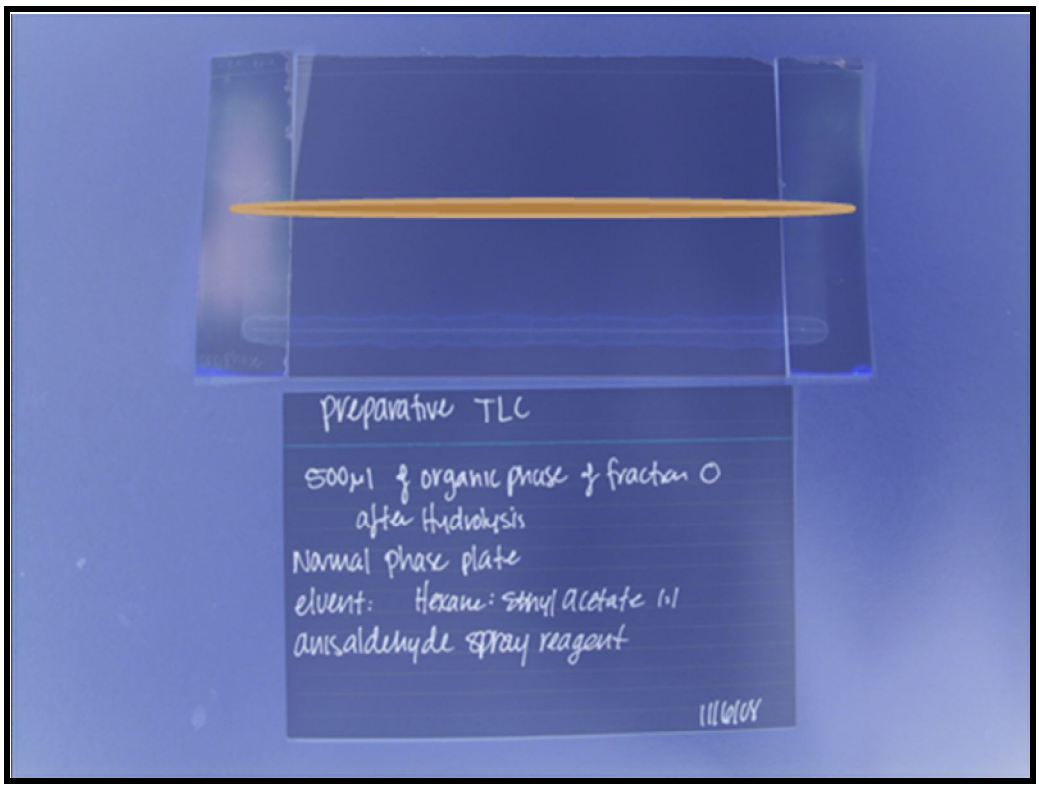

Figure 10. Preparative TLC plate before scraping off band (indicated in blue).

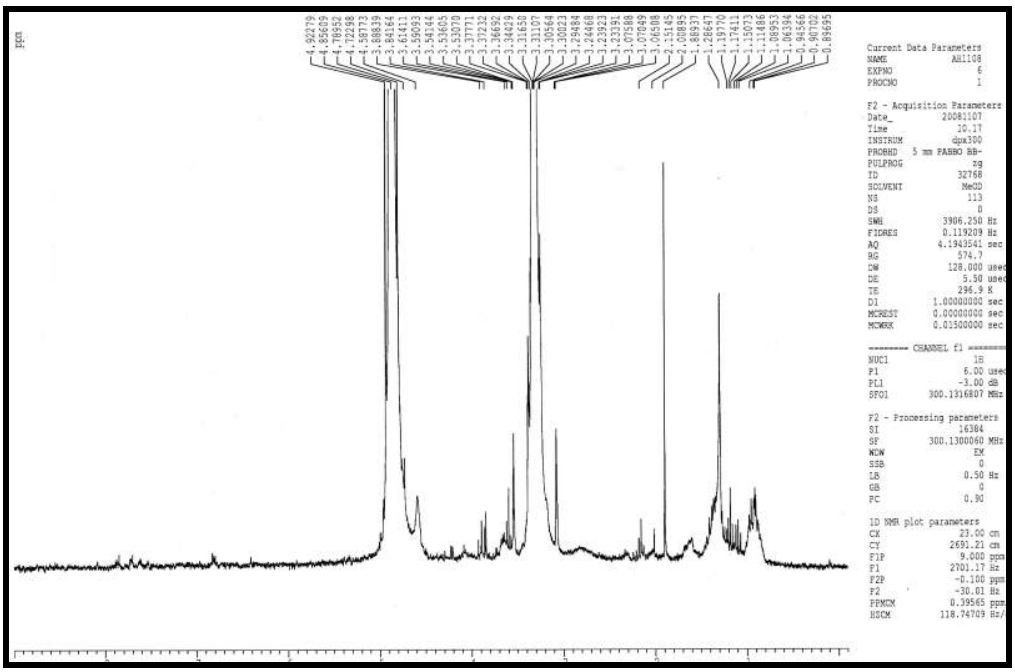

Figure 11. NMR spectrum of the final isolate indicating a further unidentified lipid.