Antioxidants in *Sutherlandia frutescens*, a medicinal plant in South Africa

By

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1. Summary

Sutherlandia frutescens (syn. *Lessertia frutescens*)has been used as traditional medicine in South Africa for numerous ailments. In this study, plant material was extracted with dichloromethane (DCM), methanol (MeOH) in a Soxhlet apparatus. And then the methanol extract was partitioned into ethyl acetate (EtOAc), 1-butanol (BuOH) and water. The dichloromethane extract, the ethyl acetate extract and the butanol extracts were subjected to further separation; several compounds were isolated by Versaflash normal phase column and reverse phase column, low pressure column chromatography with Sephadex LH20, Toyopearl and MCI CHP20P, and preparative high pressure liquid chromatography (HPLC). 1H- and 13C-NMR-spectroscopy were used to elucidate the structure of these isolated compounds. Antioxidant activity was measured by 1,1- diphenyl-2-picrylhydrazyl radical (DPPH)-radical scavenging, and inhibition of 15-lipoxygenase enzyme (15-LO) from soybeans.

Flavonoids and triterpenoids were suggested to be present by our South African collaborators, some of these substances have been isolated and identified from the EtOAc- and BuOH-extracts. A series of flavonoid glycosides with a hydroxymethylglutaryl moiety and a kaempferol or quercetin aglycone and the known triterpene glucoside sutherlandioside C were isolated. In addition, a substance which appears to be a C-24-epimer or a regioisomer of sutherlandioside C with the glucose moiety bound to C-24 instead of C-25) has been isolated. This is not reported in *Sutherlandia frutescens* before, and appears to be a new natural product. From preliminary data, the known sutherlandiosides B and D and their 24-epimers / regioiomers appear to be present, as well. In the DCM extract we found 4-hydroxybenzaldehyde. This is not an uncommon natural product, but it has not been reported previously from the genus *Lessertia* or *Sutherlandia*. And also in the DCM extract there was found an unknown compound, which might be 6- or 7-methoxylated chromanone, isocoumarin or dihydrobenzofuran. Calculated NMR spectra for these compounds are, however, not in accord with observed data.

The DCM extract and the EtOAc extracts showed higher 15-LO activity than other extracts. In the DPPH-test all extracts had low radical scavenging activity.

2. Acronyms

Arconyms	Meaning
Aceton-d6	Deuteroacetone
ACN	Acetonitrile
CD_3OD	Deuteromethanol
$CDCl_3$	Deuterochloroform
$CHCl_3$	Chloroform
COSY	Correlated spectroscopy
COX	Cyclooksygenase
D_2O	Deuteriumoxide
DAG	Diacylglycerol
DFR	Dihydroflavonol reductase
DMSO	Dimethylsulfoxide
DNA	Deoxyribonucleic acid
DPPH	1,1-Diphenyl-2-picrylhydrazyl
EDRF	Endothelium-derived relaxation factor
EtOAc	Ethyl acetate
EtOH Ethanol	
GSH	Glutathion (reduced form)
GSSG	Glutathion (oxidized form)
13-HPODE	13-hydroperoxy-(9Z,11E)-octadecadienoic acid
H_2O_2	Hydrogen peroxide
HCl	Hydrogen chloride
HETE	Hydroeicosatetraenoic acid
HPETE	Hydroperoxyeicosatetraenoic acid
HPLC	High performance liquid chromatography
ICD_{50}	The concentration which shows 50 % inhibition of 15-lipoxygenase enzyme
LDL	Low density lipoprotein
LO	Lipoxygenase
MeOH	Methanol
N_2-gas	Nitrogen gas
NADPH	Nicotinamide adenine dinucleotide phosphate
NMR	Nuclear magnetic resonance
$^1O_2$	Singlet oxygen
$O_2^{\cdot -}$	Superoxide
OH·	Hydroxy radical
ppm	Parts per million
ROS	Reactive oxygen species
SD_{50}	The concentration which shows 50% radical scavenging activity
SD	Standard deviation
SOD	Superoxide dismutase
TLC	Thin layer chromatography
TMS	Tetramethylsilane

UV	Ultra violet
δ	Chemical shift

3. Aim

The aim of this study was to isolate and identify compounds in *Sutherlandia frutescens*, and to study the biological activity of the extracts and also isolated compounds of this plant.

Free radicals and reactive oxygen species (ROS) are involved in several pathological states and 15-lipoxygenase enzyme (15-LO) is believed to be involved in the development of atherosclerosis. These are reasons why it is of interest to find compounds with antioxidant character and inhibition of 15-LO which may influence these pathological states.

4. Introduction

4.1 Sutherlandia frutescens (syn. Lessertia frutescens)

Sutherlandia frutescens belongs to the Fabaceae (Leguminosa) family which is the third largest family of flowering plants. It is a small lax spreading shrub, which can be about 1.2 meters in height. The branches are hanging down, and the leaflets are from slightly to densely hairy. The plant has red flowers, and blooms between July and December. The species is found in areas of the South Western and Northern Cape Provinces and can also be found in Botswana, Zimbabwe and Namibia (Mncwangi and Viljoen 2007).

4.2 Traditional uses of Sutherlandia frutescens

In 1918, the plant was use to treat pandemic flu in South Africa. The root and particularly the leaves of *Sutherlandia frutescens* are widely use in traditional medicine in southern Africa. It has been used by several cultural groups like Zulu, Cape Dutch, San, Khoi, Sotho and Nguni-speaking people. *S.frutescens* can also calls ”cancer bush” in Afrikaans ”kankerbos”. After large scale cultivation by a company called Phyto Nova since 2000, *S. frutescens* became popular as an adaptogenic tonic, and is as said to have an appetite stimulant effect (Mncwangi and Viljoen 2007; Van Wyk and Albrecht 2008).

S.frutescens is used for a wide diversity of ailments like diabetes mellitus, internal cancer, stress, lack of appetite, indigestion, heart burn, reflux esophagi, stomach ulcer, wounds, antithrombotic, kidney and liver problems and many more. More recently the plant is used in therapy for anorexia, HIV/AIDS, tuberculosis and cancer (Munk 1997; Duggan et al. 2001; Colebunders, Dreezen et al. 2003; Dalvi 2003; Fernandes et al. 2004; Chinkwo 2005; Katerere 2005; Harnett et al. 2005; Mills, Cooper et al. 2005; Mills, Foster et al. 2005; Chadwick et al. 2007; Van Wyk and Albrecht 2008)

4.3 Chemistry

Van Wyk (1997) has found that *S. frutescens* contains bioactive compounds including GABA (γ-aminobutyric acid), L-canavanine and pinitol. Tai et al. (2004) used GC-MS and LC-MS methods, and have confirmed the appearance of canavanine, but also found other compounds such as L-arginine, pinitol, γ-aminobutyric acid (GABA), asparagine and secondary plant metabolites like saponins (triterpene glycosides). Flavonol glycosides were reported by Moshe et al. (1998).

4.3.1 Potential effects of the compounds found in *S. frutescens*

- Canavanine (a non-protein amino acid) and L-arginine: L-canavanine is a guanidinooxy structure analog of L-arginine. The anticancer effect and anti-metabolite effect of L-canavanine has been confirmed in several studies (Rosenthal 1977; Rosenthal 1997). It has been shown that L-canavanine is a selective inhibitor of inducible nitric oxide synthase, during rodent endotoxaemia (Liaudet et al. 1996). Canavanine is a growth inhibitor in some bacteria cultures (Volcani and Snell 1948), has antiviral activity and antitumor effect (Green et al. 1980; Green 1988).

- γ-Aminobutyric acid (GABA): GABA is the main inhibitory transmitter in the brain (Rang et al. 2003). GABA may have an inhibitory effect of tumor cell migration (Ortega 2003).

- Pinitol: D-pinitol, the 3-methoxy analogue of D-chiroinositol, known as an antidiabetic agent, exerts an acute and chronic insulin-like anti-hyperglycemic effect in STZ-diabetic mice, and might be involved in an interaction with a part of cellular signal pathway that links insulin to glucose transport (Bates et al. 2000). It is also found that (+)-pinitol has anti-inflammatory effect (Singh et al. 2001).

- Saponins: see section 5.8, p. 23 Saponins (triterpene glycosides).
- Flavonoids: see section 5.7, p. 22 Flavonoids.

5. Antioxidants and free radicals

5.1 Antioxidants

Antioxidants are substances which are capable of slowing or preventing the oxidation of other molecules, e.g. inhibition of lipid peroxidation (Gutteridge and Halliwell 1994). The human body contains substrates for peroxidation like proteins, lipids, nucleic acids and carbohydrates. Antioxidants are important, because of reactive oxygen species (ROS) are formed as a result of pathological processes as well as normal cellular metabolic reactions. Ions from metals can catalyze redox reaction of a reactive oxidant. Antioxidants may prevent oxidative damage by removing catalysts, a process in which they are used up as the reaction proceeds, repair damage to the target, and destroying badly damage target and replace with a new one (Gutteridge and Halliwell 1994; Halliwell 2007).

5.2 Free radicals

A free radical is a molecule with one or more unpaired electron in outer orbit, which has ability to exist as an independent molecule. There are many types of free radicals and theirs chemical reactivity varies, but in general free radicals are more reactive than normal molecules. Examples of oxygen free radicals are superoxide ($O_2^{\cdot -}$) and hydroxyl radical ($OH^{\cdot}$). ROS are oxygen radicals and also non-radical derivatives of oxygen, such as singlet oxygen ($^1O_2$) and hydrogen peroxide (H_2O_2). ROS are important because they may be as highly reactive as free radicals. ROS may be involved in process generating free radicals (Halliwell 2007).

5.2.1 Formation of free radicals and reactive oxygen species

In aerobe metabolism, oxygen reacts with many different molecules and makes oxygen-centered radicals. Oxygen is a major constituent of the human body, and hemoglobin in the red blood cells, a transport protein, is evolved to carry oxygen round in the human body. Oxygen free radicals are constantly generated in the human body in process like in making energy, decomposition of lipids and proteins, catecholamine response and in inflammation process. Superoxide (O_2^-) and hydroxyl radical ($OH^{\cdot}$) are formed when oxygen attracts one

or three electrons. Mitochondria are cell organelles that consume oxygen during energy production (Gutteridge and Halliwell 1994; Kerr et al. 1996) .

Some reactive free radicals are made by 'accidents of chemistry'; for example, leakage of electrons directly onto oxygen from the intermediate electron carriers of the mitochondrial electron transport chain generates a steady stream of $O_2^{\cdot -}$. Exposure of living organisms to ionizing radiation splits the O – H bond in water (an important constituent of living cells) homolytically to generate $OH^{\cdot}$ and $H^{\cdot}$. $OH^{\cdot}$ will damage whatever it is generated next to, and the harmful effects can be seen is when $OH^{\cdot}$ attacks proteins, DNA and lipids (Stogner and Payne 1992; Halliwell 2007).

Other free radicals, except $OH^{\cdot}$, may be useful *in vivo*. For example superoxide ($O_2^{\cdot -}$) is produced by phagocytic cells and helps them to kill bacteria. $NO^{\cdot}$ is involves in many process such as killing of parasites by macrophages and helps to regulate blood pressure ($NO^{\cdot}$ is identical to EDRF; endothelium derived relaxation factor). And $NO^{\cdot}$ is synthesized from the amino acid L-arginine by vascular endothelial cells, phagocytes and many other cell types. H_2O_2 can be produced by the action of several oxidase enzymes in cells, and is used by the enzyme to help make thyroid hormones, and sometimes H_2O_2 can act like a second messenger (Kerr et al. 1996; Halliwell 2007).

5.2.1 Antioxidant defences

The human body has formidable antioxidant defence systems which can scavenge and minimize the formation of ROS, but they aren't 100 % effective. Therefore there are repair systems which exist to heal or manage molecules which have been oxidatively damaged (Halliwell 2005). The defence system consists of several enzymes and low-molecular-mass free radical compounds. Many enzymes are able to repair or remove the unwanted products involved in oxidative damage, like superoxide dismutase enzymes (SODs) which removes $O_2^{\cdot -}$ by convert it to H_2O_2. These enzymes are found in mitochondria and cytosol (Halliwell 1994; Halliwell 2007).

5.2.2 Enzyme defence:

- Superoxide dismutase (SOD)

  SOD is one of the most effective intracellular antioxidant enzymes, and catalyzes converting of superoxide to hydrogen peroxide (Halliwell 2005).

  Conversion of superoxide to hydrogen peroxide:

  $$2O^{\bullet}_2{}^- + 2\ H^+ \rightarrow H_2O + O_2$$

  The three major classes of antioxidant enzymes are the superoxide dismutase enzymes (SOD), the catalases, and glutathione (GSH) peroxidases. These specialized antioxidant enzymes react with and, in general, detoxify oxidants. For example, glutathione peroxidases (GSHPX) remove H_2O_2 by using it to oxidize reduced glutathione (GSH) to the oxidized form, glutathione disulphide (GSSG). Glutathione reductase (GSSH), a flavoprotein enzyme, regenerates GSH from GSSG, using NADPH (reduced nicotinamide-adenine dinucleotide phosphate) as a source of reducing power (Halliwell 2005).

- Catalase enzymes

They convert hydrogen peroxide to water and oxygen (Halliwell 2005).

Reduction of hydrogen peroxide to water:

$$2\ H_2O_2 \rightarrow 2\ H_2O + O_2$$

- Glutathione peroxidases

These enzymes are selenoenzymes, since they require selenium for their action and remove hydrogen peroxide by oxidizing reduced glutathione (GSH) to oxidized glutathione (GSSG). Glutathione reductase regenerates GSSG to GSH by using NADPH as source of reducing power (Halliwell 2005).

$$2\ GSH + H_2O_2 \rightarrow GSSG + 2\ H_2O$$

$$GSSH + NADPH + H^+ \rightarrow 2\ GSH + NADP^+$$

5.2.3 Low-molecular-mass free radical scavengers:

In addition to enzymes, many low-molecular-mass free radical scavengers exist. α-Tocopherol (derived from diet, as vitamin E) occurs in membranes and lipoproteins. It blocks the chain reaction of lipid peroxidation by scavenging intermediate peroxyl radicals. From the diet, the body gets several other antioxidants, including vitamin C and flavonoids (Halliwell 2005).

5.3 Oxidative stress

In the body there is always a balance between antioxidants and generation of free radicals. Oxidative stress occurs when this balance is disturbed in favor of free radicals and ROS, and the body has not enough antioxidant to defend itself (Halliwell 2005). The term is referred by (Halliwell 2005) as "the situation of serious imbalance between production of reactive species and antioxidant defence". Most cells can tolerate mild oxidative stress, since the cells have repairing mechanisms which recognize and remove free radical-damaged molecules and replace them. Severe oxidative stress can lead to disturbed cell metabolism leading to increasing of intracellular Ca^{2+}, DNA fragmentation, lipid peroxidation and protein damage. All of these processes can induce cell damage or cell death as necrosis or apoptosis (Gutteridge and Halliwell 1994; Sies 1997; Halliwell 2007).

Oxidative stress can result from diminished antioxidants e.g. mutation affecting antioxidant defence enzymes, or from increasing production of reactive species e.g. by exposure of toxins that are themselves reactive species (Halliwell 2005).

5.4 Lipid peroxidation

Lipid peroxidation is oxidative attacks on cholesterol and phospholipids containing unsaturated fatty acyl moieties. Biological membrane structures contain lipid bilayers of phospholipids. The polyunsaturated fatty acids are vulnerable to radical-attack leading to lipid peroxidation. Peroxidation may cause damage on membrane lipids and proteins, and releasing of antioxidant storage in membranes (Gutterigde and Halliwell 1994).

There are two types of lipid peroxidation, enzymatic and non-enzymatic lipid peroxidation. Enzymatic lipid peroxidation is controlled peroxidation of fatty acids and gives hydro-peroxides and endo-peroxides as its outcome. The enzymes cyclooxygenase and lipoxygenases catalyze these reactions. Non-enzymatic peroxidation may be caused by ROS, metal ions and hydrogen peroxides (Halliwell 1995; Sies 1997).

Non-enzymatic peroxidation starts when a radical which is reactive enough removes one hydrogen atom from a methylene group between two double bonds in a fatty acid. It will generate a fatty acid radical which is unstable, and the molecule is then stabilized by rearrangement to a conjugated diene. The fatty acid radical reacts with oxygen and generates peroxide radical. A peroxide radical can attack another fatty acid, starting a chain reaction by a formation of a new fatty acid radical and a lipid hydroperoxide. Lipid hydroperoxides can generate cyclic peroxides and cyclic endo-peroxides, these molecules can continue fragmentation and give rise to different aldehydes like malondialdehyde (Halliwell 2007).

5.5 Pathological importance

The damage on the body tissues can lead to further free radicals and ROS generation. Oxidative damage can play an essential role in several diseases. In most cases, increased oxidative activity is a consequence of, and not a cause of the disease. Oxidative damage can contribute to exacerbate the state of the disease (Halliwell 2005; Halliwell 2007).

5.5.1 Atherosclerosis

It is suspected that atherosclerosis starts with damage on endothelial walls in blood vessels, which may involve oxygen free radicals (Kerr et al. 1996). Monocytes are attracted to the injury and move from blood into the endothelial tissue, where they transform to macrophages. Macrophages secrete O_2, H_2O_2 and cause a local oxidative stress, leading to peroxidation of LDL (low density lipoprotein). Oxidized LDL is recognized by macrophage scavenger receptors, and internalized to form “foam cells”. Foam cells are a pre-state of

atherosclerosis in the arterial wall. The oxidized LDL has direct chemotactic activity for monocytes and stimulates the binding of monocytes to the endothelium. It also stimulates macrophages to excrete growth factors which again stimulate smooth-muscle cell formation at the injury spot. Accretion of smooth muscle cells and lesions in arterial walls lead to constricting of arteries, and reduces blood supply to organs like heart and brain. Stroke or heart failure occurs when a blood vessel is completely blocked, usually by thrombosis formation around lesions (Halliwell 2005; Thomson et al. 2007).

5.5.2 Rheumatoid arthritis

Rheumatoid arthritis is a chronic inflammation in joints, which causes a painful swelling and loss of functioning and mobility. The rheumatoid joint is a center for intense oxidative stress. Macrophages and neutrophiles are present and generate O_2, H_2O_2, HOCl, NO· and other potential prooxidants. Bleeding in joints can increase iron concentration, and lead to formation of OH· (Gutteridge and Halliwell 1994).

5.5.3 Cancer

The first step in the development of cancer is a reaction between evoking substances and DNA, which induces injury to the genome. The damage on DNA in regions which regulate cell formation and cell growth can lead to uncontrolled cell proliferation and formation of cancerous tumors. ROS can react with DNA and make different modified purine- og pyrimidine bases. ROS do not initiate cell formation, but cancer can develop if these injuries are not repaired and cells are exposed to stimuli which start cell formation (Halliwell 2007).

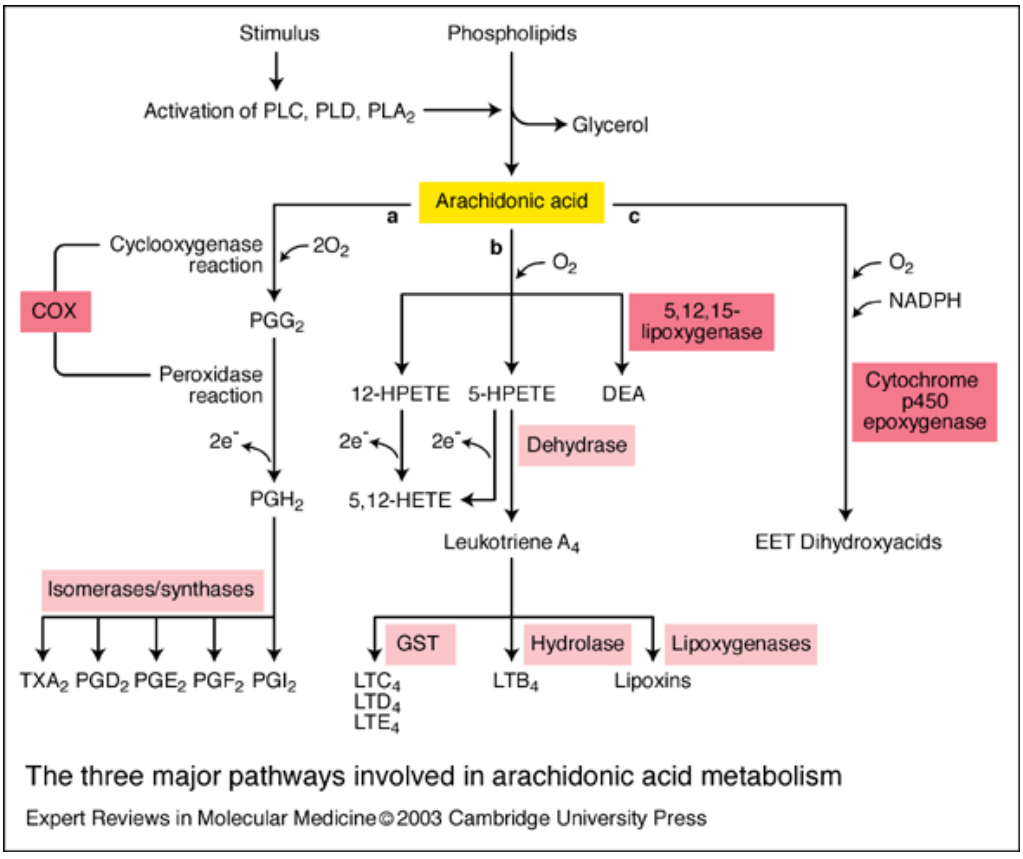
5.5.4 Alzheimer

Alzheimer´s disease is a major dementing disorder among elderly patients. An important element of the pathology is decrease of cholinergic transmission. It is known that formation of neurofibrillary tangles, containing polymerized and hyperphosphorylated tau protein, and senile plaques containing β-amyloid peptide are involved in Alzheimer. It is suspected that reactive oxygen species might be involved in Alzheimer´s disease, since oxidative stress in Alzheimer is manifested by for example lipid peroxidation which has been detected with

various indices, advanced glycation end products and free radical formation (Casetta, Govoni et al. 2005).

5.6 Eicosanoid biosynthesis and 15-lipoxygenase

Eicosanoids is a term for physiologically active derivatives which are made by metabolism of arachidonic acid, a C_{20}-unsaturated fatty acid which contains four double bonds. They are prostaglandins, leukotrienes, tromboxanes, hydroperoxyeicosatetraenoic acids (HPETE), and hydroxyeicosatetraenoic acids (HETE) (Smith 1989; Rang, Dale et al. 2003; Samuelson 2004). Eicosanoids are produced *de novo* from phospholipids in the human body, and are involved in many physiological processes. Eicosanoids are some of the most important mediators and modulators in inflammation processes (Rang, Dale et al. 2003).



The three major pathways involved in arachidonic acid metabolism

Releasing of arachidonic acid from phospholipids is the initial and rate determining step in eicosanoid synthesis. This can happen in three ways: (1) by the action of phospholipase A_2

directly, (2) by the action of phospholipase C followed by diacylglycerol lipase or (3) by the action of phospholipase D following by phospholipase A_2. This release of arachidonic acid may be caused by different stimuli for example proteolytic or hormonal stimuli. The kind of stimulus depends on cell type, since prostanoids are stored by cells. Thrombin in bloodplates, bradykinin in fibroblasts, antigen-antibody reactions in mast-cells and cell damage may initiate arachidonic acid release (Smith 1989; Fonteh et al. 1994; Rang et al. 2003)

Arachidonic acid will be transformed by several enzymes into different eicosanoids. Cyclooxygenase-1 and -2 (COX) catalyze the formation of prostaglandins, prostacyclin and thromboxanes, while lipoxygenases (5-, 12- og 15-LO) act on arachidonic acid to form hydroperoxy fatty acid, which will give leukotrienes and lipoxins.

Eicosanoids have many different effects; depending on which tissue types and receptors they react with. Prostaglandins have effects like vasodilation (PGI2, PGD2), inhibition of platelet aggregation (PGI2, PGD2), contraction or relaxation of smooth muscles (PGE2), inhibiting of acid secretion in the stomach (PGE2) and increasing segregation of mucus in the stomach (PGE2). Thromboxane (TXA2) gives platelet aggregation and vasoconstriction. Leukotrienes trigger contraction of bronchial muscles, vasodilation in most vessels, coronary vasoconstriction, activating of monocytes, and they stimulate proliferation and cytokine production from macrophages and lymphocytes. Lipoxins are involved in inflammation responses (Rang et al. 2003)

Research has showed that 15-LO has an important role in the progression of human diseases like cancer, psoriasis and atherosclerosis (Steinberg 1999; Schneider and Bucar 2005). 15-LO has the ability to oxidize esterified fatty acids in biological membranes and in LDL, which is an important step in the formation of atherosclerotic lesions. Furthermore, inhibition of 15-HPETE, an intermediate in production of lipoxins, leads to increased synthesis of prostacyclin which promotes vasodilation and counteracts platelets aggregation (Schneider and Bucar 2005). 15-LO inhibors without antioxidant effect have been shown to inhibit atherosclerosis progression in research animals (Sendobry 1997).

5.7 Flavonoids

Flavonoids constitute a large, natural group of phenolic compounds. They are widely distributed in Nature and are found in almost all higher plants (Malterud 1998). Their main structure contains two aromatic rings, bound together by a 3-carbon chain, which is usually cyclized to a pyrane ring. Flavonoids usually contain several phenolic hydroxyl groups, and are classified in several subgroups, depending on structure variation. The most common subgroups are flavones, flavonols, flavandiols, anthocyanins and isoflavonoids.

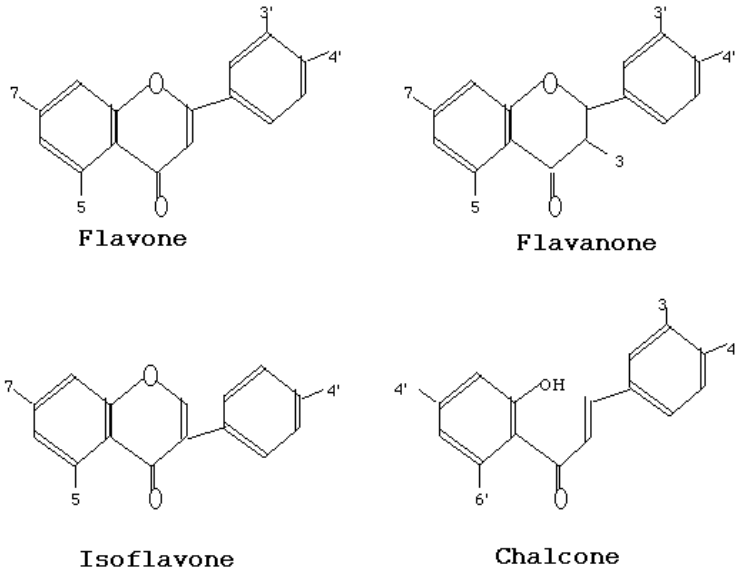


Fig. 1. Structure of some flavonoids.

Flavonoids are known as antioxidants, and that is why they are an important part of diet, with a potential to prevent diseases. They have the ability to scavenge free radicals like superoxide and hydroxyl, and inhibit any damage caused by them (Havsteen 1983). Flavonoids have many other valuable effects, like decreasing leakage through capillary vessel walls, counteraction of aggregation of blood platelets and inhibition of 15-LO which is suspected to play a major role in progression of atherosclerosis. Other interesting biological activities of flavonoids are anti-inflammatory, anti-hepatotoxicity, antitumor, antimicrobial, antiviral and estrogen effects (Malterud 1998; Pietta 2000; Samuelson 2004).

The most important relationships between structure and antioxidant / radical scavenging activity for flavonoids have been defined as follows (Rice-Evans, Miller et al. 1996):

- Ortho 3',4'-dihydroxy substitution on B-ring (give the most stable phenoxyl radical due to electron dislocation).
- Meta 5,7-dihydroxy substitution in A-ring.
- 2,3-double bound in combination with both 4-keto group og 3-hydroxyl group in the C-ring (for electron dislocation) as long as there is an ortho dihydroxy structure in the B-ring
- Changes in substitution position of hydroxyl groups and substitution of hydroxyl groups by glycosylation usually will lead to reduced antioxidant activity.

5.8 Saponins - Triterpenes

Saponins are complex compounds in plants and animals, which are composed of a saccharide attached to a steroid or triterpene. The name saponin is derived from a latin name meaning "soap", since they were used to make soap for hundreds of years. They are characterized by their surfactant and cholesterol binding properties, and give stable foam when shaken with water (Osbourn 1996; Lacaille-Dubois 1999). They have many biological and pharmaceutical activities like anti-allergic, cytotoxic, anticancer, antimicrobial, immune modulating, anti-hepatotoxic, antifungal properties.

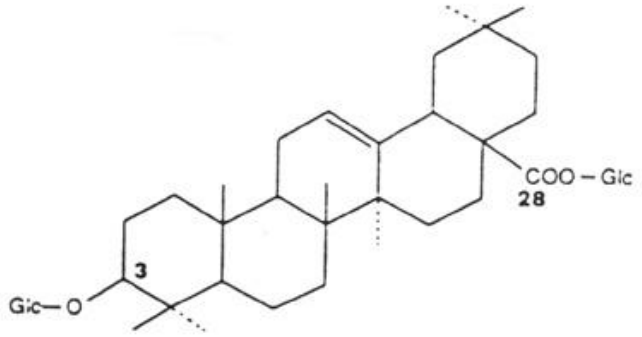


Fig. 2. Structure of a typical saponin

They have been used as adjuvant in vaccines, since they can increase the specific immune response for antigen and induce immune defense. Saponins are used as adjuvants in vaccines for animals. Saponins as adjuvants in human vaccines are currently not available, because of their hemolytic effect and their propensity to give skin reactions (Lacaille-Dubois 1999).

6. Methods

6.1 Separation and identification methods

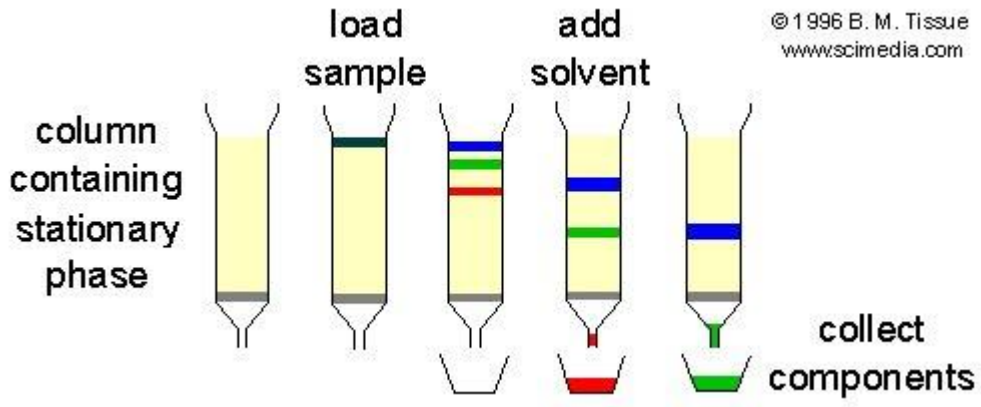
6.1.1 Liquid-liquid extraction (LLE)

Liquid-liquid extraction is a classic method which is often a part of processing of plant extracts of unknown composition. LLE is easy to perform, and its significant savings in operating costs can be achieved by fine-tuning extraction systems. The separations of two or more components are due to their unequal solubility in two immiscible liquid phases. A feed solution containing one or more solutes is thoroughly mixed with an immiscible solvent having a different density. Two phases will then be formed – the continuous phase (usually water) and the dispersed phase (usually organic solvent). The assumption is that the wanted analyte(s) has high affinity in the dispersed phase, so that it can be removed from the continuous phase. Therefore the choice of solvent for dispersed phase is essential in liquid – liquid extraction. And also, the solvent in dispersed phase shall have high selectivity and high affinity so that unwanted components remain in the continuous phase. The solvent should have low toxicity and preferably not be flammable, since a large amount of solvent is usually used in LLE. Bases are extracted into the dispersed phase with pH 2-3 units higher than their pKa-value, from the continuous phase, while acids in continuous phase are extracted into the dispersed with pH 2-3 units lower than their pKa-value (Greibrokk et al. 1998; Pedersen-Bjergaard and Rasmussen 2004).

6.1.2 Low-pressure column chromatography (LPCC) and Versaflash.

Chromatography is a separation method that relies on differences in partitioning behavior between a mobile phase and a stationary phase to separate the components in a mixture. Liquid chromatography (LC) is an analytical chromatographic technique that is useful for separating ions or molecules that are dissolved in a solvent. The separation is achieved on the basis of different speeds of transportation or difference in retention for different molecules (Greibrokk et al. 1998). If the sample solution is in contact with a second solid or liquid phase, the different solutes will interact with the other phase to differing degrees due to differences in adsorption, ion-exchange, partitioning, or size. These differences allow the

mixture components to be separated from each other by using these differences to determine the transit time of the solutes through a column. LPCC involves two phases (a stationary and a mobile phase), the mobile phase which is transported through the column and the stationary phase which remains in the column. When a mixture of solutes is applied in the apparatus and pumped into the column, it will be transported along the column with different speeds for different constituents, depending on their interactions with the stationary phase. The two phase system is necessary, since differences in speed of migration are caused by chemical interactions between the molecules of the two chromatographic phases. The applied solutes will distribute over the two phases. A solute with a high affinity towards the stationary phase will be transported slower through the column, and a solute which has low affinity towards the stationary phase or does not enter stationary phase at all will be transported fast or at the same speed at which the mobile phase is transported through the column. The choice of mobile phase depends on which components are to be separated. Often, the mobile phase is a mixture of two or more solvents, and elution can be undertaken as isocratic or gradient elution.. Depending on the choice of packing material of the stationary phase, several separation mechanisms are available (Heftmann 1975; Cannell 1998; Greibrokk et al. 1998; Pedersen-Bjergaard and Rasmussen 2004).



Schematic of a simple liquid chromatographic separation

In VersaFlash column separation basic principles are the same as in LPLC, and a prepacked VersaPak column with polar or nonpolar stationary phase is employed.

VersaFlash apparatus (Sigma-Aldrich, 2009)

Here are some of the packing materials for the column:

- **Silica (VersaFlash)**

In normal phase chromatography, silica is the most used stationary phase. Silica has a very large surface area, since it is a porous material. Silica consist of silanol groups (Si-OH), which are the active groups, and give the surface polar and weak acidic character (Pedersen-Bjergaard and Rasmussen 2004). The chemical interaction with substances is through hydrogen bonds, in which the surface hydroxyl groups are the proton donor. The acidic character makes amines and other bases strongly adsorb, and they are eluted slowly (Greibrokk et al. 1998; Pedersen-Bjergaard and Rasmussen 2004).

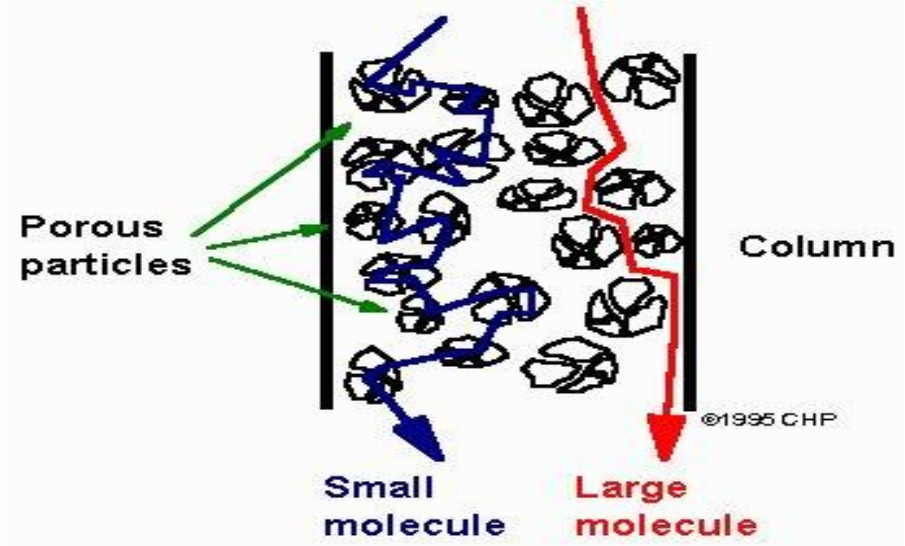
- **Reverse phase C18-bonded silica (VersaFlash)**
  Reverse phase chromatography uses a nonpolar stationary phase and a polar mobile phase. The most common chemical interaction in reverse phase chromatography is van der Waals type bonding between stationary material and solute(s) (Van der

Waals type interaction is a weak interaction, which increases with increasing molecules size). The degree of adsorption to reverse phase silica gel is proportional to the lipophilicity of the compounds being chromatographed, and mobile phase used are usually aqueous. The polar interaction is not significant here, because of the aqueous mobile phase, which counteracts this type of interaction. Substances will be eluted in order of decreasing polarity. Modified silica with $C_{18}H_{37}$-groups bonded is often used in this case (Greibrokk et al. 1998; Pedersen-Bjergaard and Rasmussen 2004).

6.1.3 Size-exclusion chromatography (SEC)

Size-exclusion chromatography (SEC), also called gel-filtration or gel-permeation chromatography (GPC), uses porous particles to separate molecules of different sizes. It is generally used to separate biological molecules, and to determine molecular weights and molecular weight distributions of polymers. Molecules that are smaller than the pore size can enter the particles and therefore have a longer path and longer transit time than larger molecules that cannot enter the particles (Cannell 1998; Pedersen-Bjergaard and Rasmussen 2004).

Schematic of a size-exclusion chromatography column



Molecules larger than the pore size can't enter the pores and elute together as the first peak in the chromatogram. This condition is called total exclusion. Molecules that can enter the pores will have an average residence time in the particles that depends on the molecules size and shape. Different molecules therefore have different total transit times through the column. This portion of a chromatogram is called the selective permeation region. Molecules

that are smaller than the pore size can enter all pores, and have the longest residence time on the column and elute together as the last peak in the chromatogram. This last peak in the chromatogram determines the total permeation limit (Pedersen-Bjergaard and Rasmussen 2004).

Following are some of the gel materials use in SEC or GPC:

- **Sephadex LH-20**

  Sephadex LH-20 is made by hydroxypropylation of Sephadex G-25, and it is a dextran gel which is made of cross-linked dextran chains to give a three dimensional polysaccharide network. The hydroxypropyl groups are attached by ether linkages to glucose units of the dextran chains. This gel has dual lipophilic and hydrophilic properties. Sephadex LH-20 swells well and is also stable in solvents with different polarity. The degree of swelling increases with increasing polarity of the solvent (= mobile phase). Sephadex LH- 20 is used in gel filtration, which separates molecules according to their size. Substances are eluted from columns of Sephadex in order of decreasing molecular size. An additional advantage with Sephadex is adsorption separation, because Sephadex LH-20 has an affinity for aromatic and cyclic compounds. These characters of Sephadex LH-20 vary due to different mobile phases (Henke 1995; Hostettmann al. 1998).

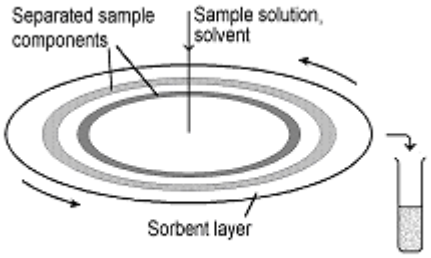
- **Toyopearl HW40**

  Toyopearl is a semi rigid, porous, hydrophilic gel for medium pressure LC. The gel consists of a matrix from oligoethyleneglycol, glycidylmethacrylate and pentaerythrodimethacrylate. Toyopearl HW40 separates substances with molecular weight in a range from 100 to 10 000 Da. It is a size exclusion separation chromatography, i.e. separates molecules by their size. The stationary phase is a porous packed material with specific pore size, this means that molecules which are too big to fit in the pores will be eluted first since they will migrate with the mobile phase front through the column. Molecules will be eluted in order of decreasing molecular size. Toyopearl HW40 has high chemical stability and is compatible with organic solvents (Greibrokk et al. 1998; Hostettmann et al. 1998).

- **MCI gel CHP20P**

A gel consisting of a polyaromatic (styrene – divinylbenzene) adsorbent resin. MCI gel CHP20P is designed for separations of aromatic compounds, peptides, steroids, desalting ect. The separation principle is like reverse phase chromatography. The potential problems caused by exposed silanols in silica-based materials is avoided (Cannell 1998; Supelco 2007).

6.1.4 Centrifugal Thin-Layer Chromatography – Chromatotron



Chromatotron separation follows the same principles as analytic thin-layer chromatography. In chromatotron separation (centrifugally accelerated thin-layer chromatography; CA-TLC), an adsorbent layer (1, 2 or 4 mm sorbent thickness) is used to coat a circular glass plate. In order to prevent breaking up of the thin layer, the sorbent is mixed with a binder, usually calcium sulphate hemihydrates (dried gypsum), and also a fluorescence in short wave UV-light compound added to sorbent. Silica gel 60 F254 for TLC is often used as sorbent in chromatotron (Hostettmann et al. 1998).

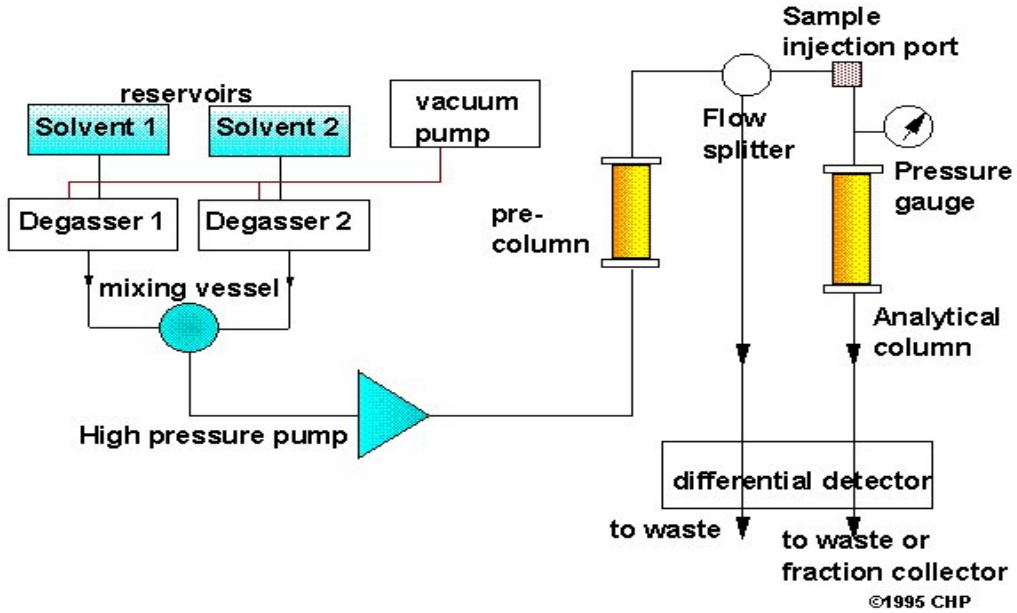
The prepared glass plate is screwed onto the hub of the electric motor and rotated at 800 rpm. When the sorbent has been washed several times with eluent (mobile phase), the

sample is introduced onto the sorbent-free centre of the plate. Thereafter, eluent is applied at the center of the plate and passes across the thin layer under the influence of the centrifugal force. At the periphery, the bands are spun off and collected through an exit tube in the chamber. The chamber is covered with a UV-transparent plastic lid; this enables the observation of colorless but UV-active substances zones with a UV lamp. A steady flow of nitrogen is passed through the chamber to prevent condensation of the eluent and to avoid oxidation of the sample (Hostettmann et al. 1998).

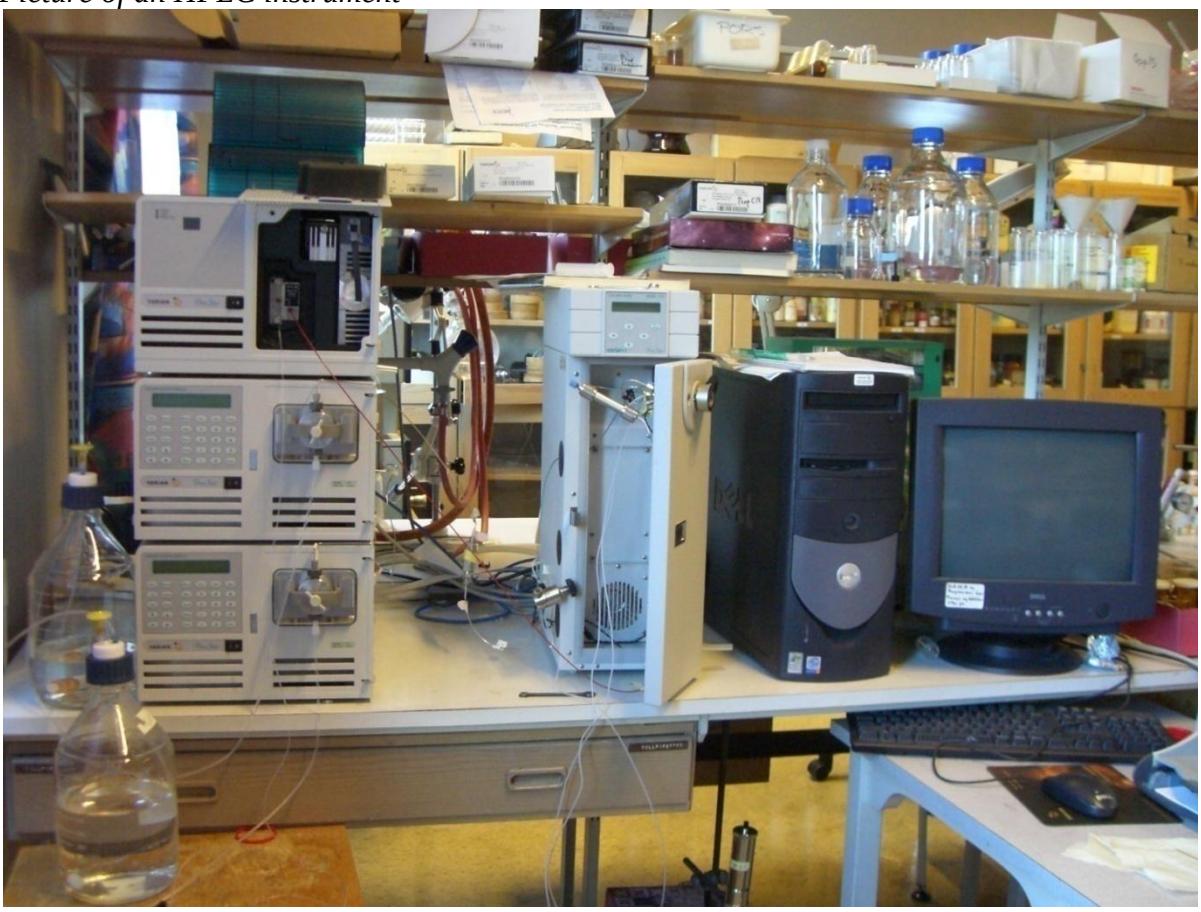
6.1.5 High performance liquid chromatography (HPLC)

High-performance liquid chromatography (HPLC) is a form of liquid chromatography to separate compounds in solution. HPLC instruments consist of a reservoir of mobile phase, a pump, an injector, a separation column, and a detector. Compounds are separated by injecting the sample mixture onto the column. The different components in the mixture pass through the column at different rates due to differences in their partitioning behavior between the mobile liquid phase and the stationary phase (Pedersen-Bjergaard and Rasmussen 2004).

Schematic of an HPLC instrument



Picture of an HPLC instrument

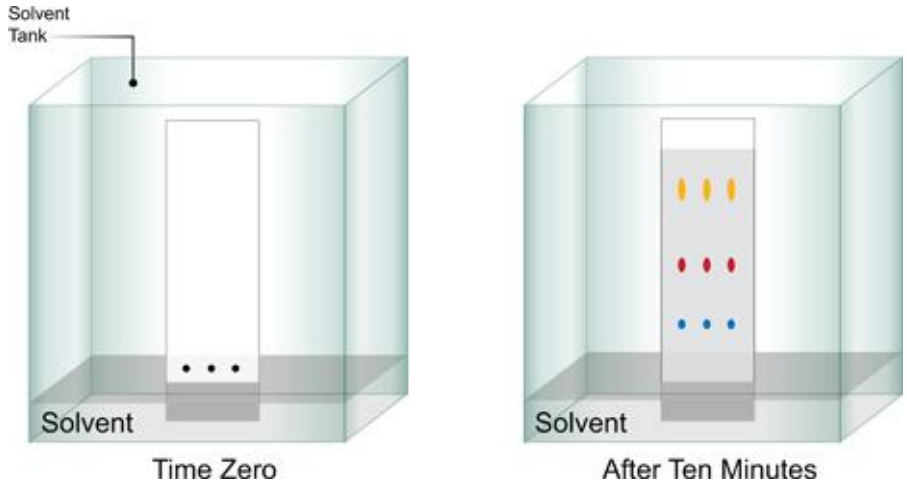


HPLC is a versatile, robust, and widely used technique for the isolation of natural products. The main difference between HPLC and common column chromatography is that the diameter of stationary phase particles is comparatively low (3-10µm), and these particles are tightly packed to give a very uniform column bed structure. The small particle diameter means that a high pressure is needed to drive the chromatographic solvent (or "eluent") through the bed. It is very important that mobile phase is clear, empty of air and also that the temperature of mobile phase should be in equilibrium with the whole system. The most common stationary phases consist of modified silica in which the surface is bonded with long chain alkyl-groups, substituted alkyl-groups or other hydrocarbons, and the mobile phase often consist of a mixture with water and an organic solvent which is miscible with water. HPLC can be used as adsorption, normal phase, reverse phase, ion-exchange, ion-pair and as size exclusion chromatography (Greibrokk et al. 1998; Pedersen-Bjergaard and Rasmussen 2004).

6.1.6 Analytical TLC

This method is used to identify substances and to check the purity of products**.** Thin-layer chromatography consists of a stationary phase immobilized on a metal, glass or plastic plate, and an organic solvent. The sample, either liquid or dissolved in a volatile solvent, is deposited as a spot on the stationary phase. Many spots can be applied on the stationary phase and be separated in a single run. The constituents of a sample can be provisionally identified by simultaneously running standards with the unknown. The stationary phase is often silica based, reverse phase materials (ex. C18), ion exchange media (cellulose or silica as matrix), or normal phase. Gypsum or an inert organic binder material is usually added to the stationary phase to increase mechanical strength (Greibrokk et al. 1998; Pedersen-Bjergaard and Rasmussen 2004).

The bottom edge of the plate is placed in a solvent reservoir, and the solvent moves up the plate by capillary action. When the solvent front has moved sufficiently upward, the plate is removed from the solvent reservoir. The separated spots are visualized with ultraviolet light or by spraying with visualization reagents, often followed by heating. The different components in the mixture move up the plate at different rates due to differences in their partitioning behavior between the mobile liquid phase and the stationary phase (Greibrokk et al. 1998; Pedersen-Bjergaard and Rasmussen 2004).



Schematic of a TLC setup.

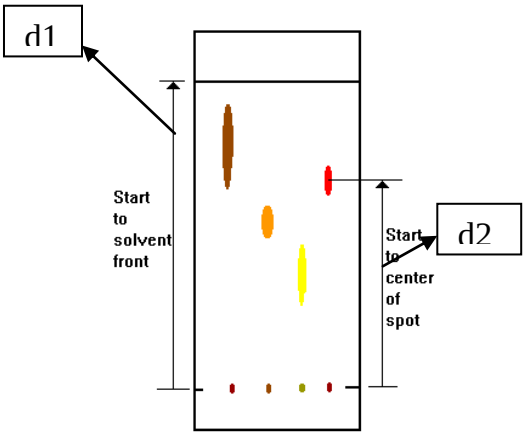
R_f = d2/d1 = 1/ 1+k

d1 = start to mobile phase front

d2 = start to center of spot

↑ k: ↑ retention

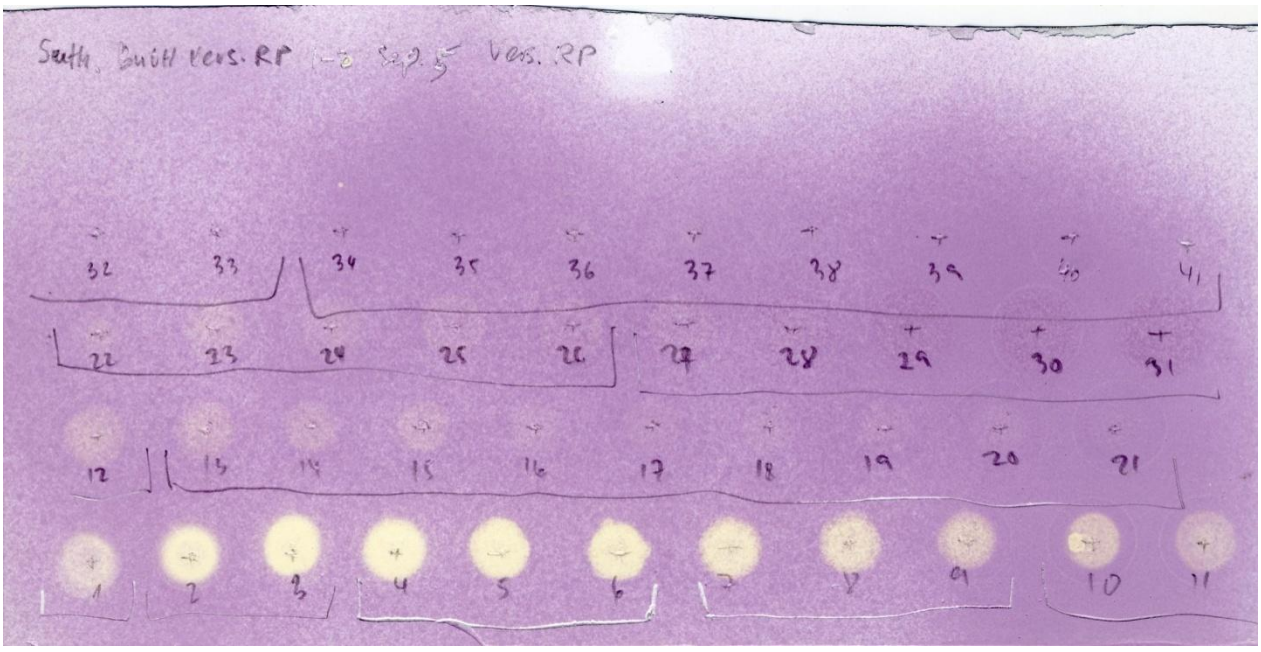
↓ R_f – value (short distance).



6.1.7 Preparative TLC

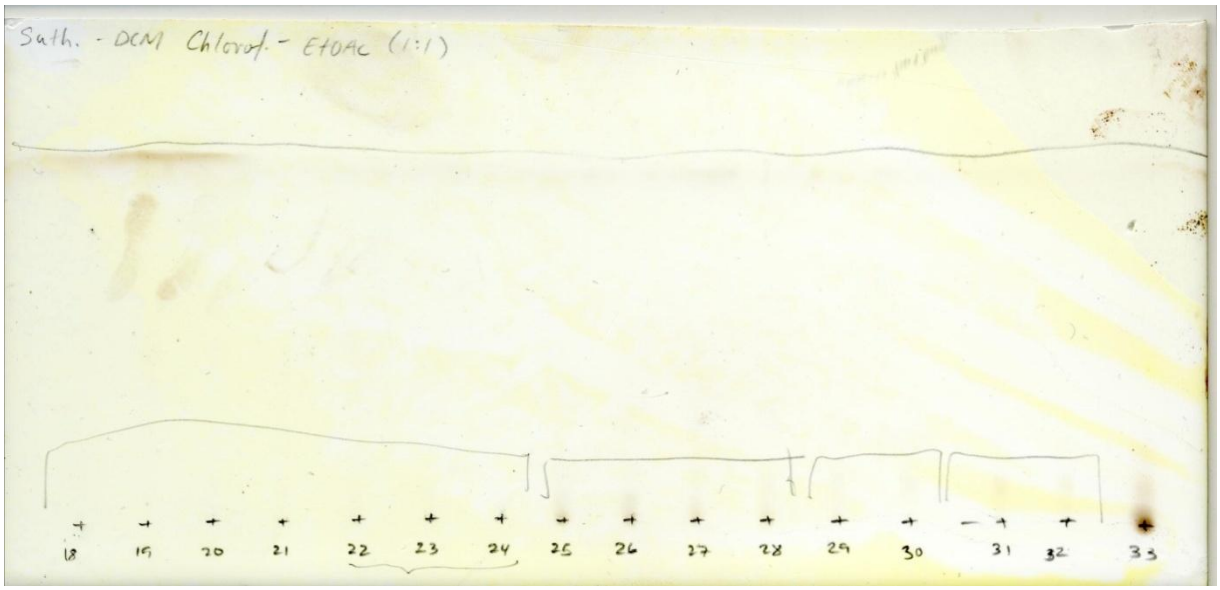
Preparative TLC is used to collect one or more substances on a preparative scale. Preparative TLC is in principle the same method as analytical TLC. But the difference is that the sample is deposited as a line, and that a concentrating zone consisting of inactivated silica may be used for concentrating the applied material into a narrow line (Pedersen-Bjergaard and Rasmussen 2004).

6.1.8 Visualization by DPPH spraying



This is described in section 1.2.1, DPPH – test. The DPPH reagent in this case was used to detect the presence of antioxidants. DPPH (diphenylpicrylhydrazyl) reacts with radical quenching groups present in the crude extract. Examples of such compounds are flavonoids, hydroxycinnamic acids, tannins etc. Thus DPPH on forming these complexes show the observed loss of coloration (from violet to yellow) on the TLC plate.

6.1.9 Ceric spraying



Ceric reagent (CAS) consist of sulfuric acid (10%) and cerium(IV)sulphate (1%) in water. When sprayed with CAS reagent, a pale yellow coloration is observed immediately after CAS spraying, turning to brown, violet or red after heating at ca 105^{o} for five minutes. CAS reagent will form a colored complex by reaction with organic compounds (Greibrokk, Lundanes et al. 1998). This method is used to decide which fractions should be combined after fractioning by column chromatography.

6.1.10 NMR – (nuclear magnetic resonance)

In chemistry, NMR-spectroscopy (nuclear magnetic resonance) is often used to elucidate the structure of a compound by identifying 1H- and 13C-atoms. This method is fast and without loss of substance.

The magnetic moment vectors of nuclei of hydrogen (1H), carbon (13C) and other isotopes which are NMR active (have spin quantum number > 0) behave like small magnets and spin around the direction of an external magnetic field. The absence of outer magnetic field gives the magnetic nuclear randomly directions. 1H and 13C have a spin quantum number of ½ and can orient their own magnetic field parallel or anti-parallel with the outer magnetic field. The parallel orientation demands less energy than anti-parallel, that is the reason why the most nuclei are oriented parallel to the external field. When the parallel oriented nuclei are irradiated with electro-magnetic radiation with correct frequency ("radio frequency field"), they will absorb the energy and flip over from a lower energy level to a higher energy level. The magnetic moment vector is in resonance with the radio frequency field when this happens, that's why the name nuclear magnetic resonance is used for this method. (McMurry, 2000)

The correct frequency which is necessary for resonance is dependent on the strength of magnetic field and nuclear identity. Absorbing frequency is not the same for all 1H or 13C-nuclei. All nuclei in molecules is surrounded by electrons. When a molecule is subjected to an outer magnetic field, the electrons form small local magnetic fields in which counteract the outer magnetic field. Then the magnetic field will affect nuclei less. The effective magnetic field on each nucleus is following:

$B_e = B_o - B_l$

(Be: Effective magnetic field; Bo: External magnetic field; Bl: Local magnetic field).

This effect is called nuclear shielding, and reduces the magnetic field at the nucleus. Each specific nucleus in a molecule is in different electron environment, and this will lead to different local magnetic fields. The NMR-instrument will detect signal positions for all nuclei.

In a NMR-spectrum, applied field strength increases from left to right. The position in spectra where a nucleus resonates is called chemical shift (δ), and has unit "parts per million (ppm). The signal at δ 0 correspond to a standard which is called tetramethylsilane (TMS), and this is used to calibrate the chemical shift scale. The nuclei which give signals to the left in the spectra are less shielded (deshielding) than signals to the right and give higher ppm (δ-value). Nuclei which are deshielded get influence of electronegative substituents, like olefins

and aromatic carbons and protons. Aliphatic carbons and protons are shielded (Solomons and Fryhle 2004).

A 1H-spektrum has signals in the interval 0-15 ppm and they give different types of information which is useful for interpreting of spectra. The following parameters are used in interpreting a 1H-spectrum:

- Integration curve

The area of a signal in a spectrum is proportional to the amount of protons giving rise to the signal. This can give information to determine how many protons in each signal.

- Signal splitting

A signal from a specific proton can be split by the interaction with the neighbour protons, which may have magnetic moment vectors parallel or antiparallel to the external magnetic field. Signal splitting happen only when there two sets of protons have different chemical shifts. Characteristic patterns like singlets, doublets, triplets etc. appear when splitting happen. The multiplicity of a signal is related to the number of protons on the neighbour carbon. A signal from one proton which has n identic neighbour protons is split into a multiplet with n+1 peaks. Chemically and magnetically equivalent protons do not give splitting.

- Coupling constant (J)

The distance between peaks in a multiplet is called the coupling constant (J). It is measured in Hz and in the interval 0-18. The coupling constant gives information about which protons are coupled to each other. Coupled signals show identical coupling constants.

A 13C-spectrum gives signals in the interval δ= 0-220 ppm, and it consists of one signal for each carbon atom. 13C – 1H coupling is usually eliminated by "proton noise decoupling". Integration of signals is usually not done, since signal area does not exactly reflect the number of carbons giving rise to that signal.

There are different types of NMR-spectra, like 1-dimensional (1-D) and 2-dimensional (2D). 1-D NMR spectroscopy methods are, e.g., 1H, 13C and APT (Attached Proton Test). 2-D NMR spectroscopy methods are, among others, COSY (Correlated Spectroscopy), NOESY (Nuclear Overhauser Effect Spectroscopy), HMBC (Heteronuclear Multiple Bond Correlation) and HSQC (Heteronuclear Single Quantum Coherence).

6.2 Methods for measuring biological activity

6.2.1 Radical scavenging (DPPH – test)

The DPPH test provides information on the reactivity of test compounds with a stable free radical. Because of its odd electron, 1,1- diphenyl-2-picrylhydrazyl radical (DPPH) gives a strong absorption band at 517 nm in visible spectroscopy, observed as a deep violet colour (Blois 1958; Malterud et al. 1993). As the electron becomes paired off in the presence of a free radical scavenger, the absorption vanishes, resulting in decolorization (from violet to yellow colour) (Glavind 1963; Malterud et al. 1993). Reduction of absorption is usually caused by formation of 1,1- diphenyl-2-picrylhydrazine (DPPH-H) which has low absorbance at 517 nm (Glavind 1963). This method of screening also allows for a bio-assay guided study of natural products.

The reduction in absorbance is due both to the amount and the activity of radical scavengers. Because of the strong absorption of the DPPH radical at 517 nm, solutions with low concentration can be measured, and so Beer-Lamberts law applies for the whole concentration interval. The free radical DPPH • scavenging (usually by reduction) activity was calculated from the equation: Activity [% of DPPH reduction] = [(A-Ax) / A] x 100%, where A – initial absorbance of DPPH • solution with methanol, Ax - absorbance of a DPPH solution at the end of the measurement period with a tested fraction solution (test) or BHA or quercetin (positive control) solution. The antiradical activity SC50, defined as the concentration of a sample showing 50% DPPH radical scavenging activity, can be determined from a graph in which concentration and reduction activity is plotted against each other, or by a computer program (Malterud et al. 1993).

6.2.2 Inhibition of 15-lipoxygenase (15-LO test)

15-lipoxygenase (15-LO) enzyme peroxidates arachidonic acid to 15-HPETE (15-hydroperoxyeicosatetraenoic acid), which can be transformed in vivo into other eicosanoids. In our assay, 15-LO from soya beans is used. It is not exactly identical to mammalian 15-LO, but there is a good correlation between inhibition of the two enzymes. Therefore soya beans lipoxygenase can use as a testing enzyme to identify inhibitor of mammalian 15-LO

(Lyckander and Malterud 1992; Gleason et al. 1995). Another reason is that soya beans 15-LO is cheap, easily assessable and quite stable (Lyckander and Malterud 1992). Enzyme solutions are kept cold during the whole experiment, so that loss of activity by oxidative denaturation is delayed. Enzyme activity is reduced linearly as a function of solution storage time. This can be adjusted for by measurement of blanks periodically through the experiment.

Linoleic acid is used as substrate for 15-LO, instead of arachidonic acid which is more expensive and less stable. Inhibition of peroxidation of linoleic acid gives comparable values to those obtained for peroxidation by arachidonic acid (Lyckander and Malterud 1990).

Linoleic acid has a 1,4-diene-type structure, and forms 13-hydroperoxy-(9Z,11E) octadecadienoic acid (13-HPODE) (Gleason et al. 1995; Lyckander and Malterud 1996).

CH_3-...-CH=CH-CH_2-CH=CH-...-COOH + O_2 -->

CH_3-...CH(OOH)-CH=CH-CH=CH-...-COOH

13-HPODE has two conjugated double bonds, this makes the compound absorb UV-light at 230-235 nm. Linoleic acid has no conjugated double bonds and that is why it doesn't have this character. Peroxidation of linoleic acid will increase absorbance at 234 nm proportional with concentration of conjugated diene. Inhibition of 15-LO enzyme will lead to a slower rate for increase in absorbance (Gutteridge and Halliwell 1990). The increase in absorbance for samples with and without inhibitor in 30-90 seconds period after enzyme is added, is measured. Calculation is performed by the following formula (Lyckander and Malterud 1992):

100*(A2-A1)/A2

A1: Absorbance increase (AU/min) of samples with inhibitor

A2: Absorbance increase of samples without inhibitor

Linoleic acid is dissolved in borate buffer with pH 9,00. Linoleic acid has a pKa-value = 4,77 and at pH 9,00 is nearly completely ionized. And to increase solubility, linoleic acid is

first dissolved in 150 µl ethanol before buffer is added. This will give a clear solution, which is important to measure correct absorbance (Haining and Axelrod 1958).

A small amount of lipid peroxide must be present for 15-LO to perform peroxidation of linoleic acid. This can be achieved by opening the ampoule with linoleic acid and keep it at room temperature for about 1 day before experiment starts. Also, slightly peroxidised linoleic acid makes it easier to get a clear solution (Haining and Axelrod 1958).

7. General experimental methods

7.1 Low pressure column chromatography

Procedure

Before separation, the column's packing material is mixed with start eluent. After swelling of packing material, the slurry is poured into a suitable column and eluted with 1-2 column volumes of start eluent.

The sample to be separated is dissolved in a small volume of start eluent and applied to the column. Different components will be eluted via gradient – or isocratic elution, and fractions of suitable volume are collected. At last the column is washed with 70% acetone and/or 100% acetone to elute any remaining material from the sample.

Solvents and reagents

A solvent with good dissolving properties is used to dissolve samples. In this work, mixtures of water and methanol were used for elution.

7.2 HPLC

Procedure

Before applying a sample solution, the column is conditioned with mobile phase for about 30 minutes. Thereafter the solution to be separated is injected into the loop. Gradient elution was chosen in our experiments. In analytic HPLC a chromatogram will be printed out, and in preparative HPLC, the effluent is collected into fractions.

Solvents and reagents

Mixtures of acetonitrile and water or MeOH and water in different concentrations of these.

7.3 Analytical thin-layer chromatography

Procedure

Mobile phase was made and poured into the TLC tank, and the tank was closed for 15 minutes before start to make the atmosphere in the tank saturated. Samples were applied as dots on the TLC-plate with a capillary tube. The volume applied of each sample was from 10-20 µl. After applied samples are dried, the TLC-plate was placed in the tank. The TLC-plate was taken out when the mobile phase had moved a sufficient distance, and the front was marked. When the plate is dried, components are detected in UV-light at 254 nm (shortwave) and 366 nm (long wave). At last the plate can be sprayed with DPPH – or ceric reagents.

Solvents and reagents

Mixed solvents which were used contained MeOH, water, acetone, chloroform, acetonitrile and EtOAc.

7.4 DPPH – spraying

Procedure

After the solutes have been applied on TLC-plate and the plate has been developed and dried, the plate is sprayed with DPPH reagent. The results (radical scavengers showing as yellow spots on a violet background) will appear after a short time.

Solutes and reagents

DPPH-reagent is dissolved in methanol until the concentration is so strong that the TLC plate is covered with an even violet colour.

7.5 Ceric – spraying

Procedure

Similarly as above,, the plate is sprayed with ceric reagent and dried in the oven at 100ºC for 5-10 minutes. Dark spots formed are observed and marked.

Solutes and reagents

Cerium(IV)sulfate 1%, dissolved in 10% aqueous sulfuric acid.

7.6 NMR – spectroscopy

Procedure

Samples (amount varying from 1-100 mg) were dissolved in ca. 1 ml solvent, adding 1 drop of TMS. The mixture was poured into an NMR-tube and analysed in the NMR-spectrometer.

Solutes and reagents

TMS was used as reference and deuterated chloroform, acetone, MeOH, or pyridine were used to dissolve the samples.

7.7 Radical scavenging (DPPH-test)

Procedure

The spectrophotometer is reset with a blank sample which is methanol before measurements are started. DPPH-solution was made by solving 1,1- diphenyl-2-picrylhydrazyl (DPPH) in methanol with a concentration which gives an absorbance of ca. 1 at 517 nm. Absorbance was measured in 2,95 ml DPPH-solution before adding samples. 50 µl of samples was added, and the mixture stirred with a plastic spatula. Absorbance at 517 nm was measured over a 5-min period. Three parallels of each sample were measured.

7.8 Inhibition of 15-lipoxygenase (15-LO test)

Procedure

All solutions are at room temperature during measurements, except solution C which is kept on ice. Samples are dissolved in DMSO. Quartz cuvettes are used in the test, because quartz

doesn’t absorb UV-light at 234 nm. A blank sample containing 0.95 ml of solution A (see below), 2.00 ml of solution B and 0.05 ml DMSO is placed in the blank compartment of the spectrophotometer throughout the experiment period. Uninhibited samples (without test substance) are measured at the start, after each three measurements and at the end of the experiment. These samples contain 0.90 ml of solution A, 2.00 ml of solution B and 0.05 ml DMSO. After stirring the sample with a plastic spatula, 0.05 ml of solution C (enzyme solution) is added to start the reaction, the mixture is stirred again, and the increase in absorbance at 234 nm is measured from 30 to 90 s after enzyme addition. Test samples are made with DMSO solutions of test substance instead of pure DMSO and are measured similarly. Both uninhibited samples and test samples are measured as three parallels.

Solutes and reagents

DMSO is used to dissolve all substances and dilutions made from the initial DMSO solution.

A: Borate buffer, 0.2 M, pH 9.00. Made from boric acid and sodium hydroxide.

B: Substrate solution: Mix 50 µl linoleic acid and 150 µl ethanol. Add 50 ml of solution A. 15 ml of this is mixed with 225 ml A. This solution should be used the same day it is made.

C: Enzyme solution: dissolve 15-LO («Lipoxidase», Sigma) in A to a concentration of about 10 000 U/ml (this will give an increase in absorbance at 234 nm of ca 0.4 AU/min) An absorbance increase of 0,3-0,5 AU/min is acceptable. The enzyme solution should be kept on ice throughout the experimental period. The final concentration of enzyme will be 167 U/ml.

D: Inhibitors: The substance to be tested is dissolved in dimethyl sulfoxide (DMSO). If recovery of the sample is critical (or if the sample itself has a strong absorbance at 234 nm, making the sum of sample absorbance and DMSO absorbance too high for accurate measurements, methanol may be used instead. The concentration range to be measured for the test substance will depend on its inhibitory activity. This must be tested in each case, starting with a fairly strong solution (e.g. 10 mg/ml) and then making a dilution series.

8. Experimental procedure

8.1 Materials

8.1.1 Chemicals

Acetone, for HPLC	Prolab Fontenay S/Bois, France
Acetone-d6	Sigma-Aldrich St.Louis, USA
Acetonitrile for HPLC	Fluka Chemie Buchs, Switzerland
Acetonitrile, gradient grade	Merck Darmstadt, Germany
Butanol	Fluka Chemie Buchs, Switzerland
Cerium(IV)sulfat, p.a.	Merck Darmstadt, Germany
Dichloromethane	Fluka Chemie Buchs, Switzerland
DMSO, dried	Merck Darmstadt, Germany
DPPH	Sigma-Aldrich St.Louis, USA
Acetic acid, (>90%)	VWR International Oslo, Norway
Ethanol	Arcus Oslo, Norway
Ethyl acetate, p.a.	Fluka Chemie Buchs, Switzerland
Chloroform, p.a.	Merck Darmstadt, Germany
d-Chloroform (CDCl3)	Sigma-Aldrich St.Louis, USA
Linoleic acid	Sigma St.Louis, USA
Lipoxidase; 15-Lipoxygenase	Sigma St.Louis, USA
MCI CHP20P	Supelco, Bellefonte, USA
Methanol, p.a.	Chemi-Teknik Oslo, Norway
Methanol purum	Chemi-Teknik Oslo, Norway
d-Methanol (CD3OD)	Sigma-Aldrich

	St.Louis, USA
N2-gas	AGA Oslo, Norway
Pyridine	Sigma-Aldrich Steinheim, Germany
Sephadex LH20	Pharmacia Biotech Uppsala, Sweden
Silica gel	Merck, Darmstadt, Germany
TMS (tetramethylsilane)	Sigma-Aldrich Steinheim, Germany
Toyopearl HW40	Tosoh Bioscience Tokyo, Japan
Purified warer	School of Pharmacy Oslo, Norway
Deuterium oxide (D2O)	Aldrich Milwaukee, USA

8.1.2 Apparatus

Analytical TLC	
Kiselgel 60 F_{254}, aluminium plates RP-18 F_{254S}, aluminium plates Cellulose F, DC Plastikfolien	Merck Darmstadt, Germany
Filterpaper	
Whatman, in different sizes Phase separation paper Whatman phase separators, silicone treated	Whatman Maidstone, England
HPLC Varian Prostar Pump model 210 *Analytical:* Detector Analytic 9x0mm Analytic column Varian 250x4,6 mm microsorb mv 100-5 C18 Hamilton 705SN 50µl syringe *Preparative:* Detector Prep 9x1mm Preparative column Varian dynamax 250x21,4 mm microsorb 60-8 C18 Varian 1002 TLL 2,5ml syringe	Varian Walnut Creek, CA, USA

Low pressure column chromatography	
FMI Lab pump modell RP-G150	Fluid Metering inc New York, USA
VersaFlash Büchi 681 Chrom pump	Büchi Flawil, Switzerland
Curvettes Quartz cuvettes	 Starna Essex, England
Magnet stirring machine RCT basic	 Janke & Kunkel Staufen, Germany
Chromatotron apparatus: Chromatotron Model 7924 Pressure meter Glass plate	 Harrison Research USA
NMR Varian Gemini 200 DPX 300	 Varian Palo Alto, CA, USA Biospin GmbH Rheinstetten, Germany
Oil pump Edwards E-Lab 2	 Edwards High Vacuum International Sussex, England
Shaking machine IKA-VIBRAK-VXR	 Janke & Kunkel Staufen, Germany
Rotary evaporator Büchi Rotavapor-R	 Büchi Flawil, Switzerland
Spray apparatus TLC sprayer	 Camag Muttenz, Switzerland
UV-apparatus *UV-spectrophotometer:* Shimadzu UV 160A *Temperature regulator:* Shimadzu CPS-controller *Cuvette holder:* Shimadzu CPS-240A *UV-lamp:* Model UVSL-58 (254 og 366 nm)	 Shimadzu Kyoto, Japan Ultra Violet Products San Gabriel, CA, USA
Scale Sartorius model BP221S	 Sartorius Göttingen, Germany

8.2 Plant material and extraction

Plant material was pulverized from the leaves from *Lessertia frutescens*. It was provided by drs. W. Mabusela and Q. Johnson, University of Cape Town, where a herbarium voucher sample is deposited.

The pulverized leaves (543 g) were extracted in a Soxhlet apparatus, first with dichloromethane (DCM) (4 litres), followed by methanol (MeOH) (4 litres). Both extracts were dried in a rotary evaporator, weighed and subjected to NMR spectroscopy. (Flowchart 12.1, p. 84)

8.2.1 Primary DCM-extract

DCM-extract, 5 g or 10 g, was dissolved in chloroform, and then applied to a VersaFlash normal phase column. The column was washed with 200 ml chloroform before the samples were applied. First 100 % chloroform, thereafter 10, 20, 40 and 100 % ethyl acetate, and finally 100 % acetone were used as mobile phase and fractions of ca. 50 ml were collected. The column was washed with chloroform to get rid of the rest of the acetone. There were collected totally 51 fractions (5 g-sample) and 65 fractions (10 g-sample) (Tables 13.1 and 13.2, p. 93-96).

Analytical TLC
The fractions 1-51 and 1-65 were investigated by normal phase TLC (silica gel 60 F254)

Mobile phase for fractions 1-51: Chl-EtOAc (3:1) for fractions 1-17, Chl-EtOAc (1:1) for fractions 18-33, EtOAc-acetone (1:1) for fraction 34-45, and 100 % acetone for fractions 46-51.

Mobile phase for fractions 1-65: 100 % Chl for fractions 1-10, Chl-EtOAc (3:1) for fractions 11-25, Chl-EtOAc (1:1) for fractions 26-40, 100 % EtOAc for fractions 41-54, and EtOAc: acetone (2:1) for fractions 55-65.

The plates were viewed in UV-light with short- and long wave irradiation before and after developing. Spots which absorbed UV-light were marked. The plates were sprayed with Ceric reagent and the fractions which seemed to contain the same components were combined, evaporated to dryness on a rotary evaporator and on an oil pump, and weighed. These gave fractions D1-D16, and D2.1-D2.17 (Tables 13.1 and 13.2, p. 93-96).

NMR-spectroscopy

1H-NMR-spectra of all fractions and 13C-NMR-spectra of fractions D6 and D7 were recorded (spectrum 14.4-14.6, p. 127-128).

8.2.2 Further work on Chromatotron of fraction D2.7, D2.8, D2.9 and D2.10

Based on their 1H NMR spectra, these fractions were chosen for purification on a Chromatotron.

8.2.2.1 Fractionation of D2.7

Fraction D2.7 was dissolved in a small amount of dichloromethane (DCM). The Chromatotron plate was wetted with DCM before application of the samples. Mobile phase D2.7: first 80, 67 and 50 % chloroform (Chl) and ethyl acetate (EtOAc), and then 100 % EtOAc, 100 % acetone, 10 and 20 % methanol (MeOH) in acetone. At last 100 % MeOH. 36 fractions were collected (Table 13.3, p. 96-97).

8.2.2.2 Fractionation of D2.8

Fraction D2.8 was dissolved in small amount of Chl. The Chromatotron plate was wetted with Chl before application of the samples. Mobile phase D2.8: first 80, 67 and 50 % Chl and EtOAc, and then 100 % EtOAc, 100 % acetone. At last 100 % MeOH. 20 fractions were collected (Table 13.4, p. 98).

8.2.2.3 Fractionation of D2.9

This was done as described for D2.8. Mobile phase were 10, 20, 30, 40, 50, 70 and 100 % EtOAc and Chl, followed by 5, 10 and 100 % acetone and EtOAc, at last 20 % MeOH in acetone. 24 fractions were collected (Table 13.5, p. 98-99).

8.2.2.4 Fractionation of D2.10

This was done as described for D2.8. Mobile phase D2.10: first 50 % Chl and EtOAc, followed by 100 % EtOAc, 100 % acetone, 20 % MeOH in acetone. At last 100 % MeOH. 20 fractions were collected (Table 13.6, p. 99-100).

Analytical TLC

The fractions collected after Chromatotron fractioning were investigated by normal phase TLC (silica gel 60 F254)

Mobile phase for D2.7: Chl-EtOAc (1:1) for all fractions.

Mobile phase for D2.8: Chl-EtOAc (1:1) for all fractions.

Mobile phase for D2.9: Chl-EtOAc (1:1) for all fractions.

Mobile phase for D2.10: Chl-EtOAc (1:1) for all fractions.

The plates were studied in UV-light with short- and long waves before and after developing. The spots which absorbed UV-light were marked. The plates were the sprayed with Ceric reagent and the fractions which seems to contain the same components were combined, evaporated to dryness on a rotary evaporator and on an oil pump, and weighed. These gave fractions D2.7.1-D2.7.10, D2.8.1-D2.8.11, D2.9.1-D2.9.10, D2.10.1-D2.10.8 (Tables 13.3-13.6, p. 96-100).

NMR-spectroscopy

1H-NMR-spectra of all fractions and 13C-NMR-spectra of fractions D2.7.2, D2.8.10, D2.9.4, D2.10.8 were recorded (spectrum 14.7-14.16, p. 129-133).

8.2.2.5 Further work with D2.7.6, D2.7.7, D2.7.8, D2.7.9, D2.8.1, D2.8.2, D2.8.5 and D2.8.6

Analytical HPLC

Analytical HPLC was used to find the right method for preparative HPLC, and also to give information about how many compounds were present in the samples. 200 µl of each sample (with concentration 200 µg/ml) were dissolved in mobile phase and injected in a reverse phase (C18) column.

Mobile phase: H_2O: MeOH

Gradient elution was used, starting with (75H_2O:25MeOH) and increased to (50:50) in 15 minutes, and then to (30:70) in 20 minutes, (10:90) in 5 minutes, then kept at the same concentration in 5 minutes before reducing to (75:25). UV absorbance was measured at 234 nm, 254 nm and 210 nm (chromatogram 15.1-15.8, p. 196-199). Flow 1 ml/min.

8.2.2.6 Further work with D2.9.7 and D2.9.8

Since 1H-NMR spectra indicated that these fractions might contain the same substances, and both fractions were small, they were combined for continuation of the purification process.

Analytic HPLC/Preparative HPLC

UV-spectroscopy was used to screen and to find which wave length had the highest absorption before going on with analytic HPLC. 200 µl of D2.9.7-8 (with concentration 200 µg/ml) was dissolved in mobile phase and injected in a reverse phase (C18) column.

In preparative HPLC, D2.10.8 was dissolved in about 2 ml mobile phase before it was injected into the column. Fractions were collected as indicated by their UV absorption.

Mobile phase: H_2O:MeOH

The gradient used was the same as described above. UV absorbance was measured at 280 nm (chromatogram 15.9, p. 200). Flow 1 ml/min.

8.2.2.7 Further work with D2.8.10

Analytic HPLC/Preparative HPLC

200 µl of D2.10.8 (with concentration 200 µg/ml) was dissolved in mobile phase and injected in a reverse phase (C18) column.

Preparative HPLC was carried out as described for D2.10.7, but the sample was dissolved in 3 ml mobile phase.

Mobile phase: H_2O:MeOH

The gradient used was the same as described above. UV absorbance was measured at 280 nm (chromatogram 15.10, p. 200). Flow 1 ml/min.

8.2.3 Primary MeOH-extract

The dried methanol extract was dissolved in about 1 l water and extracted in a separatory funnel with ethyl acetate (EtOAc) (5x0,5 litre), followed by 1-butanol (BuOH) (5x0,5 litre). The organic phases were collected and taken to dryness on a rotary evaporator followed by oil pump vacuum. When the last extraction with butanol was finished, the rest of the water

extract was evaporated to dryness like the other extracts. After all extracts were dry, they were weighed.

NMR-spectroscopy

1H-NMR-spectra and 13C-NMR-spectra of the extracts were recorded.

Bioassay

Assay for radical scavenging (DPPH-test) and inhibition of 15-lipoxygenase (15-LO test) were carried out for all extracts, in concentration 166,7 µg/ml, 83,3 µg/ml, 41,7 µg/ml, 20,8 µg/ml and 10,4 µg/ml.

8.2.4 Fractioning of EtOAc-extract

EtOAc phase, ca.10 g, was solved in 5:1 EtOAc / MeOH, and then applied to a VersaFlash normal phase column. First pure EtOAc followed by 10, 20, 50 and 100 % acetone, second 100 % EtOAc, third 1:1 EtOAc / MeOH were used as mobile phase and fractions of ca. 50 ml were collected. The column was washed with EtOAc to get rid of the rest of the EtOAc / MeOH eluent. A total of 53 fractions were collected.

Analytical TLC

Fractions 1-53 were investigated by normal phase TLC (silica gel 60 F254)

Mobile phase: EtOAc: acetone (1:1) for fractions 1-32, EtOAc: acetone (3:7) fractions 33-42, acetone for fractions 43-53.

The plates were studied in UV-light with short- and long waves before and after developing. The spots which absorbed UV-light were marked. The plates were then sprayed with Ceric reagent and the fractions which seemed to contain the same components were combined, evaporated to dryness on a rotary evaporator and an oil pump, and weighed. These gave fractions E1-E12 (Table 13.12, p. 100-102).

NMR-spectroscopy

1H-NMR-spectra of all fractions and 13C-NMR-spectra of some were recorded.

Bioassay

Assay for radical scavenging (DPPH-test) was done for fractions E2, E5, E6, E7, E8, E9 (Table 13.15, p. 102-103). The fractions were tested in concentration 166,7 µg/ml, 83,3 µg/ml, 41,7 µg/ml.

8.2.5 BuOH phase

BuOH-extract, 10 g, was dissolved in 50 ml MeOH:H_2O (1:1), and then applied to a VersaFlash reverse phase column. The column was conditioned with 200 ml MeOH:H_2O (1:1) before the samples were applied. 50, 70 and 100 % MeOH were used as mobile phase and fractions of ca. 50 ml were collected. The column was washed with MeOH to resolute any remaining material from the sample. Totally 41 fractions were collected (Table 13.16, p. 103-105).

Analytic TLC

The fractions were investigated by reverse phase TLC (silica gel 60 F254).

Mobile phase: EtOAc-acetone (3:1) for fractions 1-25, EtOAc-acetone (1:1) for fractions 26-41.

The plates were irradiated with UV-light with short and long wavelength before and after developing. The fractions which absorbed UV-light or fluoresced were marked. The plates were then sprayed with Ceric reagent and the fractions which seemed to contain the same components were combined, evaporated to dryness on a rotary evaporator and on an oil pump, and weighed. These gave fractions B1-B9 (Table 13.16, p. 103-105).

NMR-spectroscopy

1H-NMR-spectra were taken of all fractions, and for some fractions 13C-NMR-spectra were recorded, as well (spectrum 14.42-14.55, p. 146-153).

8.2.5.1 LPCC with Sephadex LH20 of fraction B1-2

Since 1H-NMR spectra for both these fractions were similar; they were combined for continuing of purification.

Fractioning of B1-2

At the bottom of the column a bit of glass wool was placed to prevent Sephadex LH20 leaking from the column. Sephadex LH20 was swelled with start eluent (10 % MeOH and H_2O), until the mixture was a slurry, and then poured into the column. The column was left until Sephadex LH20 sank to the bottom. Fraction B1-2, dissolved in small amount of 10 % MeOH and H_2O, was poured into the column. 10, 20, 50 and 100 % MeOH and H_2O were used as mobile phase. 30 fractions of 50 ml were collected.

Analytical TLC

All fractions were applied on a reverse phase TLC plate.

Mobile phase: (1:1) MeOH and H_2O.

The fractions were subjected to TLC, combined and taken to dryness as described above, giving fractions B1-2 S.1-B1-2 S.5. (Table 13.17, p. 105-106)

NMR-spectroscopy

1H-NMR-spectra of all fractions and 13C-NMR-spectra of fractions B1-2 S.3 and B1-2 S.5 were recorded (spectrum 14.56-14.61, p. 153-156).

8.2.5.2 Further work with fraction B1-2 S.4 in reverse phase VersaFlash column

B1-2 S.4 was dissolved in ca. 3 ml MeOH and H_2O (1:1) and applied to a reverse phase VersaFlash column. Elution with the following gradient (mobile phase): 50, 70 and 100 % MeOH and H_2O. 33 fractions of 15 ml were collected.

All fractions were applied on a TLC plate, and then sprayed with DPPH reagent to determine how they should be combined. These gave fractions B1-2 S.4 VRP.1 – B1-2 S.4 VRP.6. (Table 13.18, p. 106-108).

NMR-spectroscopy

1H-NMR-spectra were recorded for all fractions. 13C-NMR-spectra were taken for all fractions except fraction B1-2 S.4 VRP.1 and B1-2 S.4 VRP.2 (spectrum 14.62-14.73, p. 156-162).

8.2.5.3 Further work with fraction B1-2 S.5 in reverse phase VersaFlash column

B1-2 S.5 was dissolved in ca. 3 ml MeOH and H_2O (1:1) and applied to a reverse phase VersaFlash column. Elution with following gradient (mobile phase): 50, 70 and 100 % MeOH and H_2O. 41 fractions of 15 ml were collected.

DPPH treatment as above led to combination of fractions into B1-2 S.5 VRP.1 – B1-2 S.5 VRP.9. (Table 13.19, p. 108-109).

NMR-spectroscopy

1H-NMR-spectra were recorded for all fractions and 13C-NMR-spectra were taken for fractions B1-2 S.5 VRP.3, B1-2 S.5 VRP.4 and B1-2 S.5 VRP.6 (spectrum 14.74-14.80, p. 162-165).

8.2.5.4 Further work with fraction B1-2 S.4-5 VRP in LPCC with Toyopearl

NMR spectra of fractions from B1-2 S.4 VRP and B1-2 S.5 VRP indicated two types of substances (called as type A and type B). Fractions were combined based on their NMR spectra and then applied to a Toyopearl column (2,5x15 cm) **(**Flowchart 12.8, p. 91).

B1-2 S.4-5 VRP type A: B1-2 S.4 VRP.1, B1-2 S.4 VRP.2 and B1-2 S.5 VRP.3.

B1-2 S-4.5 VRP type B: B1-2 S.4 VRP.3, B1-2 S.4 VRP.4, B1-2 S.5 VRP.4 and B1-2 S.5 VRP.5.

B1-2 S.4 VRP type A and B were dissolved in small amounts of MeOH, applied to a Toyopearl column and eluted isocratically with MeOH as mobile phase.

13 fractions from type A with fraction 1: 30 ml, fraction 2: 12 ml, fractions 3-12: 8 ml and fraction 13: ca.50 ml were collected.

10 fractions from type B with fraction 1: 15 ml, fractions 2-10: 8 ml.

Analytical TLC

All fractions were applied on reverse phase TLC plates. Mobile phase: (1:1) MeOH and H_2O.

The procedure described above gave fractions B1-2 S.4-5 VRP. T1 – B1-2 S.4-5 VRP. T4. (Flowchart 12.9, p. 92).

NMR-spectroscopy

1H-NMR-spectra were recorded of all fractions.

8.2.5.5 Further work with fraction B1-2 S.4-5 VRP. T2 on LPCC with MCI CHP20P gel

The same procedure was followed as for Sephadex LH20, but with MCI gel instead. The column was moistened with start eluent. B1-2 S.4-5 VRP. T2, dissolved in 30 % MeOH and H_2O, was applied to the column. Gradient elution with following gradients; 30, 50, 70 and 100 % MeOH and H_2O. 29 fractions were collected of 15 ml each.

Analytical TLC

All fractions were applied on a reverse phase TLC plate.

Mobile phase: (1:1) MeOH and H_2O.

Using the procedure described above, fractions B1-2 S.4-5 VRP. T2 M.1 – B1-2 S.4-5 VRP. T2 M.8 were obtained. (Table 13.20, p. 109-111).

NMR-spectroscopy

1H-NMR-spectra were taken of all fractions. 13C-NMR spectra were taken of fraction B1-2 S.4-5 VRP. T2 M.3 and B1-2 S.4-5 VRP. T2 M.5 (spectrum 14.88-14.95, p. 169-173).

8.2.5.6 Further work with fraction B1-2 S.4-5 VRP. T3 on LPCC with MCI CHP20P gel

Same procedure as with Sephadex LH20, but with MCI gel instead. B1-2 S.4-5 VRP. T2 dissolved in 30 % MeOH and H_2O, was applied to the column. Gradient elution with following gradients; 30, 50, 70 and 100 % MeOH and H_2O. 21 fractions were collected of 15 ml each.

Analytical TLC

All fractions were applied on reverse phase TLC plate.

Mobile phase: (1:1) MeOH and H_2O.

Similarly to the above, fractions B1-2 S.4-5 VRP. T3 M.1 – B1-2 S.4-5 VRP. T3 M.7 were obtained. (Table 13.21, p. 111-112).

NMR-spectroscopy

1H-NMR-spectra were taken of all fractions. 13C-NMR spectra were taken of fractions B1-2 S.4-5 VRP. T3 M.4 and B1-2 S.4-5 VRP. T3 M.5 (spectrum 14.96-14.105, p. 173-178).

8.2.5.7 Further work with fractions B1-2 S.4-5 VRP. T2 M.1 – B1-2 S.4-5 VRP. T2 M.5 on HPLC

Analytical HPLC/Preparative HPLC

UV-spectroscopy was used to find which wave length had the highest absorption for the samples before going on with analytical HPLC. 200 µl of each fraction (with concentration 200 µg/ml), dissolved in mobile phase, was injected in a reverse phase C18 column.

In preparative HPLC, all fractions were dissolved in about 3 ml mobile phase, were injected separately into the column. Fractions were collected based on their absorbance.

Mobile phase: H20:MeOH

Gradient elution was used, starting with (70:30), increased to (50:50) in 15 minutes, and then to (30:70) in 20 minutes, (10:90) in 5 minutes, keeping the same concentration in 5 minutes before reduction to (75:25) in 2 minutes. UV absorbance was measured at 265 nm (chromatogram 15.11-15-15, p. 201-203). Flow 1 ml/min.

NMR-spectroscopy

1H-NMR-spectra were taken of all fractions which weigh over 1 mg, and for some interesting fractions 13C-NMR-spectra were registered, as well.

8.2.5.8 Further work with B1-2 S.4-5 VRP. T3 M.1 – B1-2 S.4-5 VRP. T3 M.6 on HPLC

The optimal detection wavelength was determined as above. 200 µl of fractions B1-2 S.4-5 VRP. T3 M.1 – B1-2 S.4-5 VRP. T3 M.6 (with concentration 200 µg/ml), dissolved in mobile phase, was injected in a reverse phase C18 column.

In preparative HPLC B1-2 S.4-5 VRP. T3 M.1 – B1-2 S.4-5 VRP. T3 M.6 dissolved in about 3 ml mobile phase, was injected into the column. Fractions were collected based on their absorbance.

Mobile phase: H20:MeOH

The same gradient as described above was employed. UV absorbance was measured at 265 nm (chromatogram 15.16-15.21, p. 203-206). Flow 1 ml/min.

NMR-spectroscopy

1H-NMR-spectra were taken of all fractions which weigh over 1 mg, and for some interesting fractions 13C-NMR-spectra were registered, as well.

8.2.5.9 Further work with fraction B5-6 on LCCP with SephadexLH20

The procedure described in section 8.1.5.1 “LPCC with Sephadex LH20 of fraction B1-2” was followed. Fraction B5-6 dissolved in a small amount of 20 % MeOH and H_2O was applied to the column. 20, 50, 70 and 100 % MeOH and H_2O were used as mobile phase. 30 fractions of 50 ml were collected.

Analytical TLC

All fractions were applied on reverse phase TLC plates.

Mobile phase: hypophase of MeOH, chloroform and H_2O (65:35:10).

Using the methods described above, fractions B5-6 S.1 – B5-6 S.6 were obtained. (Table 13.34, p. 118-119) (Flowchart 12.7, p. 90).

NMR-spectroscopy

1H-NMR spectra were taken for all fractions. 13C-NMR spectra were taken for fraction B5-6 S.3 – B5-6 S.5 (spectrum 14.115-120, p. 183-186).

8.2.5.10 Further work with B5-6 S.3 on VersaFlash normal phase column

B5-6 S.3 was dissolved in hypophase of MeOH, chloroform and H_2O (65:35:10), and then applied to a VersaFlash normal phase column. The column was conditioned with chloroform, MeOH and hypophase of MeOH, chloroform and H_2O (65:35:10), before the samples were applied. Isocratic elution with hypophase of MeOH, chloroform and H_2O (65:35:10) as mobile phase was used. Fractions of ca. 17 ml were collected. There was collected totally 22 fractions (Table 13.35, p. 119-121).

Analytic TLC

All fractions were applied on reverse phase TLC plate.

Mobile phase: hypophase of MeOH, chloroform and H_2O (65:35:10).

Using the methods described above, fractions B5-6 S.3 VNP.1 – B5-6 S.3 VNP.8 were obtained (Table 13.35, p. 119-121).

NMR-spectroscopy

1H-NMR spectra were taken for all fractions. 13C-NMR spectra were taken for fraction B5-6 S.3 VNP.2 – B5-6 S.3 VNP.4 (spectra 14.121-14.126, p. 186-189).

8.2.5.11 Further work with fraction B5-6 S.3 VNP.2 on Chromatotron

Fraction B5-6 S.3 VNP.2 was dissolved in a small amount of DCM. The Chromatotron plate was moistened with DCM before the sample was applied. Mobile phase: hypophase of MeOH, chloroform and H_2O (65:35:10). 9 fractions of 7 ml were collected.

8.2.5.12 Further work with fraction B5-6 S.3 VNP.2 CHR 1-3 on HPLC

200 µl of each fraction (with concentration 200 µg/ml), dissolved in mobile phase, was injected into a reverse phase C18 column. Wavelength 215 nm, flow 1 ml/min.

Mobile phase: 0-10 minutes 30-40 % CH_3CN-H_2O, 10-15 minutes 40 % CH_3CN-H_2O, 15-20 minutes 40-70 % CH_3CN-H_2O, 20-30 minutes 70 % CH_3CN-H_2O, 30-31 minutes 70-30 % CH_3CN-H_2O.

In preparative HPLC, each fraction was dissolved with about 3 ml mobile phase and injected into the column. Flow 20 ml/min. Fractions were obtained as indicated by their UV absorption, taken to dryness on a rotary evaporator, and weighed. This gave 14 fractions in total (Tables 13.37-13.39, p. 122-123). According to their retention times, column fractions were combined to 6 main ones (called fractions A-F) which were subjected to further work.

8.2.5.13 Further purification of fractions A-B and E-F

The same method as described in the preceding section for preparative HPLC was used. Each peak was collected, and brought to dryness on a rotary evaporator, and weighed (chromatogram 15.22-15.25, p. 206-208).

The same gradient as described above was used.

NMR-spectroscopy

1H-NMR-spectra were taken of all fractions which weigh over 1 mg, and some fractions 13C-NMR-spectra were registered, as well (spectrum 14.127-14.130, p. 189-191).

9. Results and discussion

9.1 Primary DCM-extract

1H-NMR-spectra of DCM-extract (spectra 14.1, p. 126) showed mostly signals in the aliphatic region. 13C-NMR-spectra of this extract (spectra 14.2, p. 126) confirmed the findings from the proton spectrum and showed signals the aliphatic region, as well.

Bioassay

The extract didn't have radical scavenger activity, but in 15-LO test the DCM-extract had activity with ICD50: 70 ± 2 µg/ml.

9.2 Fractioning of DCM-extract

5 g of DCM-extract was fractionated on a VersaFlash normal phase column, 51 fractions were collected, combined into fractions D1-D16 based on analytic TLC followed by irradiation with UV-light with short and long wave, and ceric spraying. The total yield was 3,575 g (Table 13.1, p. 93-94). From the NMR-spectra of the fractions, separation seemed incomplete. And the fractions which gave interesting signals were too small to continue with purification. Therefore, 10 g of DCM-extract was fractionated with the same method, and 65 fractions were collected. Fractions D2.1-D2.17 were subjected to analytic TLC followed by irradiation with UV-light with short and long wave, and ceric spraying. the total yield was 10,73 g (Table 13.2, p. 94-96) (Flowchart 12.2, p. 85).

NMR-spectroscopy

1H-NMR-spectra of D1 and D2 contains signals from aliphatic protons. D1 showed only CH_2 and CH_3 signals and was assumed to be a straight-chain hydrocarbon. From integration, an average chain length of 27 carbon atoms was calculated (spectra 14.3, p. 127). Fraction D2 showed triplets indicative of a CH_2O group (4.05 ppm) and a $CH_2C{=}O$ group (2.29 ppm) in addition to shielded CH_2 and CH_3 signals (spectra 14.4, p. 127). This might indicate a wax ester (a long-chain alcohol esterified with a long-chain fatty acid). Both long-chain

hydrocarbons and wax esters are commonly found constituents on plant surfaces. The 13C-NMR-spectra of D2 indicated a oxygen-substituted carbon at 64.4 ppm. D3-D4 did not contain any signals of interest, and fraction D5 appeared from its 1H-NMR-spectrum to contain mostly fatty acids. Fractions D6 and D7 had signals in the aromatic and olefinic region, which is 5-8 ppm in 1H-NMR-spectra. In addition, aromatic methoxyl groups could be present (singlets at 3.5 – 4 ppm) (Spectrum 14.5-14.6, p. 128).

As mentioned above, we decided to conduct a second separation with 10 g DCM-extract which was separated with the same method. A better separation was achieved, but only fractions D2.7-D2.10 appeared to be of interest from their NMR spectra (Flowchart 12.3, p. 86).

The 1H-NMR-spectrum of D2.7 had two aromatic doublet signals which later on was showed to be derived from 4-hydroxybenzaldehyde. D2.8 contained methoxy signals at 3.92 and 4.01ppm. D2.9 contained a lot of interesting signals which were in the methoxy-, aliphatic-, olefin- and aromatic regions. These findings for D2.7-D2.9 were deciding for our decision to continue with investigation on a Chromatotron. D2.10 was included for Chromatotron separation, because it might contain some substances from fraction D2.9.

9.2.1 Further work with D2.7-D2.10

Chromatotron

D2.7 was separated on the Chromatotron. Totally 36 fractions were collected and combined into fractions D2.7.1-D2.7.10 based on analytical TLC followed by screening in UV-light with short and long wave, and ceric spraying. It yielded 111,2 mg (Table 13.3, p. 96-97)

D2.8 was separated on the Chromatotron. Totally 20 fractions were collected and combined into fractions D2.8.1-D2.8.11 as above. It yielded 170,8 mg (Table 13.4, p. 98)

D2.9 was separated on the Chromatotron. Totally 24 fractions were collected and combined into fractions D2.9.1-D2.9.10 as above. It yielded 42,9 mg (Table 13.5, p. 98-99)

D2.10 was separated on the Chromatotron. Totally 20 fractions were collected and combined into fractions D2.10.1-D2.10.8 as above. It yielded 36,6 mg (Table 13.6, p. 99-100)

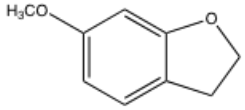
NMR-Spectroscopy

1H-NMR-spectra all D2.7 fractions are not of interest, except D2.7.2 and D2.7.3. In the NMR spectra of these fractions, a 1-H singlet at 9.9 ppm and two 2-H doublets at 7.81 and 6.96 ppm (both with J=8.6 cps) were indicative of an aromatic aldehyde. The aldehyde carbon signal at 190.7 ppm in the 13C-spectrum and a hydroxylated aromatic carbon signal at 161.6 ppm pointed towards 4-hydroxybenzaldehyde as a possible structure, although the substance was impure. This structure was confirmed by direct comparison with authentic substance (NMR, TLC). 4-Hydroxybenzaldehyde is not an uncommon natural product, but it has not been reported previously from the genus *Lessertia* or *Sutherlandia* (spectrum 14.7-14.8, p. 129)(Flowchart 12.4, p. 87).

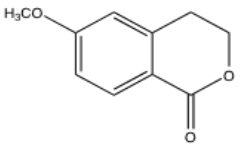
1H-NMR-spectra of the D2.8 fractions were not of interest, except D2.8.2, D2.8.6 and D2.8.10. 4-Hydroxybenzaldehyde was found in fraction D2.8.2. The spectrum of fraction D2.8.6 contained aromatic proton signals. D2.8.10 contained 2 O-methoxy groups. These fractions were subjected to further work (spectrum 14.9-14.11, p. 130-131).

1H-NMR-spectra of the D2.9 fractions were not of interest, except D2.9.7 and D2.9.8. With deuterochlorofom as solvent, a 3-H singlet at 3.97 ppm could be due to an aromatic methoxyl group. Two coupled triplets (2H each) at 4.02 and 3.19 ppm (J=5.3 cps) could be taken as an $–O-CH_2-CH_2-R$ system in which R might be an aromatic or a carbonyl substituent. In addition, the aromatic region appears to contain an AMX pattern (6.96, 7.54 and 7.81 ppm) which could indicate an aromatic ring with 1,2,4-substitution. This is, however, speculative, since other signals interfere (spectrum 14.12-14.16, p. 131-133). Since these fractions are too small it was decided to combine these two fractions into one fraction D2.9.7-8. But it seems that D2.9.7-8 may be volatile, since the mass decreased during separation process. From this, no definite structure can be suggested, although 6- or 7-methoxylated chromanons, isocoumarins or dihydrobenzofurans would appear possible. Calculated 1H NMR shift values given in the SciFinder database do not accord very well with the observed values, however.

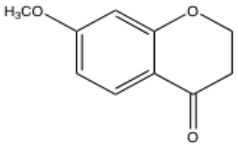
Structures of the suggested compounds:



6-methoxy-dihydrofuran



6-methoxyisocoumarin



7-methoxychromanone

1H-NMR-spectra of D2.10 fractions are not of interest, except D2.10.8. It contains the same substance as D2.9.7-8, but in a very small amount (spectra 14.17, p. 134).

9.2.2 Further work with fraction D2.7.6, D2.7.7, D2.7.8, D2.7.9, D2.8.1, D2.8.2, D2.8.5, D2.8.6, D2.9.7-8 and D2.8.10

Analytical HPLC

All fractions were studied by analytical HPLC (C18-column) and chromatograms were taken with UV-detector at 210nm, 234 nm, 254 nm, 280nm, because UV-scanning showed best absorption at these wavelengths (chromatograms 15.1-15.8, p. 192-195). Chromatogram of D2.9.7-8 contains one peak (chromatogram 15.9 p. 196, Table 13.7 p. 100), these could mean that there is one UV-absorbing substance. D2.8.10 has two peaks (chromatogram 15.10 p. 196, Table 11.8 p. 100). These two fractions were then decided to go on with by preparative HPLC. As the rest were poorly separated, we decided not to go on with these.

9.2.2.1 Further work with D2.9.7-8

Preparative HPLC

The substances in D2.9.7-8 were separated with preparative HPLC, and a chromatogram was taken with UV-detector at 280 nm, since this fraction appears to contain aromatic compound based on its NMR spectra and aromatic compound absorb UV-light at 280 nm. Fraction D2.9.7-8 HPLC5 was collected, dried in a rotary evaporator and weighed.

NMR-spectroscopy

After preparative HPLC, enough substance was available for 1H-NMR spectroscopy, but not for 13C-NMR. In deuterated methanol, a 2H multiplet at 7.53-7.58 ppm, a 1H doublet at 6.83 ppm, two 2H triplets at 3.93 and 3.16 ppm, and one 3H singlet at 3.90 ppm was observed, in good correlation with the above spectrum (spectrum 14.18-14.9, p. 134-135). This, however, does not yield further structural information.

9.2.2.2 Further work with D2.8.10

Preparative HPLC

The substances in D2.10.8 were separated with preparative HPLC, and a chromatogram was taken with UV-detector at 280 nm. Fractions D2.10.8 HPLC2 and D2.8.10 HPLC5 were collected, dried in a rotary evaporator and weighed (Table 11.8, p. 100).

NMR-spectroscopy

This fraction contains 1H NMR signals which probably can be ascribed to aromatic protons (apparently five singlets) and two aromatic methoxyl groups. We are unable to suggest a structure from the data available (spectrum 14.20-14.21, p. 135-136).

9.3 MeOH-extract

Extraction of the MeOH crude extract suspended in water with EtOAc and BuOH gave three extracts including "the aqueous rest", which was left after the other extractions.

These extractions from plant materials led to substances distributed in solutions with different polarity. The semi-polar compounds were extracted into EtOAc, the more polar in BuOH, while the most polar compounds remained in the aqueous solution. This gave a rough separation of the semipolar and polar compounds in the plant material (flowchart 12.1, p. 84).

Table 13.9. Extraction yield:

Fraction/extract	**Yield**
Primary MeOH-extract from *Sutherlandia frutescens*	Ca.98 g
EtOAc-extract	10 g
BuOH-extract	22 g
The aqueous phase	1 g

NMR-spectroscopy

1H-NMR-spectra of the MeOH-extract (spectra 14.22, p. 136) showed signals indicative of aliphatic protons and carbohydrate protons, and minor signals in the aromatic and olefinic regions. The MeOH-extract therefore seems to contain a mixture of aromatic and aliphatic substances, but mostly carbohydrates. The 13C-NMR-spectrum (spectra 14.23, p. 137) confirms the findings from the proton spectrum.

The 1H-NMR-spectrum of the BuOH-extract (spectra 14.24, p. 137) showed small peaks in aromatic- and olefin areas, aliphatic protons and carbohydrates. These findings are also confirmed by the 13C-NMR-spectrum (spectra 14.25, p. 138).

The 1H-NMR-spectrum of the EtOAc-extract (spectra 14.26, p. 138) showed signals in the aliphatic region and 2 doublets in the olefin region (5.85 and 6.77 ppm) with coupling constant 9,8 cps which probably means a cis-olefin compound. The 13C-NMR-spectrum of the EtOAc-extract (spectra 14.27, p. 139) confirmed the findings from proton spectra and also showed carbonyl signals.

Bioassay

The extracts showed low radical scavenger activity with SC50 of more than 166,7 µg/ml. 15-LO test for BuOH extract and the aqueous rest showed quite low activity with ICD50 close to 166,7 µg/ml (the highest concentration measured), but the EtOAc-extract had a significantly higher activity than the other extracts (ICD50: 57 ± 3 µg/ml) (Tables 13.10-13.11).

Table 13.10. Radical scavenging activity of extracts.

Extract	SC50 (µg/ml)	SD
EtOAc-extract	>166,7	-
BuOH-extract	>166,7	-
Aqueous rest	Not measured	-

Table 13.11. 15-LO inhibition of extracts.

Extract	ICD50 (µg/ml)	SD
EtOAc-extract	57	3
BuOH-extract	167	-
Aqueous rest	Not measured	-

Since EtOAc-extract showed quite good activity in inhibition of 15-LO, and both the EtOAc extract and the BuOH extract also had interesting signals in proton and carbon NMR, these two extracts were chosen for further investigation.

9.4 Fractionating of the EtOAc-extract

EtOAc-extract, 10 g, was fractionated on a VersaFlash normal phase column. 53 fractions were collected, gathered together to fractions E1-E12 based on analytical TLC followed by screening in UV-light with short and long wave, and ceric spraying. Total yield was 9,89 g (Table 13.12, p. 100-102) (Flowchart 12.5, p. 88).

NMR-spectroscopy

From the 1H and 13C NMR spectra of the fractions from the EtOAc extract, it seemed that glycosylated terpenoids were present in most of the fractions. A series of triterpenoid glycosides, sutherlandioside A-D, has recently been reported from *Sutherlandia* by Fu et al (2007). By comparing our NMR data with those published, we were able to identify sutherlandiosides B, C and D as well as two unidentified compounds in our column fractions.

Table 13.13. Approximate composition of Versaflash fractions of *Sutherlandia* ethyl acetate fraction

Versaflash fraction	Weight (mg)	% Suth B	% Suth C	% Suth D	% unknown 1 (δ 206.3 ppm)	% unknown 2 (δ 200.2 ppm)	% Suth D (δ 99.1 ppm)	% epi-Suth (δ 98.0 ppm)
3	449	11	19	53	0	17	42	58
4	465	10	15	45	14	23	45	55
5	730	19	17	66	0	20	49	51
6	3696	66	0	34	0	0	66	34
7	766	Much	0	Little	0	0	63	37
8	273	Much	0	Little	0	0	56	44
9	723	Much	0	Little	0	0	55	45

Assuming that carbonyls in the different sutherlandiosides have similar response factors in 13C NMR and that the carbonyl signals have a similar height: area ratio, an approximate ratio for the content of the sutherlandiosides can be estimated from the 13C NMR spectra. The shift positions observed in our fractions are in full agreement with published data (Fu, Li et al. 2007).

In the sutherlandiosides, the anomer carbon of the glucose moiety resonates at 99.0-99.1 ppm. A signal at 98.0 ppm will later be shown (by preparative HPLC) to be related to another substance which may be a stereo- or regioisomer of the sutherlandiosides. The ratio of the signals from sutherlandioside-type and putative epi-sutherlandioside is tabulated in the last two columns of the table.

It can be seen from the table that sutherlandioside B is the major compound, followed by sutherlandioside D. Sutherlandioside C and two unknown compounds appear to co-elute with sutherlandioside D in the first fractions. There seems to be a tendency for epi-compounds to elute slightly faster than the sutherlandiosides.

From the above data, a rather crude estimate can be made of the amount of the sutherlandiosides.

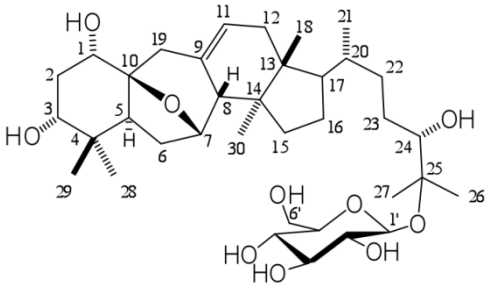
Table 13.14. Amount of substances in VersaFlash fractions form the EtOAc extract.

Fraction	Weightmg	Suth B	Suth C	Suth D	Unknown 1	Unknown 2
3	449	49	85	238	0	76
4	465	47	70	209	65	107
5	730	139	124	482	0	146
6	3696	2439	0	1257	0	0
7	766	766	0	0	0	0
8	273	273	0	0	0	0
9	723	723	0	0	0	0
Sum	7102	4436	279	2186	65	329
% of total	100	61	4	30	1	5

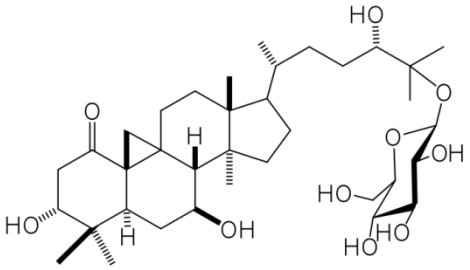
Bioassay

DPPH-test was performed with fractions E2, E5, E6, E7, E8 and E9. All fractions have SC50 >167 µg/ml (Table 11.15, p. 102-103).

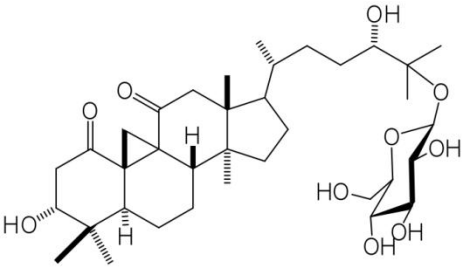
10 mg of E6 was sent to our collaborators at the University of Western Cape, Cape Town, outh Africa for studies on antiapoptotic activity. Results from their work on this fraction were not available for inclusion in this dissertation.



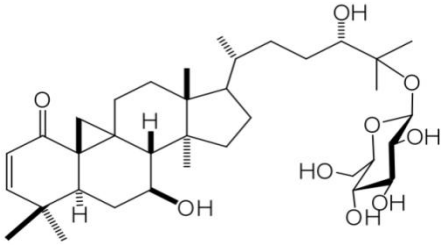
Sutherlandioside A



Sutherlandioside B



Sutherlandioside C



Sutherlandioside D

Figure… Formulas of sutherlandioside A-D.

9.5 Fractionation of the BuOH-extract

10 g BuOH-extract was fractionated on a VersaFlash reverse phase column. 41 fractions were collected, combined into 9 fractions, fractions B1-B9, based on analytical TLC followed by screened in UV-light with short and long wave, and ceric spraying. Total yield was 9,52 g (Table 11.16, p. 103-105) (Flowchart 12.6, p. 89).

NMR-spectroscopy

The 1H-NMR-spectrum of fraction B1 mostly contained signals from carbohydrates (3-5,5 ppm), with minor signals in the aromatic and olefinic region (6-8 ppm), and in the aliphatic (terpenoid?) region (0,8-3ppm). Fraction B2 and B3 looked like fraction B1, but had more of terpenoid signals. Fractions B1 and B2 were combined into one fraction for further separation with LPCC on Sephadex LH20.

Fraction B4 had signals of carbohydrates and terpenoids, but aromatic and olefinic signals were gone.

The 1H-NMR-spectrum from fraction B5 had signals which were assumed to be from the sutherlandiosides which were mentioned under the EtOAc fraction, section 9.4, p. 67. The 13C-NMR-spectrum of the same fraction had 4 carbonyl signals and one set of olefinic carbons which may be from sutherlandioside D. Fraction B6 was similar to fraction B5, but in its 13C-NMR-spectra it contained only 3 carbonyl signals. These two fractions were combined into one fraction for further separation with LPCC on Sephadex LH20 (spectrum 14.42-14.55, p. 146-153).

The spectrum of fraction B7 looked quite similar to fraction B6. Fraction B7 was small and was therefore omitted from further work.

Fractions B8 and B9 had terpenoid and carbohydrate signals, but these fractions were poorly soluble.

9.5.1 LPCC with Sephadex LH20 of fraction B1-2

Fraction B1-2 weight was 5631 mg. This fraction was separated by LPCC with Sephadex LH20. 30 fractions were collected, and combined into 5 fractions based on analytical TLC followed by screening in UV-light with short and long wave, and ceric spraying. Total yield was 4,379 g (Table 11.17, p. 105-106) (Flowchart 12.7, p. 90).

NMR-spectroscopy

The 1H-NMR-spectra of fractions B1-2 S.1 and B1-2 S.2 contained signals in the carbohydrate and aliphatic / terpenoid regions. The proton spectrum from fraction B1-2 S.3 had mostly terpenoid signals, and less in the carbohydrate region.

The 1H-NMR-spectrum of fraction B1-2 S.4 had higher peaks in the aromatic and olefin region with signals that could be from H-atoms in position 6 and 8 in the A-ring of flavonols at 6,2 ppm and 6,4 ppm. Signals at 6,8-7,8 ppm, 7,5-7,8 ppm and 8,0-8,2 ppm could be from B-ring protons in quercetin- and kaempferol-type flavonols. This fraction was chosen for further separation with VersaFlash reverse phase column.

The 1H-NMR-spectrum of fraction B1-2 S.5 looked similar to fraction B1-2 S.4, but had smaller peaks in the terpenoid region. It was also chosen for further investigation. (spectrum 14.56-14.61, p. 153-156)

9.5.1.1 Fraction B1-2 S.4 separation with reverse phase VersaFlash column (443 mg)

Fraction B1-2 weighed 443 mg and was separated with VersaFlash reverse phase column. 33 fractions were collected and combined into 6 fractions based on DPPH-spraying. Total yield was 369 mg (Table 11.18, p. 106-108).

NMR-spectroscopy

1H-NMR-spectra of fractions B1-2 S.4 VRP.1 and B1-2 S.4 VRP.2 had signals assumed to be from kaempferol and quercetin glycosides. Two singlets at 1,15 ppm and 2,36 ppm appeared in almost all spectra in this series. These are likely to be from a hydroxymethylglutaric acid moiety (Mabusela, pers.comm.).

1H-NMR-spectra of fraction B1-2 S.4 VRP.3 contained more kaempferol glycosides than quercetin glycosides. From the integral it could be seen that the fraction contained twice as much kaempferol glycosides as quercetin glycosides. The 13-C-NMR-spectrum confirmed the findings in the proton spectrum, with small signals in the 140-150 ppm region which were assumed to be from C-3´and C-4´ in quercetin-type flavonoids. The 1-H-NMR-spectrum of fraction B1-2 S.4 VRP.4 was fairly similar to that of fraction B1-2 S.4 VRP.3.

The 1H-NMR-spectrum of fraction B1-2 S.4 VRP.5 had only minor signals from flavonoids, but had signals from terpenoids. This was a small fraction.

The 1H-NMR-spectrum of fraction B1-2 S.4 VRP.6 had mostly signals from triterpene glycosides. Two doublets which could be from sutherlandioside D were observed in the olefinic region. The 13-C-NMR-spectrum had one carbonyl signal, as would be expected for sutherlandioside D, but also a signal from an anomeric carbohydrate carbon which may be a stereo- or regioisomer of the sutherlandiosides as mentioned in section 9.4 “Fractioning of EtOAc-extract”. (spectrum 14.62-14.73, p. 156-162).

9.5.1.2 Fraction B1-2 S.5 separation with reverse phase VersaFlash column (303 mg)

Fraction B1-2 weighed 303 mg and was separated with VersaFlash reverse phase column. 41 fractions were collected and combined into 9 fractions based on DPPH-spraying. Total yield was 317,8 mg (Table 11.19, p. 108-109).

NMR spectroscopy

The 1H-NMR-spectrum of fraction B1-2 S.5 VRP.1 contained mostly carbohydrate signals.

The 1H-NMR-spectrum of fraction B1-2 S.5 VRP.2 had signals which may be derived from flavonoids (kaempferol and quercetin glycosides), but also large carbohydrate signals.

1H-NMR-spectra of fractions B1-2 S.5 VRP.3 and B1-2 S.5 VRP.4 contained about equal amounts of kaempferol and quercetin glycosides. 13C-NMR-spectra of these two fractions confirmed the findings in the 1H-NMR-spectra.

The 1H-NMR-spectrum of fraction B1-2 S.5 VRP.5 contained more kaempferol than quercetin glycoside signals, but was otherwise quite like fraction B1-2 S.5 VRP.4.

Fraction B1-2 S.6 VRP.6 contained a substance which from comparison of spectral data with literature(Zhang, DeWitt et al. 2004) was identified as shikimic acid. Shikimic acid is ubiquous in higher plants, but is rarely found in high concentrations. It has not been reported previously from the genus *Sutherlandia* / *Lessertia*.

The last three fractions (B1-2 S.5 VRP.7-B1-2 S.5 VRP.9) appeared to contain a complex mixture of terpenoids. (spectrum 14.74-14.80, p. 162-165).

9.5.1.3 Determine combination of fraction B1-2 S.4 VRP. And B1-2 S.5 VRP.

All flavonoid-containing fractions from B1-2 S.4 and B1-2. S.5 contained different amounts of kaempferol and quercetin glycosides, and our aim was to separate these two glycosides with LPCC on Toyopearl HW40. After investigation with 1H-NMR-spectroscopy of all fractions, we decided to combine these fractions into two groups; B1-2 S4-5 VRP. Type A and B1-2 S4-5 VRP. Type B (Flowchart 12.8, p. 91).

Fractions which contained equal amount of kaempferol- and quercetin glycosides were combined into B1-2 S4-5 VRP. Type A

Fractions which contained kaempferol glycosides more than quercetin glycosides were combined into B1-2 S4-5 VRP. Type B

B1-2 S4-5 VRP. Type A contained the following fractions:

- B1-2 S.4 VRP.1
- B1-2 S.4 VRP.2
- B1-2 S.5 VRP.3

B1-2 S4-5 VRP. Type B contained the following fractions:

- B1-2 S.4 VRP.3
- B1-2 S.4 VRP.4
- B1-2 S.5 VRP.4
- B1-2 S.5 VRP.5

9.5.1.4 Fraction B1-2 S.4-5 VRP in LPCC with Toyopearl type A and type B

Ten fractions from type A and 13 fractions from type B were collected. All fractions from type A and type B were combined into 4 fractions (T1-T4) (flowchart 12.9, p. 92) based on analytical TLC and NMR spectroscopy.

NMR-spectroscopy

The 1H-NMR-spectrum of fraction B1-2 S.4-5 VRP. T1 contained mostly kaempferol glycosides, but had a small amount of quercetin glycosides. Two singlets from hydroxymethylglutaric acid and several signals which were assumed to be anomer signals from disaccharides were observed.

The 1H-NMR-spectrum of fraction B1-2 S.4-5 VRP. T2 was of a poorer quality than fraction B1-2 S.4-5 T1. It contained some signals similar to fraction B1-2 S.4-5 T1, but the region from 5 to 5.5 ppm of this spectrum (assumed to be the region of anomeric protons in

carbohydrates) showed two set of overlapping signals, which could be from disaccharides, although mixtures of monosaccharides cannot be excluded.

The 1H-NMR-spectrum of fraction B1-2 S.4-5 T3 appears to contain disaccharides of kaempferol and quercetin.

Fraction B1-2 S.4-5 T4 appears from its 1H-NMR-spectrum to contain mostly quercetin glycosides. IThis was confirmed by its 13-C-NMR-spectrum, which was similar to that of fraction B1-2 S.4-5 T3.

Fractions B1-2 S.4-5 T2 and B1-2 S.4-5 T3 contained mixtures of quercetin- and kaempferol glycosides, and these were therefore chosen for further separation on MCI CHP20P. (spectrum 14.81-14.87, p. 166-169)

9.5.1.5 Separation of fraction T2 with LPCC on MCI CHP20H

8 fractions were collected. These fractions were brought separately to dryness on a rotary evaporator and weighed. (Table 11.20, p. 109-111)

NMR-spectroscopy

Fraction B1-2 S.4-5 T2 M1 was quite small, and the 1H-NMR-spectrum showed that there were mixtures of quercetin- and kaempferol glycosides.

From the 1H-NMR-spectrum of fraction B1-2 S.4-5 T2 M2 we could see that it contained almost only kaempferol glycosides, and an anomer proton integrating for only 1 H-atom.

The 1H-NMR-spectrum of fraction B1-2 S.4-5 T2 M3 was similar to that of the previous fraction.

1H-NMR-spectra of fractions B1-2 S.4-5 T2 M4, B1-2 S.4-5 T2 M5 and B1-2 S.4-5 T2 M6 were not very different from fraction B1-2 S.4-5 T2 M3, but obviously these fractions contain a complex mixture of many substances.

B1-2 S.4-5 T2 M7 and B1-2 S.4-5 T2 M8 were small, and 1H-NMR-spectra of these two fractions was indicative of impure fractions and a poor signal to noise ratio.

Fractions B1-2 S.4-5 T2 M1 - B1-2 S.4-5 T2 M5 were chosen for further separation with HPLC, since these fractions were big enough for further work and also had better quality than the rest. (spectrum 14.88-14.95, p. 169-173)

9.5.1.6 Separation of fraction T3 with LPCC with MCI CHP20H

7 fractions were collected. These fractions were brought separately to dryness on a rotary evaporator and weighed. (Table 11.21, p. 111-112)

NMR-spectroscopy

In the 1H-NMR-spectrum of the first fraction, B1-2 S.4-5 T3 M1, we could see signals from quercetin glycosides and also a mixture of two substances. 13C-NMR-spectra had signals from two carbonyl carbons (probably from C4) at ca 178 ppm.

The second fraction B1-2 S.4-5 T3 M2 appeared to contain mostly quercetin glycosides, but a small amount of kaempferol glycosides. Fraction B1-2 S.4-5 T3 M3 may contain an equal amount of quercetin- and kaempferol glycosides. In both fractions there is a complex signals in the anomer proton region, which could be due to a mixture of many substances.

The 1H-NMR-spectrum of fraction B1-2 S.4-5 T3 M4 had larger peaks of kaempferol- than quercetin glycosides.

Fraction B1-2 S.4-5 T3 M5 was an interesting fraction which seemed to contain only kaempferol glycosides (H- at position 6 and 8 at 6,2-6,4 ppm in 1H-NMR-spectra, and B-ring protons as two doublets at 6.9 and 8.1 ppm). There were two sets signals superposed upon each other, indicated a mixture of two substances, these could be kaempferol with disaccharides (glucose-glucose and glucose-apiose) which have been isolated previously from this plant (Mabusela, pers. comm.).

Fraction B1-2 S.4-5 T3 M6 in its 1H-NMR-spectrum looked similar to the previous fraction, but not as clear as fraction B1-2 S.4-5 T3 M4. And fraction B1-2 S.4-5 T3 M7 was a small and complex fraction. (spectrum 14.96-14.105, p. 173-178)

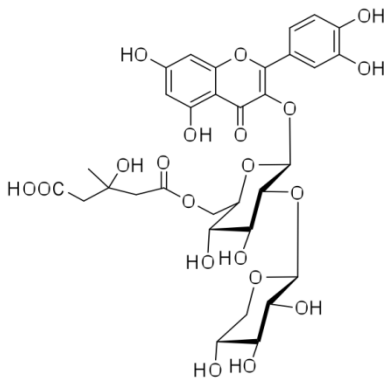
From this series, fraction B1-2 S.4-5 T3 M1 - B1-2 S.4-5 T3 M6 were chosen for separation with HPLC. The last fraction was too small and complex for further work.

9.5.1.7 Preparative HPLC of fractions from B1-2 S.4-5 T2 M1 - B1-2 S.4-5 T2 M5 and B1-2 S.4-5 T3 M1 - B1-2 S.4-5 T3 M6

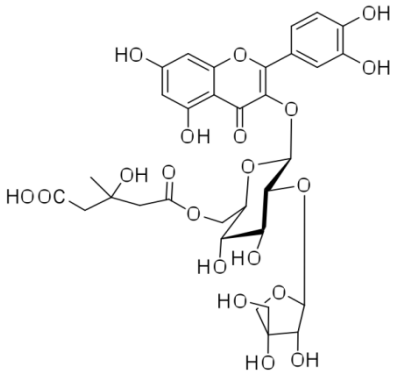
From fractions B1-2 S.4-5 T2 M1 - B1-2 S.4-5 T2 M5 and B1-2 S.4-5 T3 M1 - B1-2 S.4-5 T3 M6, 56 subfractions were obtained, based on their retention times and chromatographic patterns. Most of these subfractions weighed less than 1 mg. These small subfractions were not studied further. Larger subfractions were studied by 1H-NMR and 13C-NMR spectroscopy (Tables 13.22-13.33, p. 112-118).

NMR spectroscopy

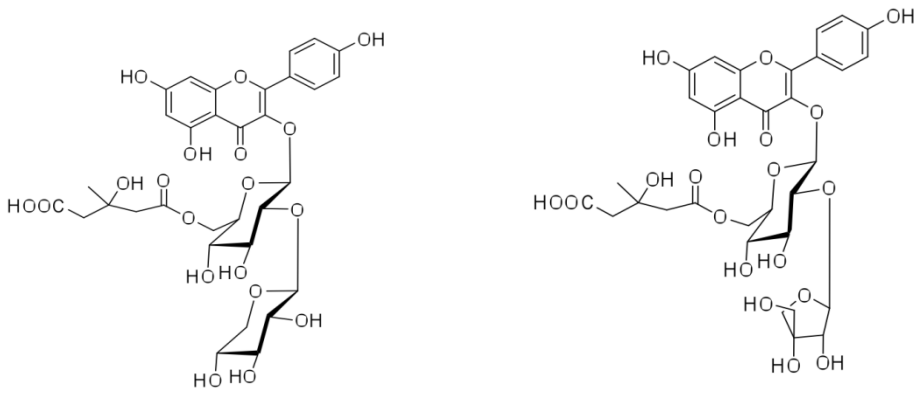
From a tabulation of NMR data of the larger subfractions (Tables 13.45, p. 125), it appears that at least three and possibly four different kaempferol glycosides are present. The quercetin glycosides were less pure and were present in smaller amounts, so these were not given priority for further studies. All of the kaempferol glycoside-containing subfractions show signals which can be ascribed to a hydroxymethylglutaryl moiety, in accordance with what has been found elsewhere (Mabusela, pers. comm.). We do not have any NMR data available for previously isolated compounds. However, since only two kaempferol glycosides have been found previously in the plant, at least one of our compounds must be different from the ones known. From a search in the SciFinder database, it appears that no hydroxymethylglutaryl derivatives of kaempferol glycosides have been reported earlier, so it would seem possible that at least one of our substances is a new natural product. Analytical HPLC shows that although our fractions are not pure compounds, all of them contain one major substance and only small amounts of contaminants. Further work is needed to elucidate the definitive structure of these flavonoids and also to carry out studies on their biological activity. (spectrum 14.106-14.114, p. 178-183)



Quercetin with glucose-glucose



Quercetin with glucose-apiose



Kaempferol with glucose-glucose Kaempferol with glucose-apiose

Figure xxx. Suggested formulas for flavonoids previously isolated from *Sutherlandia frutescens* (Mabusela, pers. comm.)

9.5.2 Separation of fraction B5-6 on LPCC with SephadexLH20

A quite big fraction with 1605,4 mg, this was chosen for separation with Sephadex LH20. There was collected 30 fractions, which were combined into 6 fractions based on analytic TLC followed by observation in UV-light with short and long wave, and ceric spraying. This yielded 1166,1 mg (Table 11.34, p. 118-119)(Flowchart 12.7,p. 90).

NMR-spectroscopy

1H-NMR-spectra of these first fractions B5-6 S.1 and B5-6 S.2 appeared to contain signals of sutherlandiosides(Fu et al. 2007).

The third fraction was similar to the two first fractions, but in addition, it had olefin signals in its 1H-NMR-spectrum. The 13C-NMR-spectrum contained 2 anomer signals from carbohydrate and 1 carbonyl signal. It was regarded as an interesting fraction for further separation.

The 1H-NMR-spectrum of fraction B5-6 S.4 was similar to that of the previous fraction, but this fraction was smaller. And the 1 H-NMR-spectrum of fraction B5-6 S.5 looked similar to fraction B5-6 S.4. (spectrum 14.115-14.120, p. 183-186)

9.5.2.1 Separation of fraction B5-6 S.3 with VersaFlash normal phase column

Total weight of this fraction was 511 mg. The fraction was applied on VersaFlash normal phase column, and 22 fractions were collected, followed by analytical TLC and screening in UV-light with short and long wavelength and ceric spraying for determination of combination into fractions. It yielded 456,6 mg (Table 13.35, p. 119-121).

NMR-spectroscopy

Fraction B5-6 S.3 VNP.1 was assumed to contain sutherlandiosides due to its 1H-NMR-spectrum.

In the next five fractions it was assumed that they contained one substance which may be sutherlandioside D (fraction B5-6 S.3 VNP.2- B5-6 S.3 VNP.6) based on comparison with literature data (Fu et al. 2007) for spectra of sutherlandioside D.

The 1H-NMR-spectrum of fraction B5-6 S.3 VNP.7 contained signals of other sutherlandiosides, probably one of them was sutherlandioside B.

9.5.2.2 Fraction B5-6 S.3 VNP.2 separation with chromatotron

This fraction (78,9 mg) was dissolved in starting eluent and then applied on the prepared glass plate. 9 fractions totally were obtained. Three fractions (fraction 5-7) were chosen for further work, because these fractions gave dark dots on analytic TLC reverse phase plate after being sprayed with ceric reagent and dried in the oven. (Table 13.36, p. 121-122)

NMR-spectroscopy

1H-NMR-spectra of all three fractions showed signals which probably were sutherlandioside D. In fraction B5-6 S.3 VNP.2 CHR 2, sutherlandioside D appeared to constitute about half of the fraction, while other triterpenoid glycosides were present in much smaller amounts.

The presence of sutherlandioside D was also confirmed by the 13C-NMR-spectrum of fraction B5-6 S.3 VNP.2 CHR.1. (spectrum 14.131-14.139,p. 192-195)

9.5.2.3 Further work of fractions B5-6 S.3 VNP.2 CHR 1- B5-6 S.3 VNP.2 CHR 3 with HPLC

All three fractions were first tested on analytical HPLC to find the optimal method for detection and preparative HPLC separation. This took some time, since the analytical HPLC column initially used was very old, did not work well, and had to be exchanged with a new one. This gave considerably better results.

Technical problems with the preparative HPLC column and a faulty injector also led to delay. It took quite long time to fix all these problems. But it was worth waiting, because we have got good separation with all three fractions.

From fraction B5-6 S.3 VNP.2 CHR 1 there were collected four fractions i.e. four peaks (Table 11.37, p. 122). Fraction B5-6 S.3 VNP.2 CHR 2 yielded 5 fractions (Table 11.38, p. 122) and the last fraction B5-6 S.3 VNP.2 CHR 3 were collected into 5 fractions (Table 11.39, p. 122-123).

These fourteen fractions were combined to major fractions A-F. And fractions A-B and E-F separated on preparative HPLC. From fraction A there were collected 2 fractions, 2 fractions collected from fraction B, 3 fractions collected from fraction E and 4 fractions collected from fraction F. (Tables 13.41-13.44, p. 189-191)

NMR-spectroscopy

Preparative HPLC of fraction A-B and E-F yielded four major fractions. Two of them were small (ca. 2 mg each) and appeared from their NMR spectra to be impure. The two largest ones, E HPLC 5 (6,7 mg) and B HPLC 6 (4,2 mg) seemed to contain one substance each. The 13C-NMR spectrum of B HPLC 6 was virtually identical to the published spectrum for sutherlandioside C (Table 13.40, p. 125-126). Since all signals seemed to be displaced 0,4 ppm relative to the literature data, a correction was made for this. This displacement has been observed previously in spectra recorded on our instrument (Malterud, pers. comm.).

Most of the signals from fraction E HPLC 5 also fit well with those from sutherlandioside C. Discrepancies were, however, observed from signals in the outer region of the side chain and in the signal from the anomeric carbon atom in the glucose moiety. Two possible explanations for this might be that the glucose is bound to C-24 instead of C-25, or that this substance is a C-24 epimer of sutherlandioside C. Conceivably, this could be decided from Heteronuclear Multiple Bond Correlation (HMBC) spectra, but amount of substance and lack of time precluded this. (spectrum 14.127-14.130, p. 189-191)

10. Conclusion

Plant material of *Sutherlandia frutescens* seems to contain many interesting compounds. Sutherlandioside C, and an epimer or regioisomer of sutherlandioside C have been identified in the BuOH extract. It appears that at least three and possibly four different kaempferol glycosides are present in the BuOH extract. Since only two kaempferol glycosides have been found previously in the plant, at least one of our compounds must be different from the ones known. From a search in the SciFinder database, it appears that no hydroxymethylglutaryl derivatives of kaempferol glycosides have been reported earlier, so it would seem possible that at least one of our substances is a new natural product. All of the kaempferol glycoside-containing subfractions show signals which can be ascribed to a hydroxymethylglutaryl moiety, in accordance with what has been found elsewhere (Mabusela, pers. comm.). The quercetin glycosides were also found in several fractions, but they were less pure and were present in smaller amounts, so these were not given priority for further studies.

In the fractions from the EtOAc extract, it seemed that glycosylated terpenoids based on kaempferol or quercetin and containing a hydroxymetylglutaryl moiety were present in most of the fractions. These appear to be hitherto undescribed products. A series of triterpenoid glycosides, sutherlandioside A-D, has recently been reported from *Sutherlandia* by Fu et al (2007). In our EtOAc extract, sutherlandiosides B, C and D and their epimers / regioiomers have been provisionally identified. The epimers/ regioisomers seem to be new natural products. In addition to these compounds, it appears from NMR data that two further unidentified compounds are present in our column fractions.

The DCM extract (ICD_{50}: 70 ± 2 µg/ml) and the EtOAc extracts (ICD_{50}: 57 ± 3 µg/ml) showed higher 15-LO activity than other extracts. In the DPPH-test, all extracts had low radical scavenging activity (SD_{50} >166,7 µg/ml). This can be explained by the high concentration of saponins or triterpenoids and the low amount of phenolic compounds in *S. frutescens.*

Plant extracts of *S. frutescens* contain many active substances which can contribute to the plant´s medicinal activity. Further work is needed to elucidate the definitive structure of several compounds and also to carry out studies on their biological activity. Due to the small amount of substances and lack of time, this was not possible within the framework of the present study.

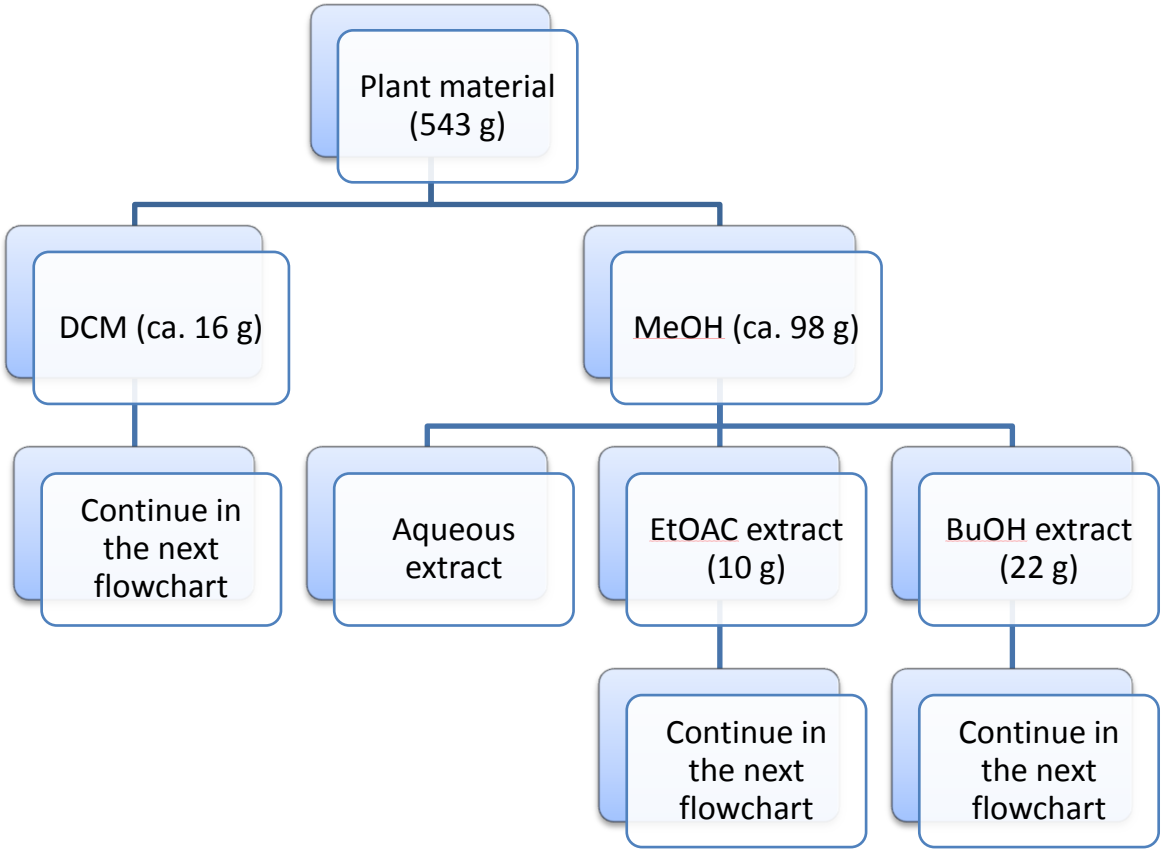
11. Suggestions for further work

Substances of interest for further work on isolation, structure elucidation and bioactivity studies appear to be present in all extracts. It would be of interest to carry out further studies on these.

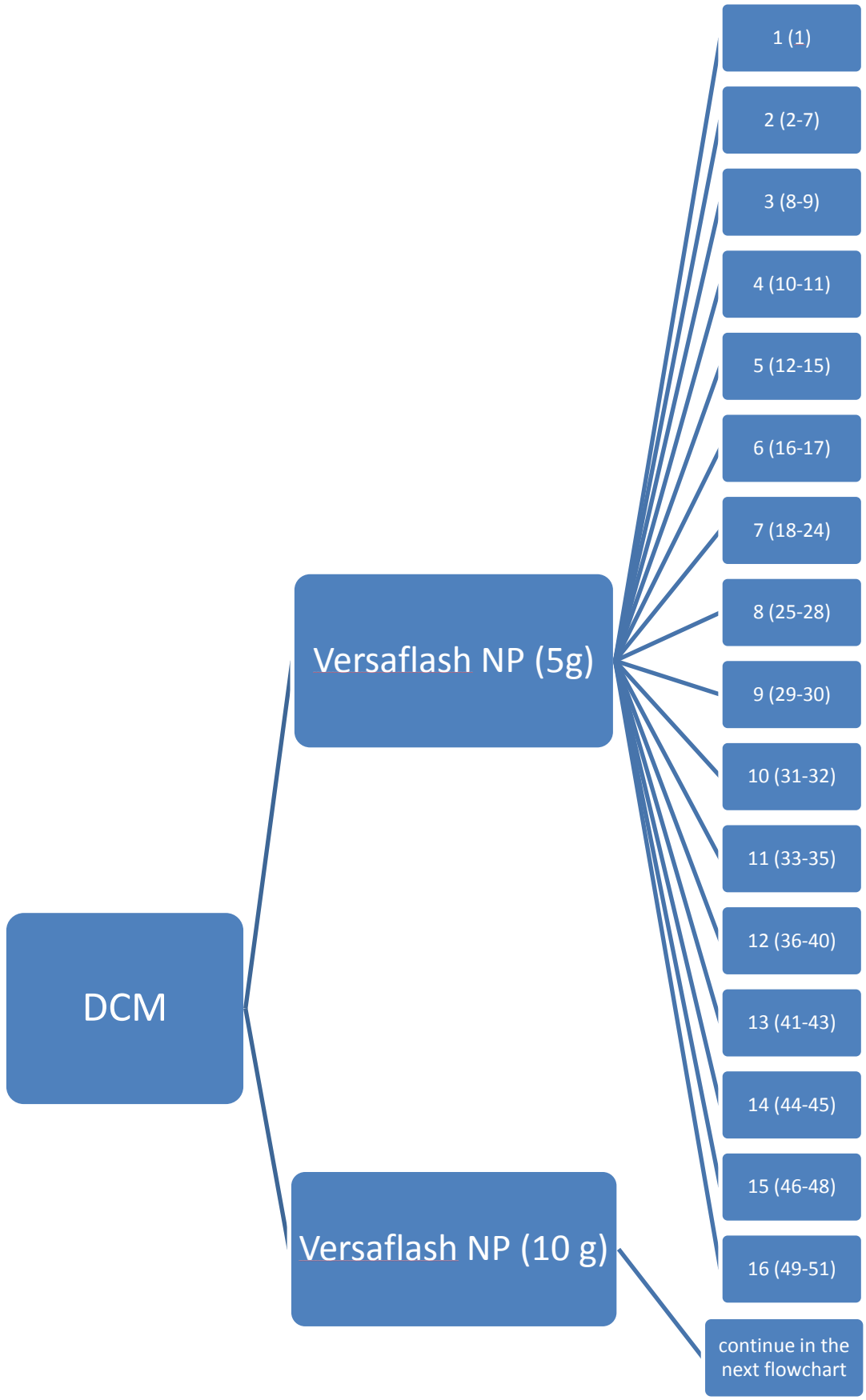
Since the EtOAc extract showed highest activity in 15-LO test, it could be a high priority to separate this extract further.

12. Flowcharts

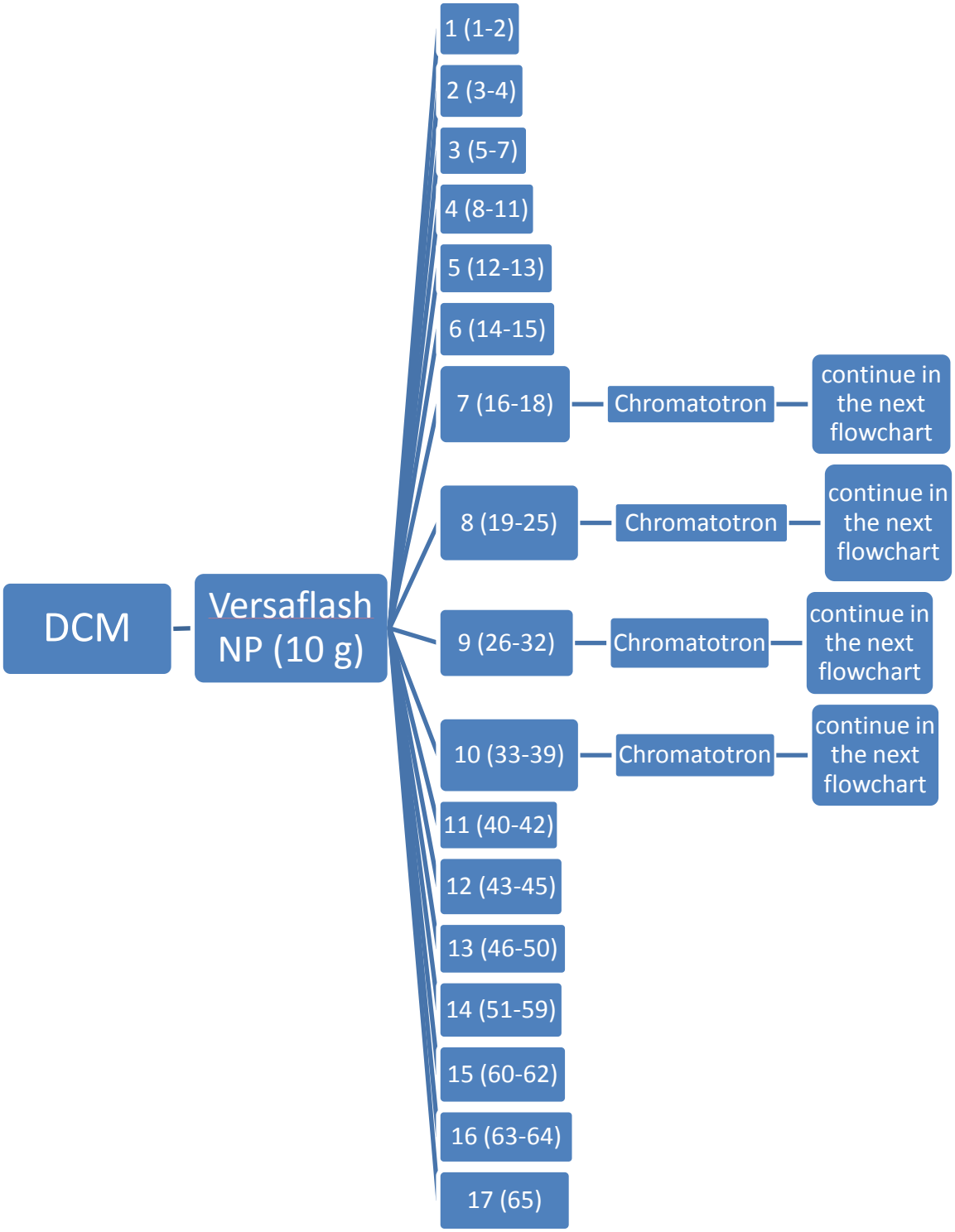
Flowchart 12.1



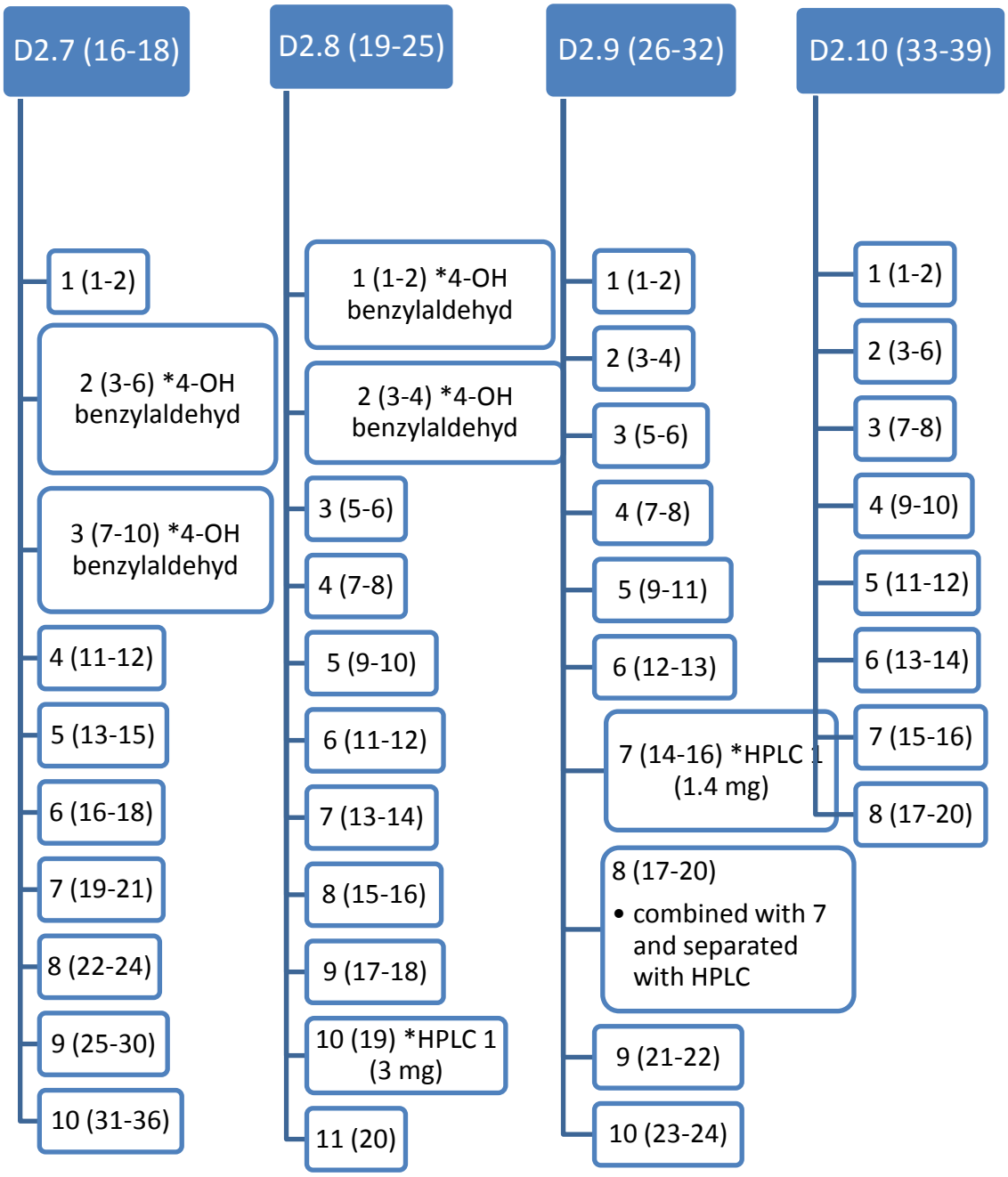
Flowchart 12.2



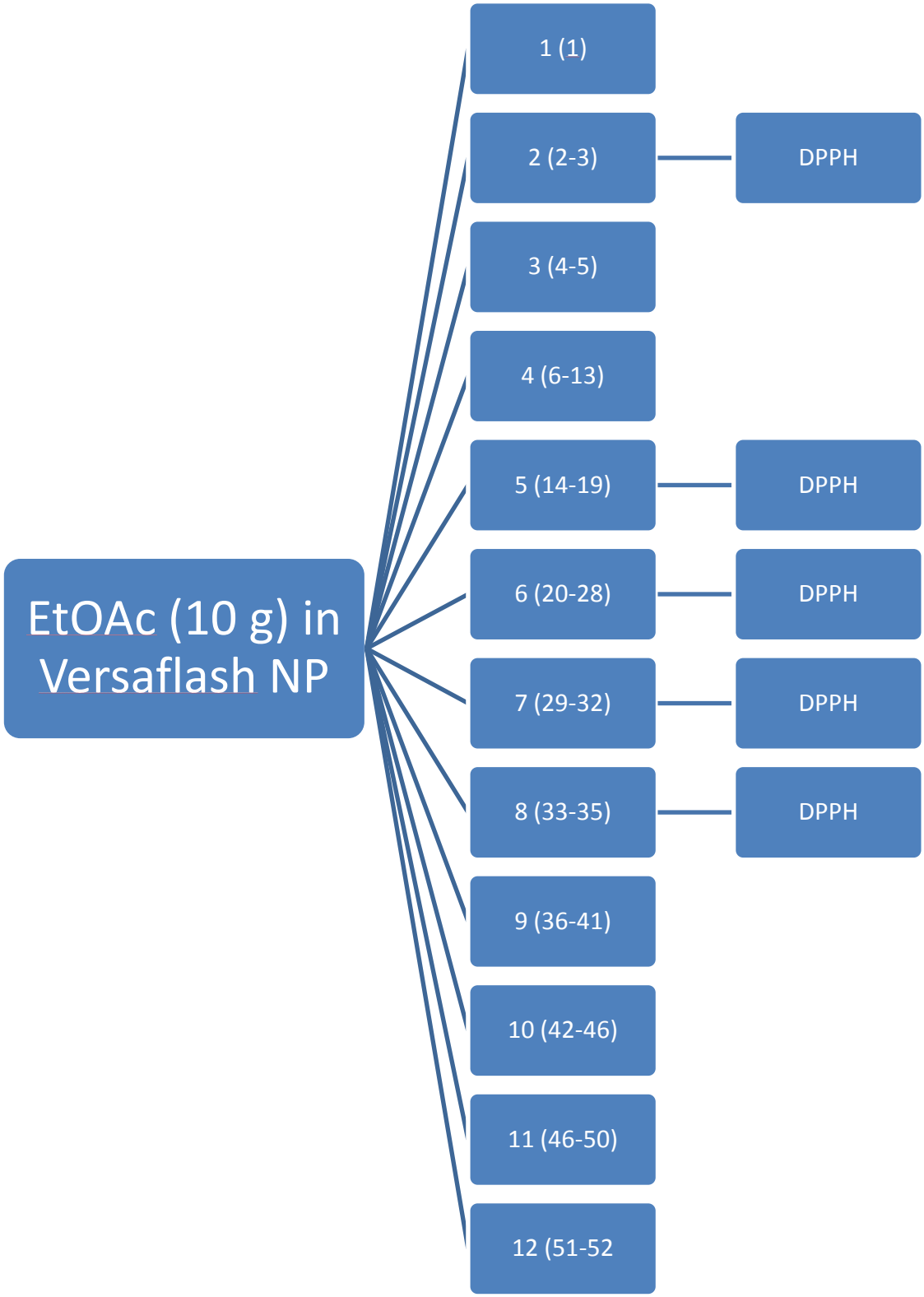
Flowchart 12.3



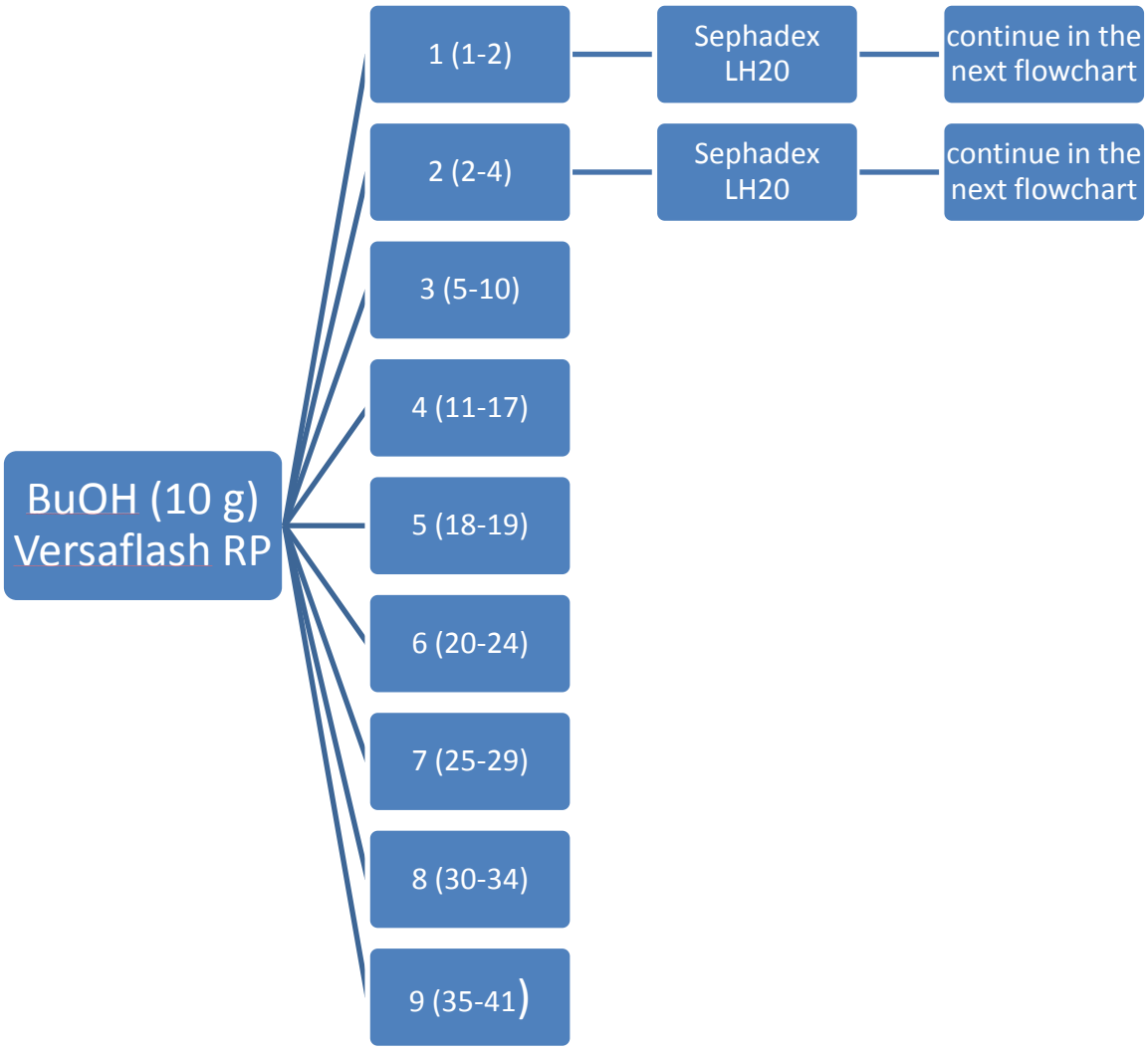
Flowchart 12.4



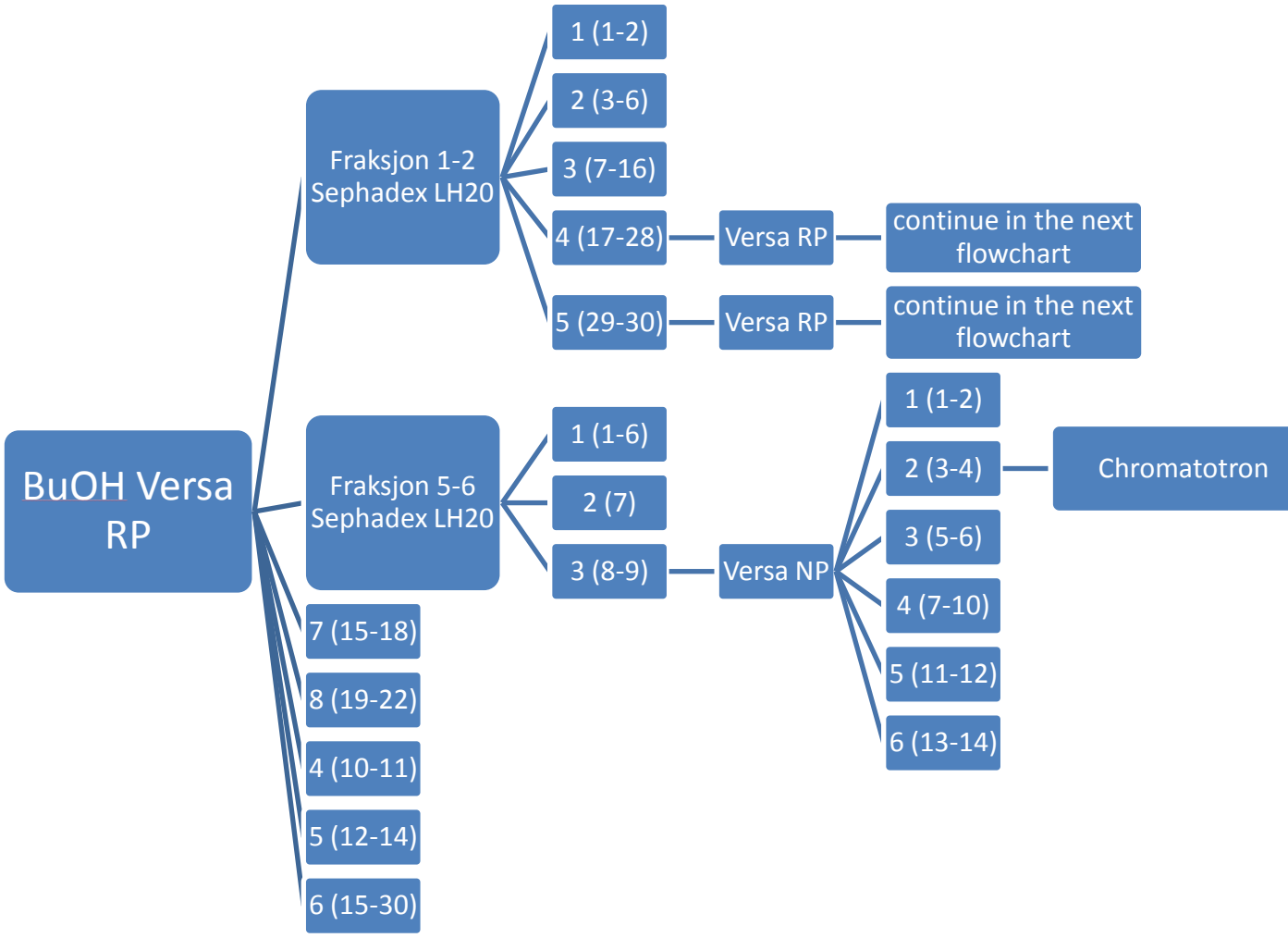
Flowchart 12.5



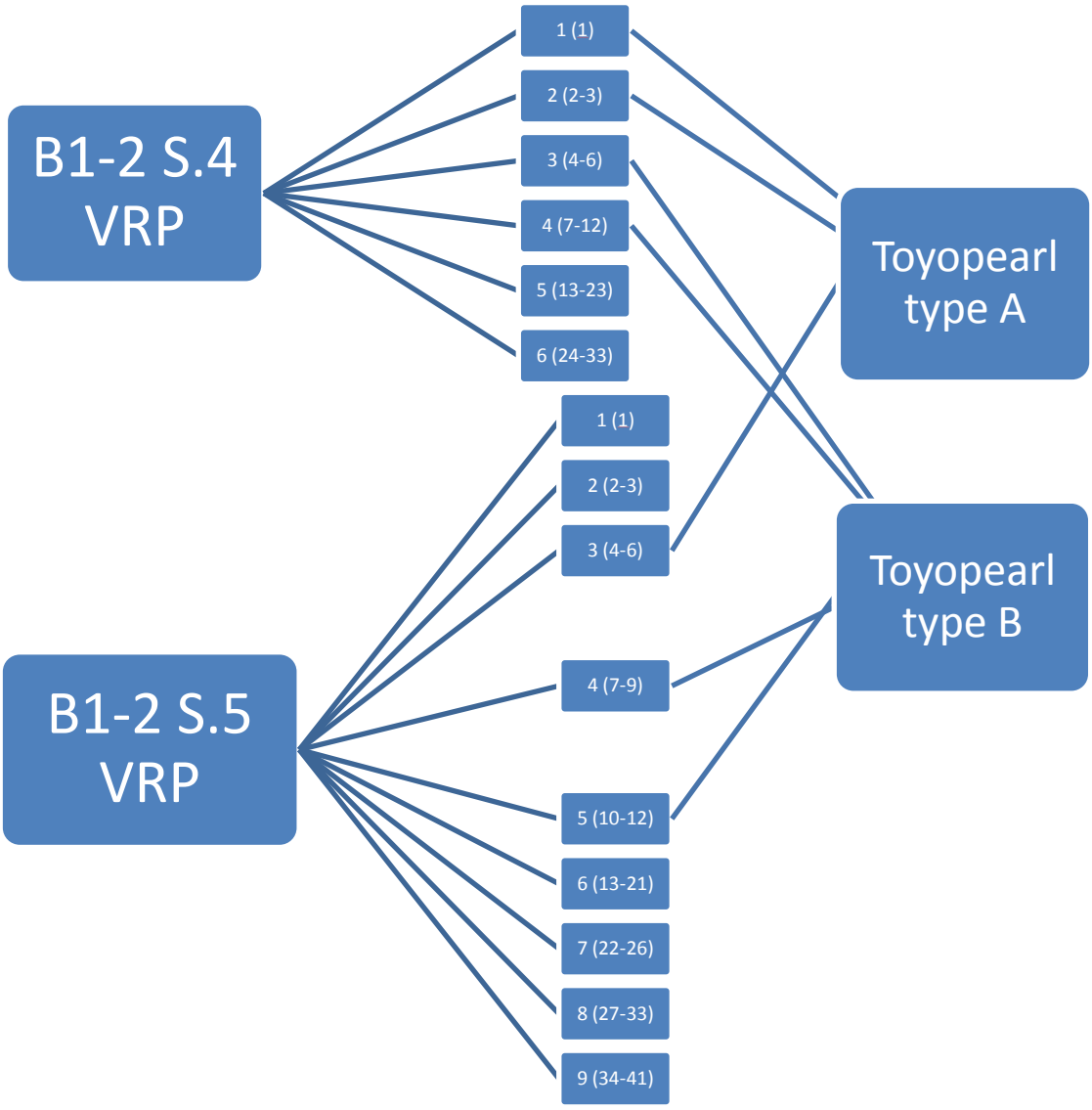
Flowchart 12.6



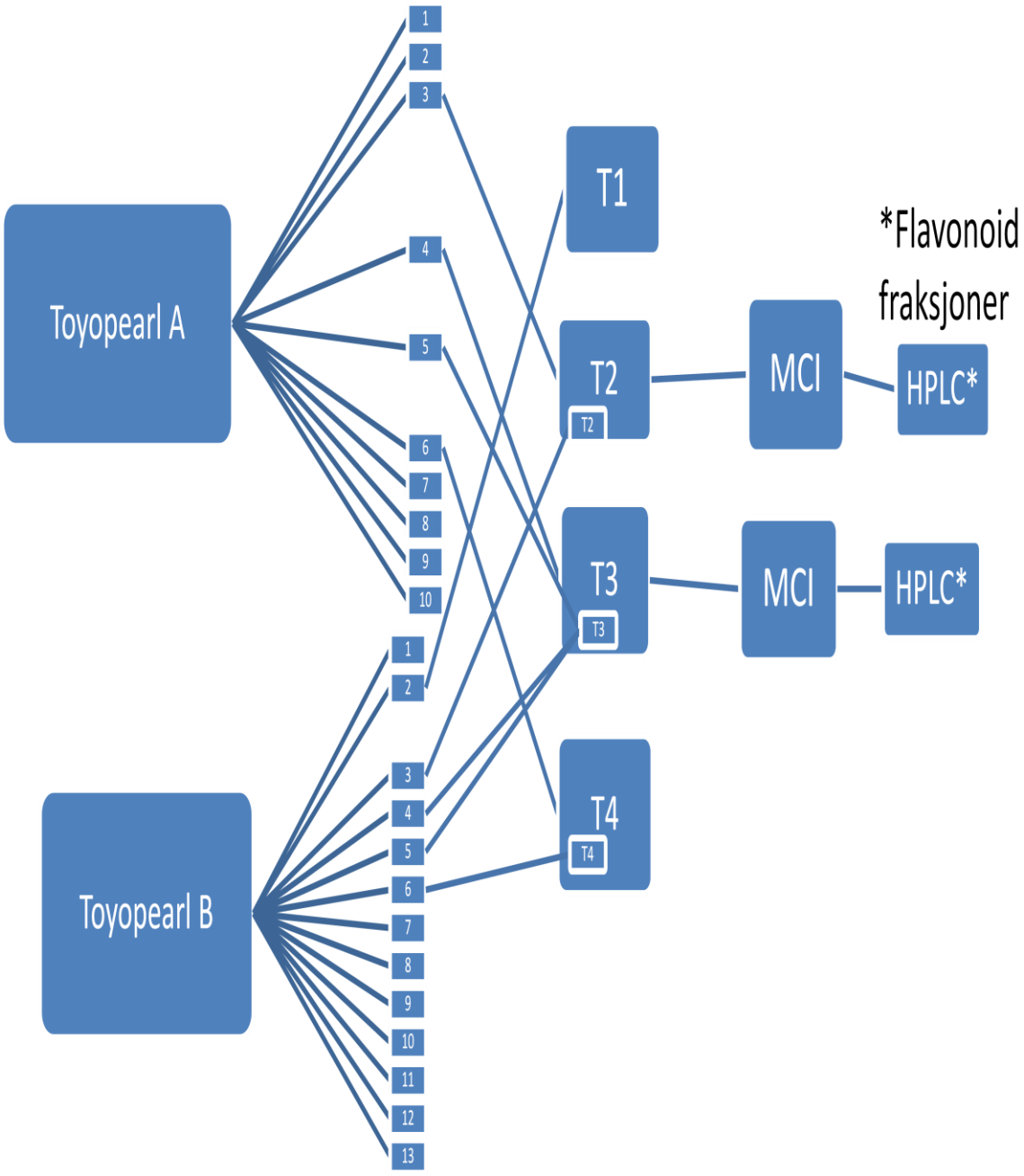
Flowchart 12.7



Flowchart 12.8. Combination of fractions B1-2 S.4 VRP ad B1-2 S.5 VRP into Toyopearl type A and B.



Flowchart 12.9



13. Tables

Table 13.1. VersaFlash chromatography of DCM extract (5 g)

Fraction	Amount (ml)	Eluent	Fraction	Amount (ml)	Mass (mg)
1	50	Chl + 25 % EtOAc	1	50	1022,3
2	50	Chl + 25 % EtOAc			
3	50	Chl + 25 % EtOAc			
4	50	Chl + 25 % EtOAc			
5	50	Chl + 25 % EtOAc			
6	50	Chl + 25 % EtOAc			
7	50	Chl + 25 % EtOAc	2	300	364,4
8	50	Chl + 25 % EtOAc			
9	50	Chl + 25 % EtOAc	3	100	617,2
10	50	Chl + 25 % EtOAc			
11	50	Chl + 25 % EtOAc	4	100	369,7
12	50	Chl + 25 % EtOAc			
13	50	Chl + 25 % EtOAc			
14	50	Chl + 25 % EtOAc			
15	50	Chl + 25 % EtOAc	5	200	69
16	50	Chl + 25 % EtOAc			
17	50	Chl + 25 % EtOAc	6	100	14,6
18	50	Chl + 50 % EtOAc			
19	50	Chl + 50 % EtOAc			
20	50	Chl + 50 % EtOAc			
21	50	Chl + 50 % EtOAc			
22	50	Chl + 50 % EtOAc			
23	50	Chl + 50 % EtOAc			
24	50	Chl + 50 % EtOAc	7	350	43,5
25	50	Chl + 50 % EtOAc			
26	50	Chl + 50 % EtOAc			
27	50	Chl + 50 % EtOAc			
28	50	Chl + 50 % EtOAc	8	200	58,8
29	50	Chl + 50 % EtOAc			
30	50	Chl + 50 % EtOAc	9	100	20,6
31	50	Chl + 50 % EtOAc			
32	50	Chl + 50 % EtOAc	10	100	15,3
33	50	Chl + 50 % EtOAc			
34	50	Acetone + 50 % EtOAc			
35	50	Acetone + 50 %	11	150	136,4

		EtOAc			
36	50	Acetone + 50 % EtOAc			
37	50	Acetone + 50 % EtOAc			
38	50	Acetone + 50 % EtOAc			
39	50	Acetone + 50 % EtOAc			
40	50	Acetone + 50 % EtOAc	12	250	46,8
41	50	Acetone + 50 % EtOAc			
42	50	Acetone + 50 % EtOAc			
43	50	Acetone + 50 % EtOAc	13	150	280,8
44	50	Acetone + 50 % EtOAc			
45	50	Acetone + 50 % EtOAc	14	100	209,2
46	50	Acetone			
47	50	Acetone			
48	50	Acetone	15	150	221,5
49	50	Acetone			
50	50	Acetone			
51	50	Acetone	16	150	85,4
SUM					3575,5

Table 13.2. VersaFlash chromatography of DCM extract (10 g)

Fraction	Amount (ml)	Eluent	Fraction	Amount (ml)	Mass (mg)
1	50	Chl			
2	50	Chl	1	100	4026,5
3	50	Chl			
4	50	Chl	2	100	645,4
5	50	Chl			
6	50	Chl			
7	50	Chl	3	150	60,8
8	50	Chl			
9	50	Chl			
10	50	Chl	4	200	48,2

11	50	Chl			
12	50	Chl			
13	50	Chl	5	100	909,4
14	50	Chl			
15	50	Chl	6	100	1322,2
16	50	Chl			
17	50	Chl			
18	50	Chl	7	150	110,9
19	50	Chl			
20	50	Chl			
21	50	Chl			
22	50	Chl			
23	50	Chl			
24	50	Chl			
25	50	Chl	8	350	125
26	50	Chl			
27	50	Chl			
28	50	Chl			
29	50	Chl			
30	50	Chl			
31	50	Chl			
32	50	Chl	9	350	122
33	50	Chl			
34	50	Chl			
35	50	Chl			
36	50	Chl			
37	50	Chl			
38	50	Chl + 10 % EtOAc	10	350	110,7
39	50	Chl + 10 % EtOAc			
40	50	Chl + 10 % EtOAc			
41	50	Chl + 10 % EtOAc			
42	50	Chl + 10 % EtOAc	11	150	162,7
43	50	Chl + 10 % EtOAc			
44	50	Chl + 10 % EtOAc			
45	50	Chl + 10 % EtOAc	12	150	25,7
46	50	Chl + 20 %	13	250	6,5

		EtOAc			
47	50	Chl + 20 % EtOAc			
48	50	Chl + 20 % EtOAc			
49	50	Chl + 20 % EtOAc			
50	50	Chl + 20 % EtOAc			
51	50	Chl + 20 % EtOAc			
52	50	Chl + 20 % EtOAc			
53	50	Chl + 20 % EtOAc			
54	50	Chl + 40 % EtOAc			
55	50	Chl + 40 % EtOAc			
56	50	Chl + 40 % EtOAc			
57	50	Chl + 40 % EtOAc			
58	50	Chl + 40 % EtOAc			
59	50	Chl + 40 % EtOAc	14	450	238,7
60	50	EtOAc			
61	50	EtOAc			
62	50	EtOAc	15	150	430,5
63	50	EtOAc			
64	50	Acetone	16	100	100,8
65	250	Acetone	17	250	2267,7
SUM				10713,7	~ 9 g

Table 13.3. Chromatotron of fraction D2.7 (110,9 mg)

Fraction	Amount (ml)	Eluent	Fraction	Amount (ml)	Mass (mg)
1	15	Chl + 20 % EtOAc			
2	15	Chl + 20 % EtOAc	1	30	7
3	15	Chl + 20 % EtOAc			
4	15	Chl + 20 % EtOAc	2	60	27

5	15	Chl + 33,34 % EtOAc			
6	15	Chl + 33,34 % EtOAc			
7	15	Chl + 33,34 % EtOAc			
8	15	Chl + 33,34 % EtOAc			
9	15	Chl + 50 % EtOAc			
10	15	Chl + 50 % EtOAc	3	60	22
11	15	Chl + 50 % EtOAc			
12	15	Chl + 50 % EtOAc	4	30	3,4
13	15	Chl + 50 % EtOAc			
14	15	Chl + 50 % EtOAc			
15	15	EtOAc	5	45	6,4
16	15	EtOAc			
17	15	EtOAc			
18	15	EtOAc	6	45	19,1
19	15	EtOAc			
20	15	EtOAc			
21	15	Acetone	7	45	2,3
22	15	Acetone			
23	15	Acetone			
24	15	Acetone	8	45	2,6
25	15	Acetone			
26	15	Acetone			
27	15	Acetone + 10 % MeOH			
28	15	Acetone + 10 % MeOH			
29	15	Acetone + 10 % MeOH			
30	15	Acetone + 20 % MeOH	9	90	5,1
31	15	Acetone + 20 % MeOH			
32	15	Acetone + 20 % MeOH			
33	15	MeOH			
34	15	MeOH			
35	15	MeOH			
36	15	MeOH	10	90	16,3
SUM					111,2

Table 13.4. Chromatotron of D2.8 (125 mg)

Fraction	Amount (ml)	Eluent	Fraction	Amount (ml)	Mass (mg)
1	25	Chl + 20 % EtOAc			
2	25	Chl + 20 % EtOAc	1	50	10,1
3	25	Chl + 33,34 % EtOAc			
4	25	Chl + 33,34 % EtOAc	2	50	24,8
5	25	Chl + 50 % EtOAc			
6	25	Chl + 50 % EtOAc	3	50	12,5
7	25	Chl + 50 % EtOAc			
8	25	EtOAc	4	50	36,6
9	25	EtOAc			
10	25	EtOAc	5	50	10,5
11	25	EtOAc			
12	25	Acetone	6	50	21,1
13	25	Acetone			
14	25	Acetone	7	50	24,9
15	20	Acetone			
16	20	Acetone	8	40	7,3
17	20	Acetone			
18	20	Acetone	9	40	1,7
19	40	MeOH	10	40	12,8
20	15	MeOH	11	15	8,5
SUM					170,8

Table 13.5. Chromatotron of D2.9 (122 mg)

Fraction	Amount (ml)	Eluent	Fraction	Amount (ml)	Mass (mg)
1	25	Chl + 10 % EtOAc			
2	25	Chl + 10 % EtOAc	1	50	2,4
3	25	Chl + 20 % EtOAc			
4	25	Chl + 20 % EtOAc	2	50	2,9
5	25	Chl + 20 % EtOAc	3	50	8,6

6	25	Chl + 30 % EtOAc			
7	25	Chl + 30 % EtOAc			
8	25	Chl + 40 % EtOAc	4	50	10,8
9	25	Chl + 40 % EtOAc			
10	25	Chl + 50 % EtOAc			
11	25	Chl + 50 % EtOAc	5	75	6,5
12	25	Chl + 70 % EtOAc			
13	25	Chl + 70 % EtOAc	6	50	1,5
14	25	EtOAc			
15	25	EtOAc			
16	25	EtOAc + 5 % Acetone	7	75	3,1
17	25	EtOAc + 5 % Acetone			
18	25	EtOAc + 10 % Acetone			
19	25	EtOAc + 10 % Acetone			
20	25	EtOAc + 10 % Acetone	8	100	3,3
21	25	EtOAc + 10 % Acetone			
22	12,5	Acetone	9	37,5	1,1
23	25	Acetone			
24	25	Acetone + 20 % MeOH	10	50	2,9
SUM					42,9

Table 13.6. Chromatotron of fraction D2.10 (110,7 mg)

Fraction	Amount (ml)	Eluent	Fraction	Amount (ml)	Mass (mg)
1	15	Chl + 50 % EtOAc			
2	15	Chl + 50 % EtOAc	1	30	4,1
3	15	Chl + 50 % EtOAc			
4	15	Chl + 50 % EtOAc			
5	15	EtOAc			
6	15	EtOAc	2	60	9,9
7	15	EtOAc			
8	15	EtOAc	3	30	5,3
9	15	EtOAc			
10	15	EtOAc	4	30	4

11	15	Acetone			
12	15	Acetone	5	30	3,1
13	15	Acetone			
14	15	Acetone + 20 % MeOH	6	30	2,1
15	15	Acetone + 20 % MeOH			
16	15	Acetone + 20 % MeOH	7	30	4
17	15	Acetone + 20 % MeOH			
18	15	Acetone + 20 % MeOH			
19	15	Acetone + 20 % MeOH			
20	15	MeOH	8	60	4,1
SUM					36,6

Table 13.7. HPLC of fraction D2.CHR.9.7-8

Peak	Fraction	Mass (mg)
1	D2.9CHR.7-8 HPLC 5	1,4
SUM		1,4

Table 13.8. HPLC of fraction D2.CHR.8.10

Peak	Fraction	Mass (mg)
1	D2.8CHR.10 HPLC 2	0,9
2	D2.8CHR.10 HPLC 5	3
SUM		3,9

Table 13.12. VersaFlash normal phase column fractioning of EtOAc extract from primary MeOH extract

Fraction	Amount (ml)	Eluent	Fraction	Amount (ml)	Mass (mg)
1	50	EtOAc	1	50	120,1

2	50	EtOAc			
3	50	EtOAc	2	100	1705,5
4	50	EtOAc			
5	50	EtOAc	3	100	449,4
6	50	EtOAc			
7	50	EtOAc			
8	50	EtOAc			
9	50	EtOAc			
10	50	EtOAc			
11	50	EtOAc + 10 % Acetone			
12	50	EtOAc + 10 % Acetone			
13	50	EtOAc + 10 % Acetone	4	400	464,6
14	50	EtOAc + 10 % Acetone			
15	50	EtOAc + 20 % Acetone			
16	50	EtOAc + 20 % Acetone			
17	50	EtOAc + 20 % Acetone			
18	50	EtOAc + 20 % Acetone			
19	50	EtOAc + 50 % Acetone	5	250	729,6
20	50	EtOAc + 50 % Acetone			
21	50	EtOAc + 50 % Acetone			
22	50	EtOAc + 50 % Acetone			
23	50	EtOAc + 50 % Acetone			
24	50	EtOAc + 50 % Acetone			
25	50	EtOAc + 50 % Acetone			
26	50	EtOAc + 50 % Acetone			
27	50	EtOAc + 50 % Acetone			
28	50	EtOAc + 50 %	6	450	3696,5

		Acetone			
29	50	EtOAc + 50 % Acetone			
30	50	EtOAc + 50 % Acetone			
31	50	EtOAc + 50 % Acetone			
32	50	EtOAc + 50 % Acetone	7	200	765,8
33	50	EtOAc + 50 % Acetone			
34	50	Acetone			
35	50	Acetone	8	150	272,4
36	50	Acetone			
37	50	Acetone			
38	50	Acetone			
39	50	Acetone			
40	50	Acetone			
41	50	Acetone	9	300	723,2
42	50	Acetone			
43	50	Acetone			
44	50	Acetone			
45	50	Acetone	10	200	151,3
46	50	Acetone			
47	50	Acetone			
48	50	EtOAc			
49	50	EtOAc	11	200	81,9
50	50	EtOAc			
51	500	EtOAc + 50 % MeOH			
52	50	EtOAc			
53	50	EtOAc	12	650	731,2
SUM					**9891,5**

Table 13.15. DPPH-test of EtOAC fractions

Fraction	Concentration (µg/ml)	% Radical scavenger	SD	SC50 (µg/ml)
E1	Do not measure			
E2	41,7	14,1	1,8	
	83,3	20,8	0,8	
	166,7	35,9	0,8	>167

E3	Do not measure			
E4	Do not measure			
E5	41,7	3,5	0,5	
	83,3	5,8	0,4	
	166,7	10,2	0,9	>167
E6	166,7	8,3	0,2	>167
E7	166,7	5,8	1,1	>167
E8	166,7	8,5	0,6	>167
E9	166,7	14,5	0,6	>167
E10	Do not measure			
E11	Do not measure			
E12	Do not measure			

Table 13.16. VersaFlash chromatography with reverse phase column of BuOH extract

Fraction	Amount (ml)	Eluent	Fraction	Amount (ml)	Mass (mg)
1	50	Water + 50 % MeOH	B1	50	558,6
2	25	Water + 50 % MeOH			
3	25	Water + 50 % MeOH			
4	25	Water + 50 % MeOH	B2	75	5072,4
5	50	Water + 50 % MeOH			
6	50	Water + 50 % MeOH			
7	50	Water + 50 % MeOH			
8	50	Water + 50 % MeOH			
9	50	Water + 50 % MeOH			
10	50	Water + 50 % MeOH	B3	300	899,7
11	50	Water + 50 % MeOH			
12	50	Water + 50 % MeOH	B4	350	317,9

13	50	Water + 50 % MeOH			
14	50	Water + 50 % MeOH			
15	50	Water + 50 % MeOH			
16	50	Water + 50 % MeOH			
17	50	Water + 50 % MeOH			
18	50	Water + 50 % MeOH			
19	50	Water + 70 % MeOH	B5	100	774,9
20	50	Water + 70 % MeOH			
21	50	Water + 70 % MeOH			
22	50	Water + 70 % MeOH			
23	50	Water + 70 % MeOH			
24	50	Water + 70 % MeOH	B6	250	830,5
25	50	Water + 70 % MeOH			
26	50	Water + 70 % MeOH			
27	50	Water + 70 % MeOH			
28	50	Water + 70 % MeOH			
29	50	Water + 70 % MeOH	B7	250	171,6
30	50	MeOH			
31	50	MeOH			
32	50	MeOH			
33	50	MeOH			
34	50	MeOH	B8	250	631,3
35	50	MeOH			
36	50	MeOH			
37	50	MeOH			
38	50	MeOH			
39	50	MeOH	B9	350	265,2

40	50	MeOH			
41	50	MeOH			
SUM					9522,1

Table 13.17. LPCC with Sephadex LH20 of fraction B1-2 (5631 mg)

Fraction	Amount (ml)	Eluent	Fraction	Amount (ml)	Mass (mg)
1	60	Water + 10 % MeOH			
2	60	Water + 10 % MeOH	1	120	38
3	60	Water + 10 % MeOH			
4	60	Water + 10 % MeOH			
5	60	Water + 10 % MeOH			
6	60	Water + 10 % MeOH	2	240	2924
7	60	Water + 10 % MeOH			
8	60	Water + 10 % MeOH			
9	60	Water + 10 % MeOH			
10	60	Water + 10 % MeOH			
11	60	Water + 10 % MeOH			
12	60	Water + 10 % MeOH			
13	60	Water + 10 % MeOH			
14	60	Water + 10 % MeOH			
15	60	Water + 10 % MeOH			
16	60	Water + 10 % MeOH	3	600	671
17	60	Water + 20 % MeOH			
18	60	Water + 20 %	4	720	443

		MeOH			
19	60	Water + 20 % MeOH			
20	60	Water + 20 % MeOH			
21	60	Water + 20 % MeOH			
22	60	Water + 50 % MeOH			
23	60	Water + 50 % MeOH			
24	60	Water + 50 % MeOH			
25	60	Water + 50 % MeOH			
26	60	Water + 50 % MeOH			
27	60	MeOH			
28	60	MeOH			
29	60	MeOH			
30	60	MeOH	5	120	303
SUM					4379

Table 13.18. Fraction B1-2 S.4 in reverse phase VersaFlash column (443 mg)

Fraction	Amount (ml)	Eluent	Fraction	Amount (ml)	Mass (mg)
1	15	Water + 50 % MeOH	1	15	6,9
2	15	Water + 50 % MeOH			
3	15	Water + 50 % MeOH	2	30	57,9
4	15	Water + 50 % MeOH			
5	15	Water + 50 % MeOH			
6	15	Water + 50 % MeOH	3	45	87,9
7	15	Water + 50 % MeOH	4	90	42,4

8	15	Water + 50 % MeOH			
9	15	Water + 50 % MeOH			
10	15	Water + 50 % MeOH			
11	15	Water + 50 % MeOH			
12	15	Water + 70 % MeOH			
13	15	Water + 70 % MeOH			
14	15	Water + 70 % MeOH			
15	15	Water + 70 % MeOH			
16	15	Water + 70 % MeOH			
17	15	Water + 70 % MeOH			
18	15	Water + 70 % MeOH			
19	15	Water + 70 % MeOH			
20	15	Water + 70 % MeOH			
21	15	Water + 70 % MeOH			
22	15	Water + 70 % MeOH			
23	15	Water + 70 % MeOH	5	165	60,6
24	15	Water + 70 % MeOH			
25	15	Water + 70 % MeOH			
26	15	Water + 70 % MeOH			
27	15	MeOH			
28	15	MeOH			
29	15	MeOH			
30	15	MeOH			
31	15	MeOH			
32	15	MeOH	6	150	113,8

33	15	MeOH			
SUM					369,5

Table 13.19. Fraction B1-2 S.5 in reverse phase VersaFlash column (303 mg)

Fraction	Amount (ml)	Eluent	Fraction	Amount (ml)	Mass (mg)
1	15	Water + 50 % MeOH	1	15	5,4
2	15	Water + 50 % MeOH			
3	15	Water + 50 % MeOH	2	30	47,1
4	15	Water + 50 % MeOH			
5	15	Water + 50 % MeOH			
6	15	Water + 50 % MeOH	3	45	94,3
7	15	Water + 50 % MeOH			
8	15	Water + 50 % MeOH			
9	15	Water + 50 % MeOH	4	45	57,9
10	15	Water + 50 % MeOH			
11	15	Water + 50 % MeOH			
12	15	Water + 50 % MeOH	5	45	24,9
13	15	Water + 50 % MeOH			
14	15	Water + 50 % MeOH			
15	15	Water + 50 % MeOH			
16	15	Water + 50 % MeOH			
17	15	Water + 70 % MeOH			
18	15	Water + 70 % MeOH	6	135	41,5

19	15	Water + 70 % MeOH			
20	15	Water + 70 % MeOH			
21	15	Water + 70 % MeOH			
22	15	Water + 70 % MeOH			
23	15	Water + 70 % MeOH			
24	15	Water + 70 % MeOH			
25	15	Water + 70 % MeOH			
26	15	Water + 70 % MeOH	7	75	16,1
27	15	Water + 70 % MeOH			
28	15	Water + 70 % MeOH			
29	15	Water + 70 % MeOH			
30	15	Water + 70 % MeOH			
31	15	Water + 70 % MeOH			
32	15	MeOH			
33	15	MeOH			
34	15	MeOH	8	105	10
35	15	MeOH			
36	15	MeOH			
37	15	MeOH			
38	15	MeOH			
39	15	MeOH			
40	15	MeOH			
41	30	MeOH	9	120	20,6
SUM					317,8

Table 13.20. Fraction B1-2 S.4-5 VRP. T2 on LPCC with MCI CHP20P gel (61,8 mg)

Fraction	Amount (ml)	Eluent	Fraction	Amount (ml)	Mass (mg)
1	15	Water + 30 %			

		MeOH			
2	15	Water + 30 % MeOH			
3	15	Water + 30 % MeOH			
4	15	Water + 30 % MeOH			
5	15	Water + 30 % MeOH			
6	15	Water + 30 % MeOH			
7	15	Water + 50 % MeOH			
8	15	Water + 50 % MeOH			
9	15	Water + 50 % MeOH			
10	15	Water + 50 % MeOH			
11	15	Water + 50 % MeOH			
12	15	Water + 50 % MeOH			
13	15	Water + 50 % MeOH			
14	15	Water + 70 % MeOH			
15	15	Water + 70 % MeOH			
16	15	Water + 70 % MeOH			
17	15	Water + 70 % MeOH	1	60	6,6
18	15	Water + 70 % MeOH	2	15	7,4
19	15	Water + 70 % MeOH	3	15	15
20	15	Water + 70 % MeOH	4	15	16,4
21	15	Water + 70 % MeOH	5	15	12,4
22	15	Water + 70 % MeOH	6	15	4,8
23	15	Water + 70 % MeOH	7	30	2,6

24	15	MeOH			
25	15	MeOH			
26	15	MeOH	8	30	1,4
27	15				
28	15				
29	50				
SUM					66,6

Table 13.21. Fraction B1-2 S.4-5 VRP. T3 on LPCC with MCI CHP20P gel (154 mg)

Fraction	Amount (ml)	Eluent	Fraction	Amount (ml)	Mass (mg)
1	7	Water + 30 % MeOH			
2	7	Water + 30 % MeOH			
3	7	Water + 50 % MeOH			
4	7	Water + 50 % MeOH			
5	7	Water + 50 % MeOH			
6	7	Water + 50 % MeOH			
7	7	Water + 50 % MeOH			
8	7	Water + 70 % MeOH			
9	7	Water + 70 % MeOH			
10	7	Water + 70 % MeOH			
11	7	Water + 70 % MeOH			
12	7	Water + 70 % MeOH	1	28	10,3
13	7	Water + 70 % MeOH	2	7	5,5
14	7	MeOH	3	7	27,3
15	7	MeOH	4	7	38,6
16	7	MeOH	5	7	31,4
17	7	MeOH			
18	7	MeOH	6	14	20,7

19	7	MeOH			
20	7	MeOH			
21	7	MeOH	7	21	10,2
22	7	MeOH			
23	60	MeOH			
SUM					144

Table 13.22. Fraction B1-2 S.4-5 VRP. T2 M.1 with HPLC (6,6 mg)

Peak	Fraction	Mass (mg)
1	B1-2 S.4-5 VRP.T2M1HPLC 5	3,1
2	B1-2 S.4-5 VRP.T2M1HPLC 6-7	4,9
3	B1-2 S.4-5 VRP.T2M1HPLC rest	1,2
SUM		9,2

Table 13.23. Fraction B1-2 S.4-5 VRP. T2 M.2 with HPLC (7,4 mg)

Peak	Fraction	Mass (mg)
1	B1-2 S.4-5 VRP.T2M2 HPLC 5	0,5
2	B1-2 S.4-5 VRP.T2M2 HPLC 6	2
3	B1-2 S.4-5 VRP.T2M2 HPLC 7	0,7
4	B1-2 S.4-5 VRP.T2M2 HPLC 8	0,6
5	B1-2 S.4-5 VRP.T2M2 HPLC 10	1,1
SUM		4,9

Table 13.24. Fraction B1-2 S.4-5 VRP. T2 M.3 with HPLC (15 mg)

Peak	Fraction	Mass (mg)
1	B1-2 S.4-5 VRP.T2M3 HPLC 6-7	2

2	B1-2 S.4-5 VRP.T2M3 HPLC 8	0,4
3	B1-2 S.4-5 VRP.T2M3 HPLC 9	2,5
4	B1-2 S.4-5 VRP.T2M3 HPLC 10	0,1
5	B1-2 S.4-5 VRP.T2M3 HPLC 11	0,4
SUM		6,4

Table 13.25. Fraction B1-2 S.4-5 VRP. T2 M.4 with HPLC (16,4 mg)

Peak	Fraction	Mass (mg)
1	B1-2 S.4-5 VRP.T2M4 HPLC 5	1,5
2	B1-2 S.4-5 VRP.T2M4 HPLC 7	3
3	B1-2 S.4-5 VRP.T2M4 HPLC 8	2,6
4	B1-2 S.4-5 VRP.T2M4 HPLC 9	6,5
SUM		13,6

Table 13.26. Fraction B1-2 S.4-5 VRP. T2 M.5 with HPLC (12,4 mg)

Peak	Fraction	Mass (mg)
1	B1-2 S.4-5 VRP.T2M5 HPLC 5	1,5
2	B1-2 S.4-5 VRP.T2M5 HPLC 6	1,4
3	B1-2 S.4-5 VRP.T2M5 HPLC 7	0,7
4	B1-2 S.4-5 VRP.T2M5 HPLC 8-9	7,1
SUM		10,7

Table 13.27. Fraction B1-2 S.4-5 VRP. T3 M.1 with HPLC (10,3 mg)

Peak	Fraction	Mass

		(mg)
1	B1-2 S.4-5 VRP.T3M1 HPLC 3	3,5
2	B1-2 S.4-5 VRP.T3M1 HPLC 4	3,6
3	B1-2 S.4-5 VRP.T3M1 HPLC 5	4
4	B1-2 S.4-5 VRP.T3M1 HPLC 6	0,8
5	B1-2 S.4-5 VRP.T3M1 HPLC 7	0,7
SUM		12,6

Table 13.28. Fraction B1-2 S.4-5 VRP. T3 M.2 with HPLC (5,5 mg)

Peak	Fraction	Mass (mg)
1	B1-2 S.4-5 VRP.T3M2 HPLC 2	1,6
2	B1-2 S.4-5 VRP.T3M2 HPLC 3	2,8
3	B1-2 S.4-5 VRP.T3M2 HPLC 4	1
4	B1-2 S.4-5 VRP.T3M2 HPLC 6	1,4
5	B1-2 S.4-5 VRP.T3M2 HPLC 7	0,5
SUM		7,3

Table 13.29. Fraction B1-2 S.4-5 VRP. T3 M.3 with HPLC (27,3 mg)

Peak	Fraction	Mass (mg)
1	B1-2 S.4-5 VRP.T3M3 HPLC 4	1,9
2	B1-2 S.4-5 VRP.T3M3 HPLC 5	1,9
3	B1-2 S.4-5 VRP.T3M3 HPLC 6	1,1
4	B1-2 S.4-5 VRP.T3M3 HPLC 7	0,4
5	B1-2 S.4-5 VRP.T3M3	1,3

	HPLC 8	
SUM		6,6

Table 13.30. Fraction B1-2 S.4-5 VRP. T3 M.4 with HPLC (38,6 mg)

Peak	Fraction	Mass (mg)
1	B1-2 S.4-5 VRP.T3M4 HPLC 2	3,3
2	B1-2 S.4-5 VRP.T3M4 HPLC 4	1,0
3	B1-2 S.4-5 VRP.T3M4 HPLC 5-6	2,5
4	B1-2 S.4-5 VRP.T3M4 HPLC 7	1,0
5	B1-2 S.4-5 VRP.T3M4 HPLC 8	3,6
6	B1-2 S.4-5 VRP.T3M4 HPLC 9-10	12,1
7	B1-2 S.4-5 VRP.T3M4 HPLC 11	5,7
8	B1-2 S.4-5 VRP.T3M4 HPLC 12	3,6
SUM		31,3

Table 13.31. Fraction B1-2 S.4-5 VRP. T3 M.5 with HPLC (31,4 mg)

Peak	Fraction	Mass (mg)
1	B1-2 S.4-5 VRP.T3M5 HPLC 3	0,5
2	B1-2 S.4-5 VRP.T3M5 HPLC 5	0,8
3	B1-2 S.4-5 VRP.T3M5 HPLC 6	1,3
4	B1-2 S.4-5 VRP.T3M5 HPLC 7	1,2
5	B1-2 S.4-5 VRP.T3M5 HPLC 8	1,2
6	B1-2 S.4-5 VRP.T3M5 HPLC 9-10	7,4
7	B1-2 S.4-5 VRP.T3M5 HPLC 11	11,9
8	B1-2 S.4-5 VRP.T3M5 HPLC 12	7,1

9	B1-2 S.4-5 VRP.T3M5 HPLC 13	1,5
SUM		32,9

Table 13.32. Fraction B1-2 S.4-5 VRP. T3 M.6 with HPLC (20,7 mg)

Peak	Fraction	Mass (mg)
1	B1-2 S.4-5 VRP.T3M6 HPLC 3	14,1
2	B1-2 S.4-5 VRP.T3M6 HPLC 5-6	6,5
3	B1-2 S.4-5 VRP.T3M6 HPLC 7	1,8
4	B1-2 S.4-5 VRP.T3M6 HPLC 8-12	0,4
5	B1-2 S.4-5 VRP.T3M6 HPLC 13-14	1,6
6	B1-2 S.4-5 VRP.T3M6 HPLC 15	0,2
SUM		24,6

Table 13.33. Fractions from fra Sutherlandia - prep. HPLC

Fractions from fra Sutherlandia - prep. HPLC					
Fractions from T2					
Fraction	Weight mg	RT (min)	1H NMR	aglycon	13C NMR
T2M1HPLC5	3,1	5.93 - 7.42	x	que	x
T2M1HPLC6-7	4,9	7.42 - 13.10	x	kae	x
T2M1HPLC rest	1,2	xxx	x		
T2M2HPLC5	0,5	5.98 - 7.06			
T2M2HPLC6	2,0	7.06 - 9.88	x	kae	x
T2M2HPLC7	0,7	9.88 - 12.61			
T2M2HPLC8	0,6	12.61 - 14.10			
T2M2HPLC10	1,1	15.44 - 17.47	x	kae?	
T2M2HPLC rest	2,0	xxx	x		

T2M3HPLC6-7	2,0	8.06 - 11.33			
T2M3HPLC8	0,4	11.33 - 13.43			
T2M3HPLC9	2,5	13.43 - 17.04	x	kae	x
T2M3HPLC10	0,1	17.04 - 18.23			
T2M3HPLC11	0,4	18.23 - 21.24			
T2M3HPLC rest	1,4	xxx	x		
T2M4HPLC5	1,5	7.82 - 10.23	x	kae	
T2M4HPLC7	3,0	13.19 - 15.02	x	kae	x
T2M4HPLC8	2,6	15.92 - 16.96	x	kae	
T2M4HPLC9	6,5	16.96 - 19.10	x	kae	x
T2M4HPLC rest	4,9	xxx	x	kae	
T2M5HPLC5	1,5	10.93 - 13.33	x	kae	
T2M5HPLC6	1,4	13.33 - 16.46	x	kae	
T2M5HPLC7	0,7	16.46 - 18.10			
T2M5HPLC8-9	7,1	18.10 - 24.50	x	kae	x

Fractions from fra Sutherlandia - prep. HPLC					
Fractions from T3					
Fraction	Weight mg	RT (min)	1H NMR	aglycon	13C NMR
T3M1HPLC3	3,5	3.89 - 5.05	x	que	
T3M1HPLC4	3,6	5.05 - 9.23	x	que	
T3M1HPLC5	4,0	9.23 - 12.10	x	que++	
T3M1HPLC6	0,8	12.10 - 13.19			
T3M1HPLC7	0,7	13.19 - 13.83			
T3M2HPLC2	1,6	1.96 - 3.16	x	que	x
T3M2HPLC3	2,8	4.15 - 7.10	x	que	x
T3M2HPLC4	1,0	7.10 - 9.13	x	que?	
T3M2HPLC6	1,4	10.79 - 13.63	x	que+kae	
T3M2HPLC7	0,5	13.79 - 15.93			
T3M3HPLC4	1,9	5.40 - 7.25	x	que + kae?	
T3M3HPLC5	1,9	7.25 - 8.80	x	que + kae?	
T3M3HPLC6	1,1	8.80 - 11.26	x	que + kae?	

T3M3HPLC7	0,4	11.26 - 13.49			
T3M3HPLC8	1,3	13.49 - 15.93	x	que + kae	
T3M4HPLC2	3,3	3.00 - 5.38	x	que + kae	
T3M4HPLC4	1,0	6.84 - 8.79	x	que + kae	
T3M4HPLC5-6	2,5	8.79 - 11.42	x	que + kae	
T3M4HPLC7	1,0	11.42 - 12.05	x	que + kae	
T3M4HPLC8	2,1	12.05 - 13-29	x	que + kae	
T3M4HPLC9-10	12,1	13.29 - 15.78	x	que + kae	
T3M4HPLC11	5,7	15.78 - 16.94	x	kae	x
T3M4HPLC12	3,6	16.94 - 19.21	x	kae	
T3M5HPLC3	0,5	3.73 - 5.13			
T3M5HPLC5	0,8	8.11 - 9.41			
T3M5HPLC6	1,3	9.41 - 11.21	x	que + kae	
T3M5HPLC7	1,2	11.21 - 12.80	x	que + kae	
T3M5HPLC8	1,2	12.80 - 13.83	x	que + kae	
T3M5HPLC9-10	7,4	13.83 - 16.71	x	kae	x
T3M5HPLC11	11,9	16.71 - 17.65	x	kae	x
T3M5HPLC12	7,1	17.65 - 18.86	x	kae	x
T3M5HPLC13	1,5	18.86 - 20.60	x	kae	
T3M6HPLC3	0,4	3.73 - 5.46			
T3M6HPLC5-6	1,6	8.11 - 11.38	x	kae	
T3M6HPLC7	0,2	11.38 - 12.38			
T3M6HPLC8-12	14,0	12.38 - 17.61	x	kae + que?	x
T3M6HPLC13-14	6,5	17.61 - 19.34	x	kae	x
T3M6HPLC15	1,8	19.34 - 20.98	x	kae	

Table 13.34. LPCC with Sephadex LH20 of fraction B5-6 (1605,4 mg)

Fraction	Amount (ml)	Eluent	Fraction	Amount (ml)	Mass (mg)
1	60	Water + 20 % MeOH			
2	60	Water + 20 % MeOH			
3	60	Water + 20 % MeOH			
4	60	Water + 20 % MeOH	1	240	189,5

5	60	Water + 20 % MeOH			
6	60	Water + 20 % MeOH			
7	60	Water + 50 % MeOH	2	60	152,6
8	60	Water + 50 % MeOH			
9	60	Water + 50 % MeOH	3	120	511
10	60	Water + 50 % MeOH			
11	60	Water + 70 % MeOH	4	120	119,4
12	60	Water + 70 % MeOH			
13	60	Water + 70 % MeOH			
14	60	Water + 70 % MeOH	5	180	57,5
15	60	MeOH			
16	60	MeOH			
17	60	MeOH			
18	60	MeOH			
19	60	MeOH			
20	60	MeOH			
21	60	MeOH			
22	60	MeOH			
23	60	MeOH			
24	60	MeOH			
25	60	MeOH			
26	60	MeOH			
27	60	MeOH			
28	60	MeOH			
29	60	MeOH			
30	60	MeOH	6	1360	136,1
SUM					1166,1

Table 13.35. Fractioning of fraction B5-6 S.3 with VersaFlash normal phase column (511 mg)

Fraction	Amount (ml)	Eluent	Fraction	Amount (ml)	Mass (mg)

1	50	hypophase of MeOH, Chloroform and H_2O (65:35:10)			
2	50	hypophase of MeOH, Chloroform and H_2O (65:35:10)	1	100	11,9
3	50	hypophase of MeOH, Chloroform and H_2O (65:35:10)			
4	50	hypophase of MeOH, Chloroform and H_2O (65:35:10)	2	100	78,9
5	50	hypophase of MeOH, Chloroform and H_2O (65:35:10)			
6	50	hypophase of MeOH, Chloroform and H_2O (65:35:10)	3	100	131
7	50	hypophase of MeOH, Chloroform and H_2O (65:35:10)			
8	50	hypophase of MeOH, Chloroform and H_2O (65:35:10)			
9	50	hypophase of MeOH, Chloroform and H_2O (65:35:10)			
10	50	hypophase of MeOH, Chloroform and H_2O (65:35:10)	4	200	165,1
11	50	hypophase of MeOH, Chloroform and H_2O (65:35:10)			
12	50	hypophase of MeOH, Chloroform and H_2O (65:35:10)	5	100	28,5
13	50	hypophase of MeOH, Chloroform and H_2O (65:35:10)			
14	50	hypophase of MeOH, Chloroform and H_2O (65:35:10)	6	100	11,7
15	50	hypophase of MeOH, Chloroform and H_2O (65:35:10)			
16	50	hypophase of MeOH,	7	200	16,2

		Chloroform and H_2O (65:35:10)			
17	50	hypophase of MeOH, Chloroform and H_2O (65:35:10)			
18	50	hypophase of MeOH, Chloroform and H_2O (65:35:10)			
19	50	hypophase of MeOH, Chloroform and H_2O (65:35:10)			
20	50	hypophase of MeOH, Chloroform and H_2O (65:35:10)			
21	50	hypophase of MeOH, Chloroform and H_2O (65:35:10)			
22	200	hypophase of MeOH, Chloroform and H_2O (65:35:10)	8	350	13,3
SUM					456,6

Table 13.36. Fraction B5-6 S.3 VNP.2 on chromatotron

Fraction	Amount (ml)	Eluent	Fraction	Amount (ml)	Mass (mg)
1	7	hypophase of MeOH, Chloroform and H_2O (65:35:10)			
2	7	hypophase of MeOH, Chloroform and H_2O (65:35:10)			
3	7	hypophase of MeOH, Chloroform and H_2O (65:35:10)			
4	7	hypophase of MeOH, Chloroform and H_2O (65:35:10)			
5	7	hypophase of MeOH, Chloroform and H_2O (65:35:10)	1	7	
6	7	hypophase of MeOH, Chloroform and H_2O (65:35:10)	2	7	
7	7	hypophase of MeOH, Chloroform and H_2O (65:35:10)	3	7	
8	7	hypophase of MeOH, Chloroform and H_2O (65:35:10)			
9	15	hypophase of MeOH, Chloroform and H_2O (65:35:10)			

SUM					

Table 13.37. Fraction B5-6 S.3 VNP.2 CHR 1 on HPLC

Peak	Fraction	Mass (mg)
1	B5-6 S.3 VNP.2 CHR.1 HPLC 1	2,8
2	B5-6 S.3 VNP.2 CHR.1 HPLC 2	1,9
3	B5-6 S.3 VNP.2 CHR.1 HPLC 3	1,1
4	B5-6 S.3 VNP.2 CHR.1 HPLC 4	0,8
SUM		6,6

Table 13.38. Fraction B5-6 S.3 VNP.2 CHR 2 on HPLC

Peak	Fraction	Mass (mg)
1	B5-6 S.3 VNP.2 CHR.2 HPLC 1	2,9
2	B5-6 S.3 VNP.2 CHR.2 HPLC 2	5,6
3	B5-6 S.3 VNP.2 CHR.2 HPLC 3	7,7
4	B5-6 S.3 VNP.2 CHR.2 HPLC 4	3
5	B5-6 S.3 VNP.2 CHR.2 HPLC 5	2,6
SUM		21,8

Table 13.39. Fraction B5-6 S.3 VNP.2 CHR 3 on HPLC

Peak	Fraction	Mass (mg)
1	B5-6 S.3 VNP.2 CHR.3 HPLC 1	5,3
2	B5-6 S.3 VNP.2 CHR.3 HPLC 2	4,5
3	B5-6 S.3 VNP.2 CHR.3 HPLC 3	5,1
4	B5-6 S.3 VNP.2 CHR.3	1,2

	HPLC 4	
5	B5-6 S.3 VNP.2 CHR.3 HPLC 5	1,5
SUM		17,6

Table 13.40. Comparison of 13C NMR data for our HPLC fractions and literature values.

Comparison of 13C NMR data for our HPLC fractions and literature values						
(Fu et al 2007)						
All spectra recorded in d5-pyridine						
E HPLC5	E HPLC5		B HPLC6	B HPLC6	Suth C	Carbon
6.7 mg	corrected		4.2 mg	corrected		number
	values			values		
208,6	209,0		208,6	209,0	209,3	11
205,0	205,4		205,0	205,4	205,6	1
135.7?			135.6?			
123.7?			123.7?			
122.3?						
97,5	97,9		98,6	99,0	99,1	1´
80,5	80,9		80,9	81,3	81,3	25
80,1	80,5		80,1	80,5	80,5	3
78,9	79,3		78,7	79,1	79,1	3´
78,4	78,8		78,2	78,6	78,7	5´
75,7	76,1		78,2	78,6	78,6	24
75,5	75,9		75,3	75,7	75,7	2´
71,7	72,1		71,7	72,1	72,1	4´
62,8	63,2		62,7	63,1	63,1	6´
52,2	52,6		52,2	52,6	52,6	12
51,2	51,6		51,3	51,7	51,7	17
49,0	49,4		48,9	49,3	49,3	14
48,3	48,7		48,2	48,6	48,6	10
46,9	47,0		46,9	47,3	47,3	2
46,4	46,8		46,3	46,7	46,7	13
43,4	43,8		43,4	43,8	43,8	5
40,4	40,9		40,4	40,8	40,8	4
39,3	39,7		39,3	39,7	39,6	8
36,3	46,7		36,8	37,2	37,2	20
34,4	34,8		34,3	34,7	34,7	22
33,9	34,3		33,8	34,2	34,2	15
33,2	33,6		33,2	33,6	33,5	9
					30,8	6
28,8	29,2		29,2	29,6	29,6	23
28,1	28,5		28,0	28,4	28,4	16
25,6	26,0		25,6	26,0	26,0	28

25,2	25,6		25,2	25,6		25,6	19
			24,2	24,6		24,6	27
23,5	23,9		23,4	23,8		23,8	7
22,9	23,3						27?
22,6	23,0		21,3	21,7		21,7	26
21,2	21,6		21,2	21,6		21,5	29
19,0	19,4		19,0	19,4		19,3	21
18,8	19,2		18,7	19,1		19,1	30
18,5	18,9						
16,7	17,1		16,6	17,0		17,0	18
Conclusion:	**B HPLC6 is probably Suth C**						
	E HPLC5 is not Suth C						

Table 13.41. Fraction A on HPLC

Peak	Fraction	Mass (mg)
1	A HPLC 6	2,6
2	A HPLC 11	0,7
SUM		3,3

Table 13.42. Fraction B on HPLC

Peak	Fraction	Mass (mg)
1	B HPLC 6	4,2
2	B HPLC 7	0,1
SUM		4,3

Table 13.43. Fraction E on HPLC

Peak	Fraction	Mass (mg)
1	E HPLC 4	0,5
2	E HPLC 5	6,7
3	E HPLC 6	0,7
SUM		7,9

Table 13.44. Fraction F on HPLC

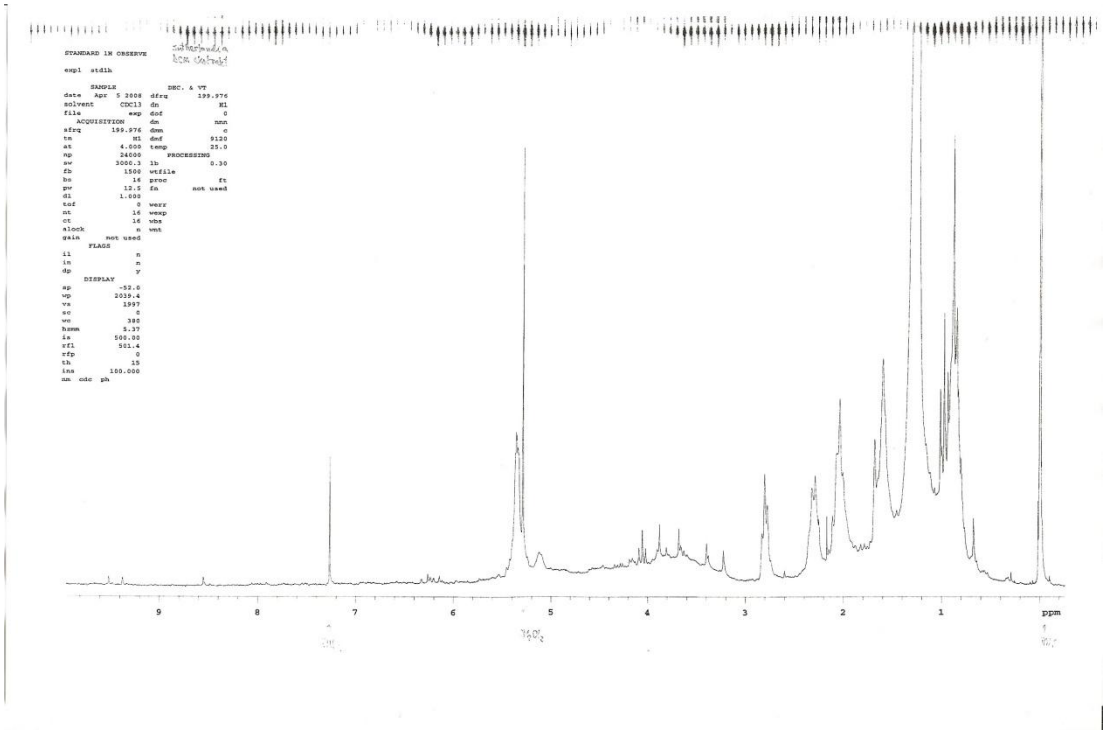
Peak	Fraction	Mass (mg)
1	F HPLC 5	0,4
2	F HPLC 6	2,1
3	F HPLC 7	0,9
4	F HPLC 8	0,9
SUM		4,3

Table 13.45. 13C-NMR data – Sutherlandia flavonoids from HPLC

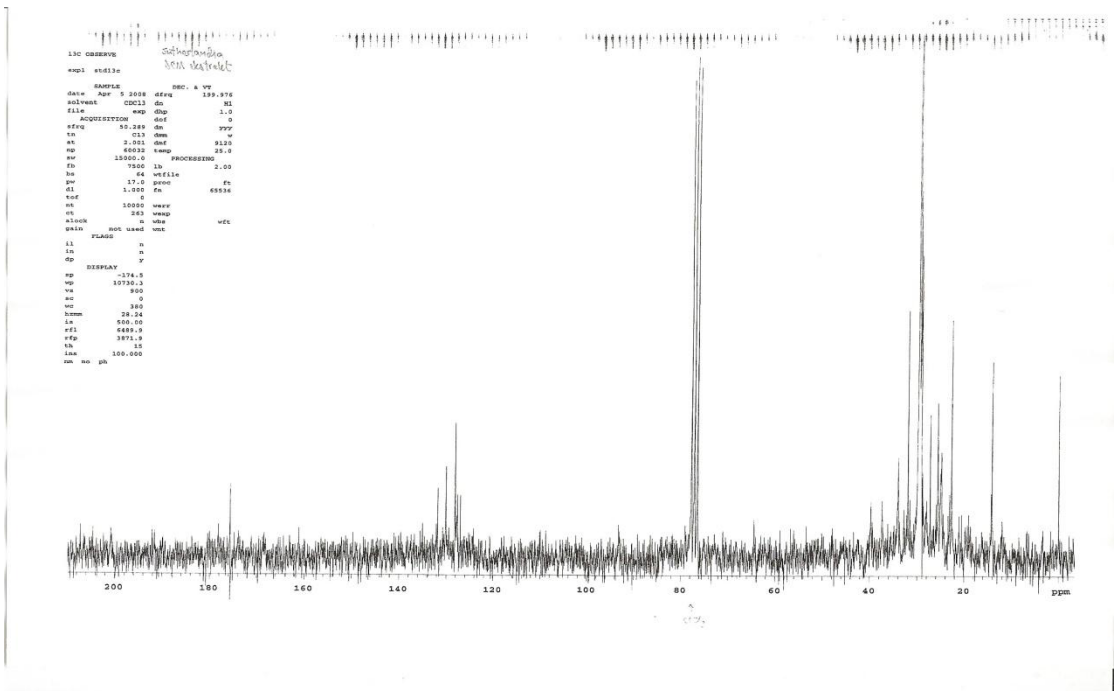
13C NMR data - Sutherlandia flavonoids from HPLC

T3M4	T3M5			T3M6		T2M1	T2M2	T2M3	T2M4	T2M5
HPLC11 5.7 mg RT 15.8-16.9	HPLC9-10 7.4 mg, RT 13.8-16.7 (thin!)	HPLC11 11.9 mg, RT 16.7-17.7	HPLC12 7.1 mg, RT 17.7-18.9	HPLC8-12 14.9 mg RT 12.4-17.6	HPLC13-14 6.5 mg RT17.6-19.3 (thin)	HPLC6-7 4.9 mg RT 7.4-13.1	HPLC6 2.0 mg RT 7.1-9.9	HPLC6 2.5 mg RT 13.4-17.0	HPLC9 6.5 mg RT 17.0-19.1	HPLC8-9 7.1 mg RT 18.1-24.5
					200,0		183,0			
179,5 176,6		179,4	179,2	179,4	179,3 173,9	179,9	180,0 176,5	179,4		179,4
		172,1	172,1	172,1	172,1	172,2	172,2 166,4	172,2	172,1	172,1
165,6	165,6	165,6	165,7	165,6	165,7			165,9	165,7	165,7
163,2	163,2	163,1	163,1	163,1	163,1	163,0		163,2	163,1	163,1
161,3	161,0	161,3	161,3	161,3	161,4	161,5	161,5	161,5	161,3	161,3
						159,3		159,3		
158,3	158,4	158,4	158,4	158,3	158,4				158,3	158,3
									149,3	
						145,6				
						144,3				
134,1		134,7	134,5	134,7		134,5	134,5	134,6	134,7	134,7
132,2	132,2	132,2	132,2	132,2	132,1	132,2	132,2	132,3	132,2	132,2
						130,2				
	125,8								126,3	
				123,8					124,5	
123,0	123,0	122,9	123,1	122,9	123,1	122,9	122,8	122,8	123,1	123,1
					122,6					
					120,7					
117,1										
116,2	116,2	116,2	116,1	116,2	116,1	116,2	116,3	116,2	116,2	116,2
	115,0									
						112,0				
110,8	110,8	110,8	110,7	110,8					110,8	110,8
					110,6					110,6
						107,6				
105,9		105,9	105,9	105,8	106,1			105,8	105,9	105,9
	104,1					104,4	104,4	104,5		
								102,7		
101,1	101,1	101,0		101,0		101,1	101,0	100,9	101,0	101,0
			100,6		100,6	100,6				100,6
99,7	99,7	99,8	99,8	99,8	99,8			100,0	99,8	99,8
	99,2									
						95,2				
94,6	94,6	94,7	94,7	94,7	94,7			94,9	94,7	94,7
							89,2			
81,9						81,8	81,9	82,1		
80,9	80,9	80,9	81,0	80,9	81,0				81,0	81,0
			78,8		78,8				78,8	78,8
			78,5		78,5					78,5
78,1	78,1	78,1	78,1	78,1	78,1		78,2	78,3	78,1	78,1
						77,9	77,8	77,9		
						77,6	77,6			
77,0	77,0	76,8		76,8					76,8	76,8
			75,6		75,6					75,6
75,4	75,5	75,5	75,5	75,5		75,4	75,4	75,5	75,5	75,4
		75,2								
		74,4		74,4			74,3		74,4	74,4
	73,9			73,8						
			71,8		71,8				71,7	71,7
						71,3	71,3	71,4		
						70,9				
70,6	70,6	70,6	70,6	70,6	70,6				70,6	70,6
										66,5
66,2	66,2	66,3	66,4	66,2	66,4				66,2	66,2
		64,6	64,4	64,5	64,4			64,2	64,5	64,4
						63,8	63,9			
62,4						62,6	62,6	62,7		
62,1	62,1									
	57,4									
54,7								54,4		
					53,0					
						47,0	47,0			
		46,3	46,3	46,4	46,3			46,6	46,4	46,5
					45,8					
								30,9		
27,7		27,7	27,7	27,7	27,6	27,7	27,7	27,7	27,7	27,7
	26,8									26,5
21,3								21,3		
							19,7			
	Kaempferol doublet in 1H NMR:									
8,10	8,10	8,09	8,04	8,09	8,04	8,00	8,01	8,02	8,09	8,04
								8,04		8,09
	Anomer proton in carbohydrate part of the molecule:							(2 signals)		(2 signals)
5,50 J 7.9	5,51 J 7.9	5,46 J 7.5	5,49 J 7.2	?	5,48 J 7.3	5,34 J 7.5	5,34 J 7.7	5,44 J 7.3	5,46 J 7.8	5,46 J ?
5,43 J ca 1	5,43 J ca 1	5,43 J ca 1	5,44 J ca 1	5,44 ?	5,47 ?			5,38 J 7.5	?	
Disaccharide	Disaccharide	Disaccharide	Disaccharide	Disaccharide?	Disaccharide	Monosaccharide	Monosaccharide	Mixed?	Disaccharide	Mixed?

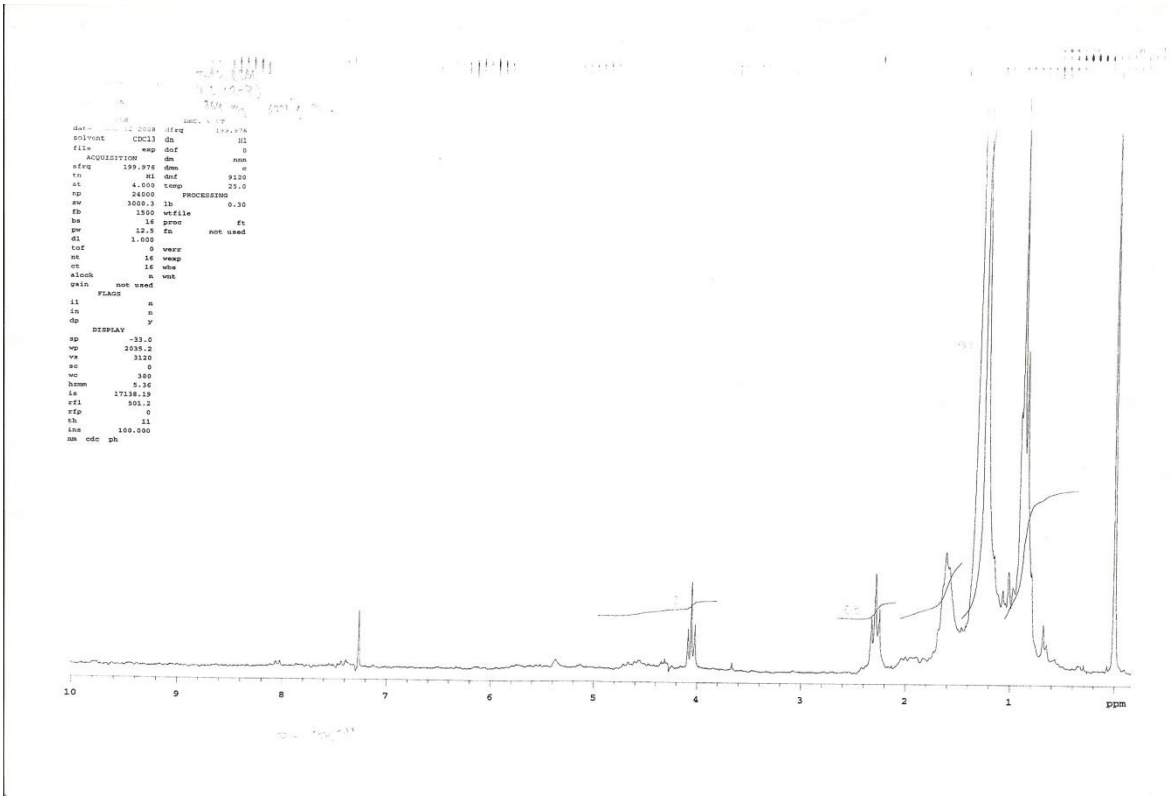
14. NMR spectrum



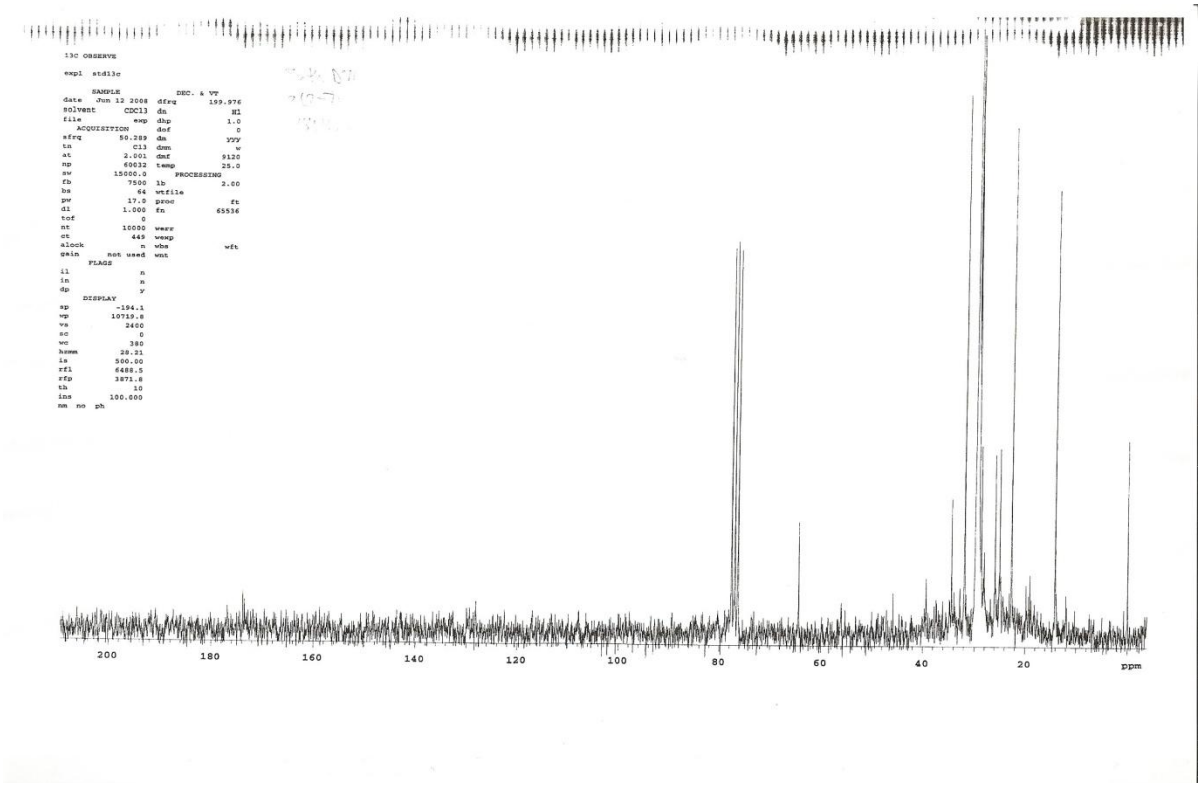
NMR spectra 14.1: 1H-NMR-spectra of DCM extract



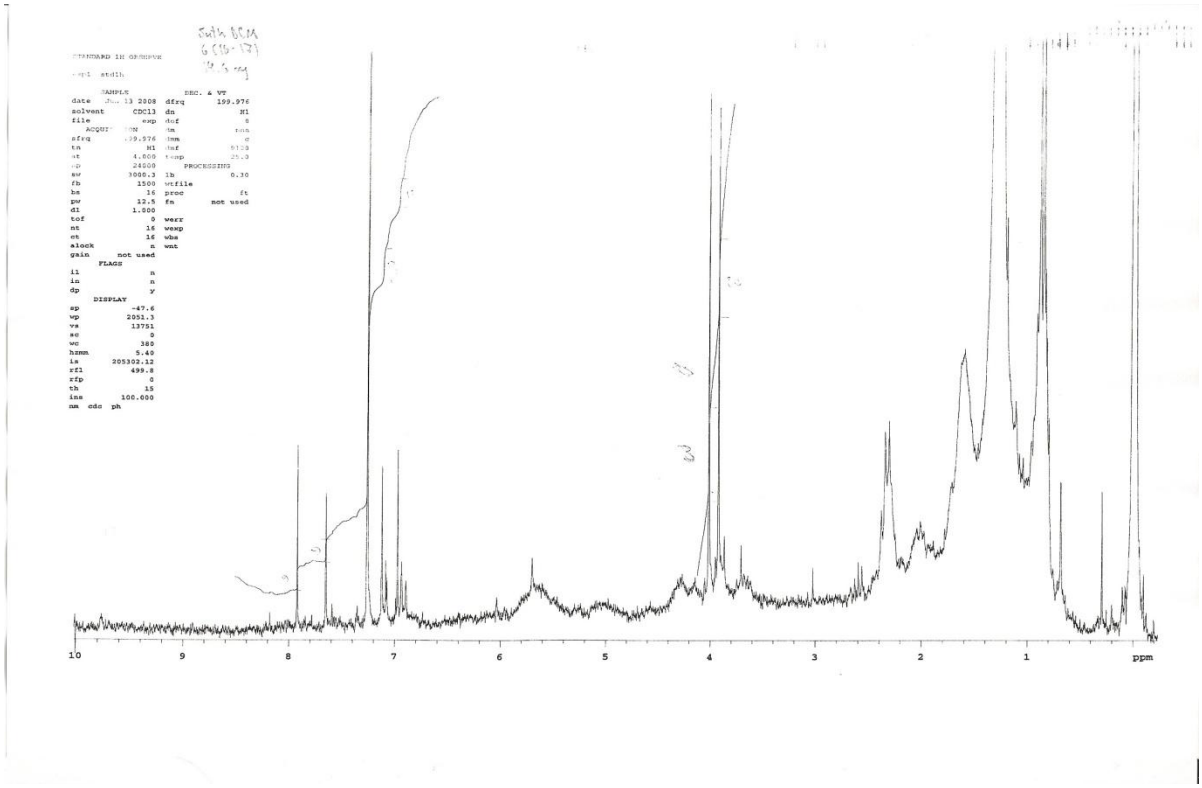
NMR spectra 14.2: 13C-NMR spectra of DCM extract



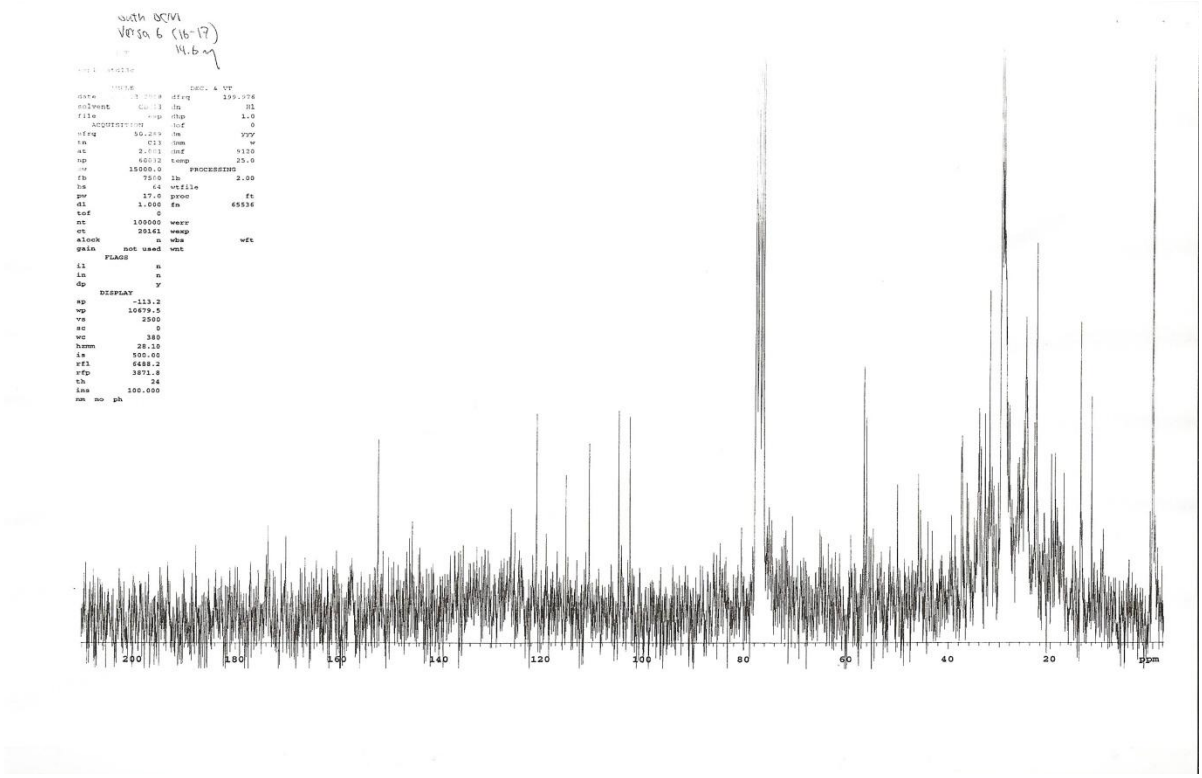
NMR spectra 14.3: 1H-NMR spectra of fraction D2.



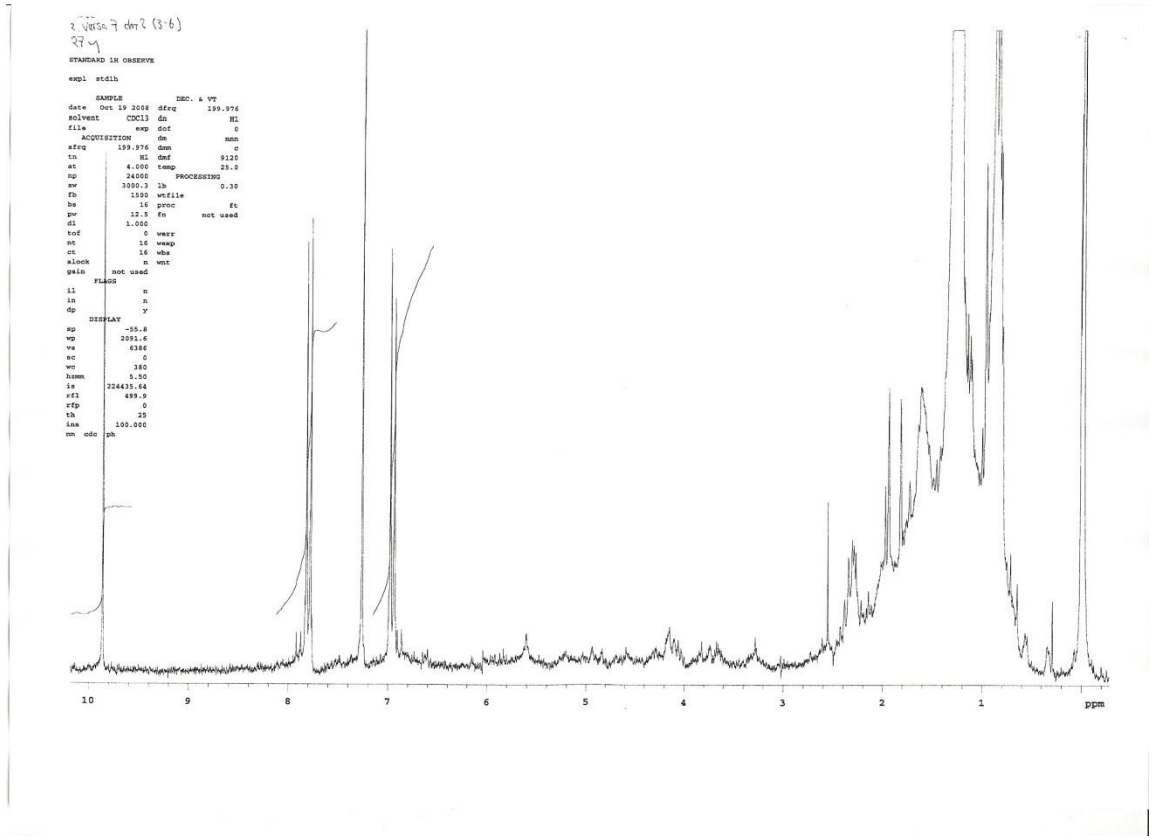
NMR spectra 14.4: 13C-NMR spectra of fraction D2



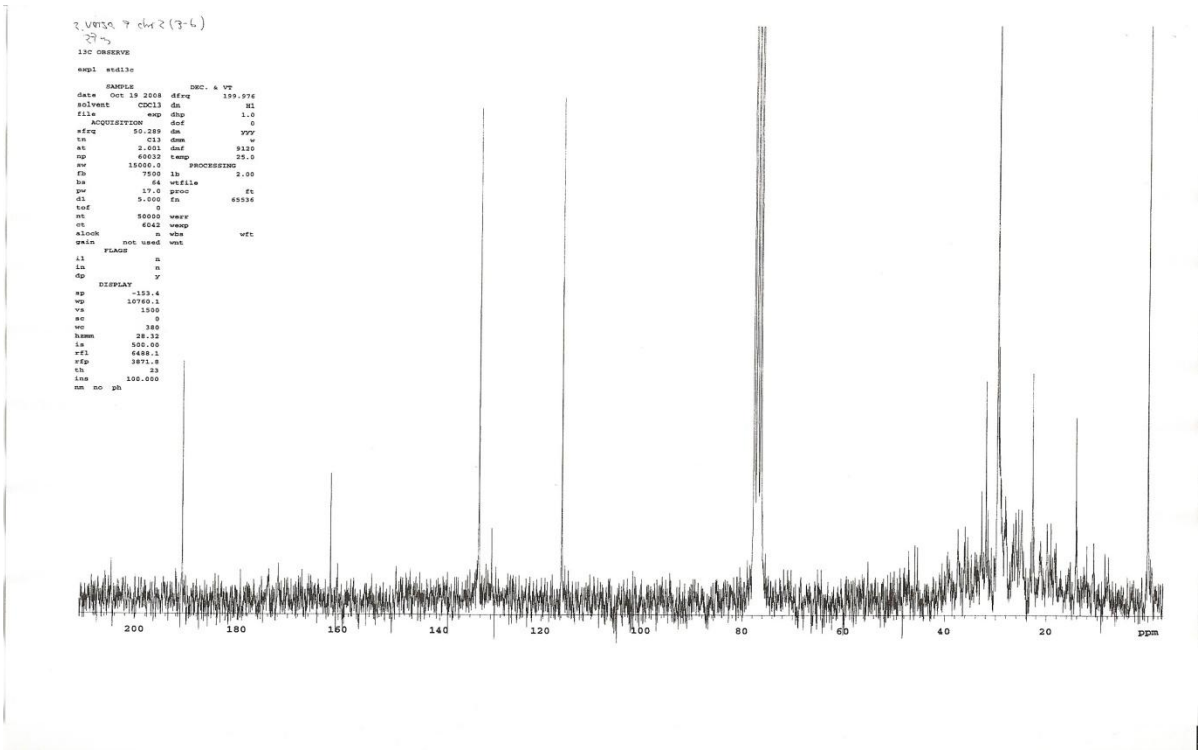
NMR spectra 14.5: 1H-NMR spectra of fraction D6.



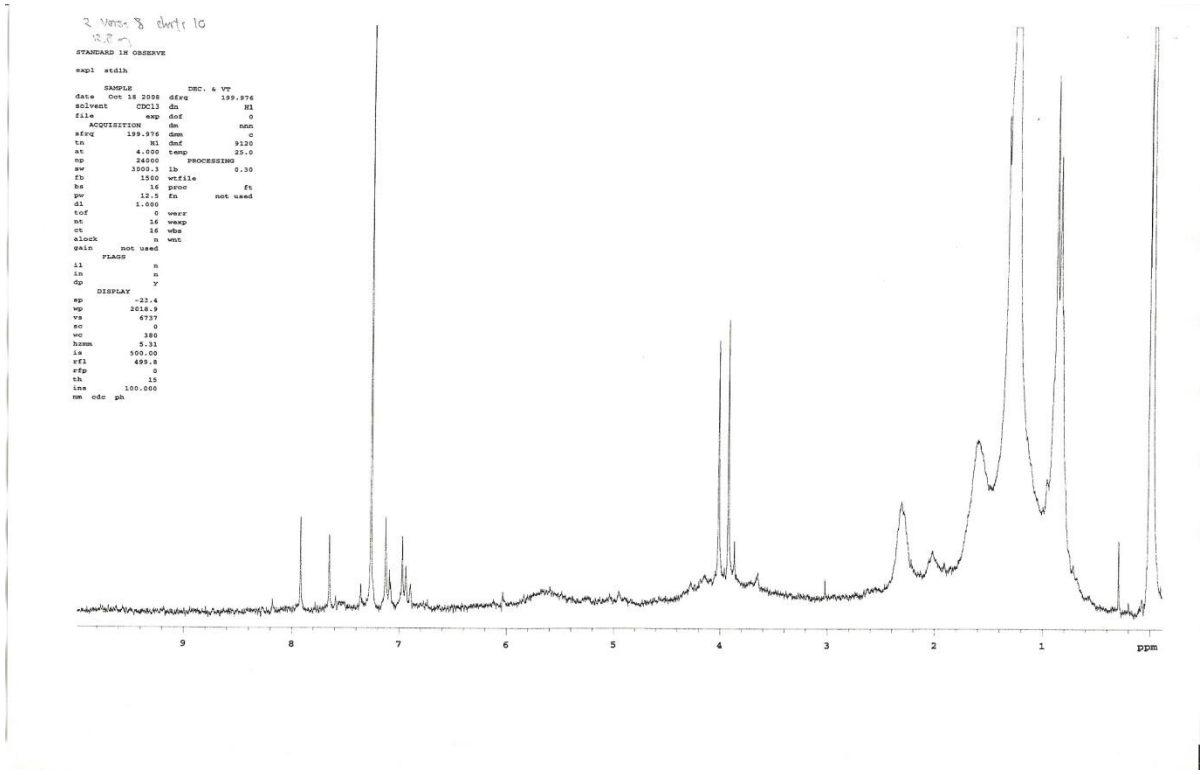
NMR spectra 14.6: 13C-NMR of fraction D6



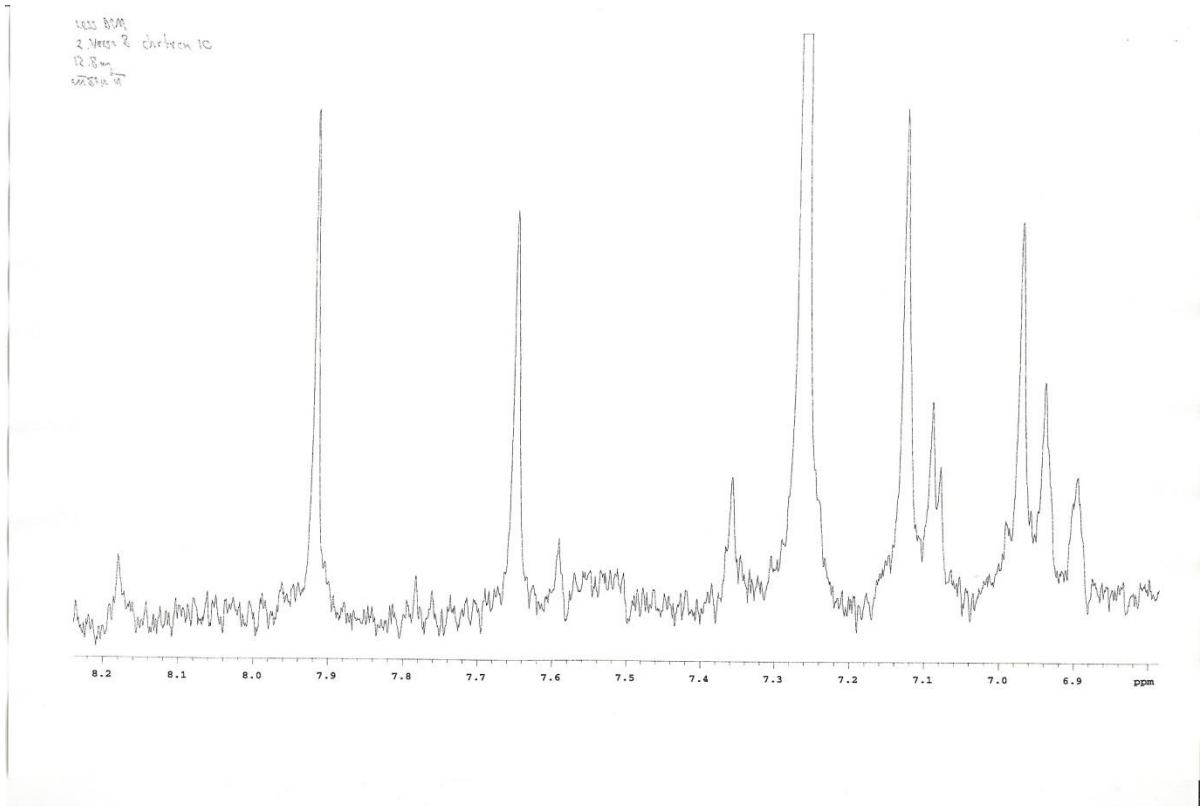
NMR spectra 14.7: 1H-NMR spectra of fraction D2.7.2.



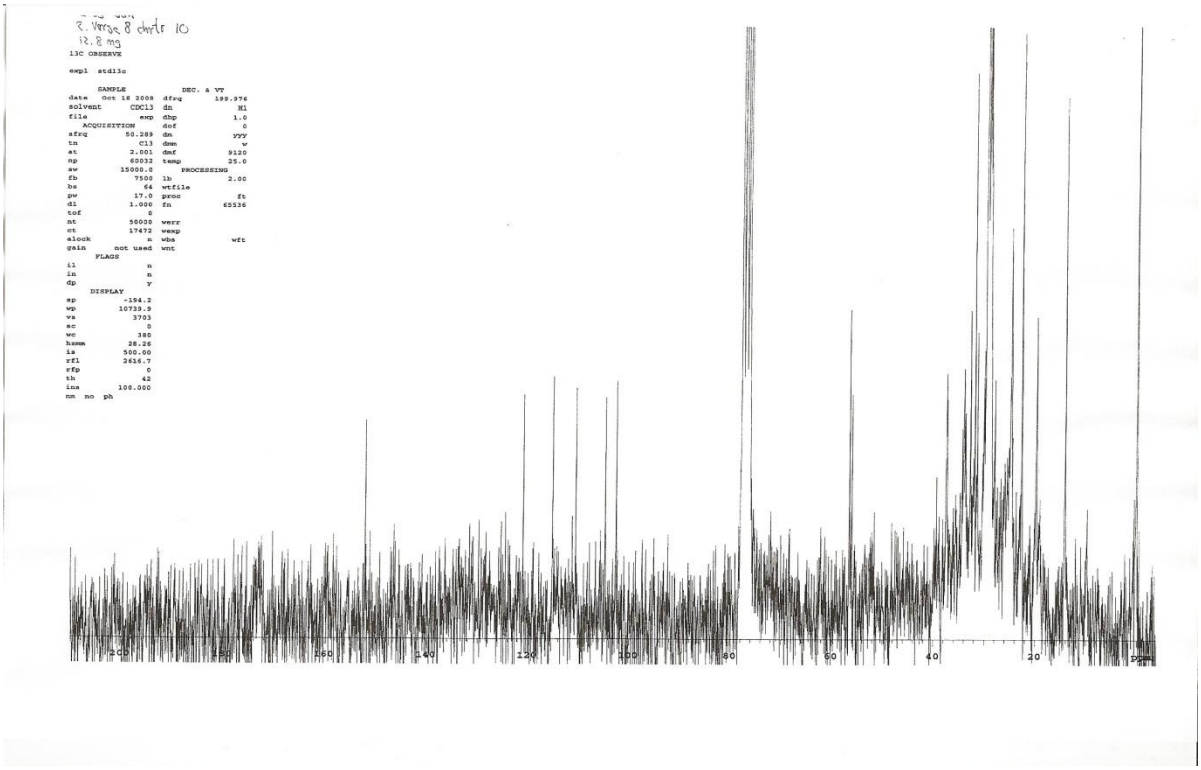
NMR spectra 14.8: 13C-NMR spectra of fraction D2.7.2



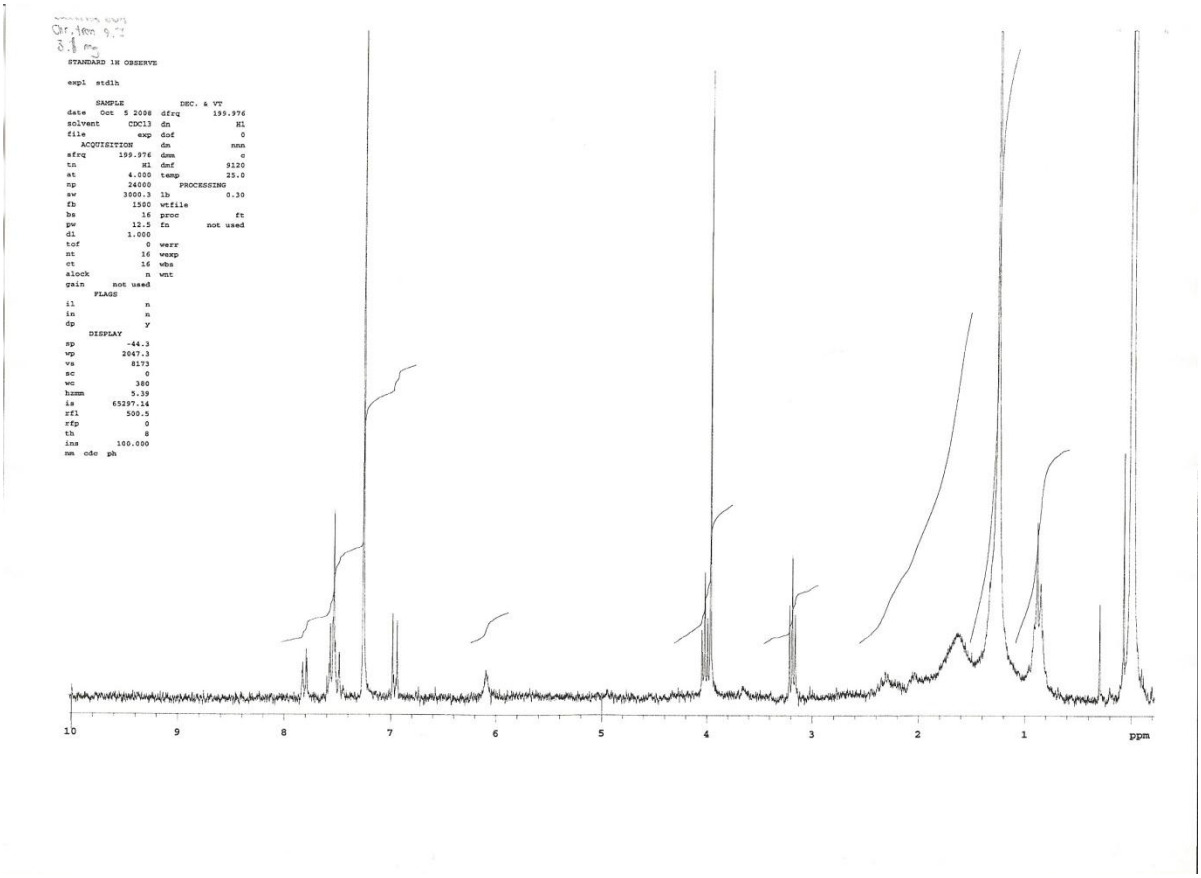
NMR spectra14.9: 1H-NMR spectra of fraction D2.8.10



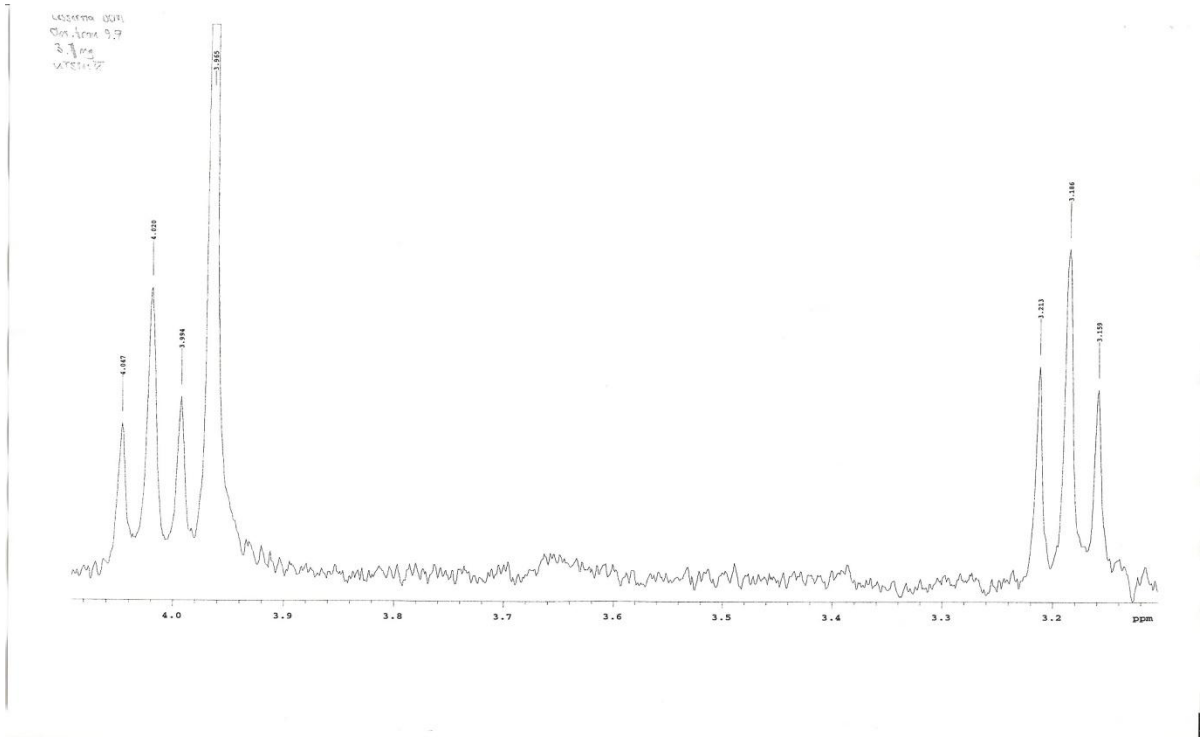
NMR spectra 14.10: “et utsnitt” from 1H-NMR spectra of fraction D2.8.10



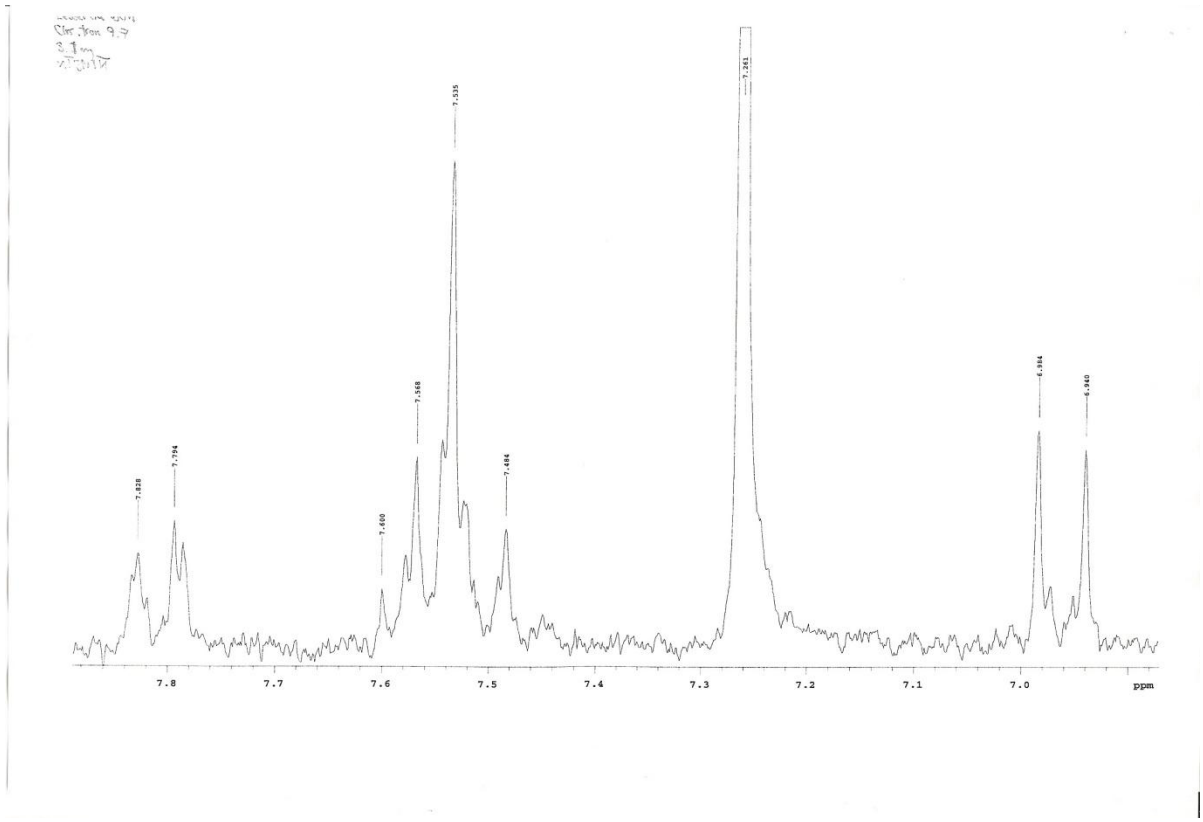
NMR spectra 14.11: 13C-NMR spectra of fraction D2.8.10



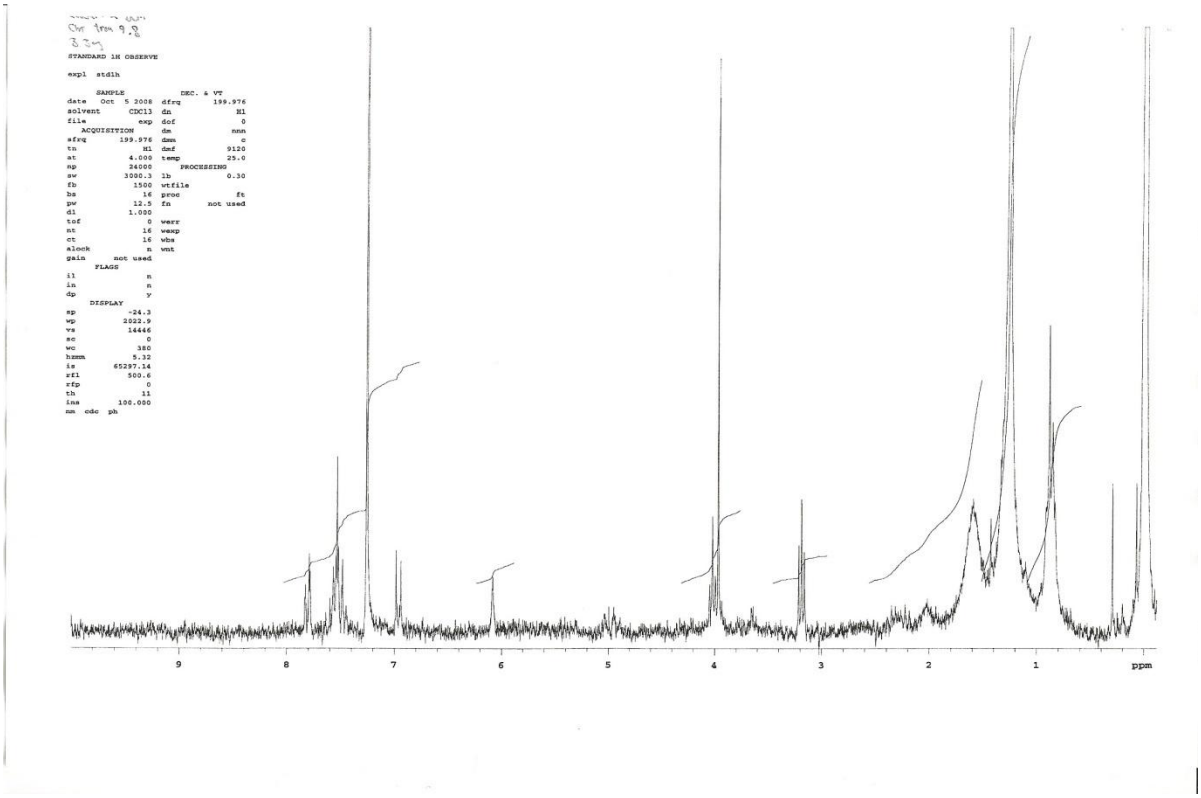
NMR spectra 14.12: 1H-NMR spectra of fraction D2.9.7



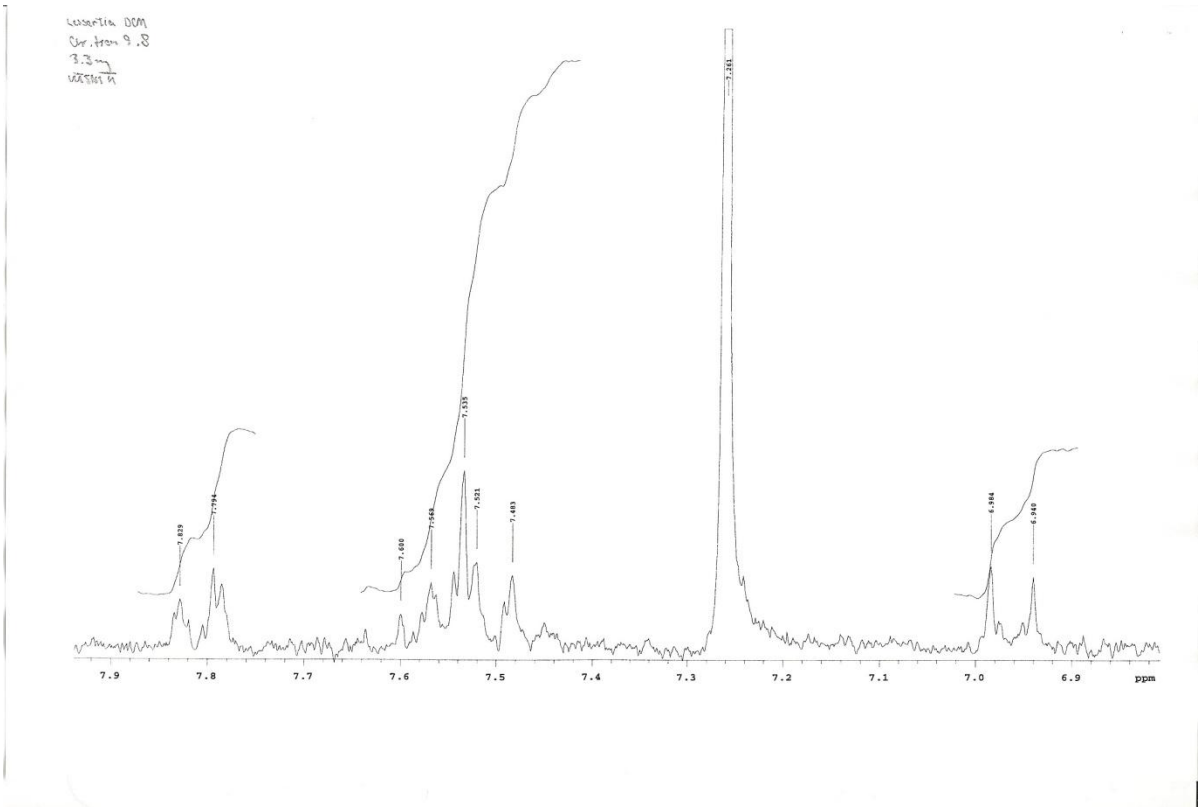
NMR spectra 14.13: partly from 1H-NMR spectra of fraction D2.9.7.



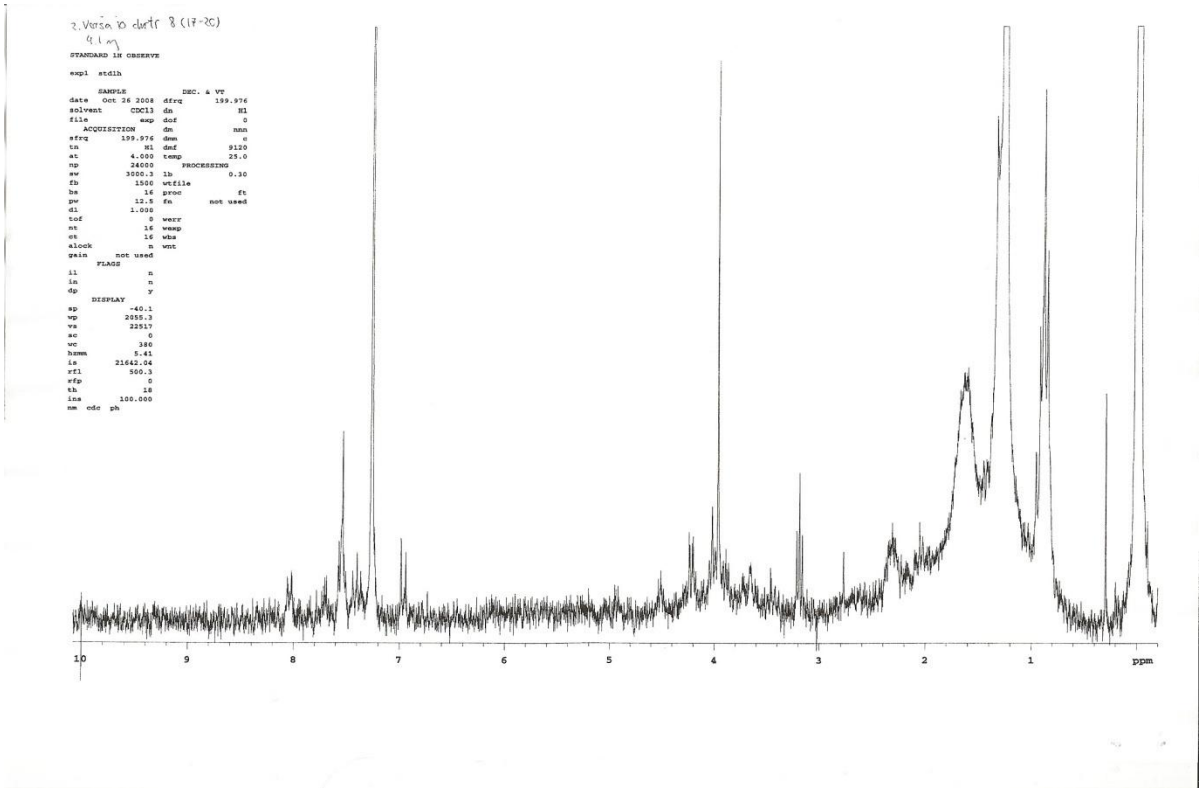
NMR spectra 14.14: partly from 1H-NMR spectra of fraction D2.9.7



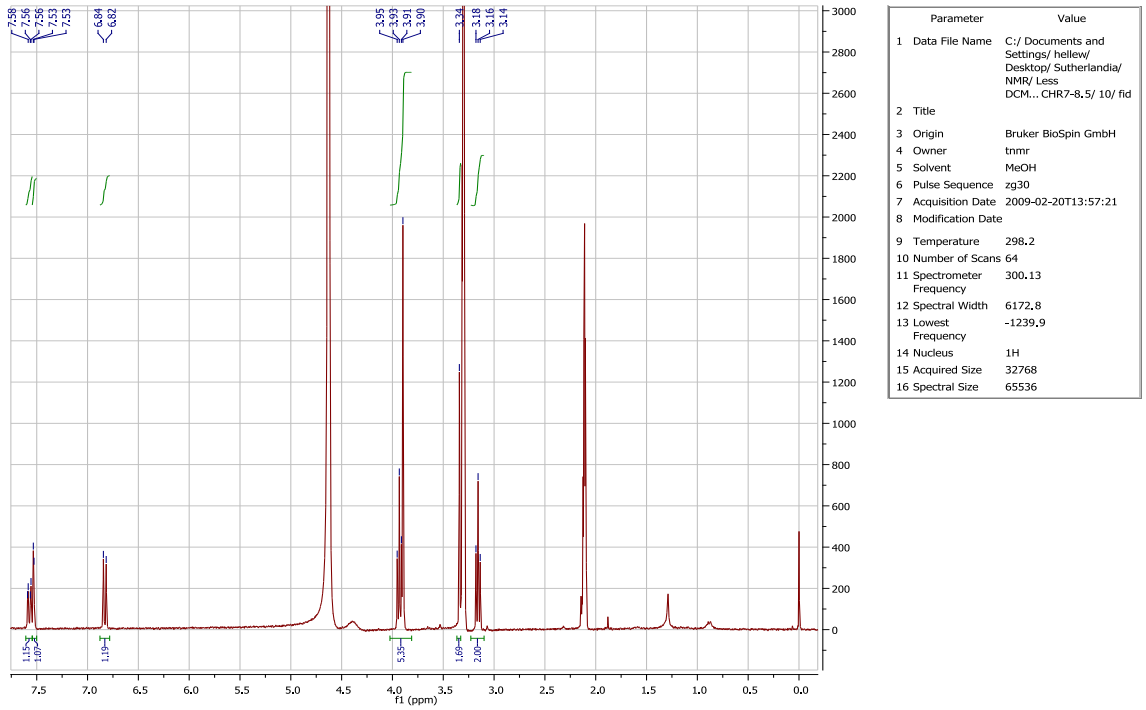
NMR spectra 14.15: 1H-NMR spectra of fraction D2.9.8



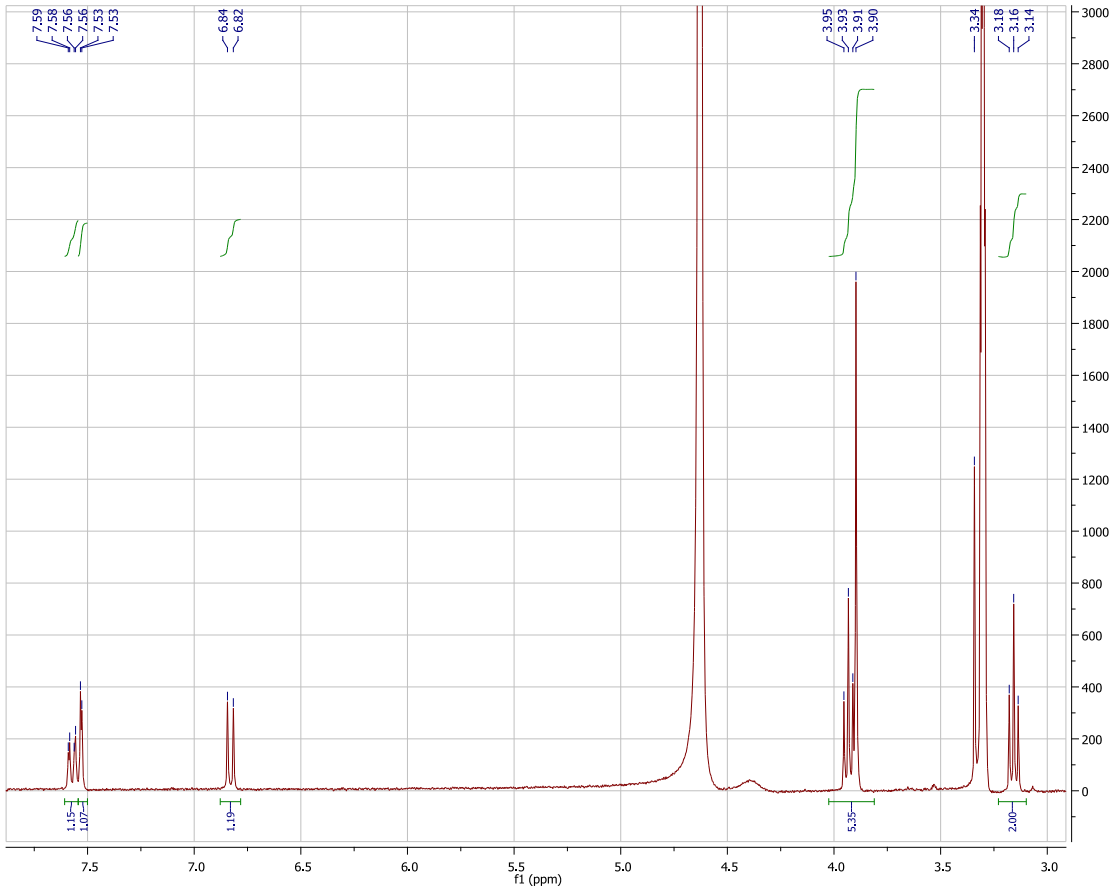
NMR spectra 14.16: partly from 1H-NMR spectra of fraction 2.9.8



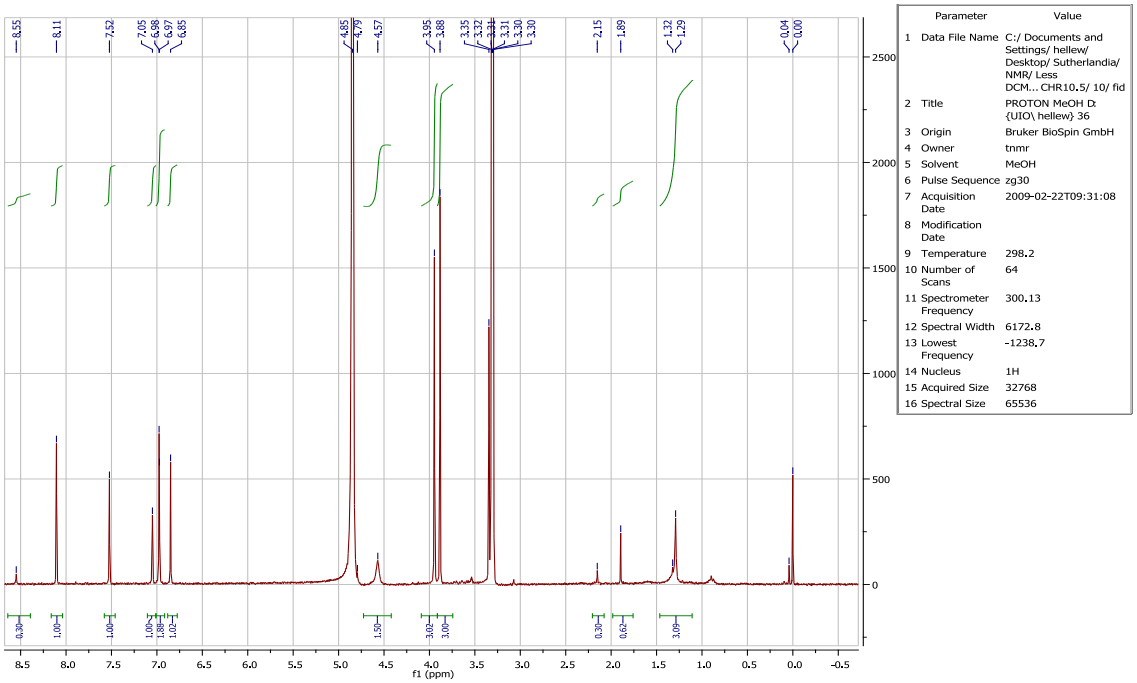
NMR spectra 14.17: 1H-NMR spectra of fraction D2.10.8



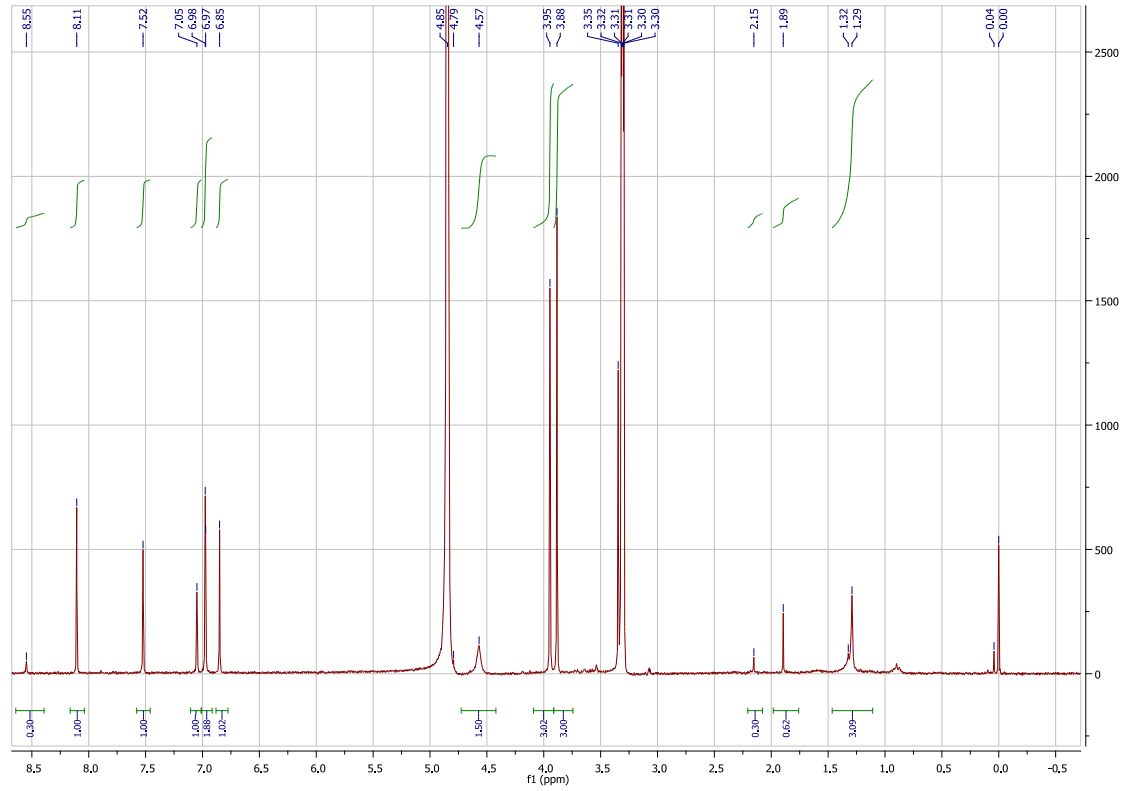
NMR spectra 14.18: 1H-NMR spectra of fraction D2.9.7-8 HPLC 5



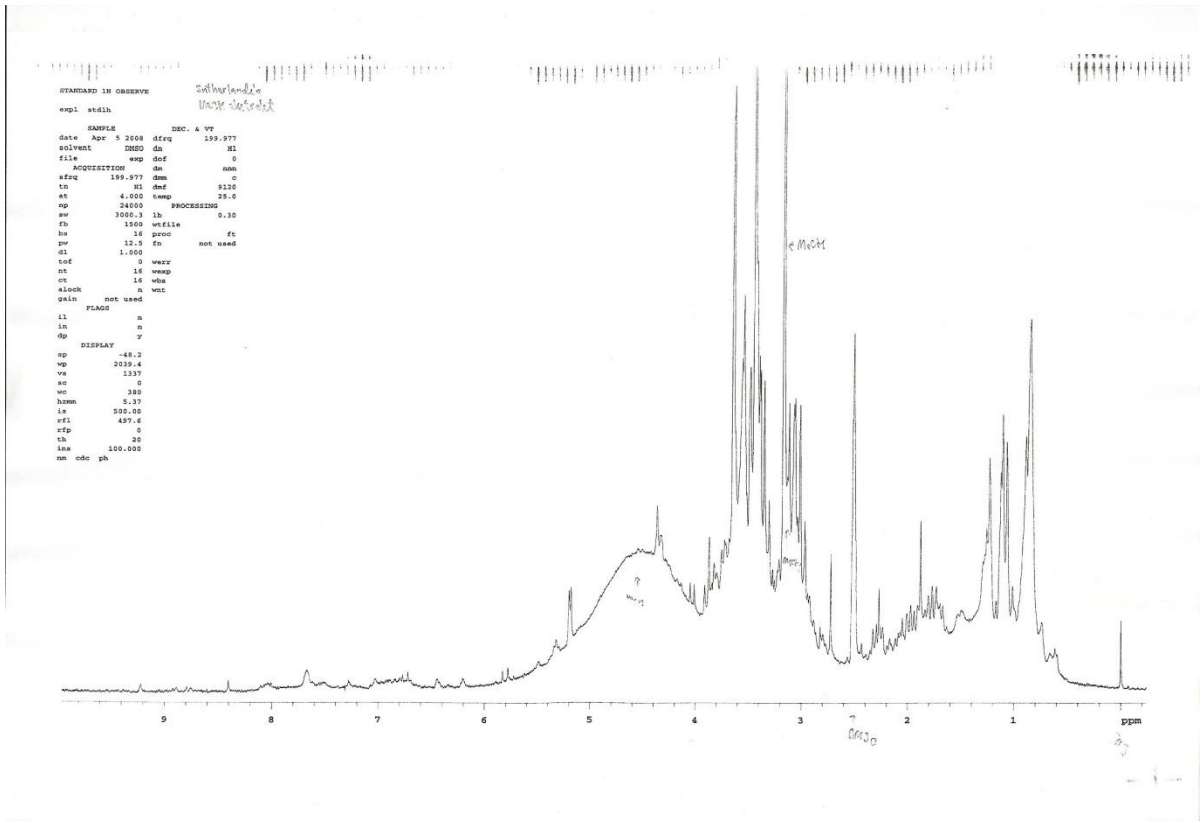
NMR spectra 14.19: partly from 1H-NMR spectra of fraction D2.9.7-8 HPLC 5



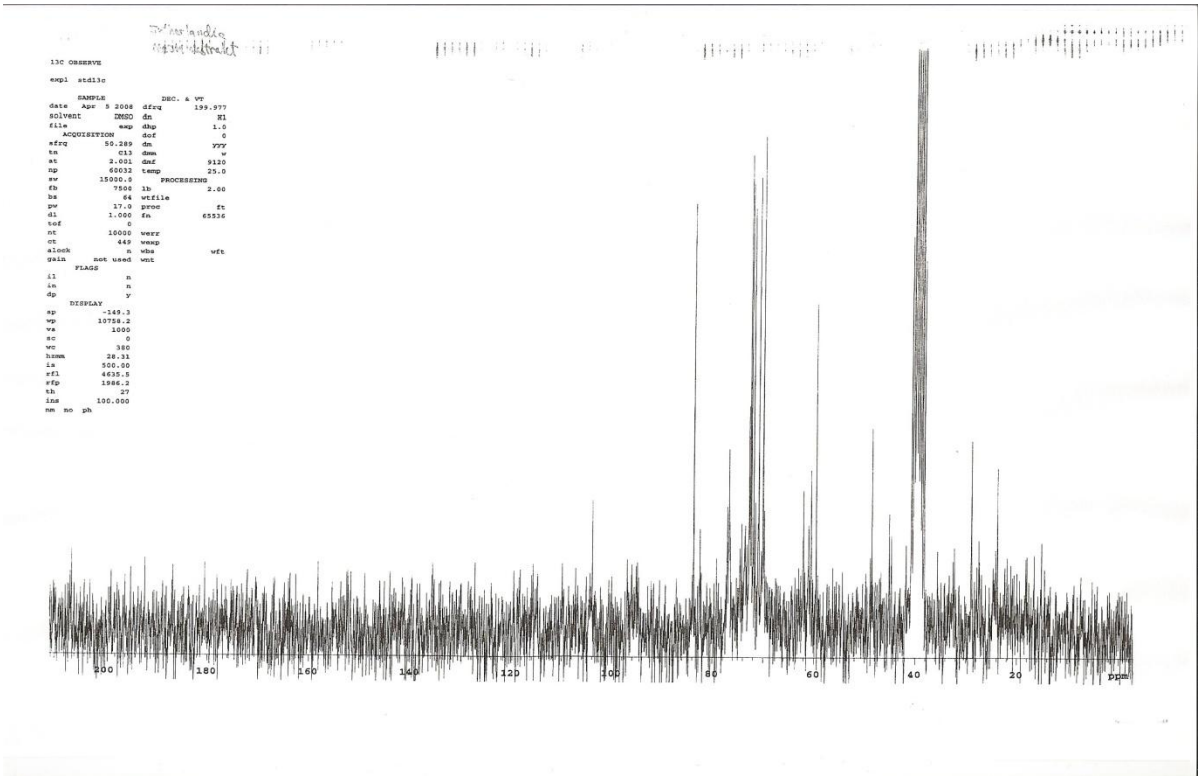
NMR spectra 14.20: 1H-NMR spectra of fraction D2.8.10 HPLC 5



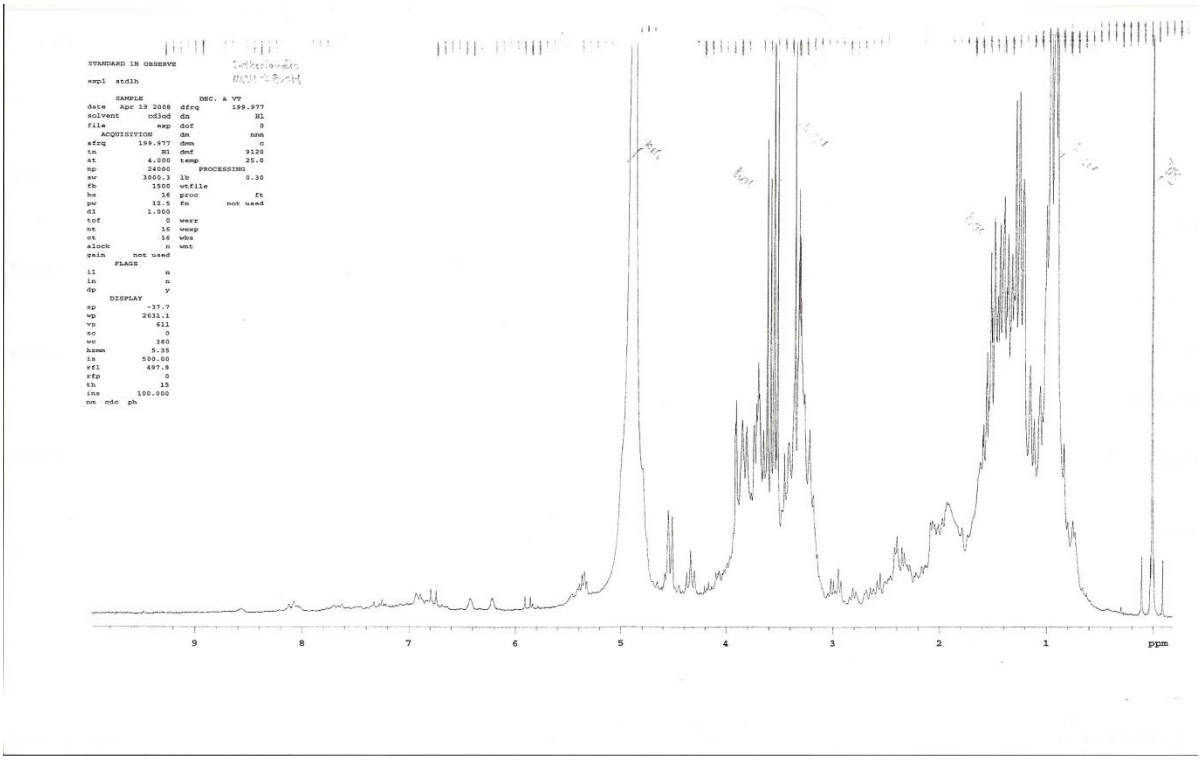
NMR spectra 14.21: 1H-NMR spectra of fraction D2.8.10 HPLC 5



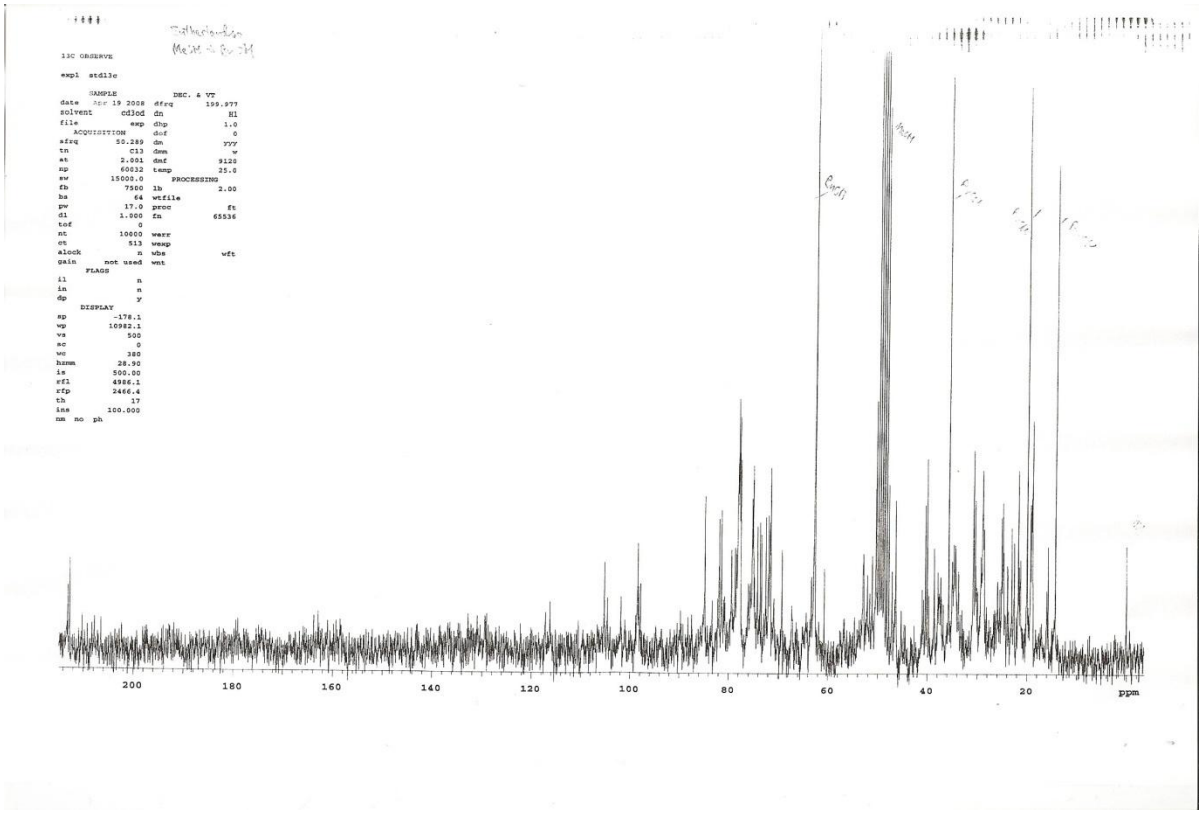
NMR spectra 14.22: 1H-NMR spectra of MeOH extract



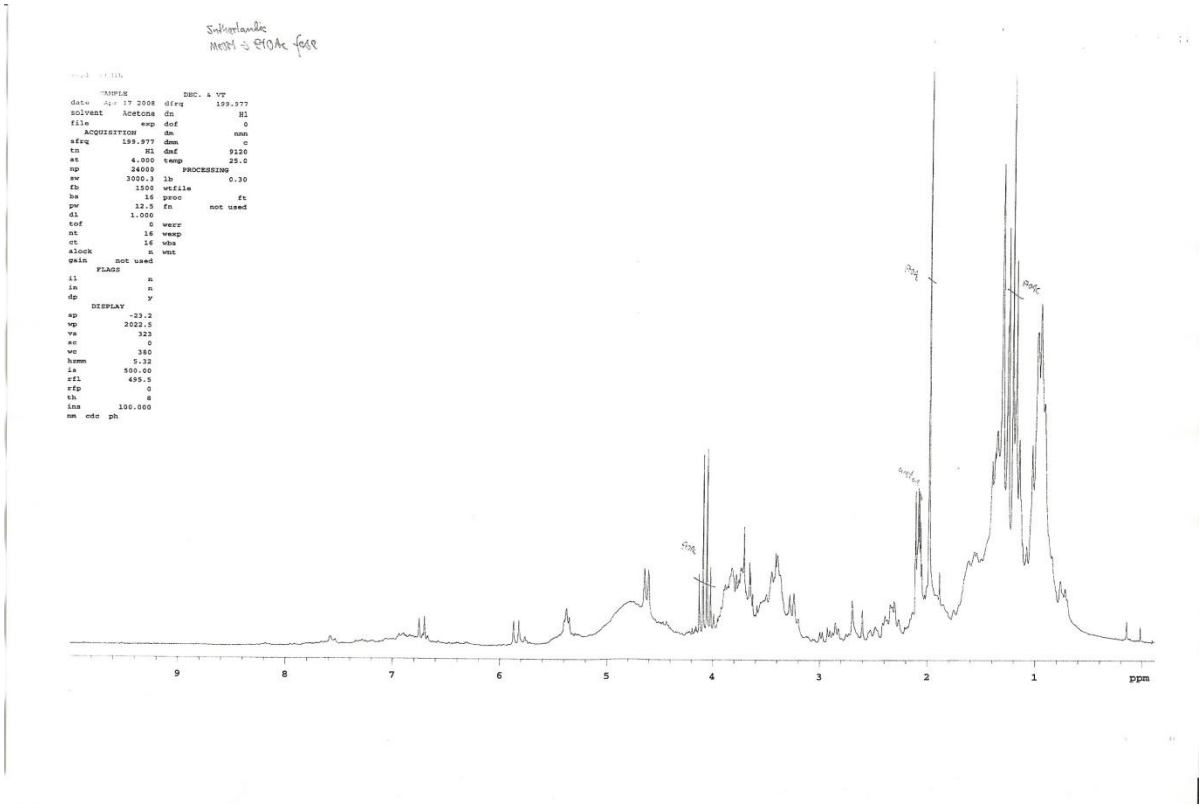
NMR spectra 14.23: 13C-NMR spectra of MeOH extract



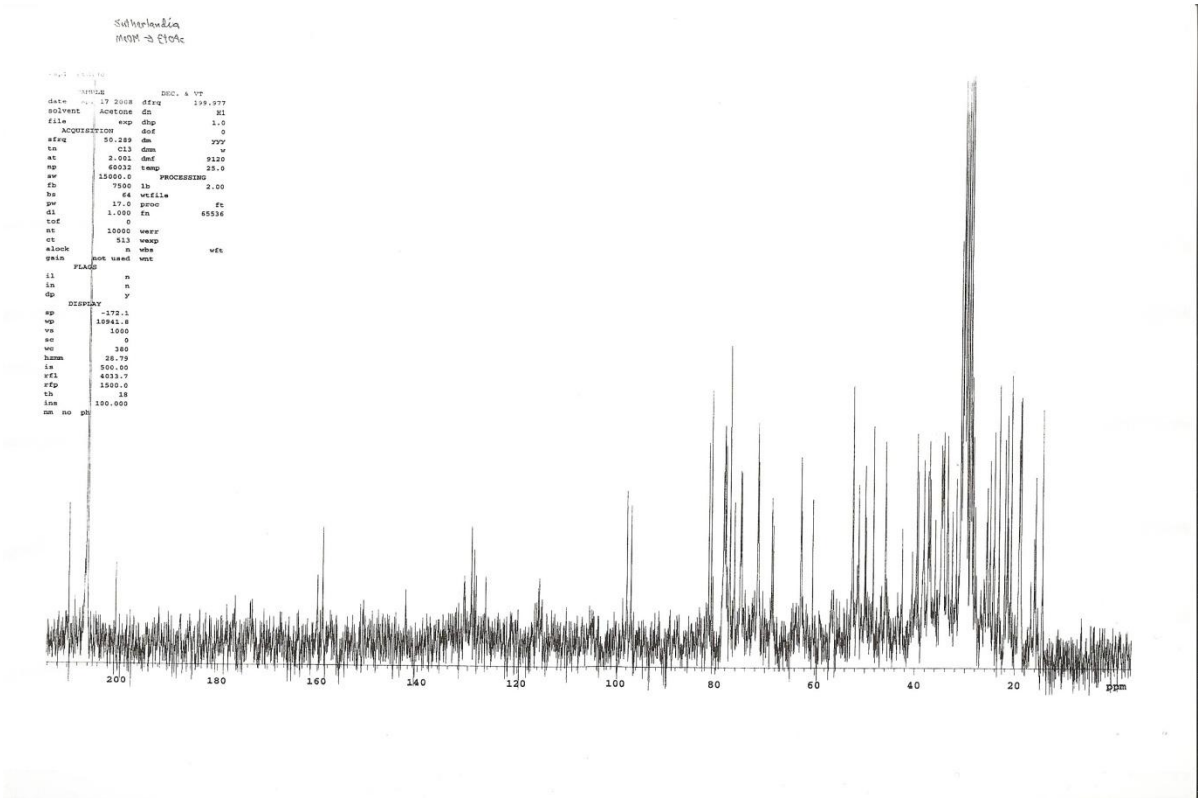
NMR spectra 14.24: 1H-NMR spectra of BuOH extract



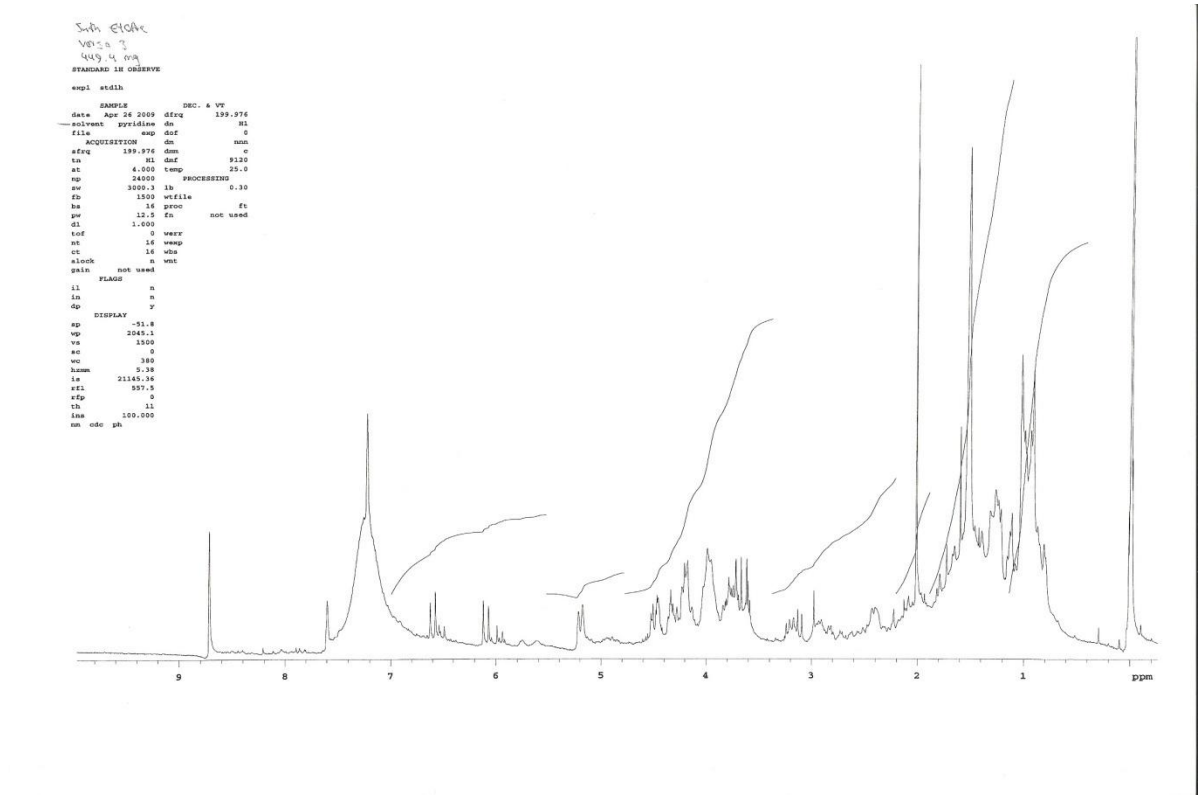
NMR spectra 14.25: 13C-NMR spectra of BuOH extract



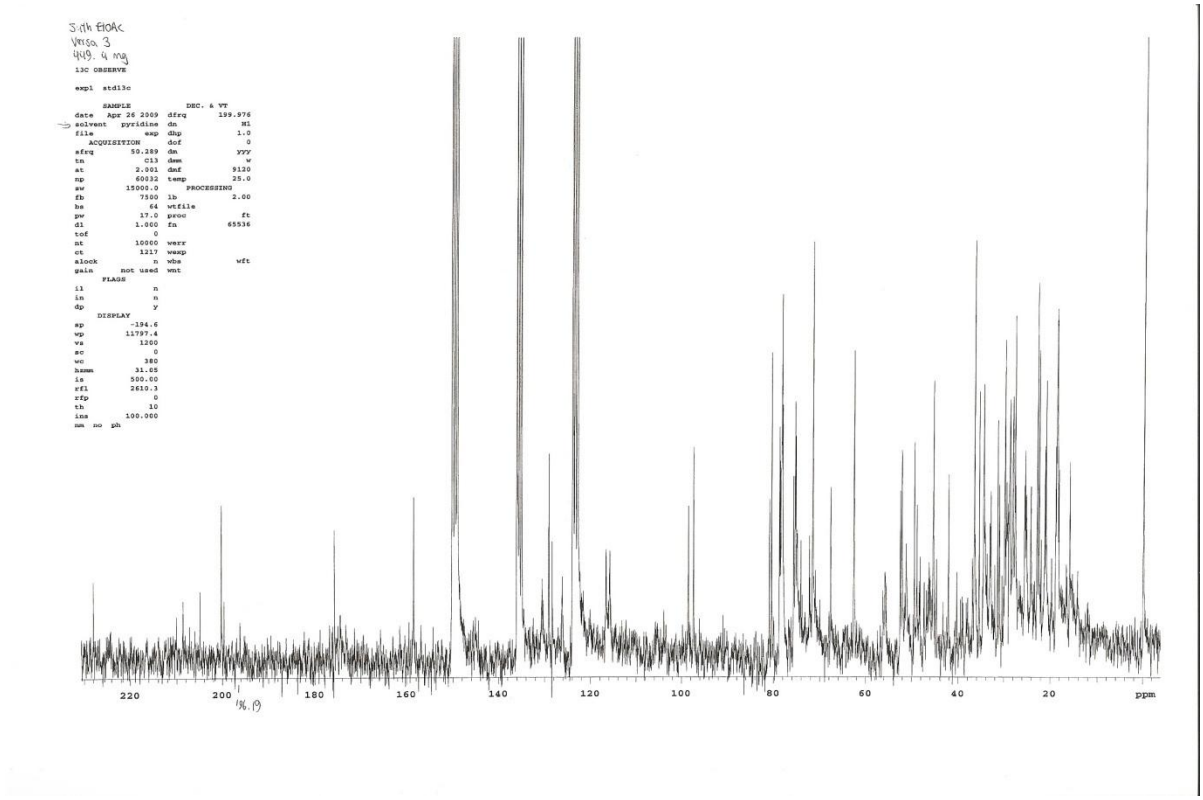
NMR spectra 14.26: 1H-NMR spectra of EtOAc extract



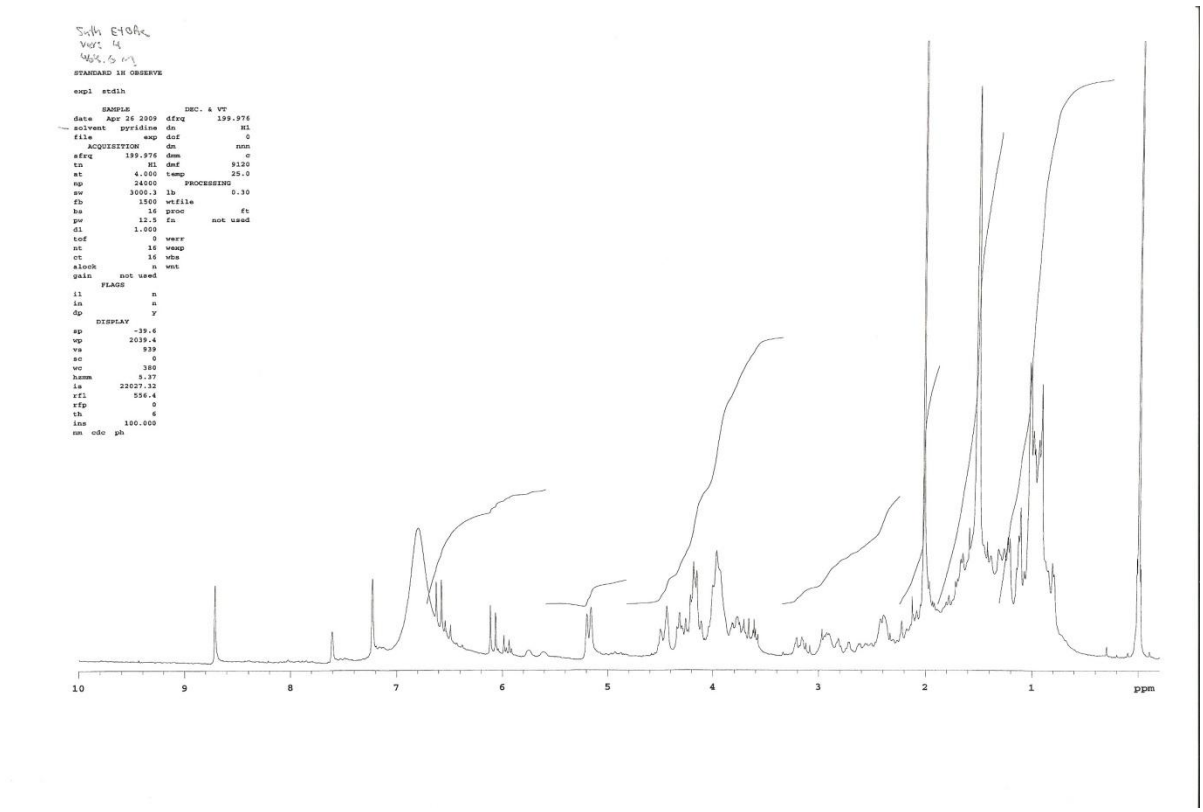
NMR spectra 14.27: 13C-NMR spectra of EtOAc extract



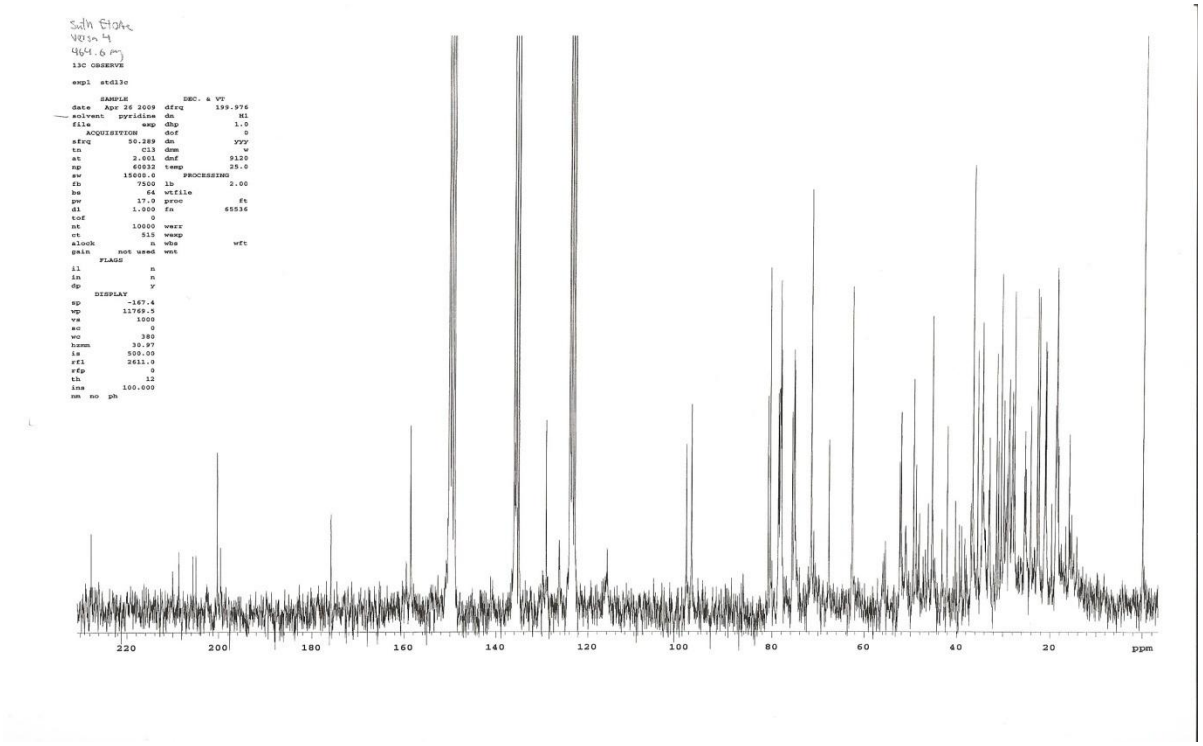
NMR spectra14.28: 1H-NMR spectra of fraction E3.



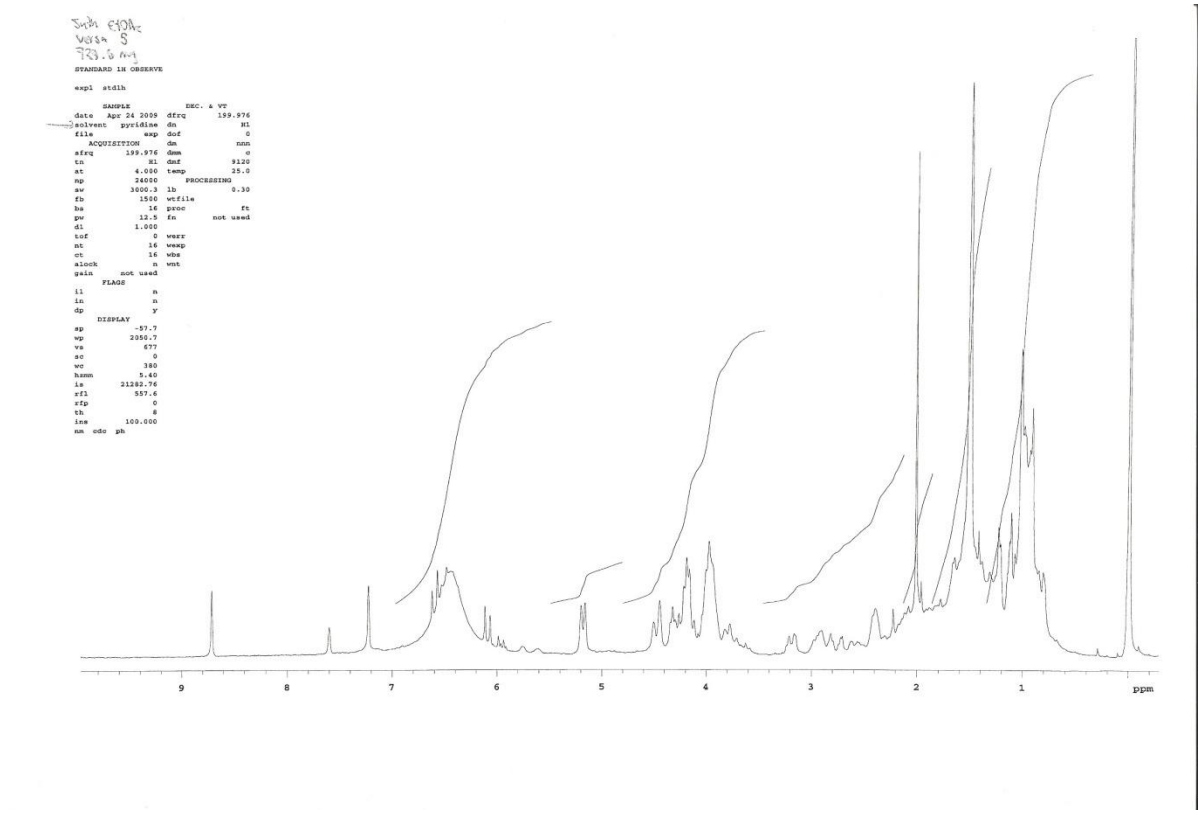
NMR spectra 14.29: 13C-NMR spectra of fraction E3.



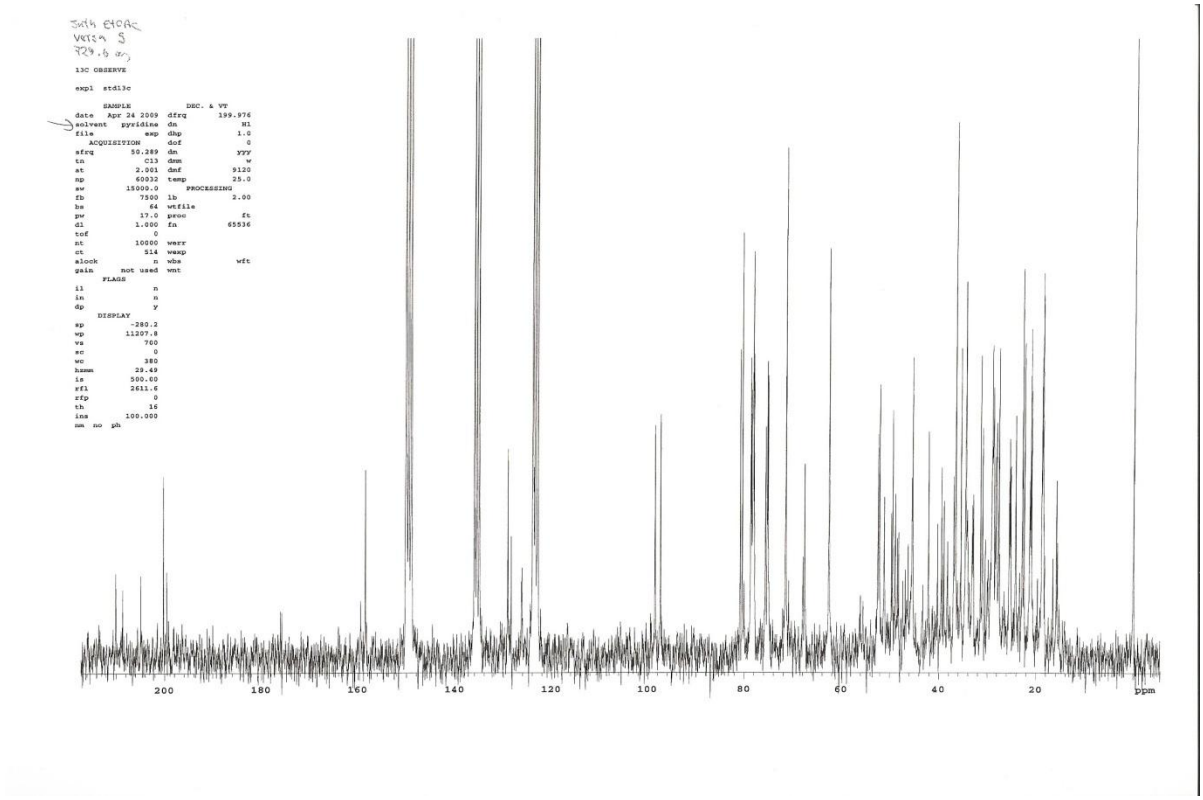
NMR spectra 14.30: 1H-NMR spectra of fraction E4



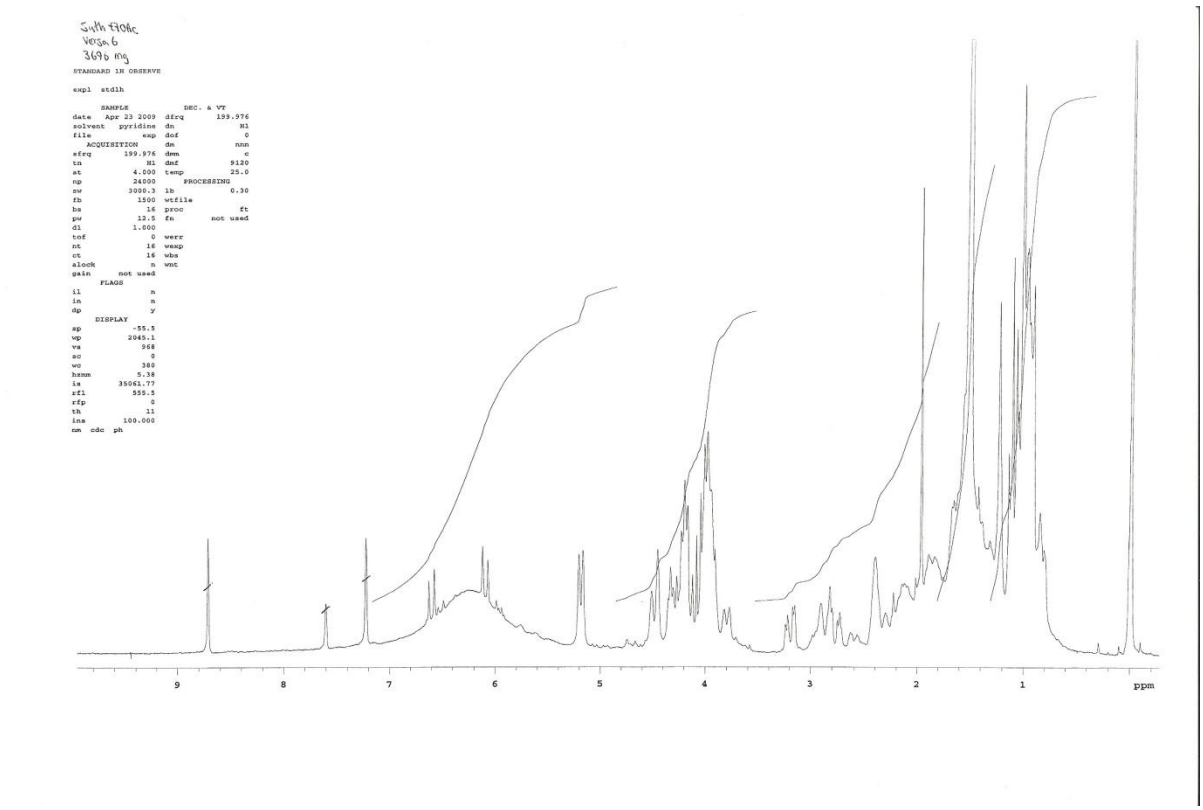
NMR spectra 14.31: 13C-NMR spectra of fraction E4.



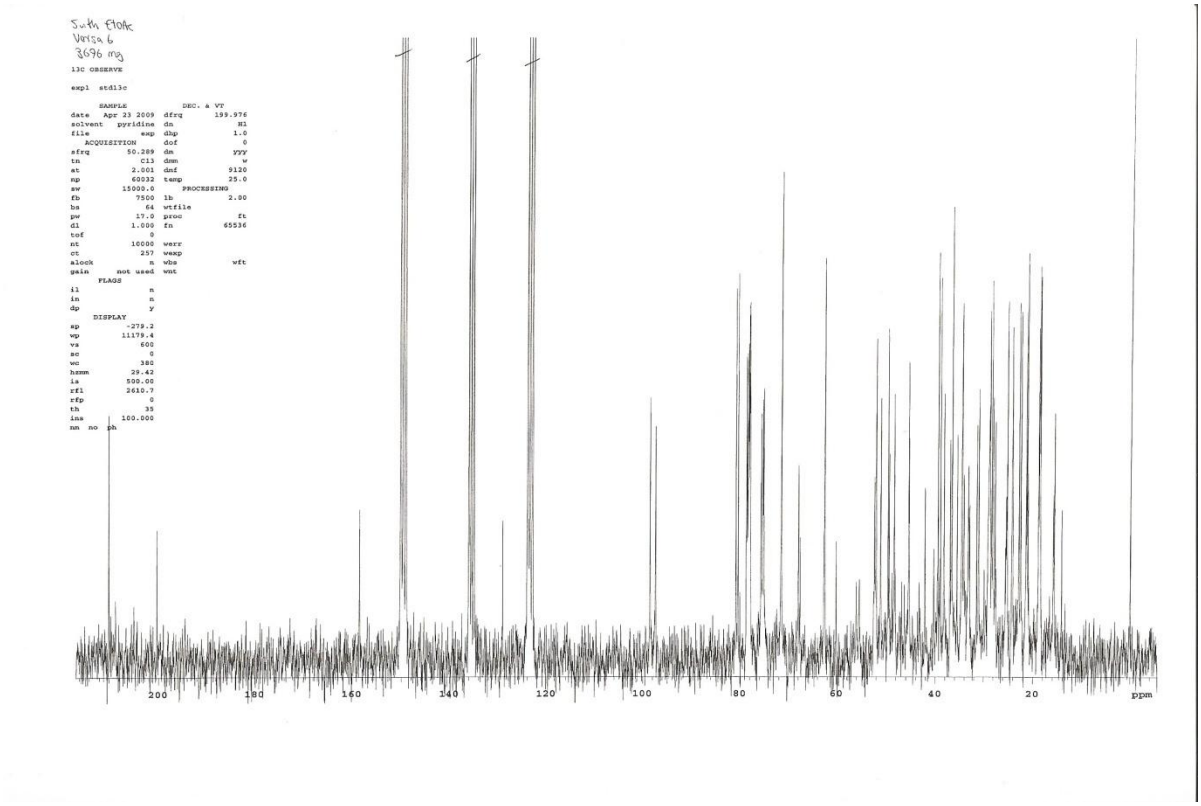
NMR spectra 14.32: 1H-NMR spectra of fraction E5



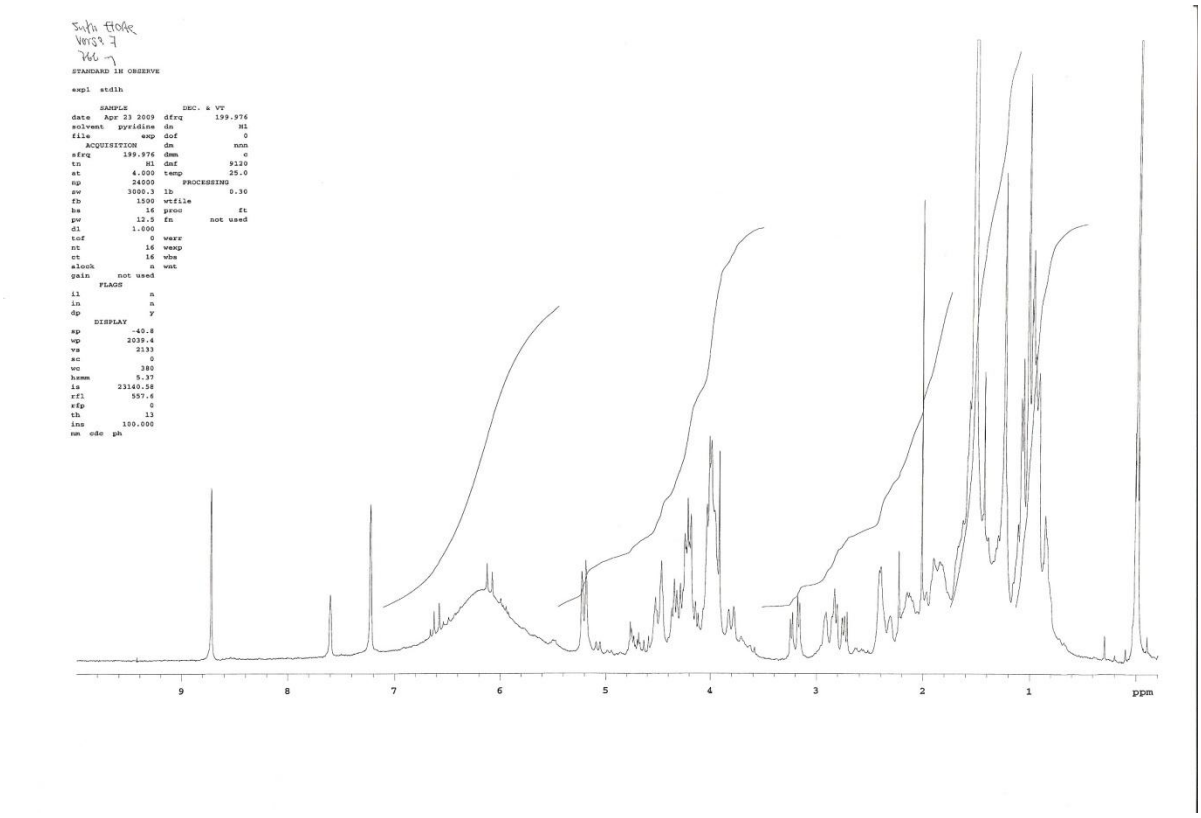
NMR spectra 14.33: 13C-NMR spectra of fraction E5.



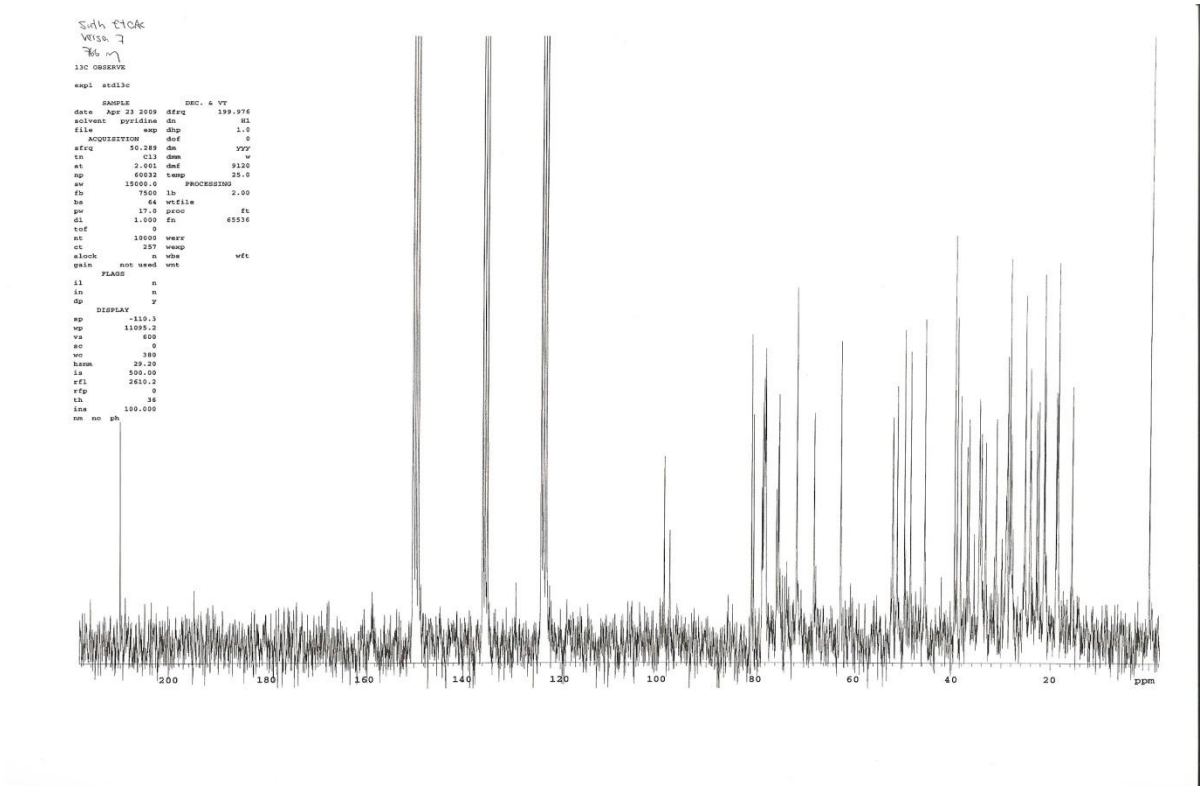
NMR spectra 14.34: 1H-NMR spectra of fraction E6.



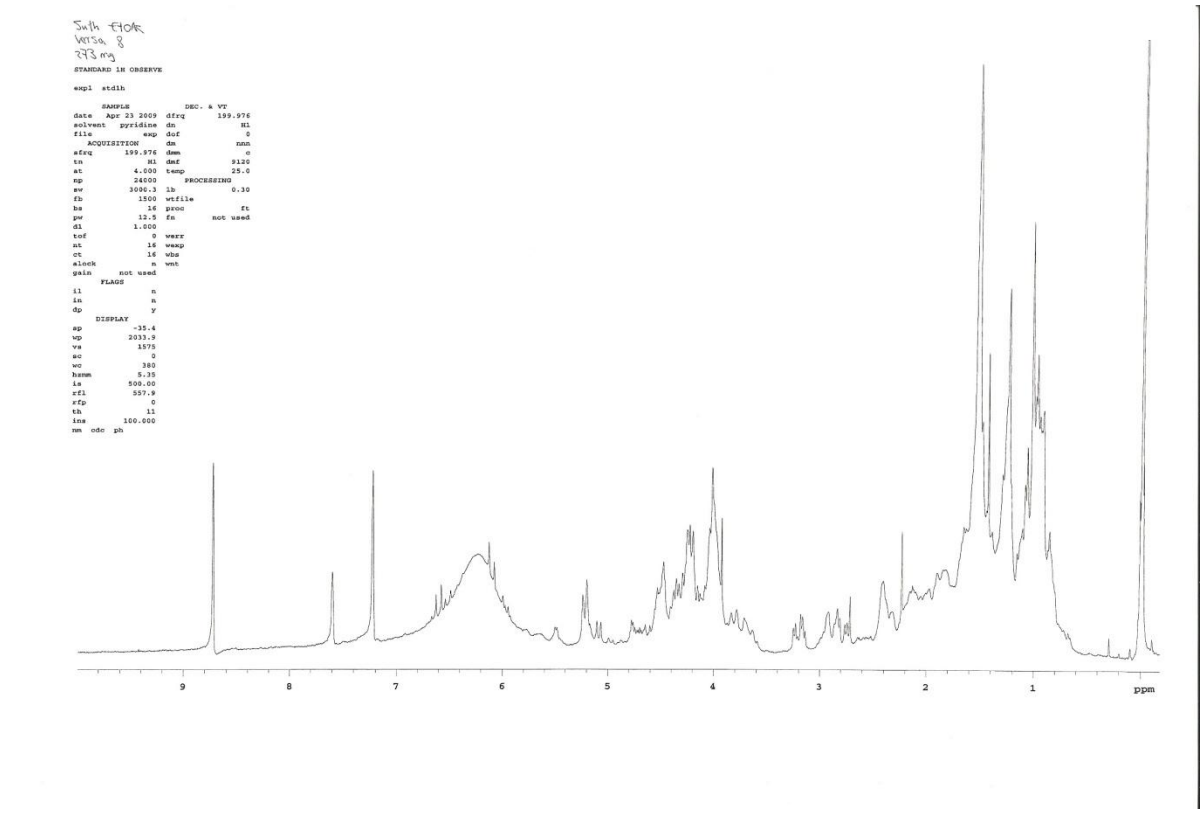
NMR spectra 14.35: 13C-NMR spectra of fraction E6.



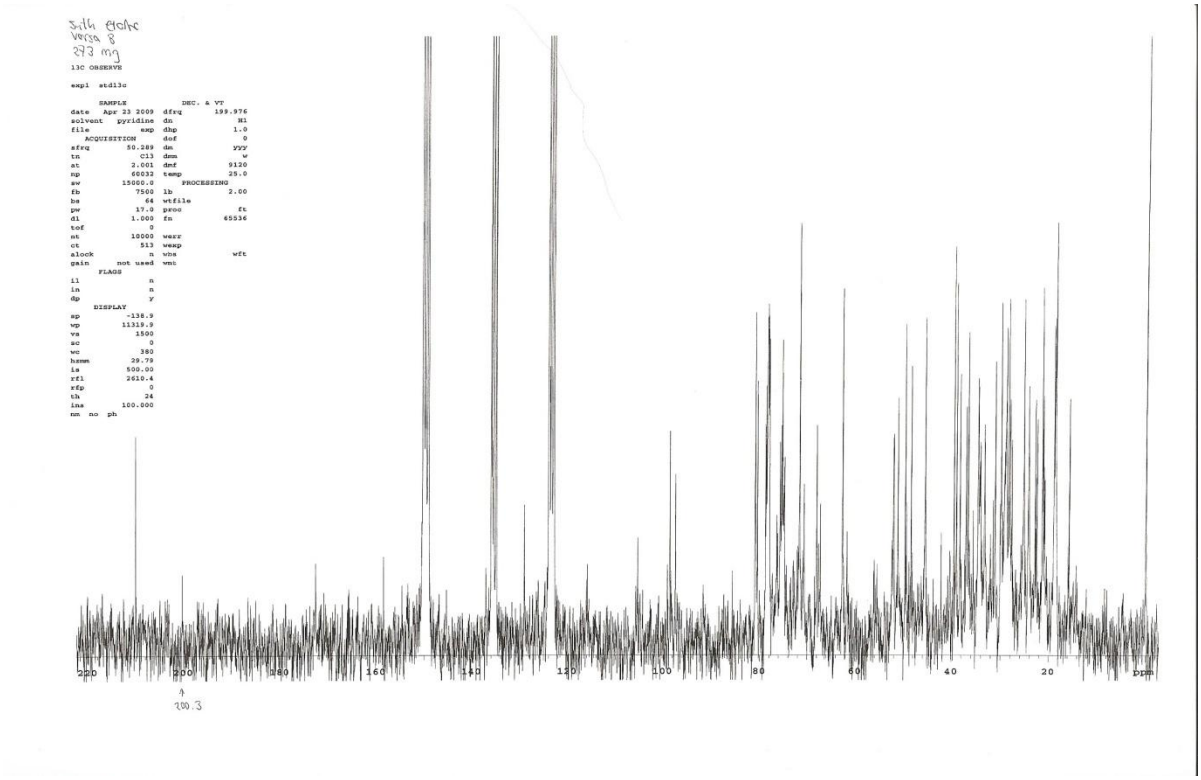
NMR spectra 14.36: 1H-NMR spectra of fraction E7.



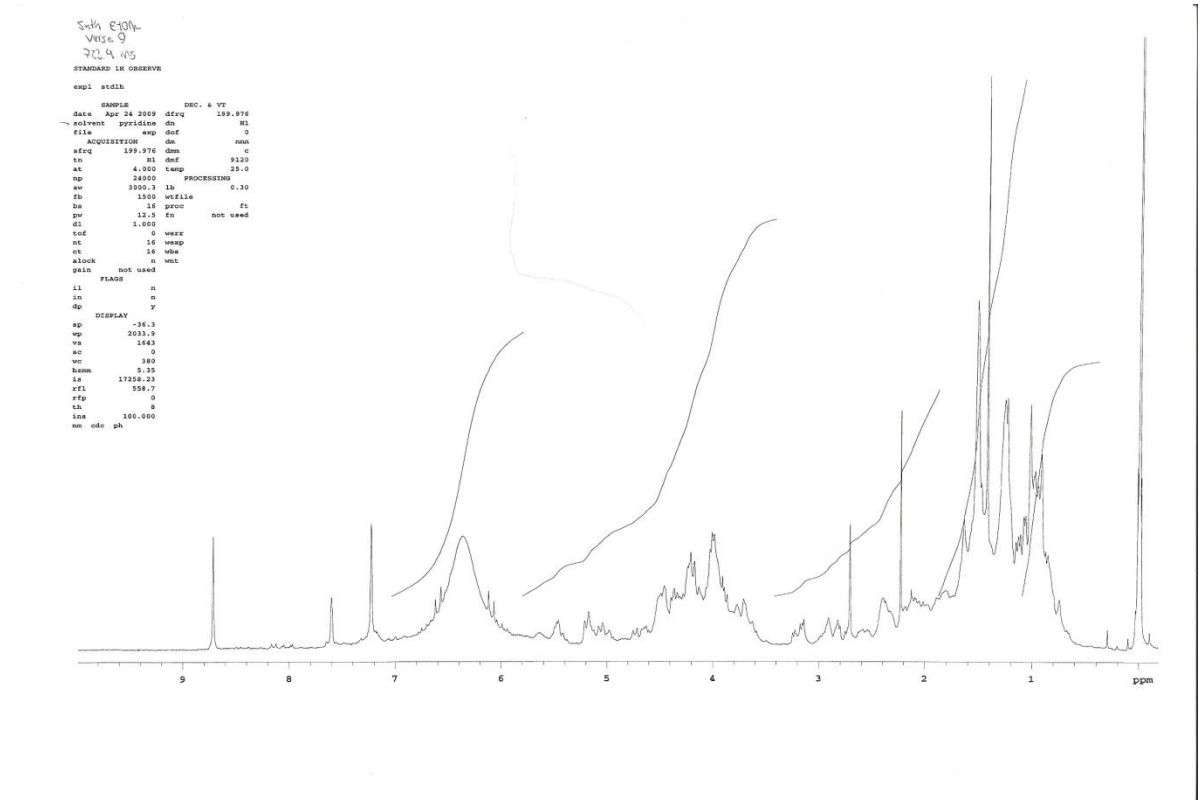
NMR spectra 14.37: 13C-NMR spectra of fraction E7.



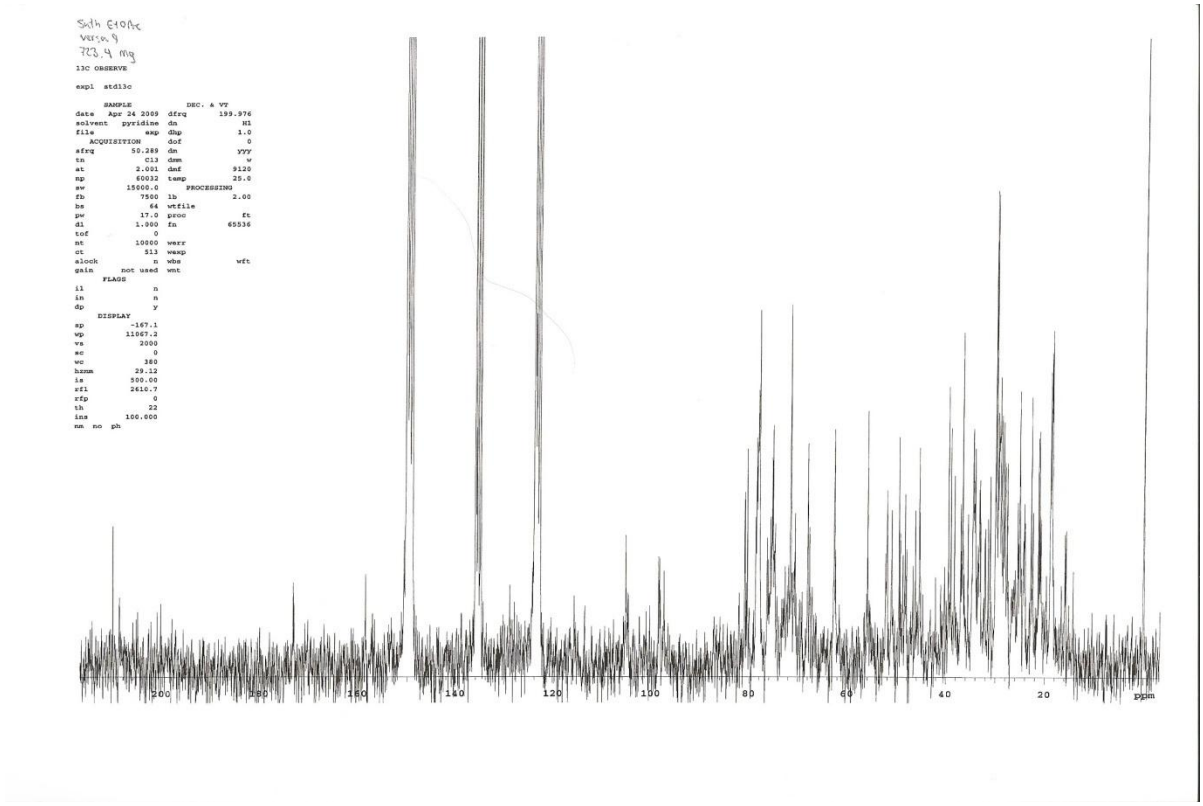
NMR spectra 14.38: 1H-NMR spectra of fraction E8.



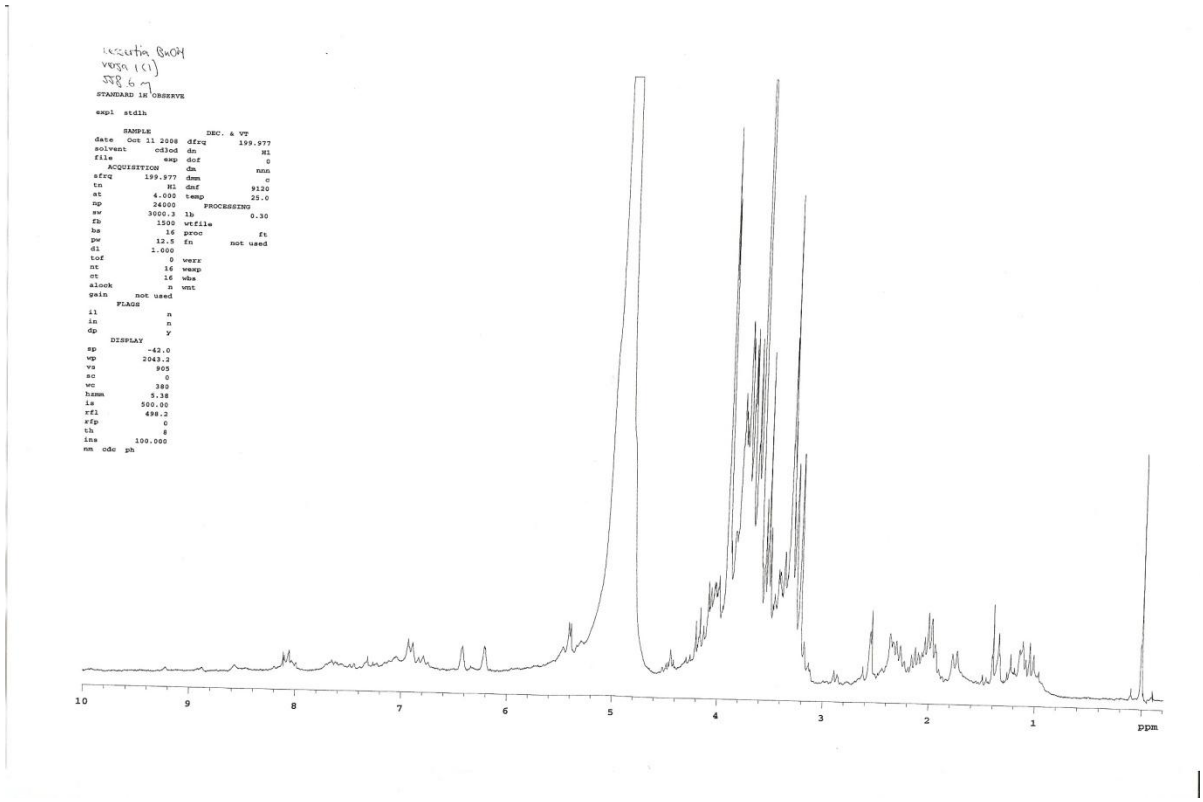
NMR spectra 14.39: 13C-NMR spectra of fraction E8.



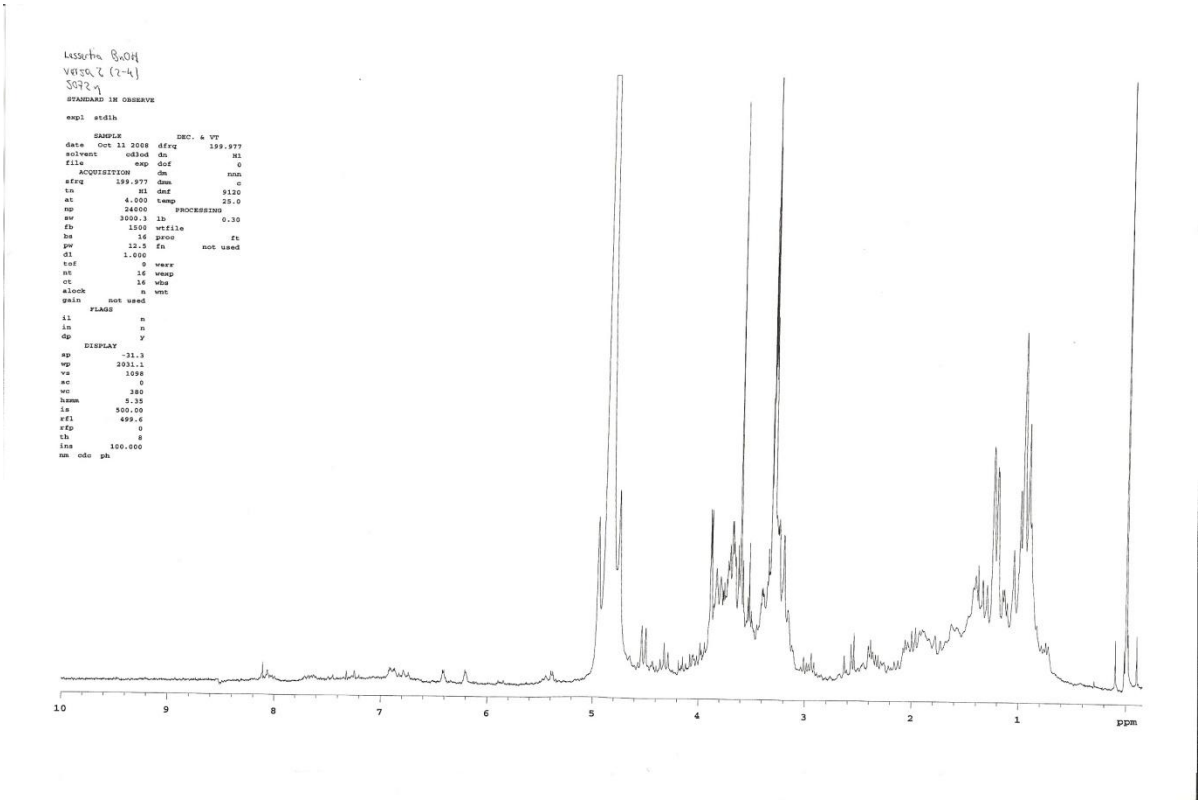
NMR spectra 14.40: 1H-NMR spectra of fraction E9.



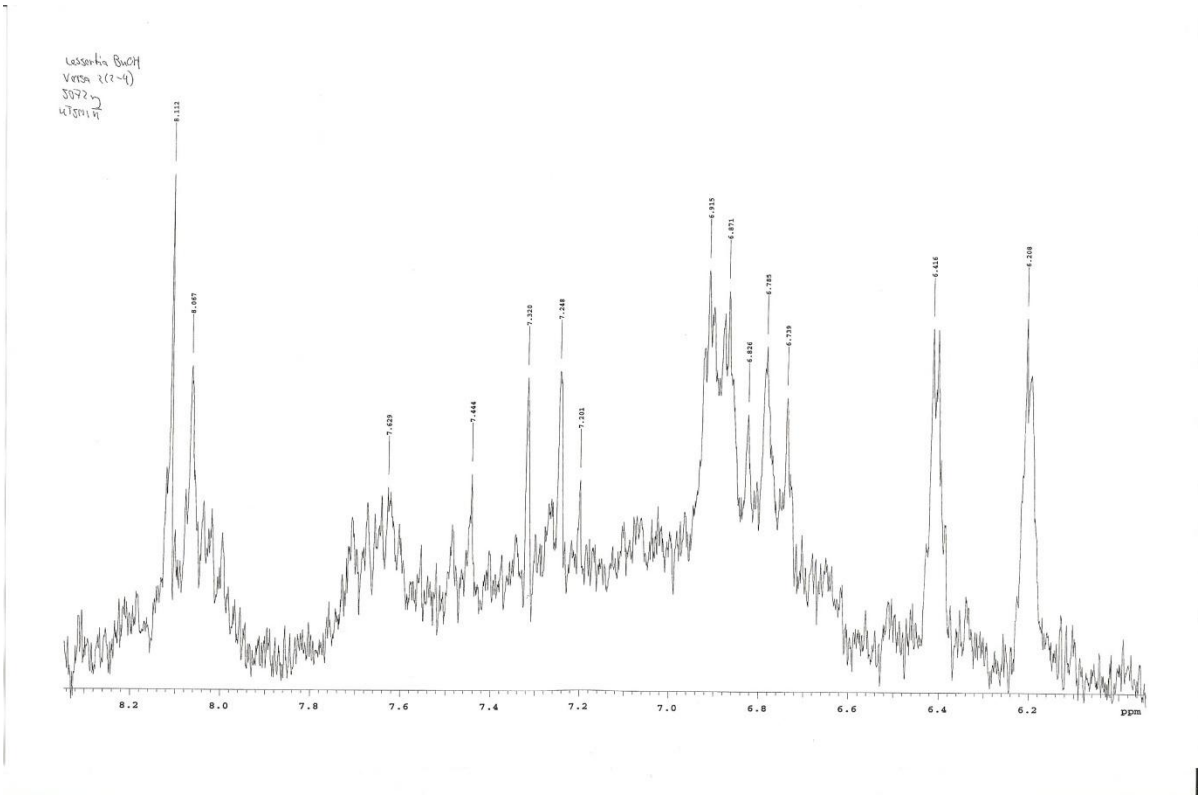
NMR spectra 14.41: 13C-NMR spectra of fraction E9.



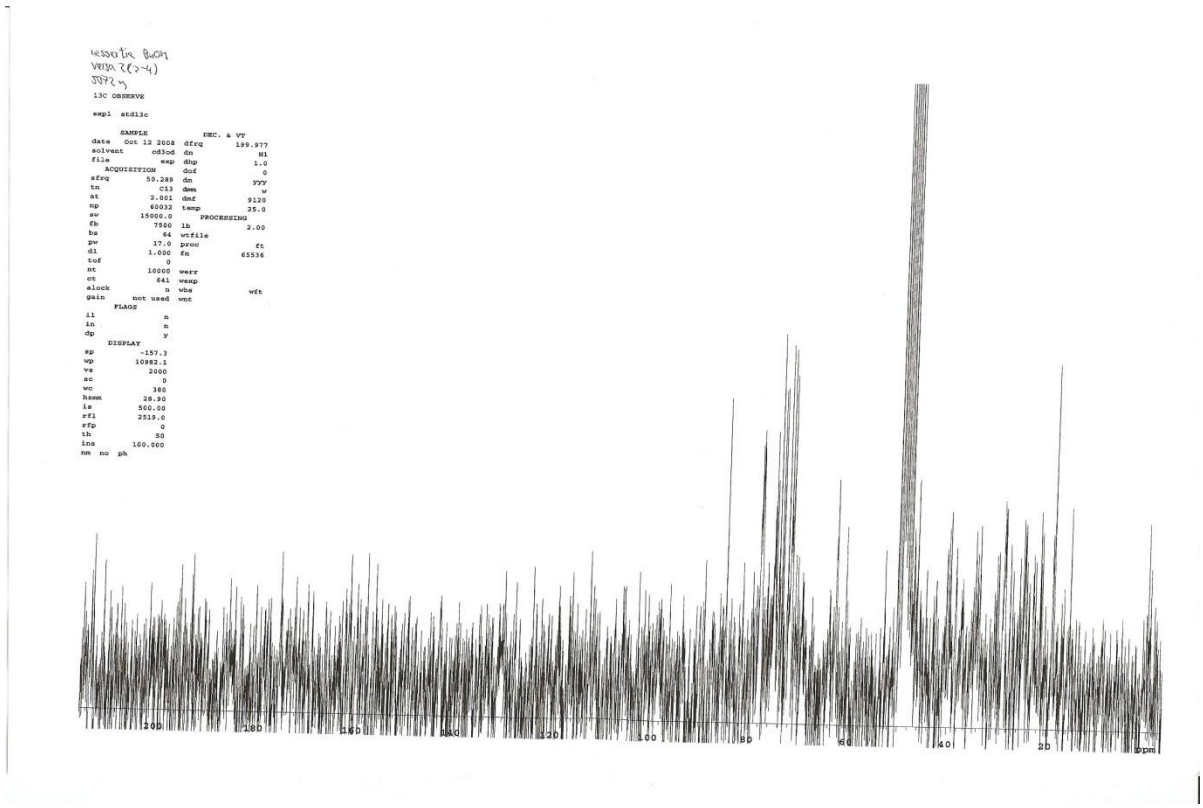
NMR spectra 14.42: 1H-NMR spectra of fraction B1



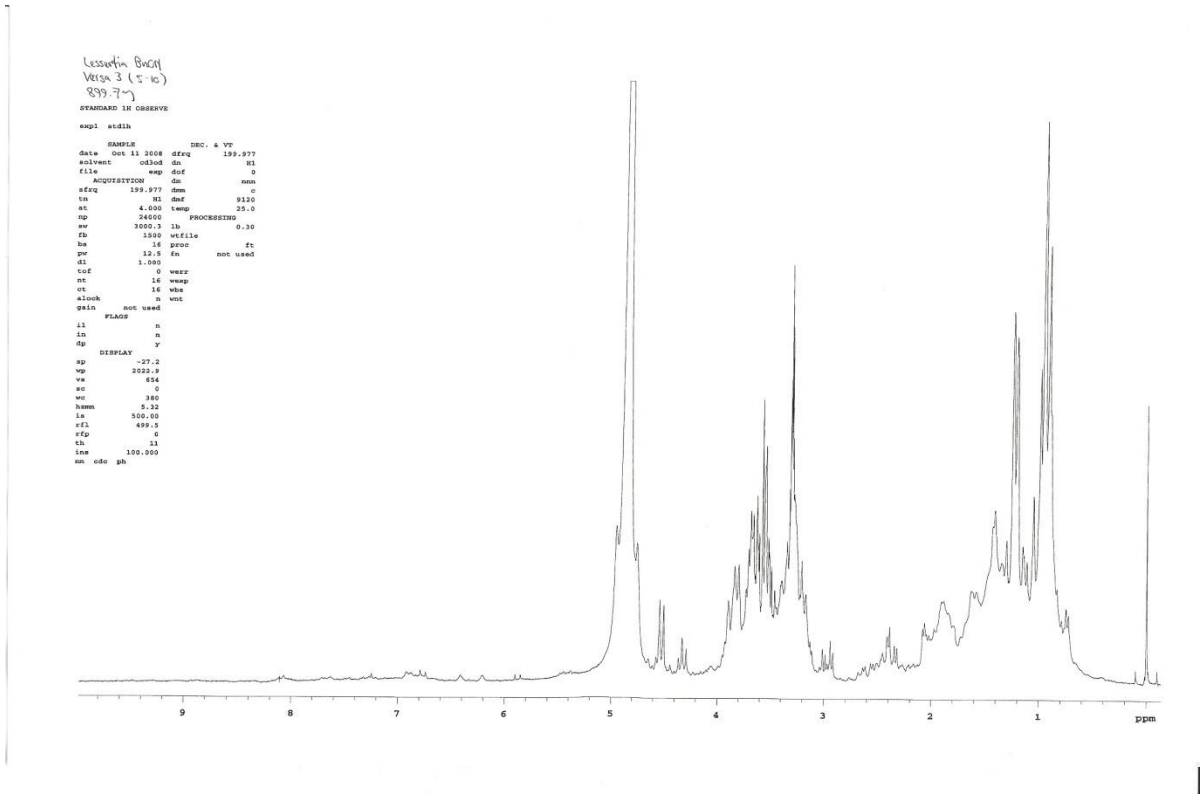
NMR spectra 14.43: 1H-NMR spectra of fraction B2



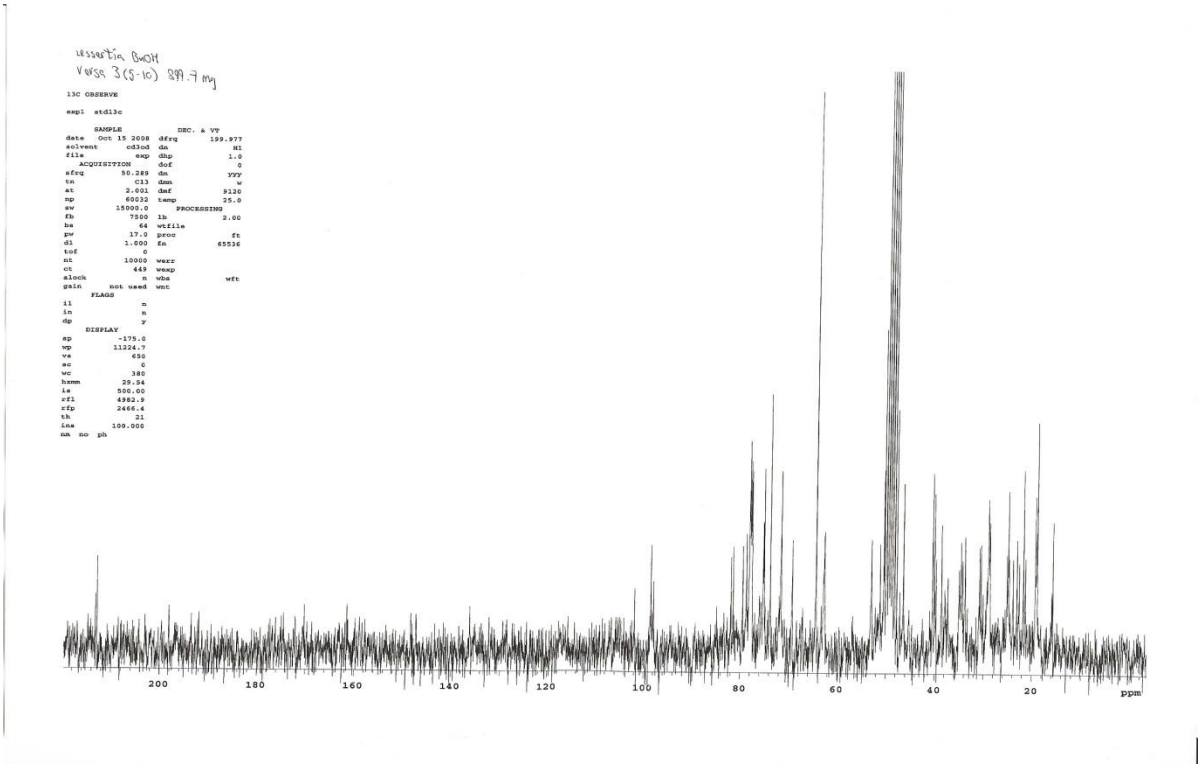
NMR spectra 14.44: partly from 1H-MNR spectra of fraction B2



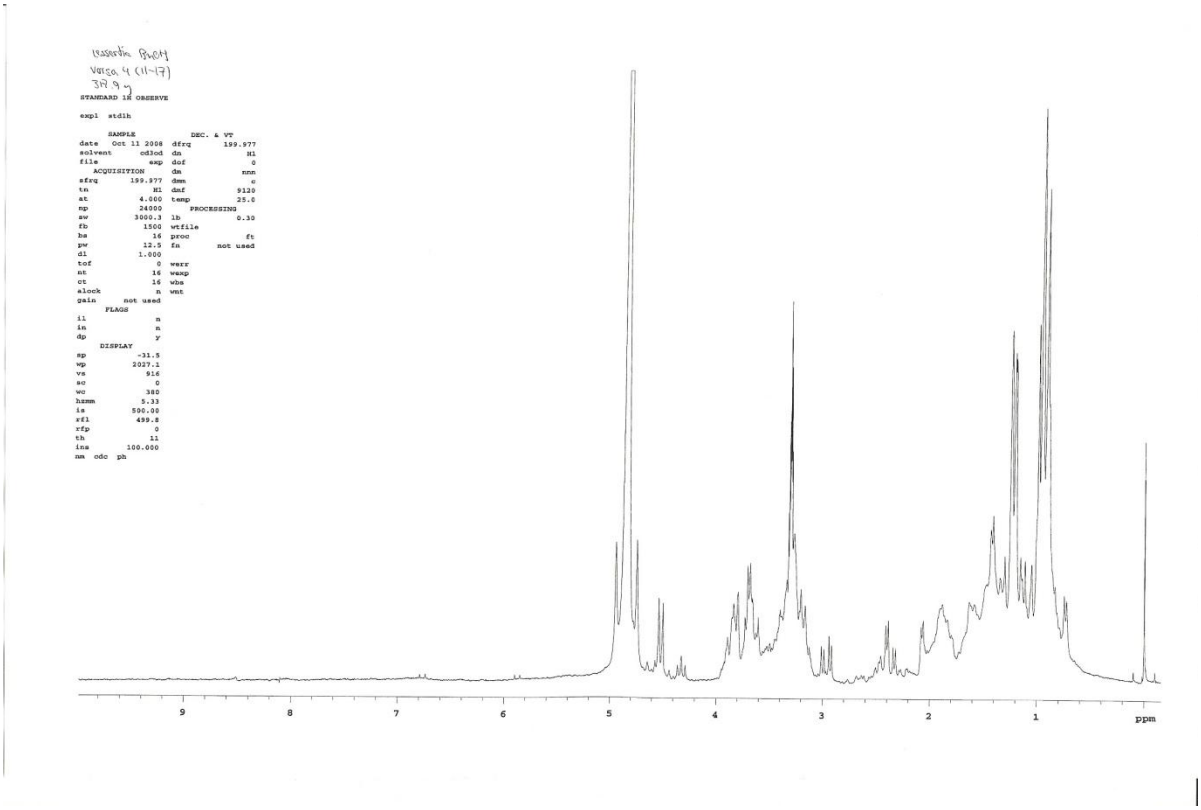
NMR spectra 14.45: 13C-NMR spectra of fraction B2.



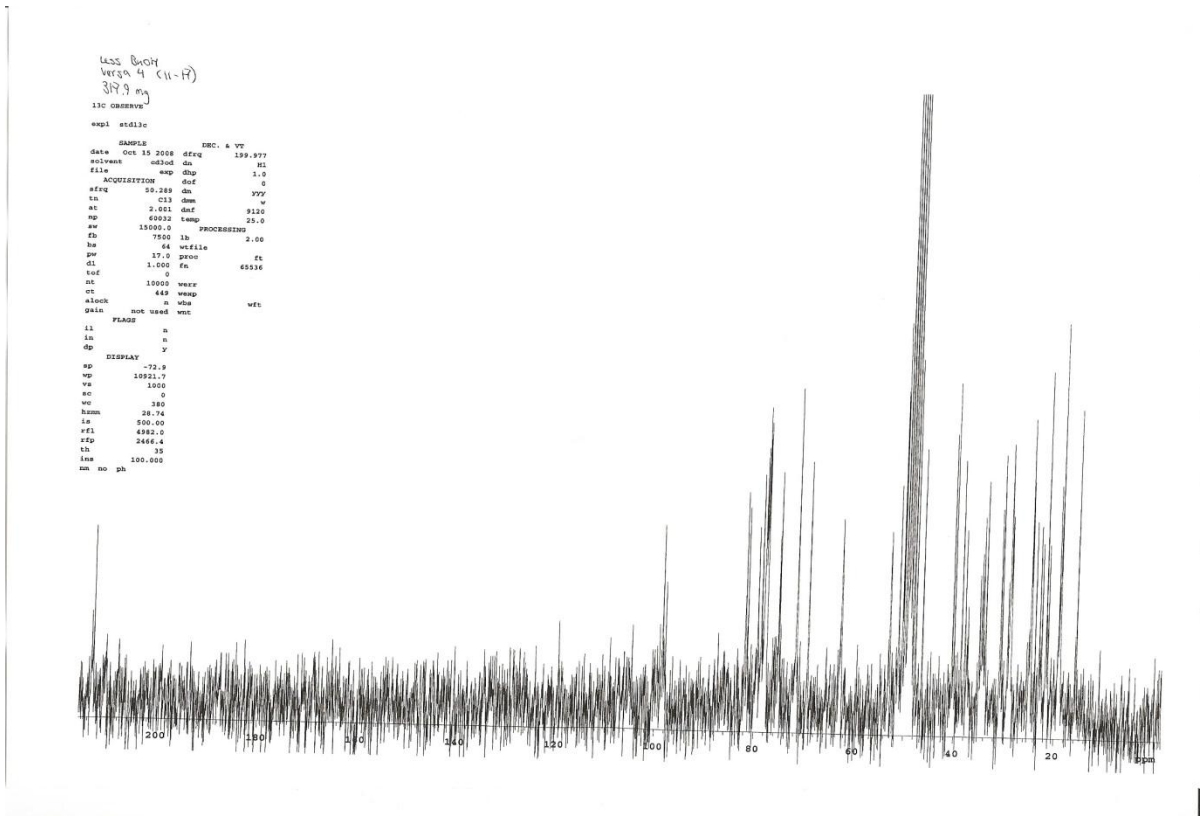
NMR spectra 14.46: 1H-NMR spectra of fraction B3.



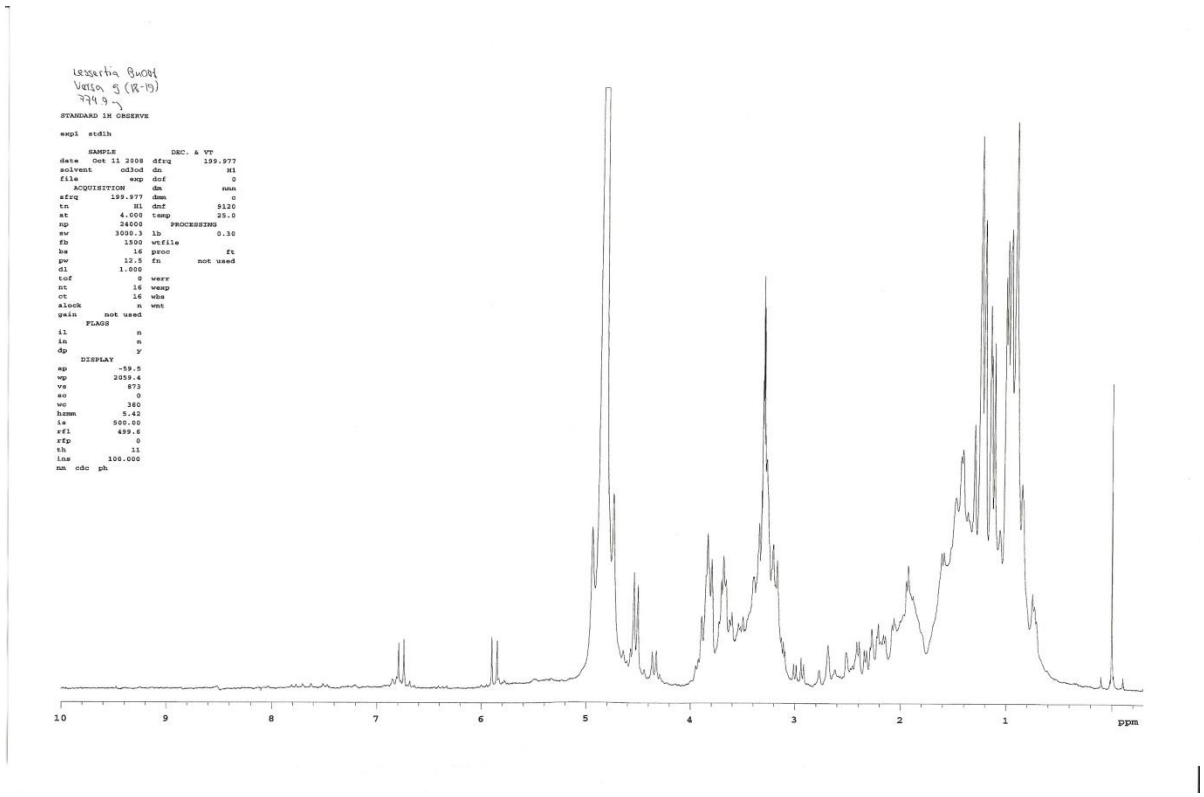
NMR spectra 14.47: 13C-NMR of fraction B3.



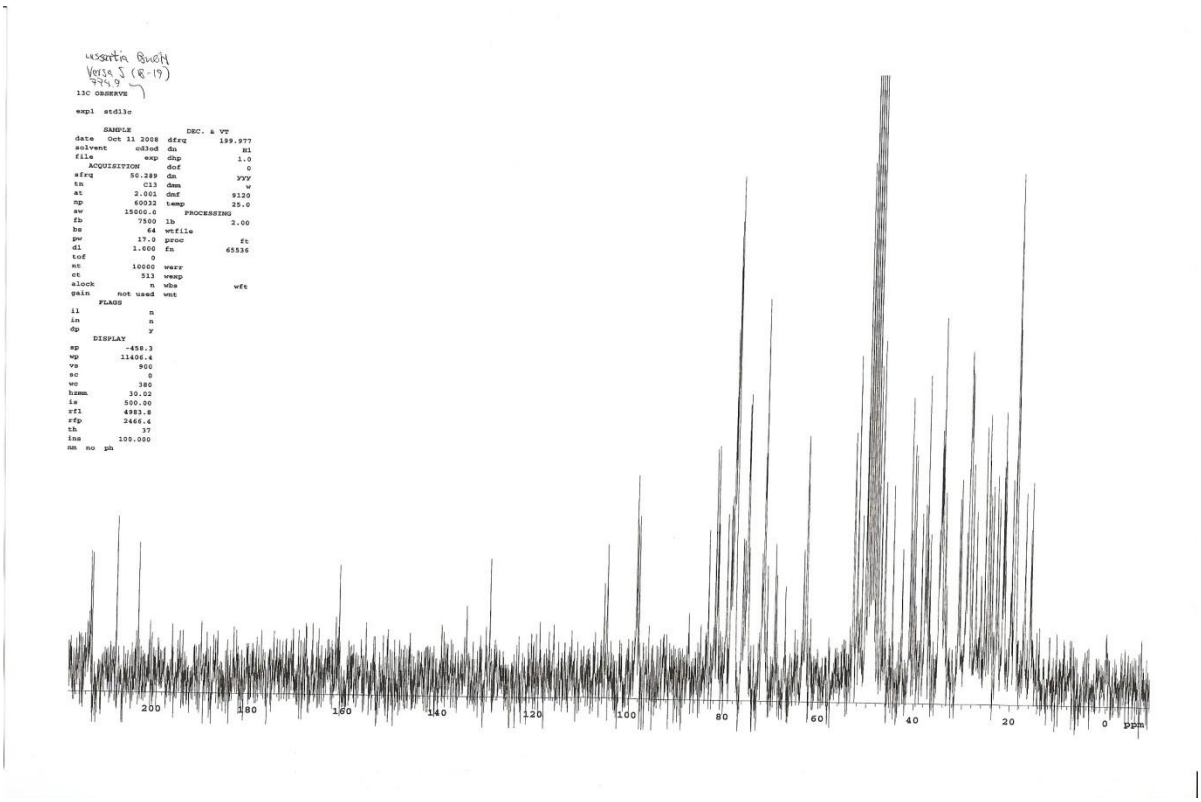
NMR spectra 14.48: 1H-NMR spectra of fraction B4.



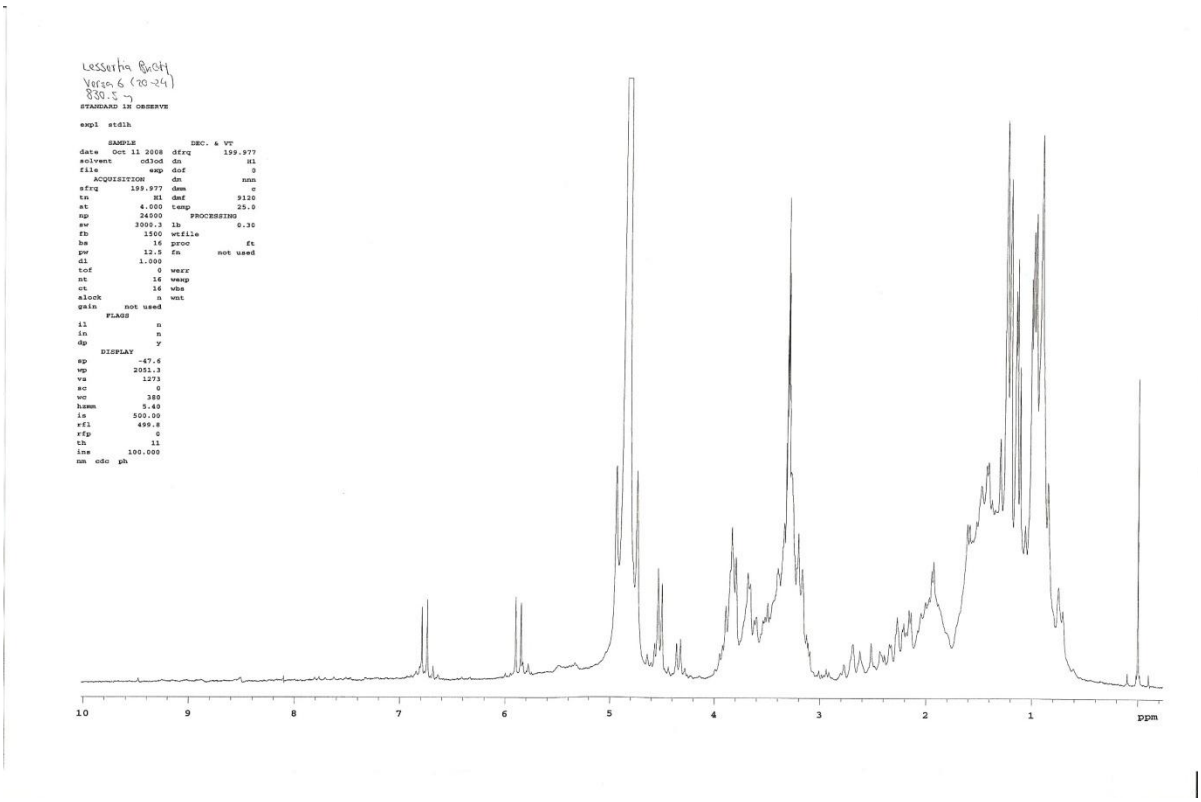
NMR spectra 14.49: 13C-NMR spectra of fraction B4.



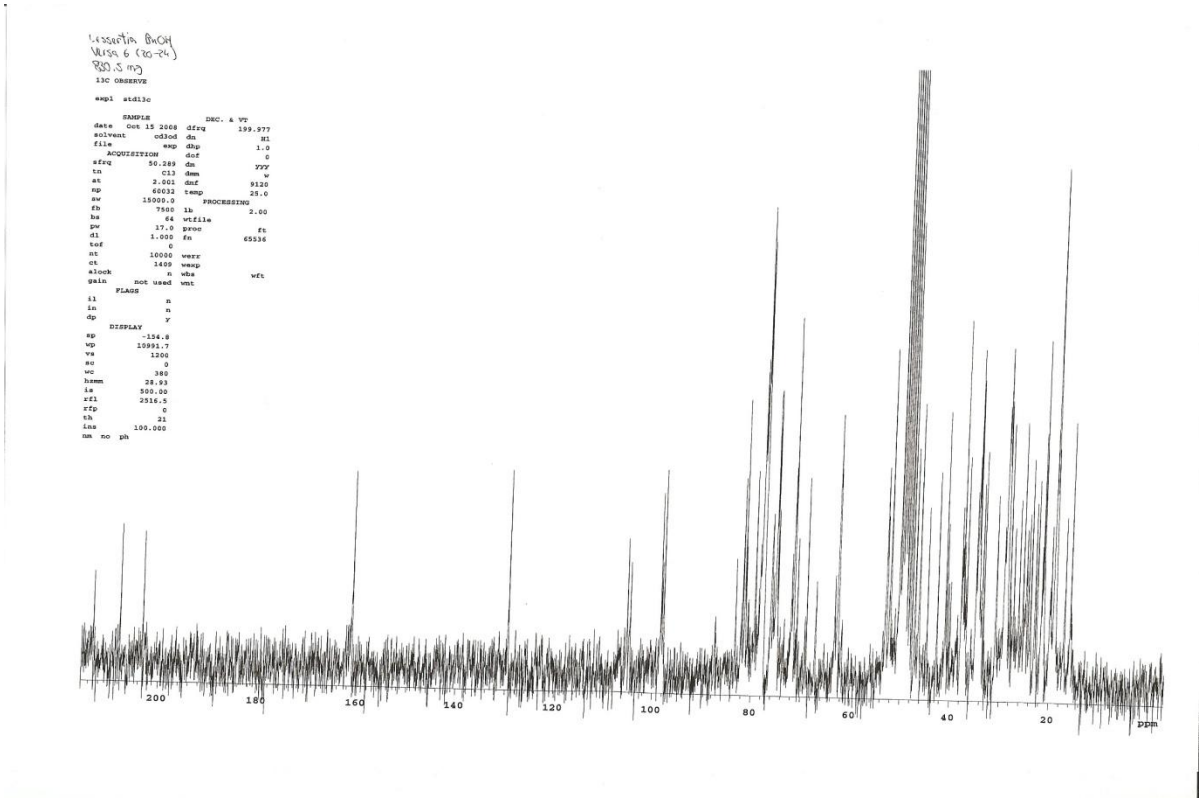
NMR spectra 14.50: 1H-NMR spectra of fraction B5.



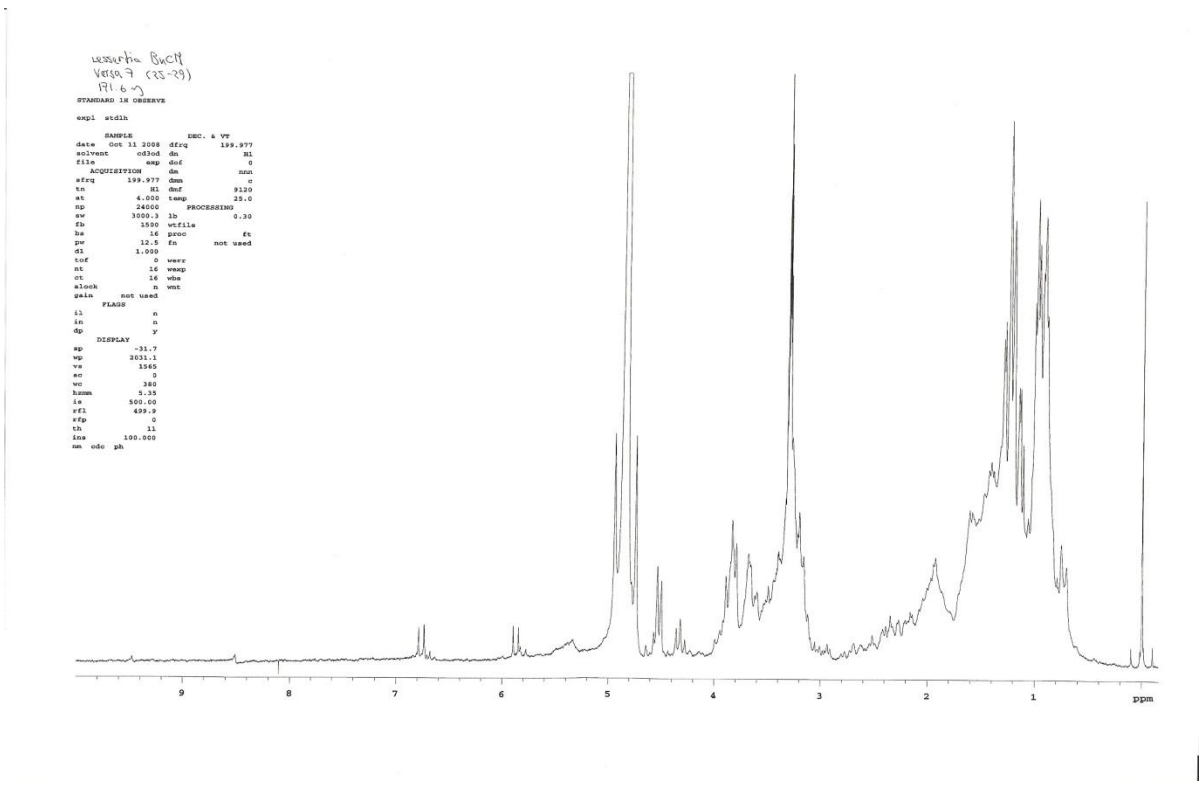
NMR spectra 14.51: 13C-NMR spectra of fraction B5.



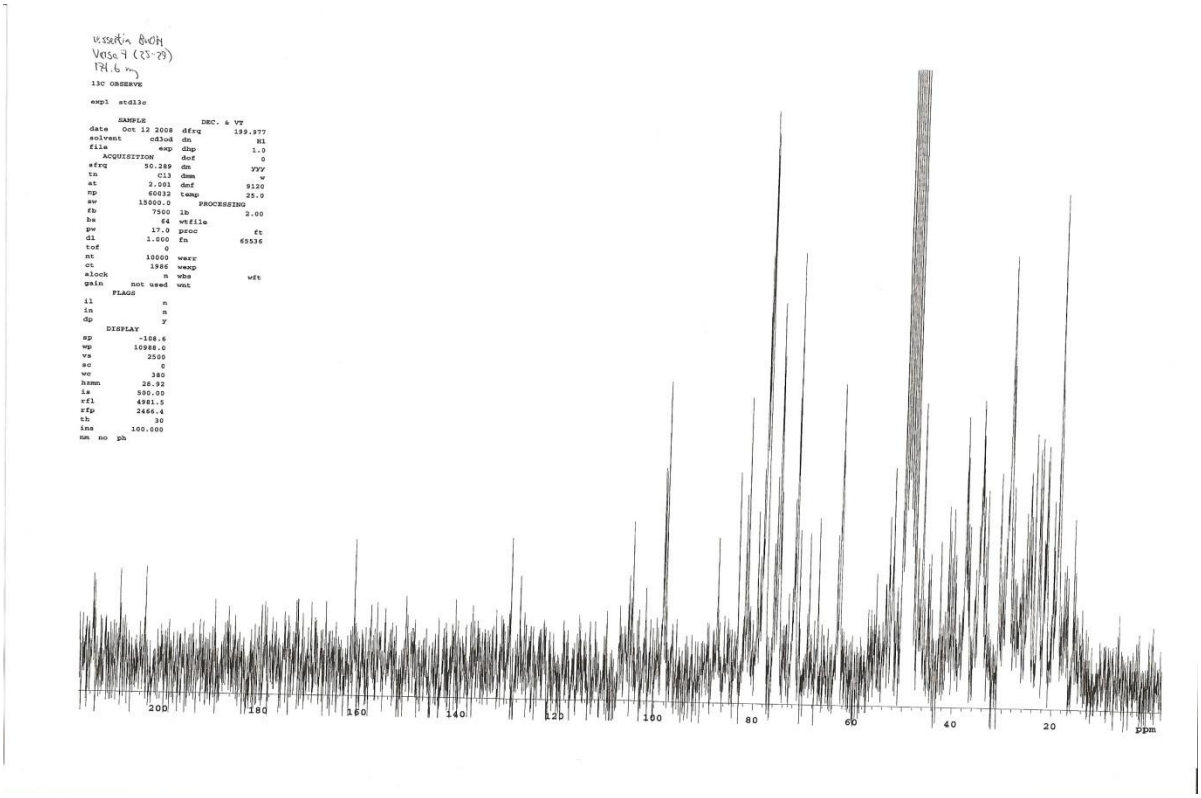
NMR spectra 14.52: 1H-NMR spectra of fraction B6.



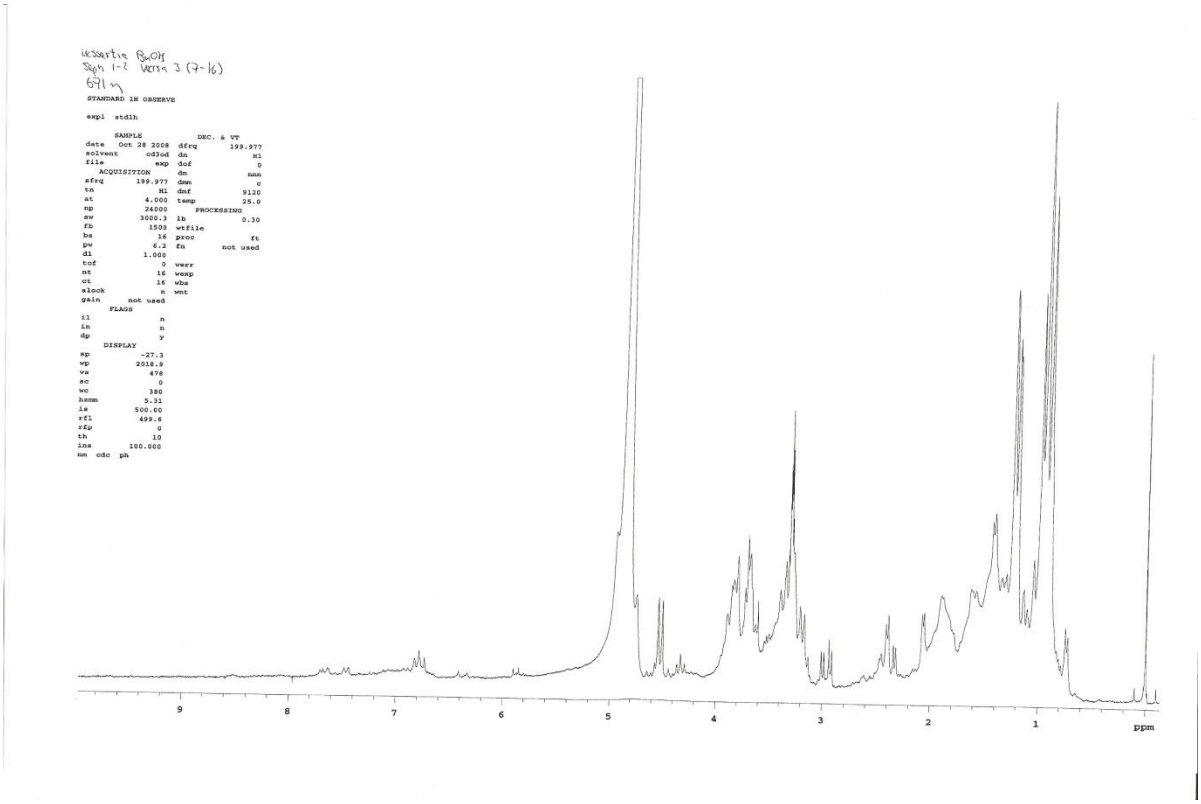
NMR spectra 14.53: 13C-NMR spectra of fraction B6.



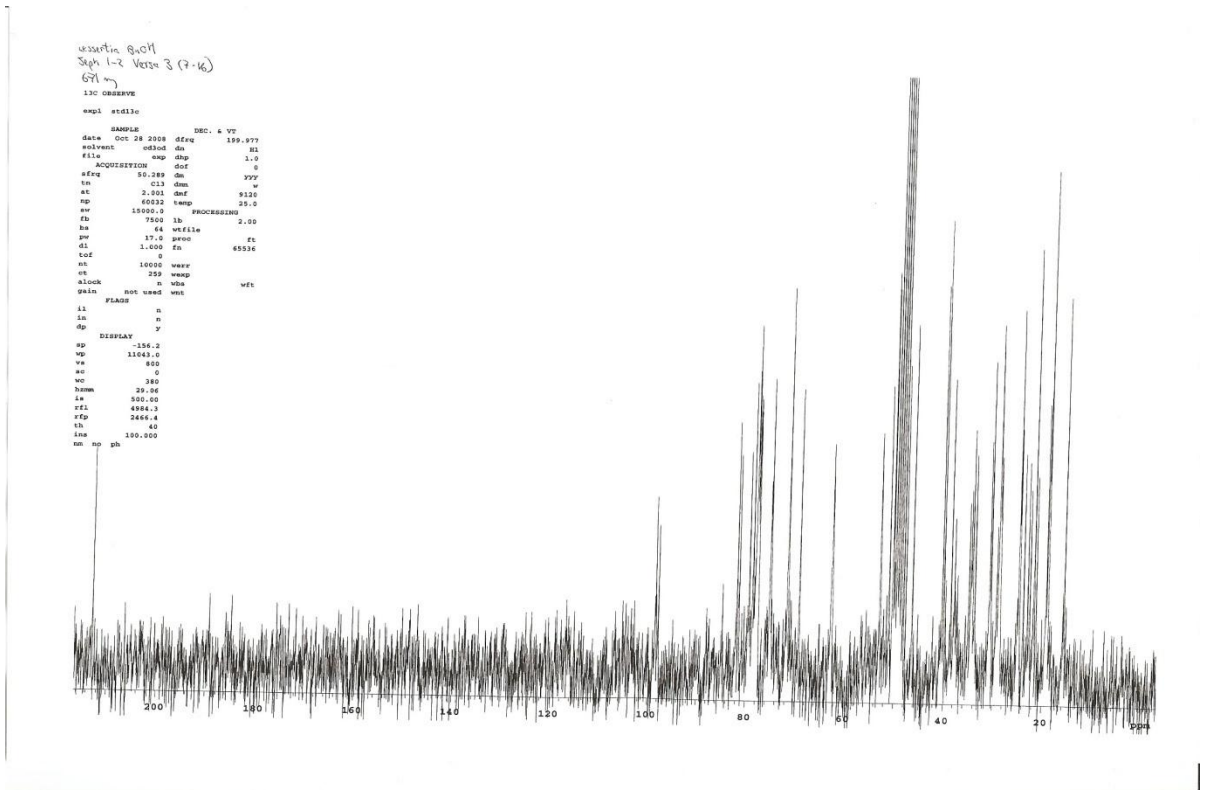
NMR spectra 14.54: 1H-NMR spectra of fraction B7.



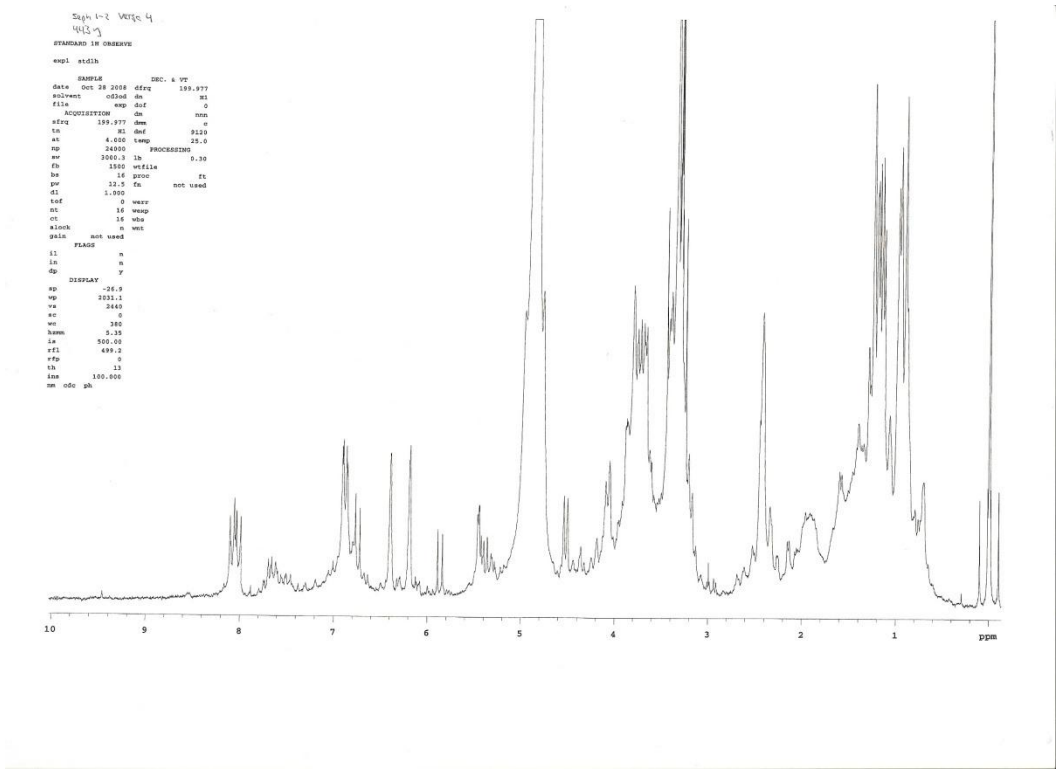
NMR spectra 14.55: 13C-NMR spectra of fraction B7.



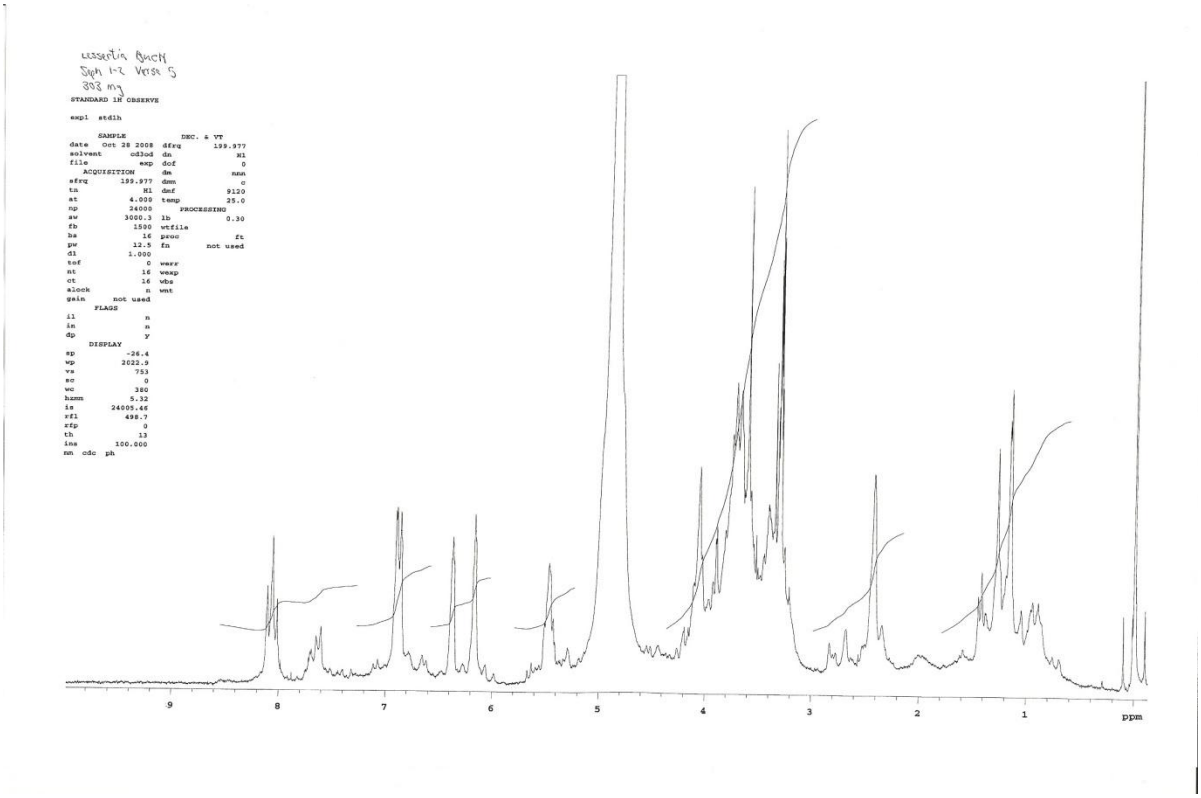
NMR spectra 14.56: 1H-NMR spectra of fraction B1-2 S.3



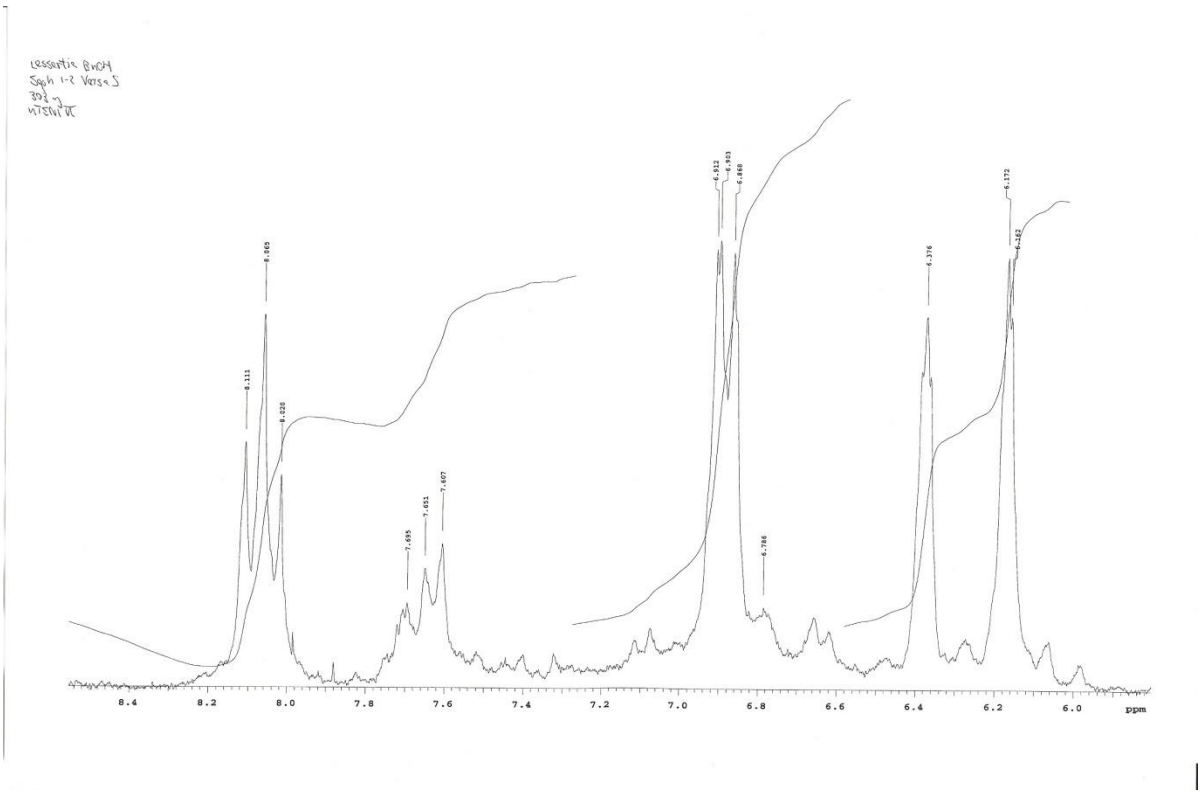
NMR spectra 14.57: 13C-NMR spectra of fraction B1-2 S.3.



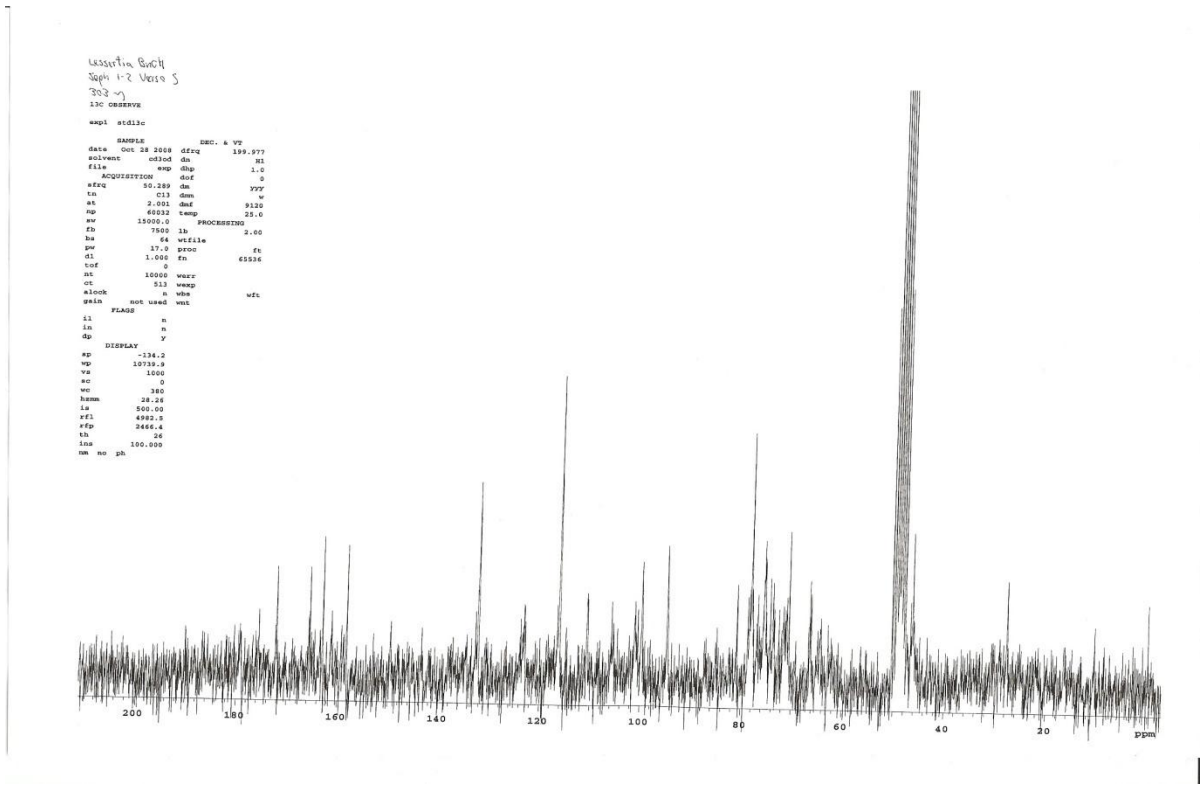
NMR spectra 14-58: 1H-NMR spectra of fraction B1-2 S.4.



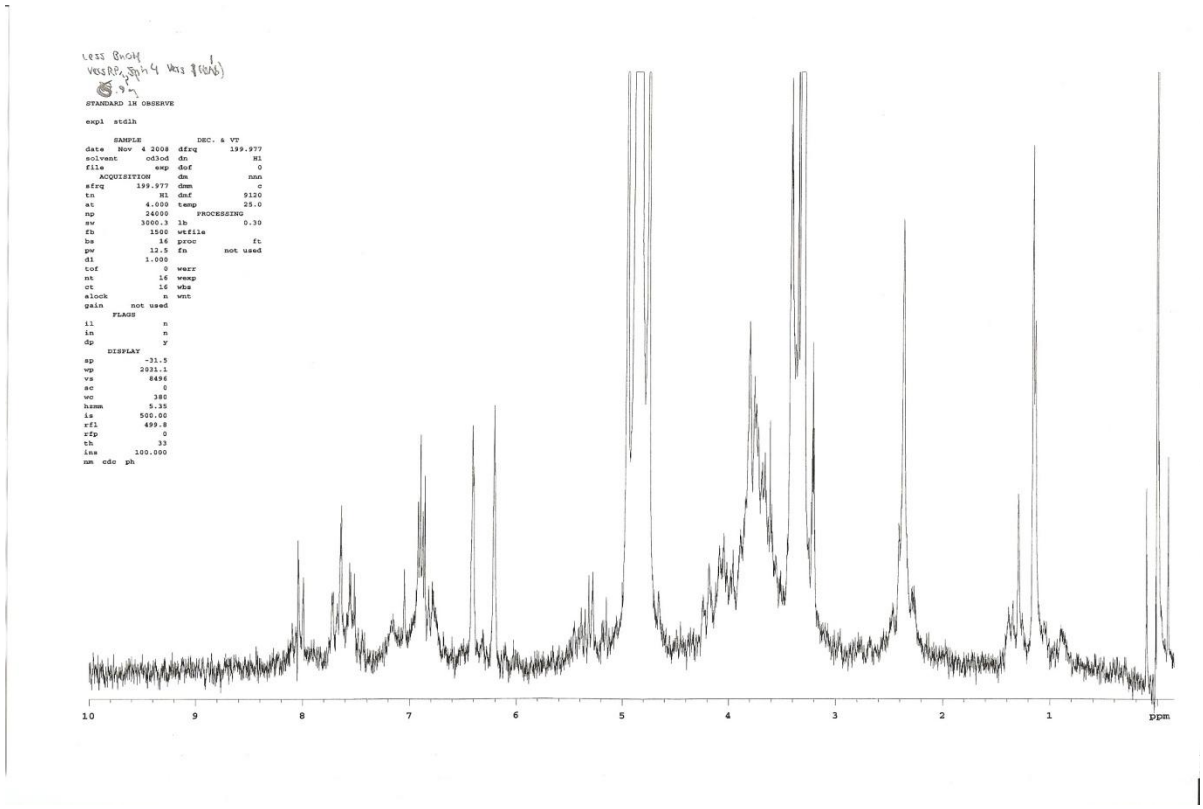
NMR spectra 14.59: 1H-NMR spectra of fraction B1-2 S.5



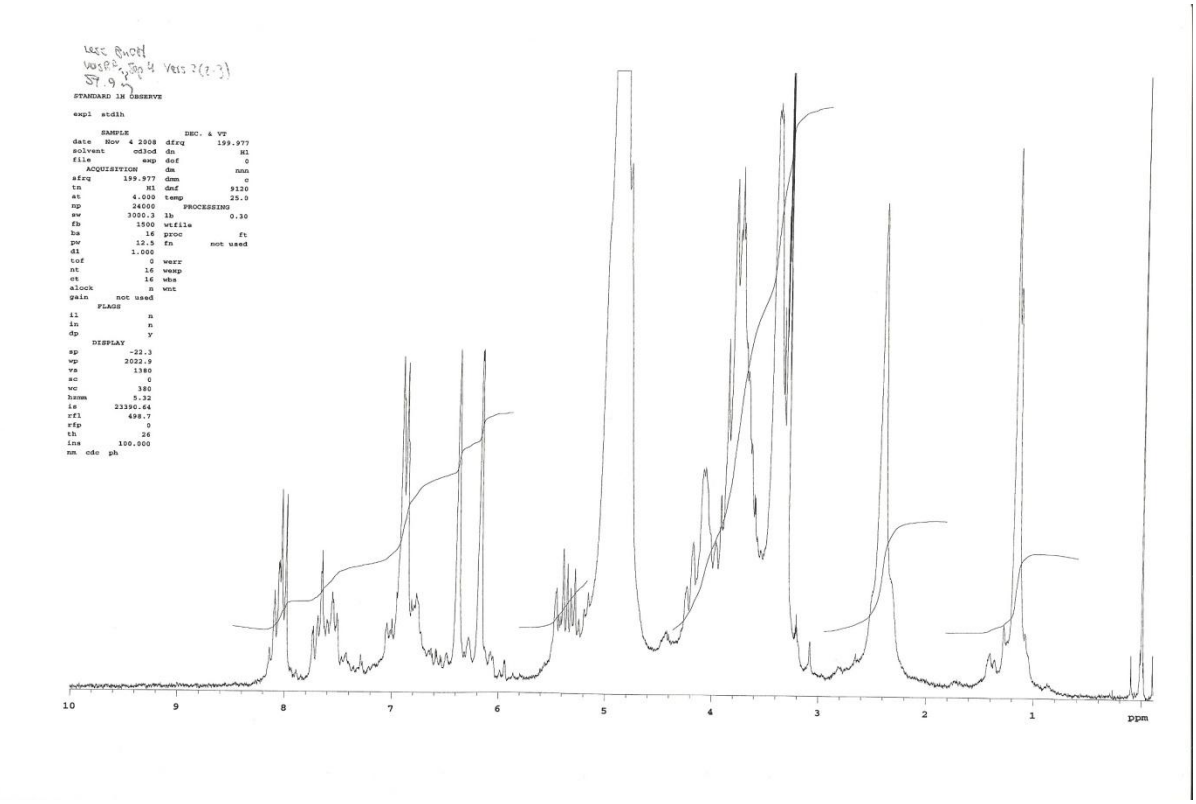
NMR spectra 14.60: partly from 1H-NMR spectra of fraction B1-2 S.5.



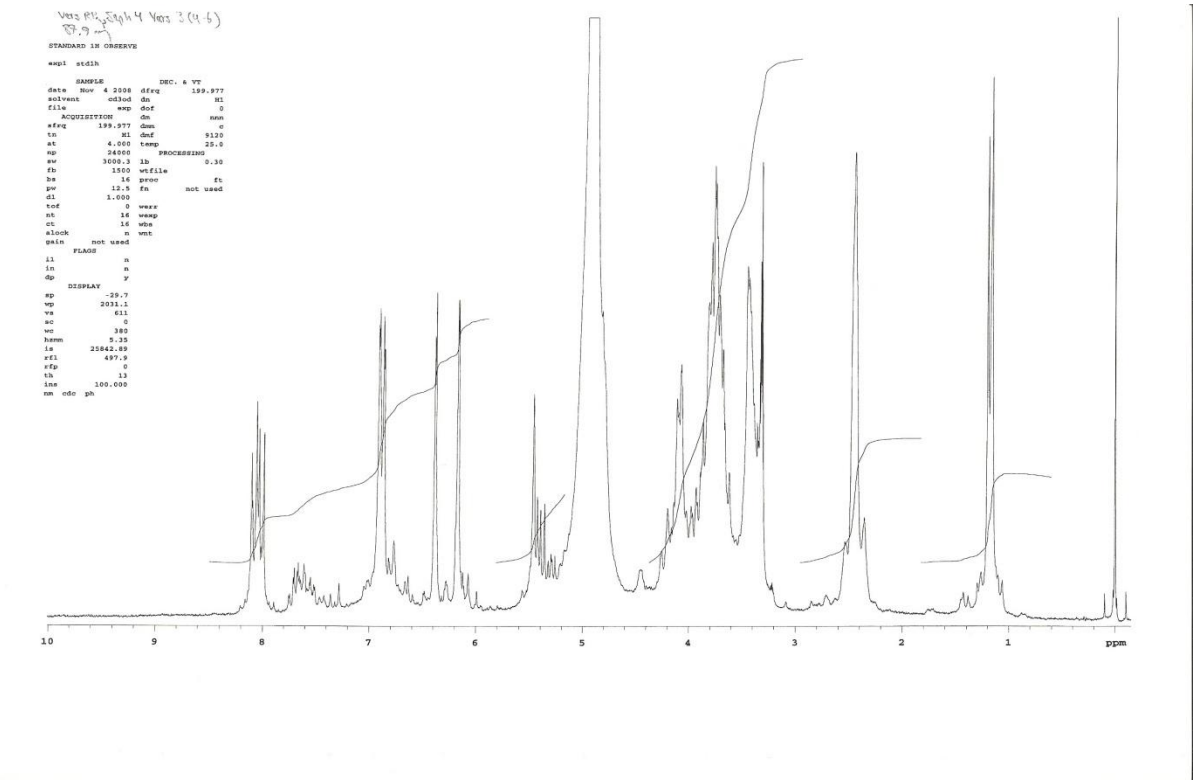
NMR spectra 14.61: 13C-NMR spectra of fraction B1-2 S.5.



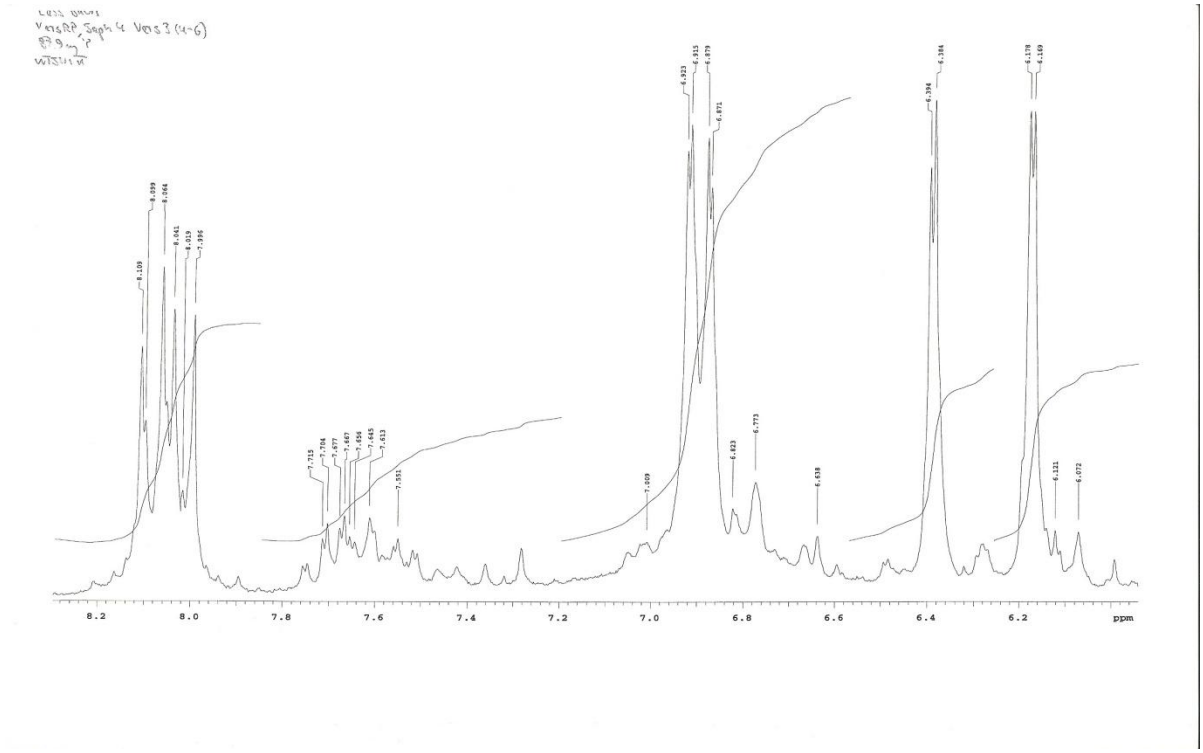
NMR spectra 14.62: 1H-NMR spectra of fraction B1-2 S.4 VRP.1.



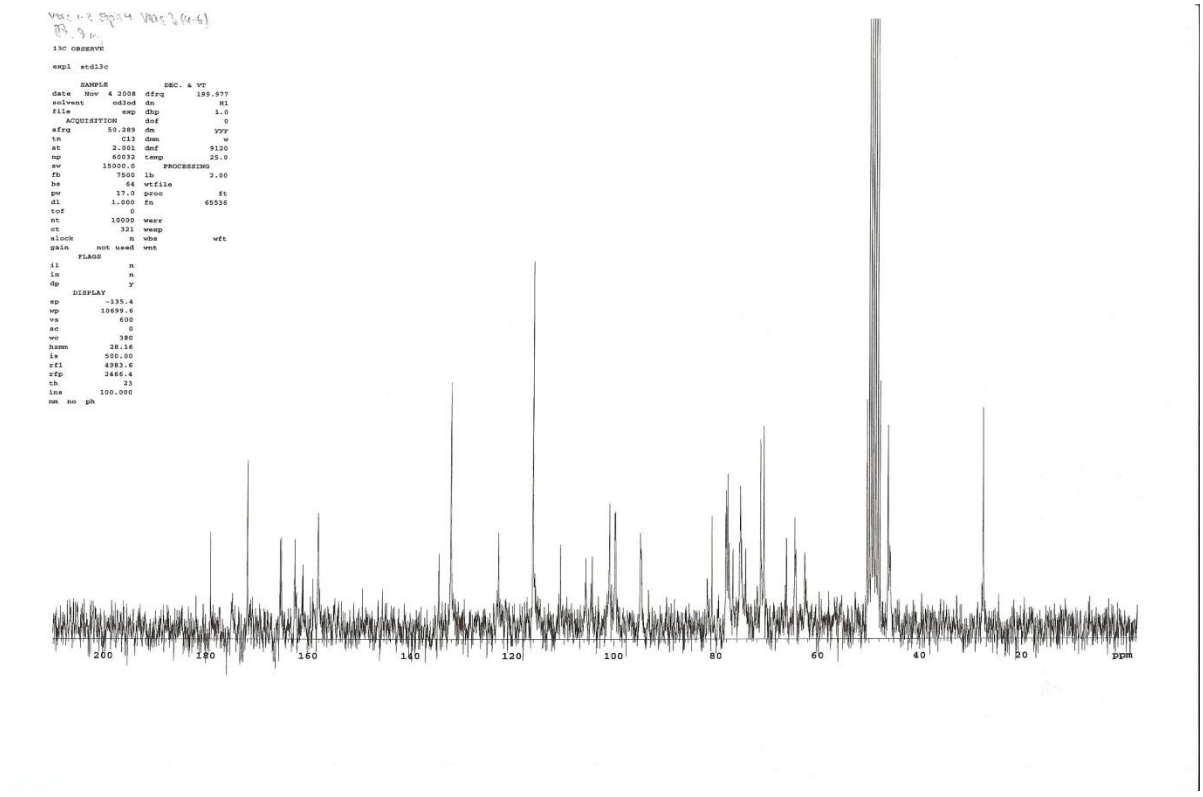
NMR spectra 14.63: 1H-NMR spectra of fraction B1-2 S.4 VRP.2.



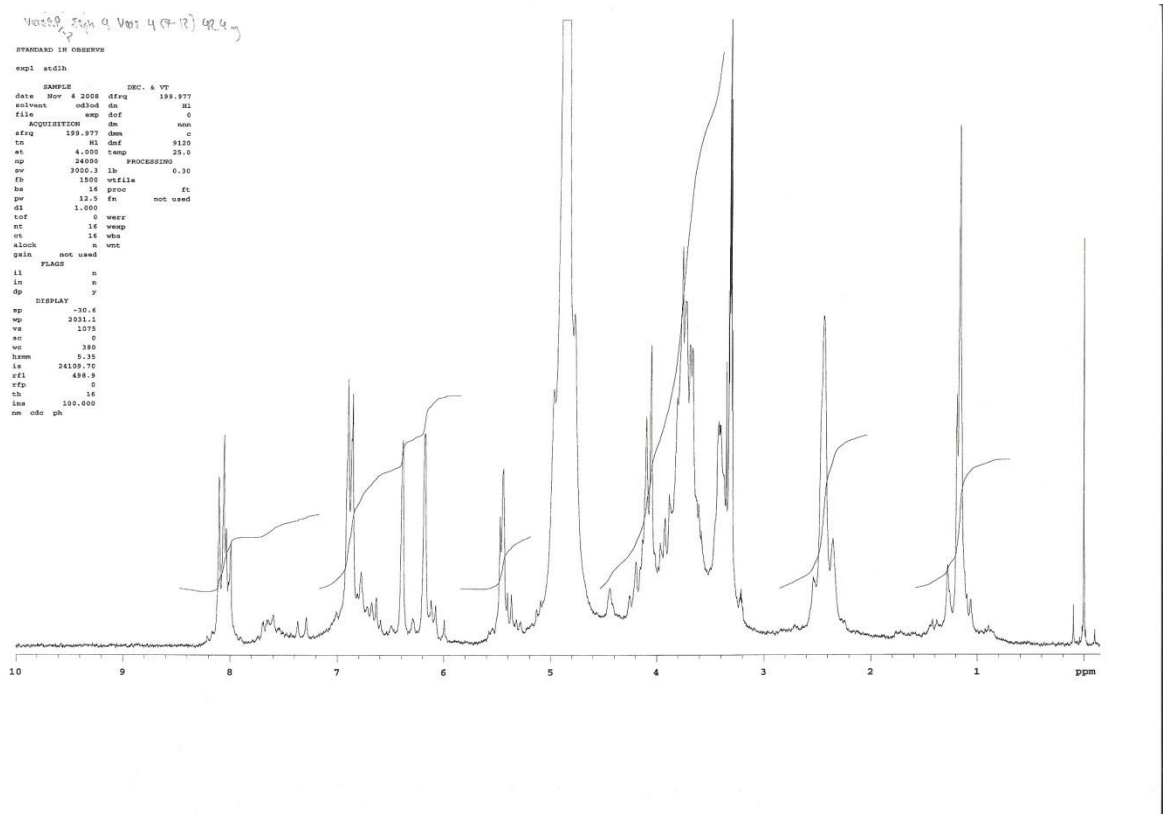
NMR spectra 14.64: 1H-NMR spectra of fraction B1-2 S.4 VRP.2.



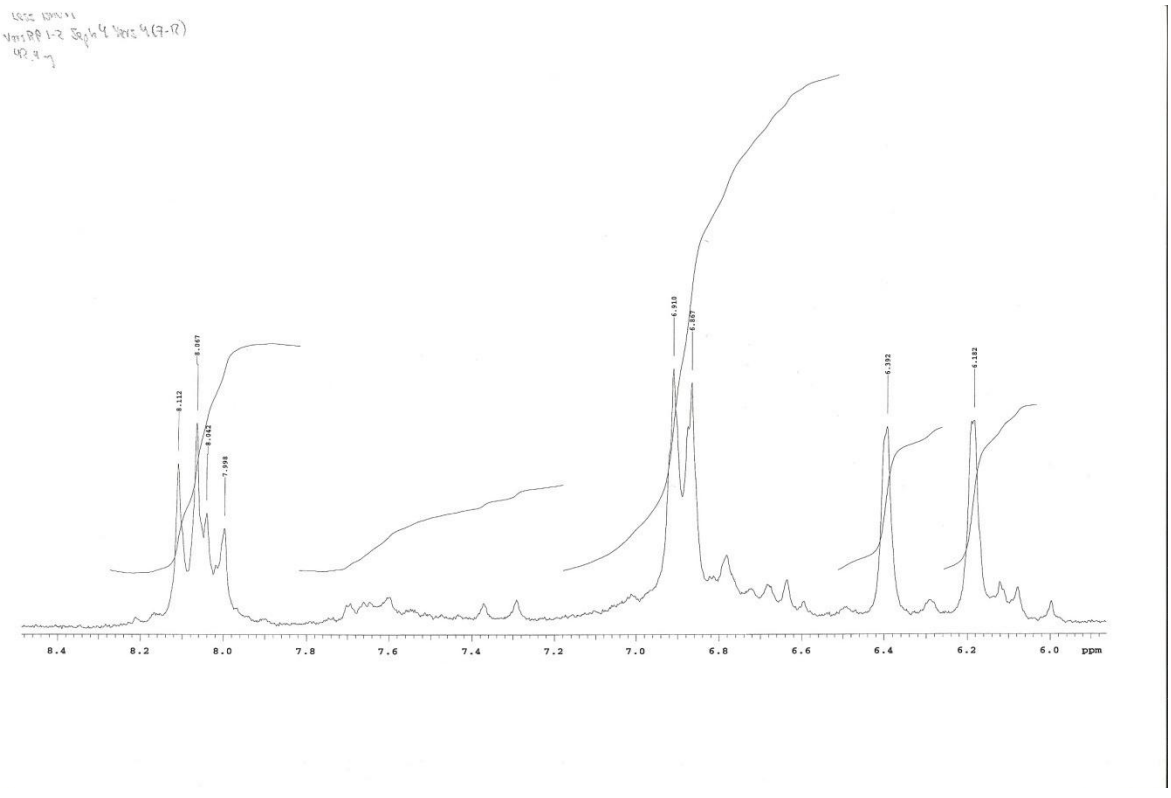
NMR spectra 16.65: partly from 1H-NMR spectra of fraction B1-2 S.4 VRP.3.



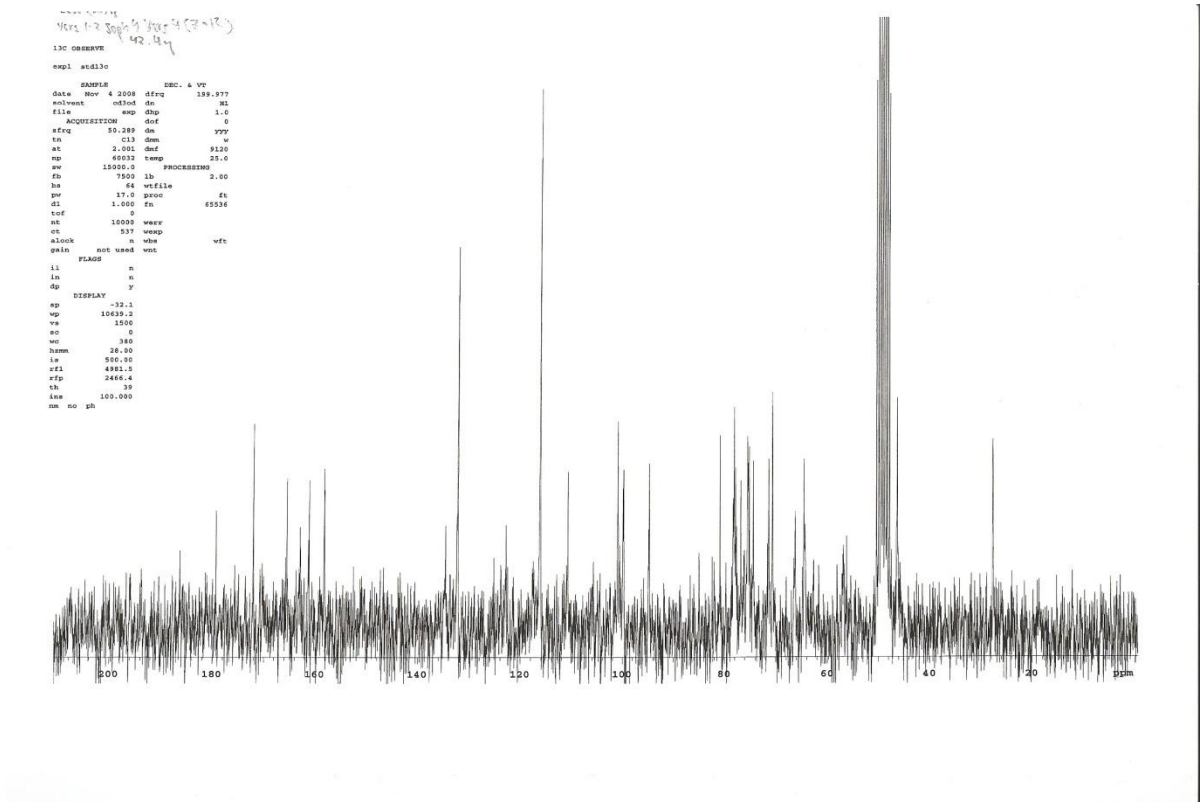
NMR spectra 16.66: 13C-NMR spectra of fraction B1-2 S.4 VRP.3.



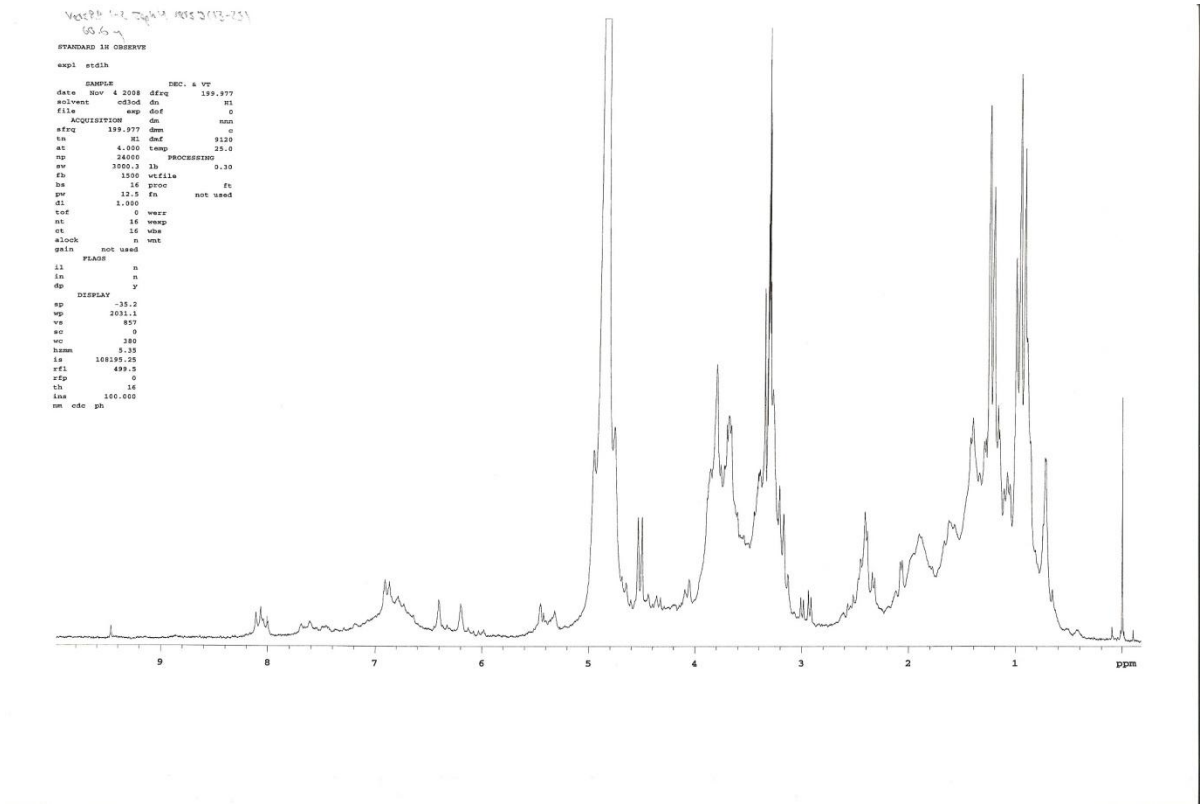
NMR spectra 16.67: 1H-NMR spectra of fraction B1-2 S.4 VRP.4.



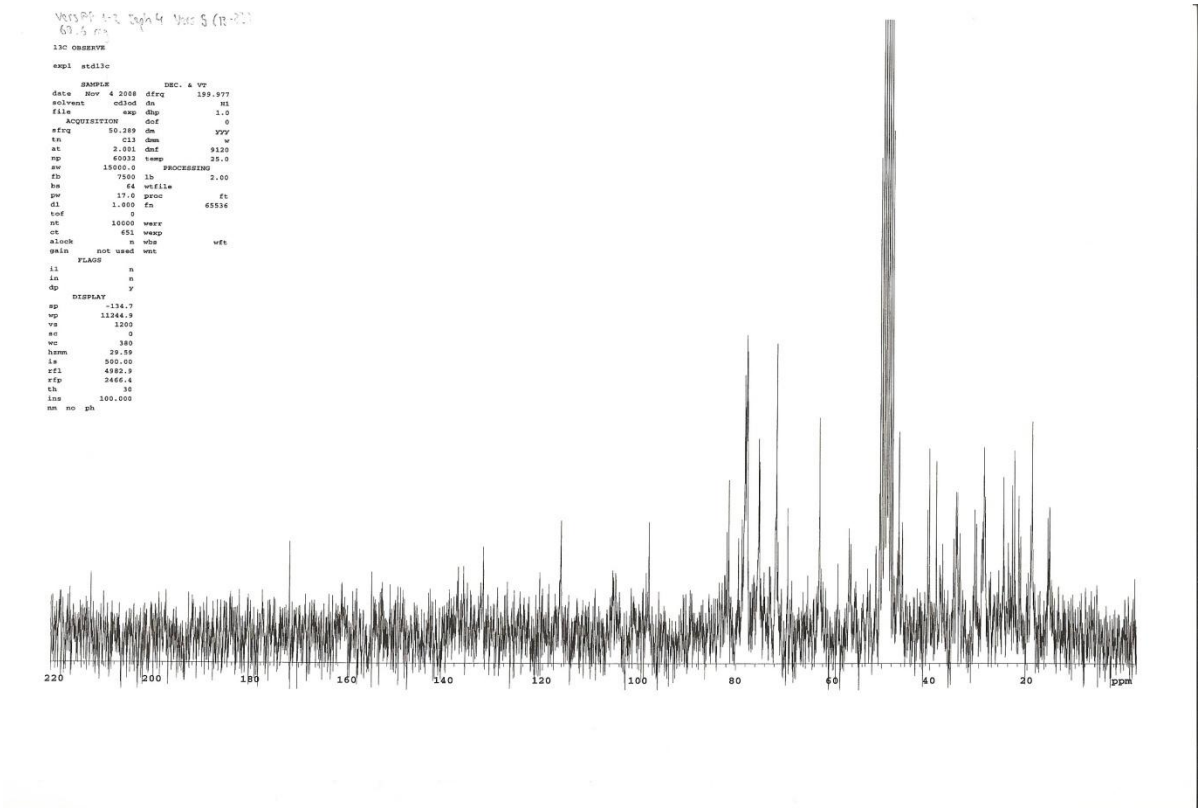
NMR spectra 16.68: partly from 1H-NMR spectra of fraction B1-2 S.4 VRP.4.



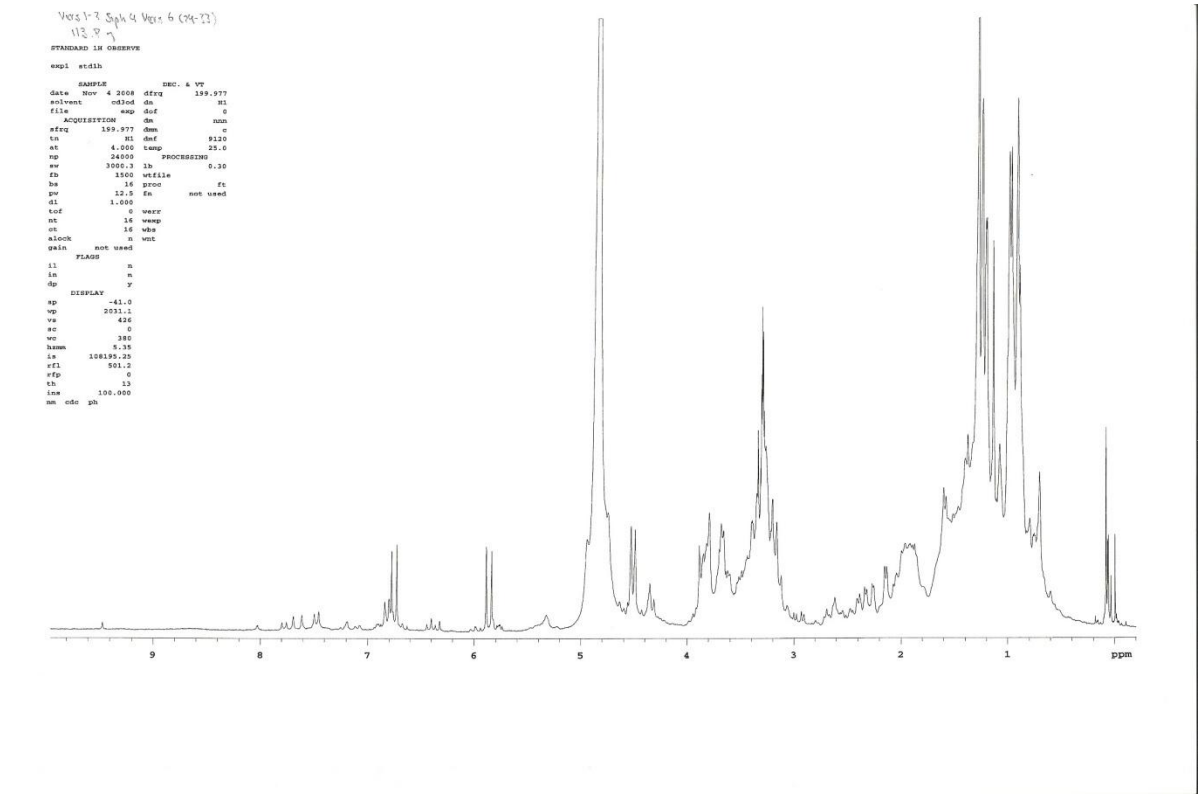
NMR spectra 14.69: 13C-NMR spectra of fraction B1-2 S.4 VRP.4.



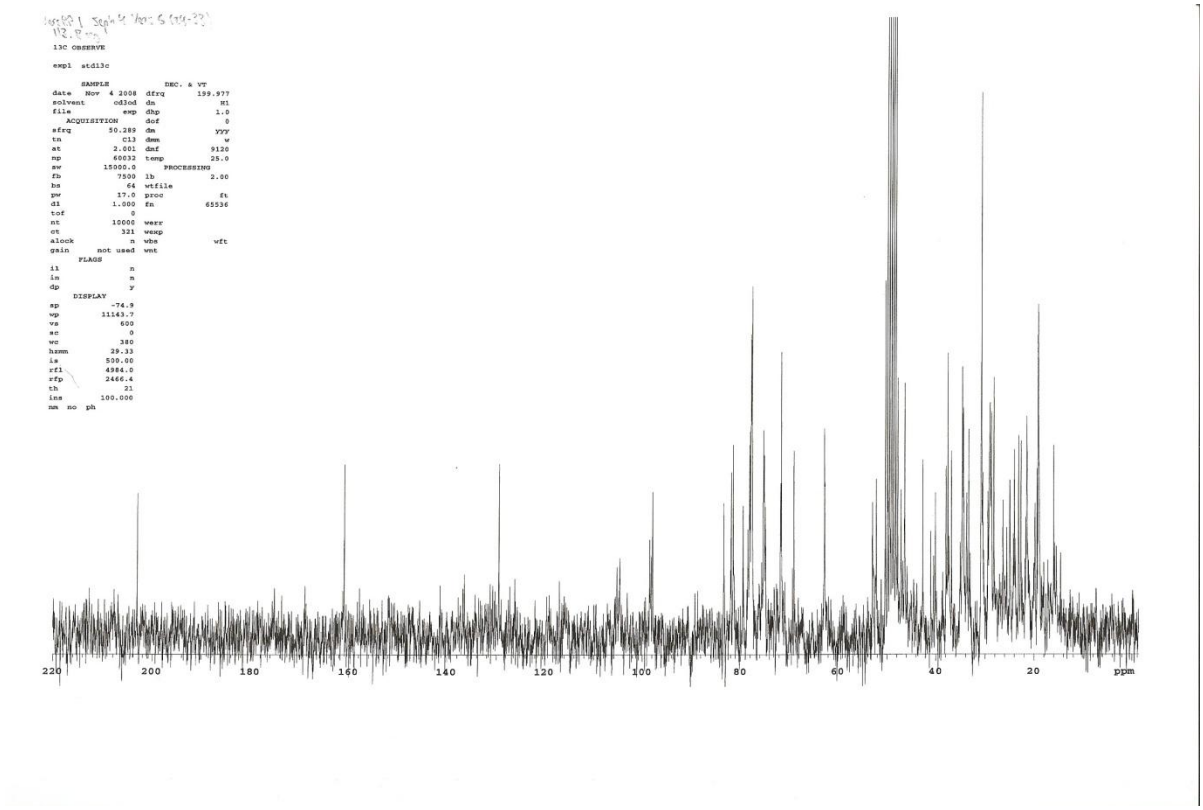
NMR spectra 14.70: 1H-NMR spectra of fraction B1-2 S.4 VRP.5.



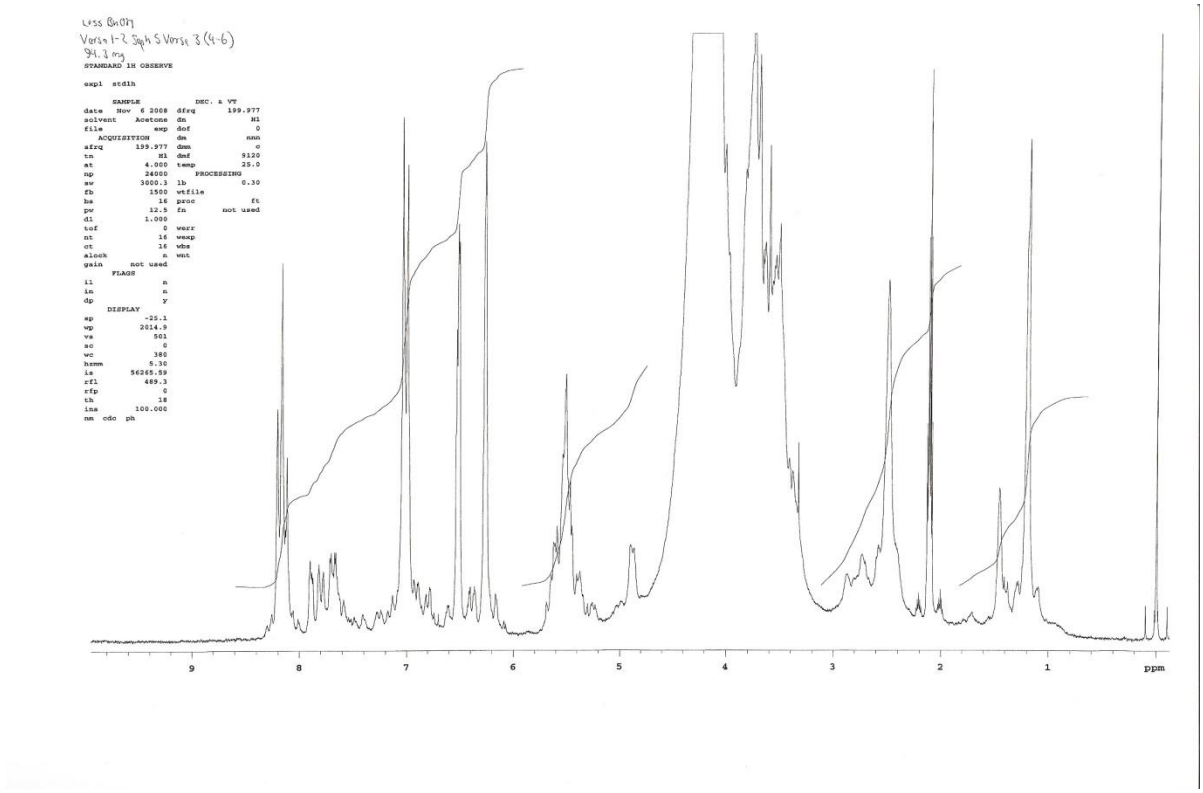
NMR spectra 14.71: 13C-NMR spectra of fraction B1-2 S.4 VRP.5.



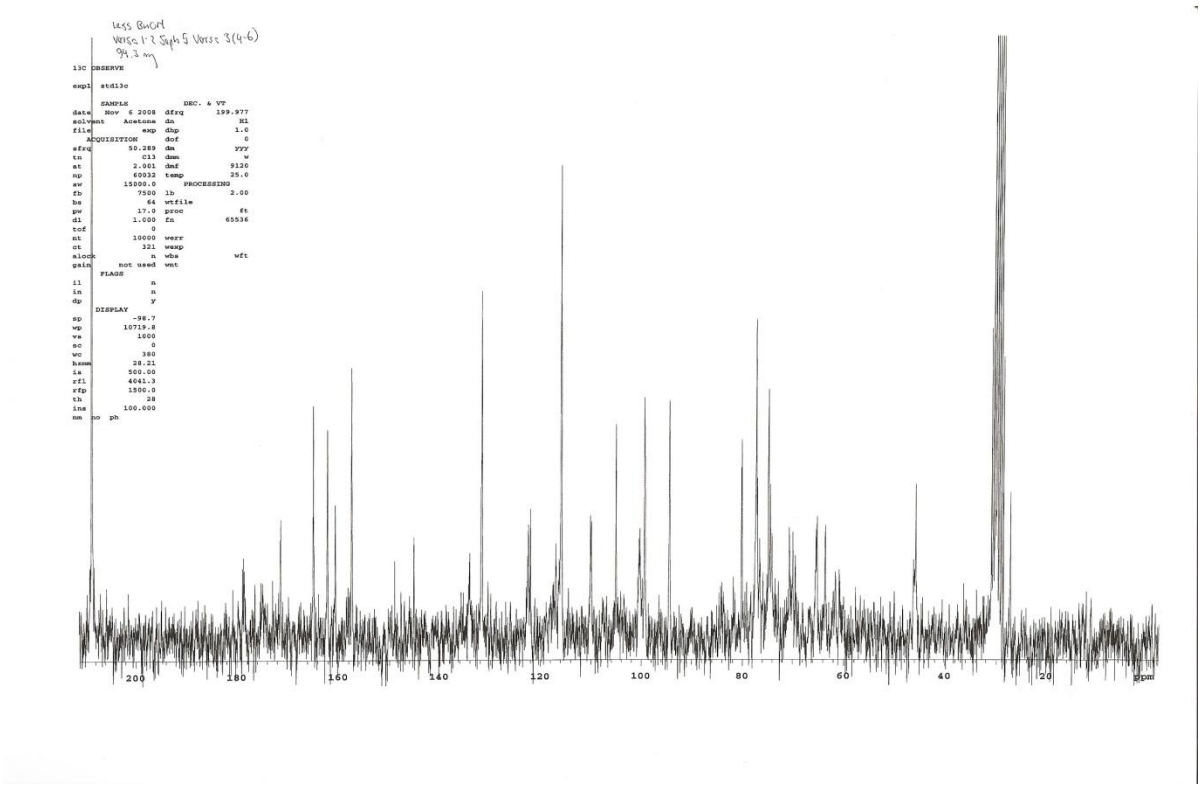
NMR spectra 14.72: 1H-NMR spectra of fraction B1-2 S.4 VRP.6.



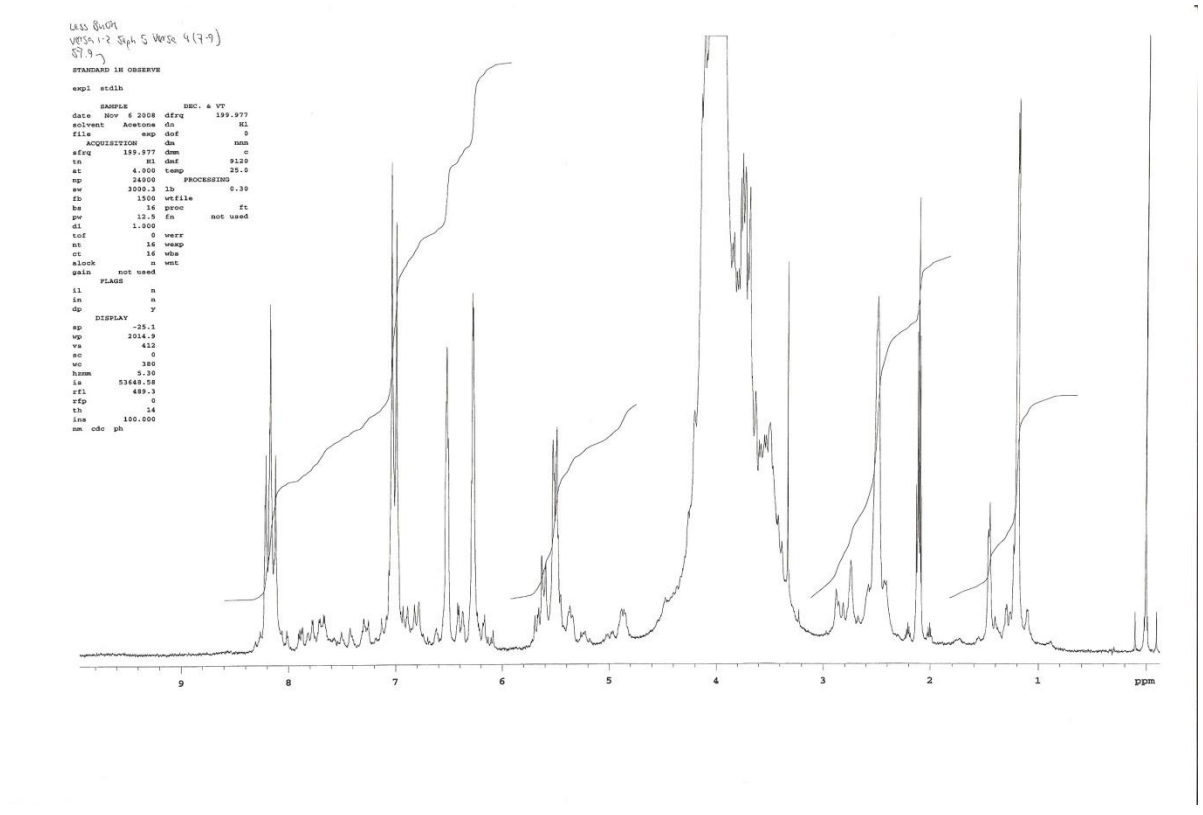
NMR spectra 14.73: 13C-NMR spectra of fraction B1-2 S.4 VRP.5.



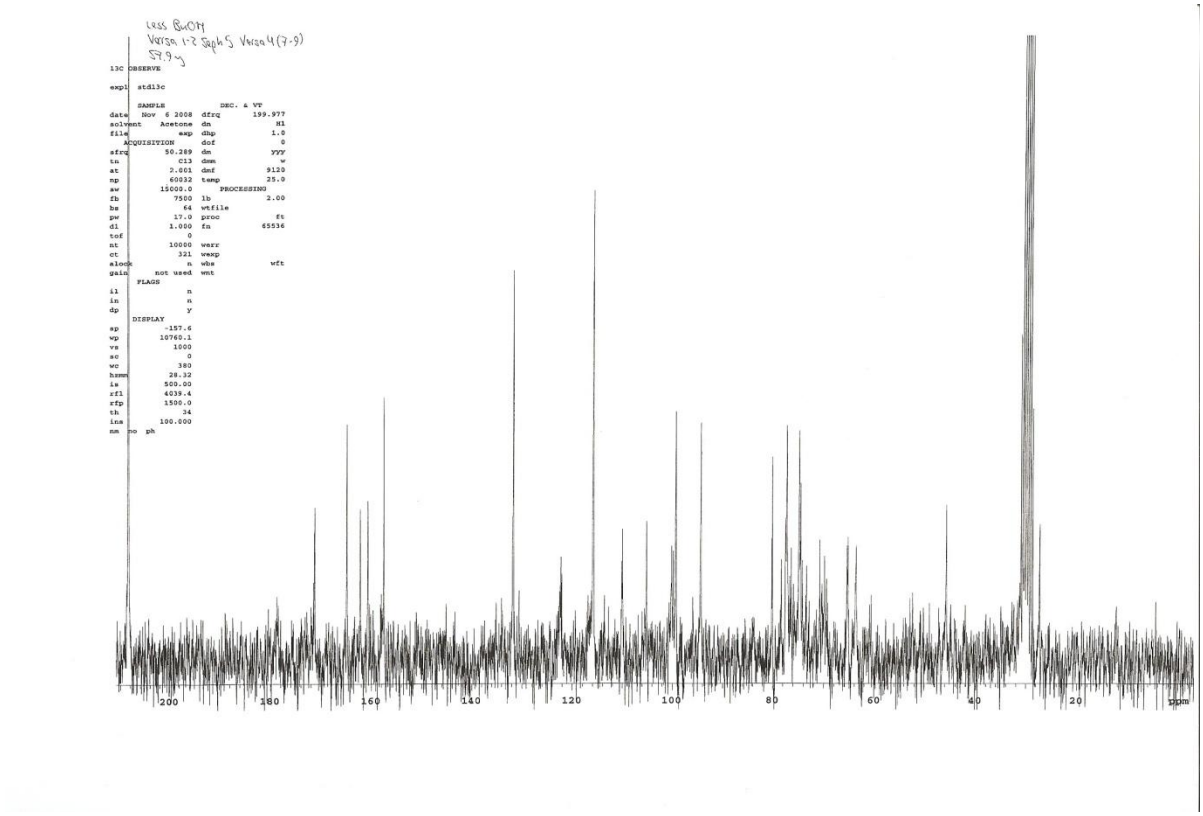
NMR spectra 14.74: 1H-NMR spectra of fraction B1-2 S.5 VRP.3.



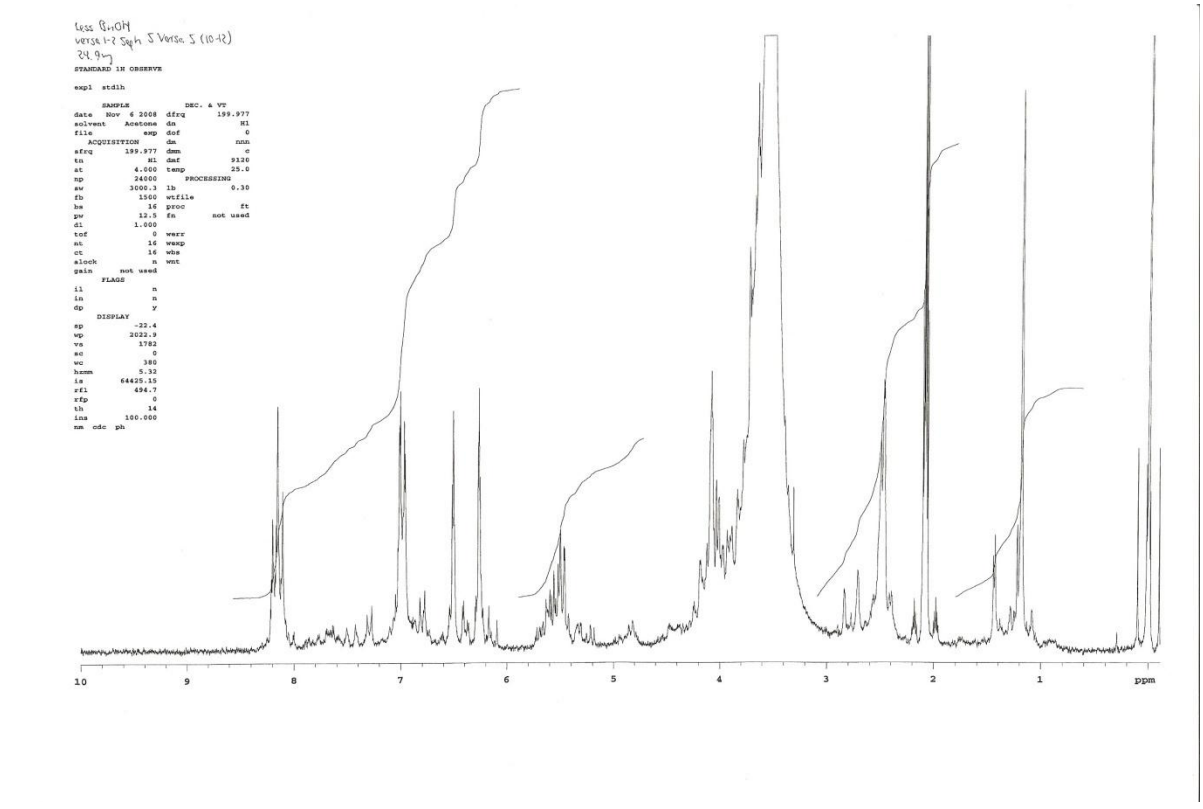
NMR spectra 14.75: 13C-NMR spectra of fraction B1-2 S.5 VRP.3.



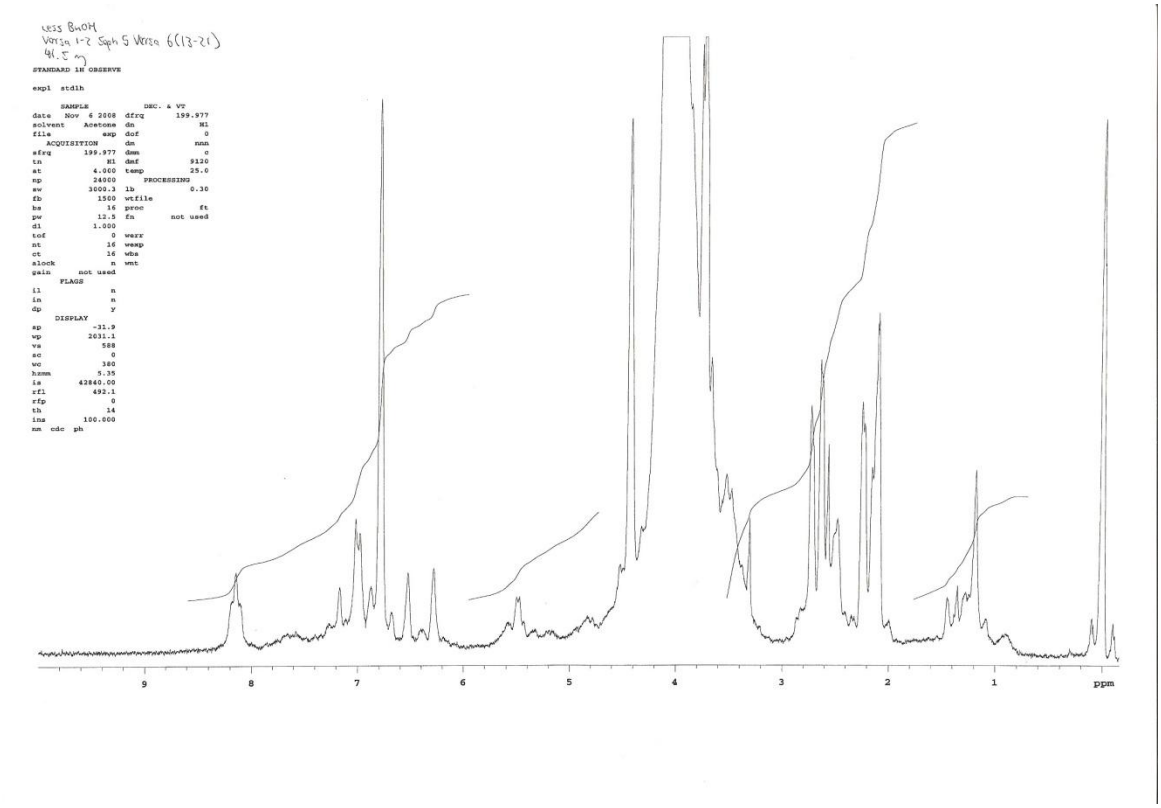
NMR spectra 14.76: 1H-NMR spectra of fraction B1-2 S.5 VRP.4.



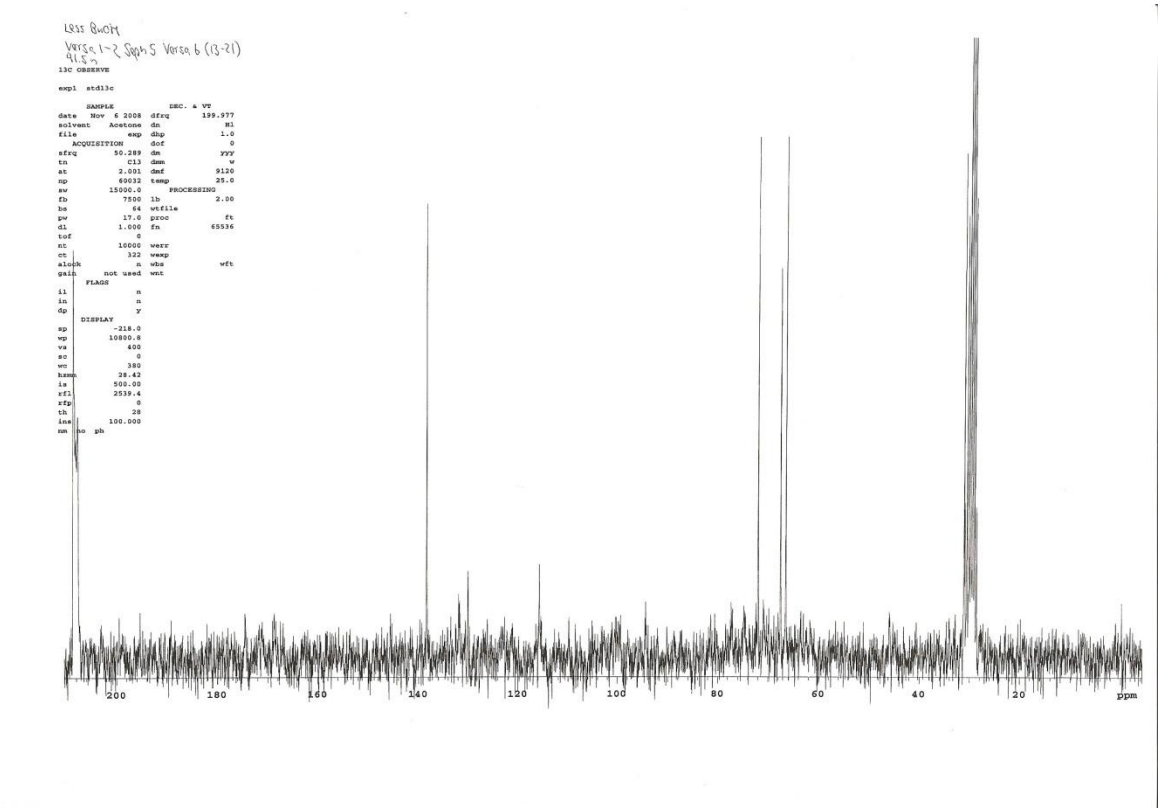
NMR spectra 14.77: 13C-NMR spectra of fraction B1-2 S.5 VRP.4.



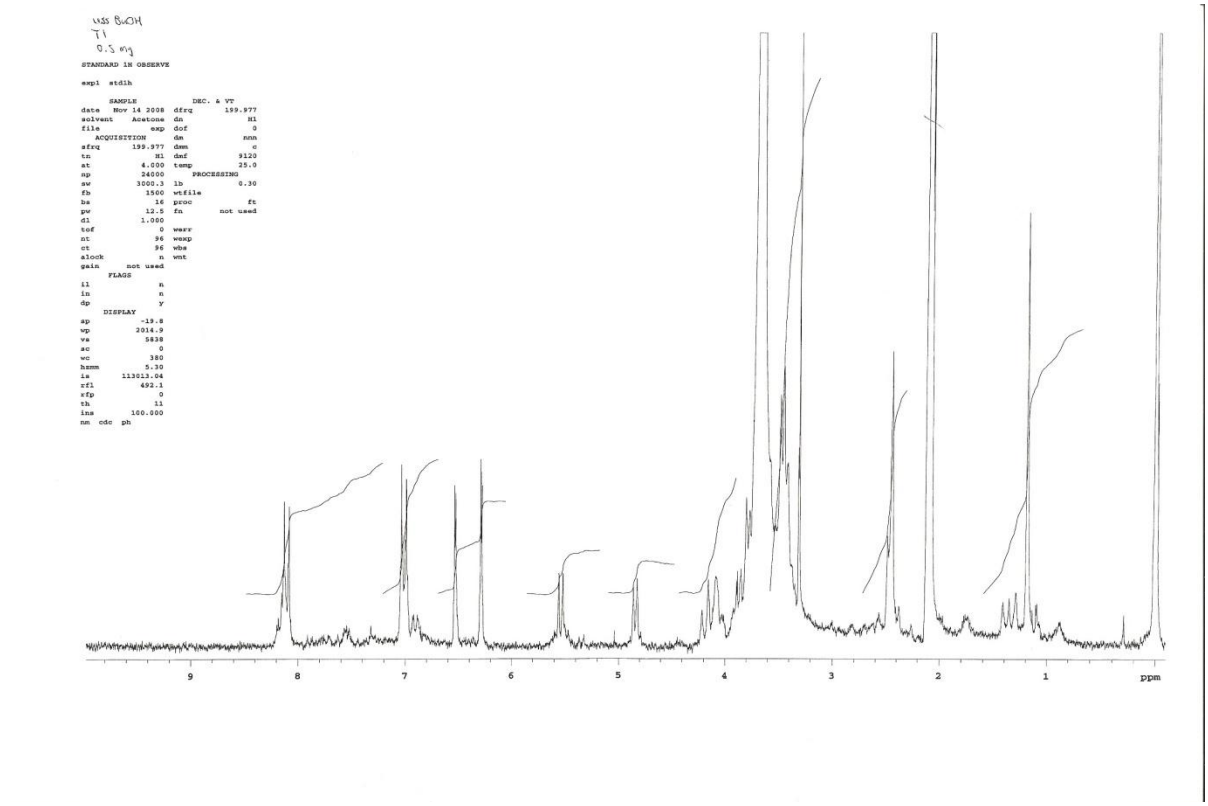
NMR spectra 14.78: 1H-NMR spectra of fraction B1-2 S.5 VRP.5.



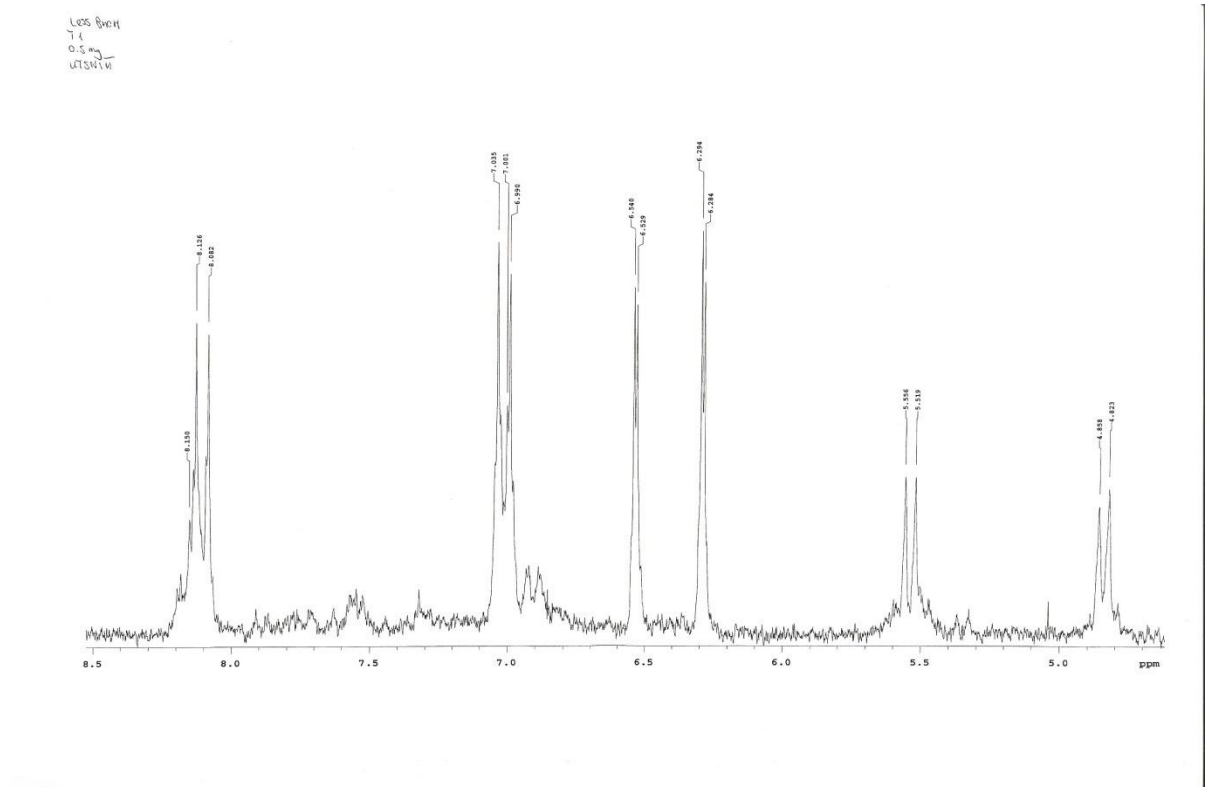
NMR spectra 14.79: 1H-NMR spectra of fraction B1-2 S.5 VRP.6.



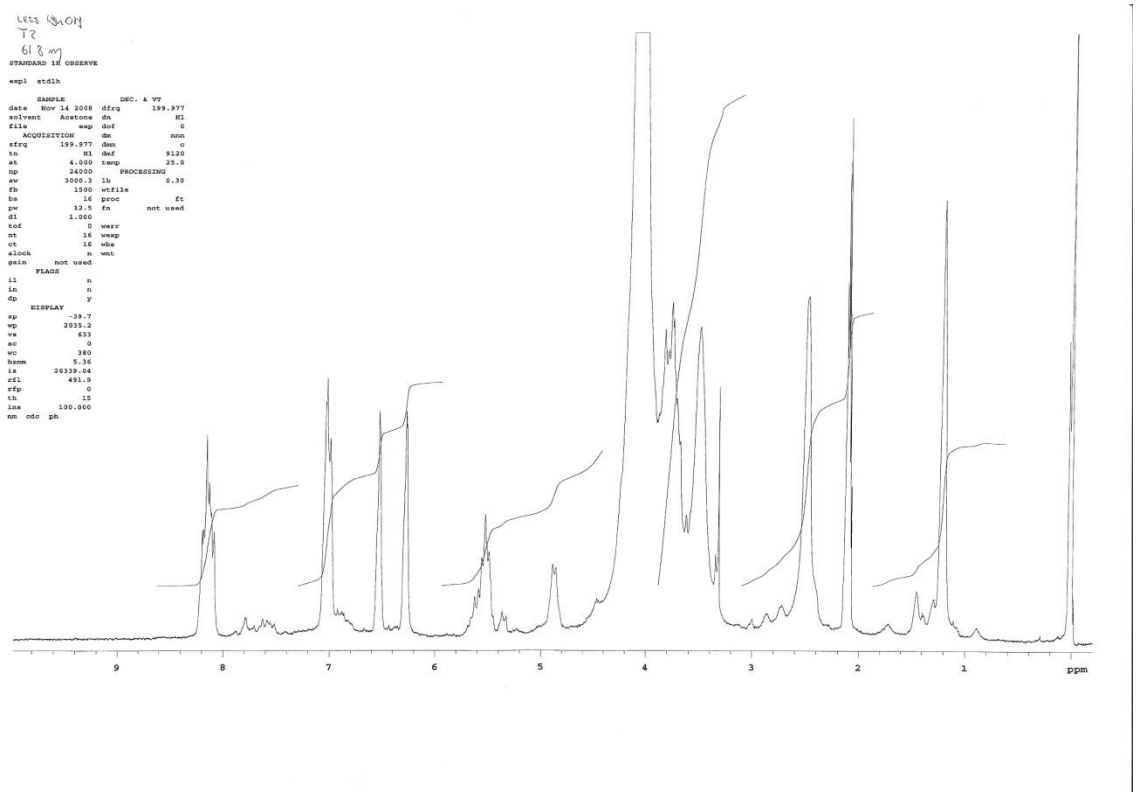
NMR spectra 14.80: 13C-NMR spectra of fraction B1-2 S.5 VRP.6.



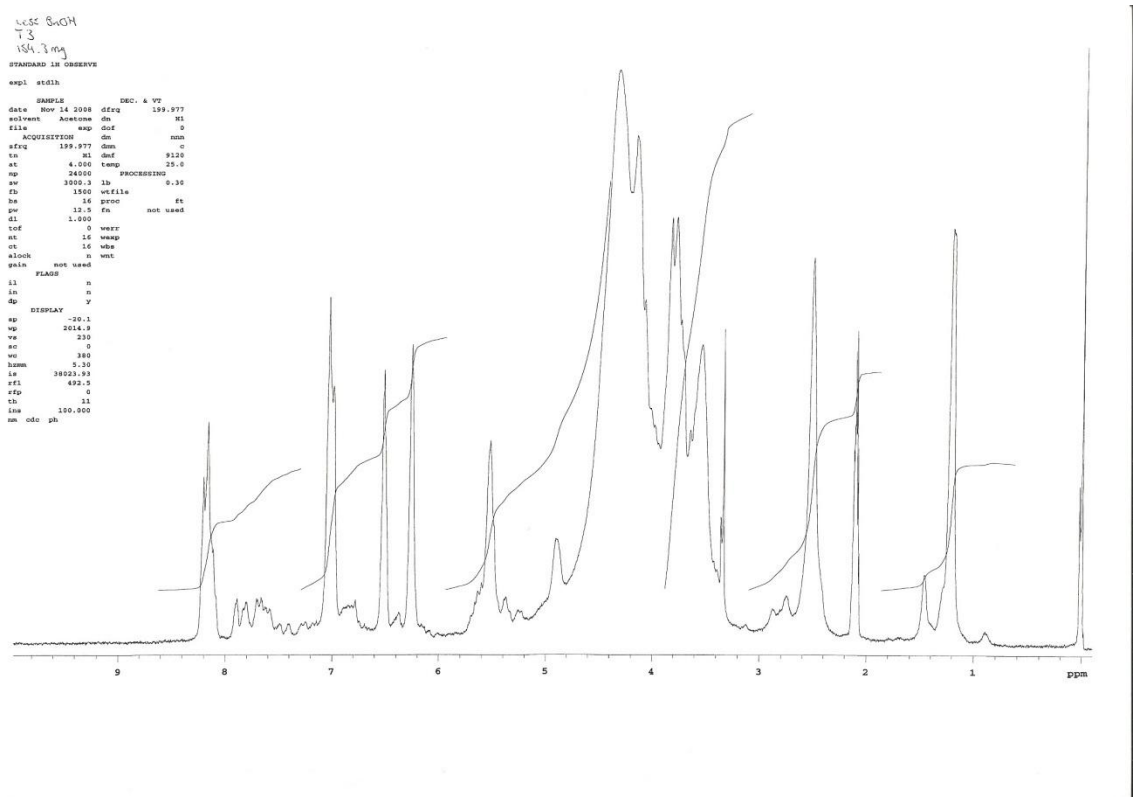
NMR spectra 14.81: 1H-NMR spectra of fraction B1-2 S.4-5 VRP T1.



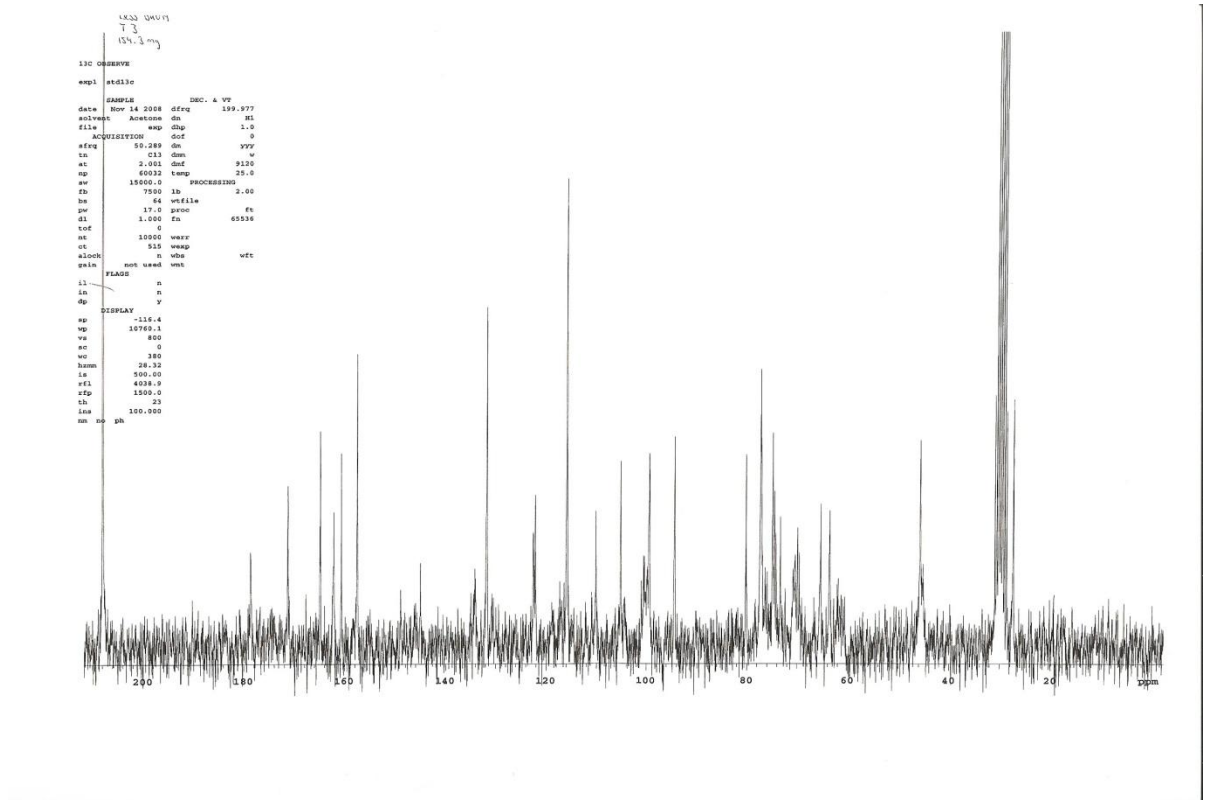
NMR spectra 14.82: partly from 1H-NMR spectra of fraction B1-2 S.4-5 VRP. T1.



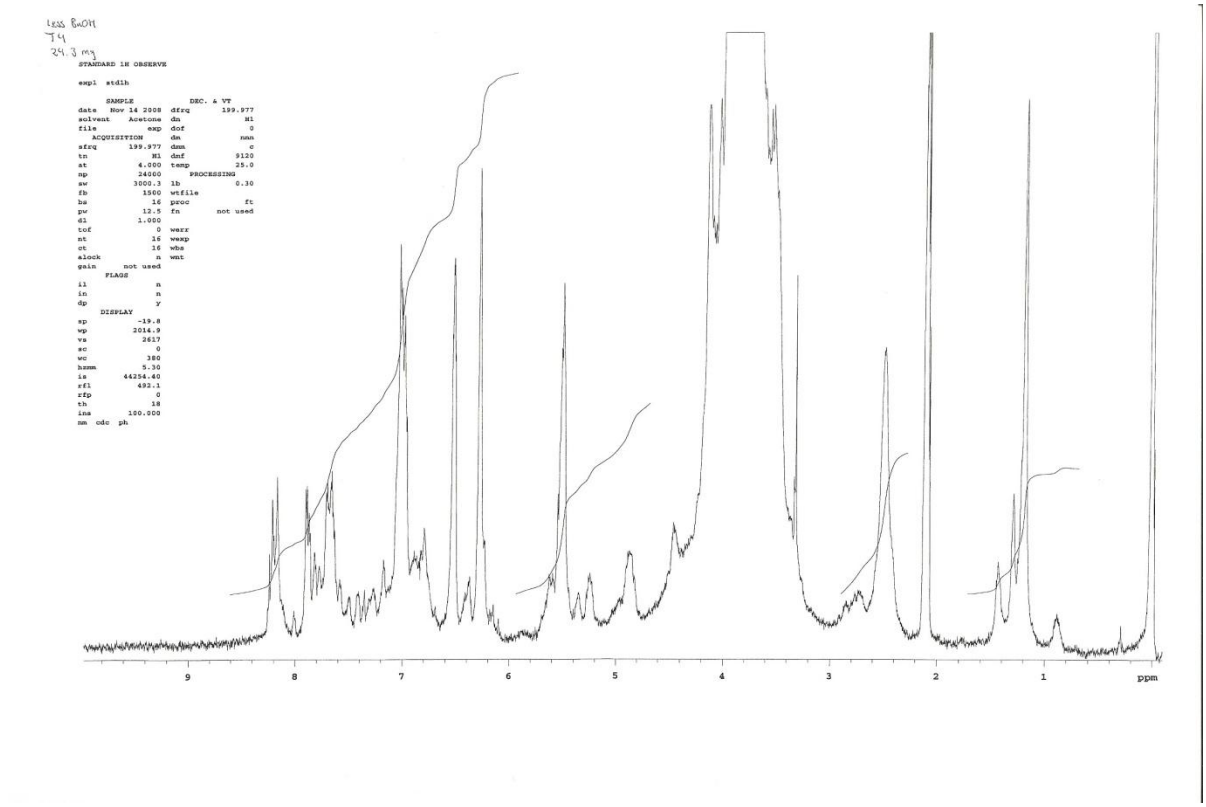
NMR spectra 14.83: 1H-NMR spectra of fraction B1-2 S.4-5 VRP. T2.



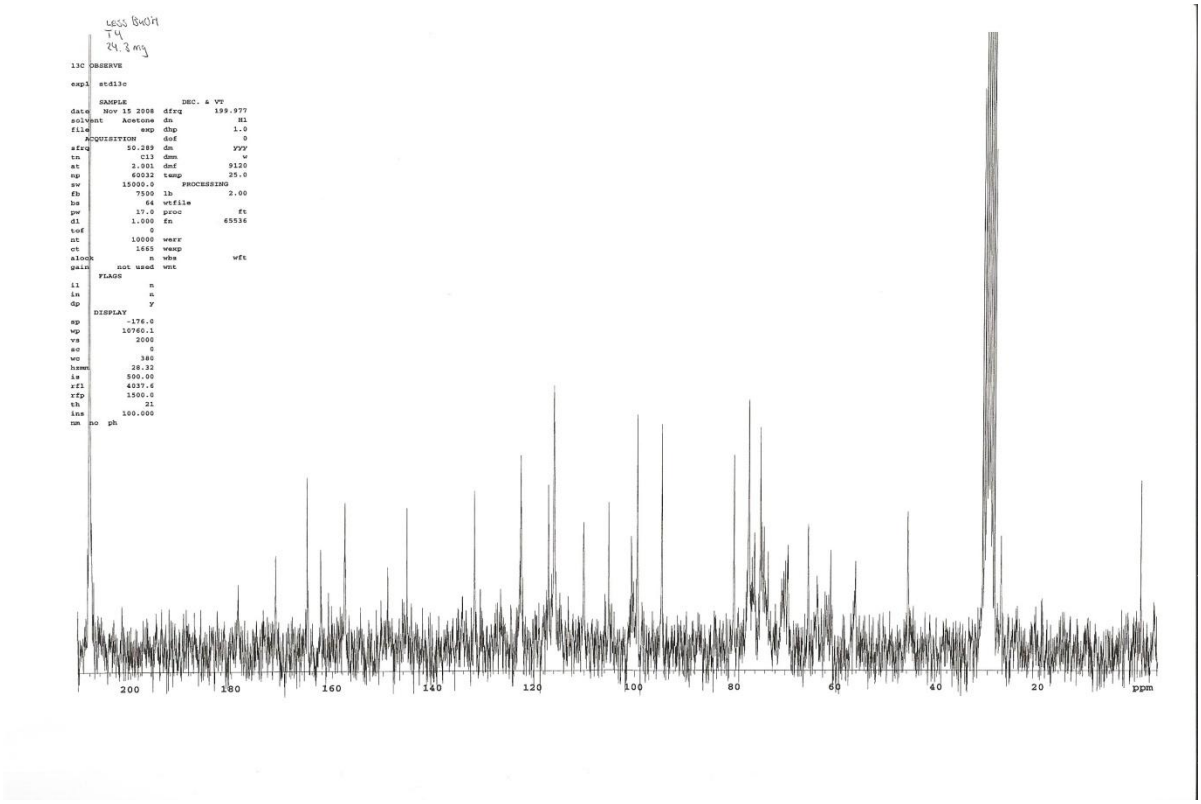
NMR spectra 14.84: 1H-NMR spectra of fraction B1-2 S.4-5 VRP. T3.



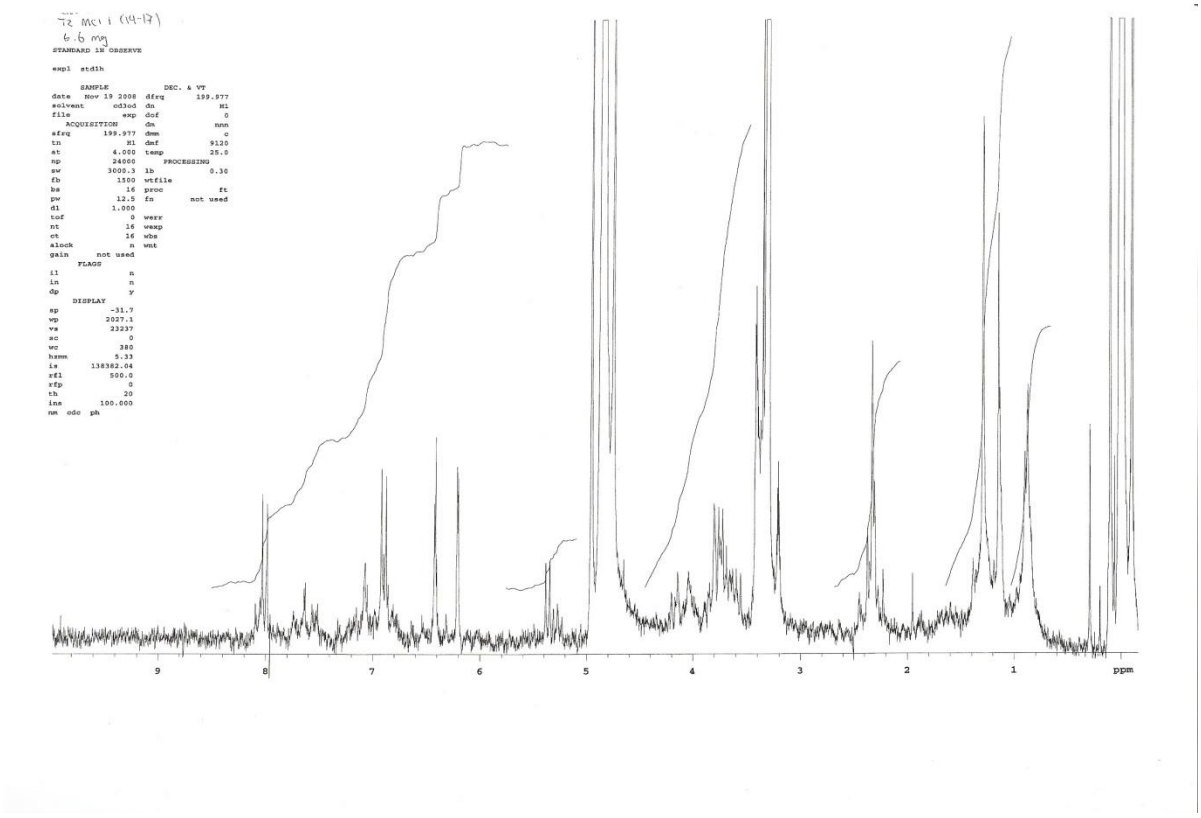
NMR spectra 14.85: 13C-NMR spectra of fraction B1-2 S.4-5 VRP. T3.



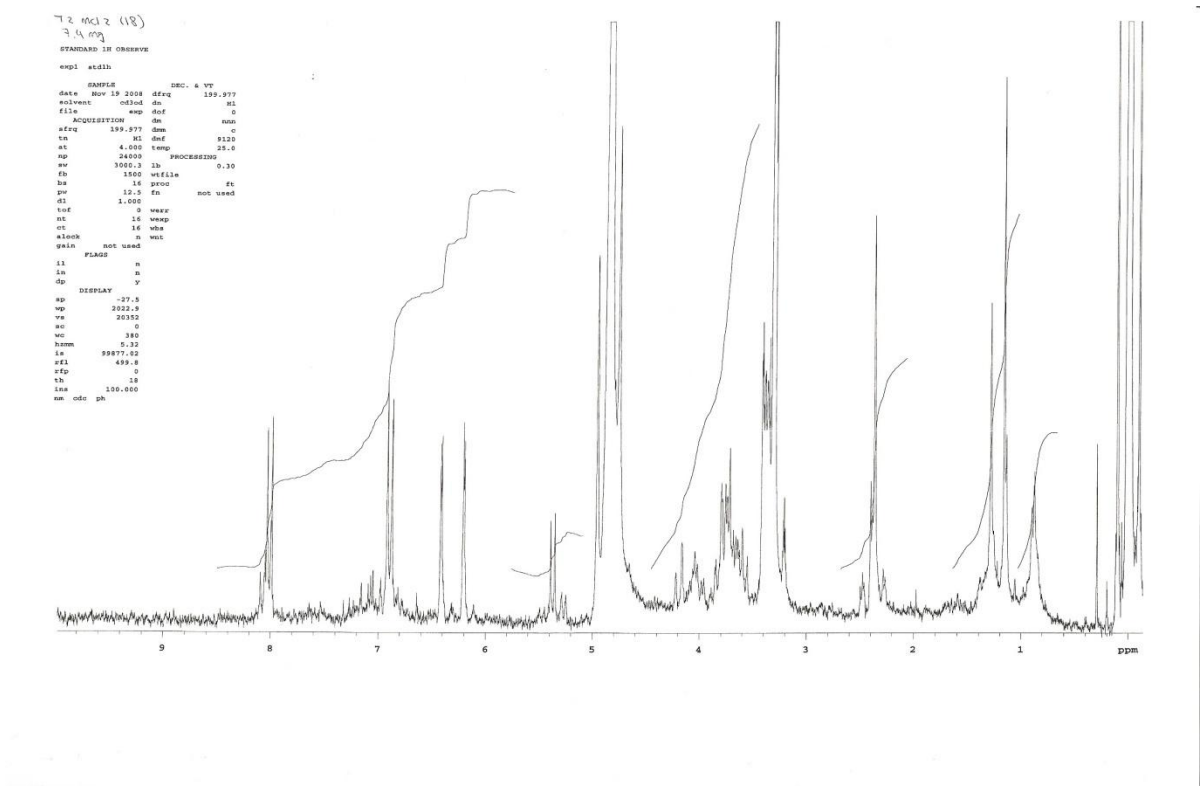
NMR spectra 14.86: 1H-NMR spectra of fraction B1-2 S.4-5 VRP. T4.



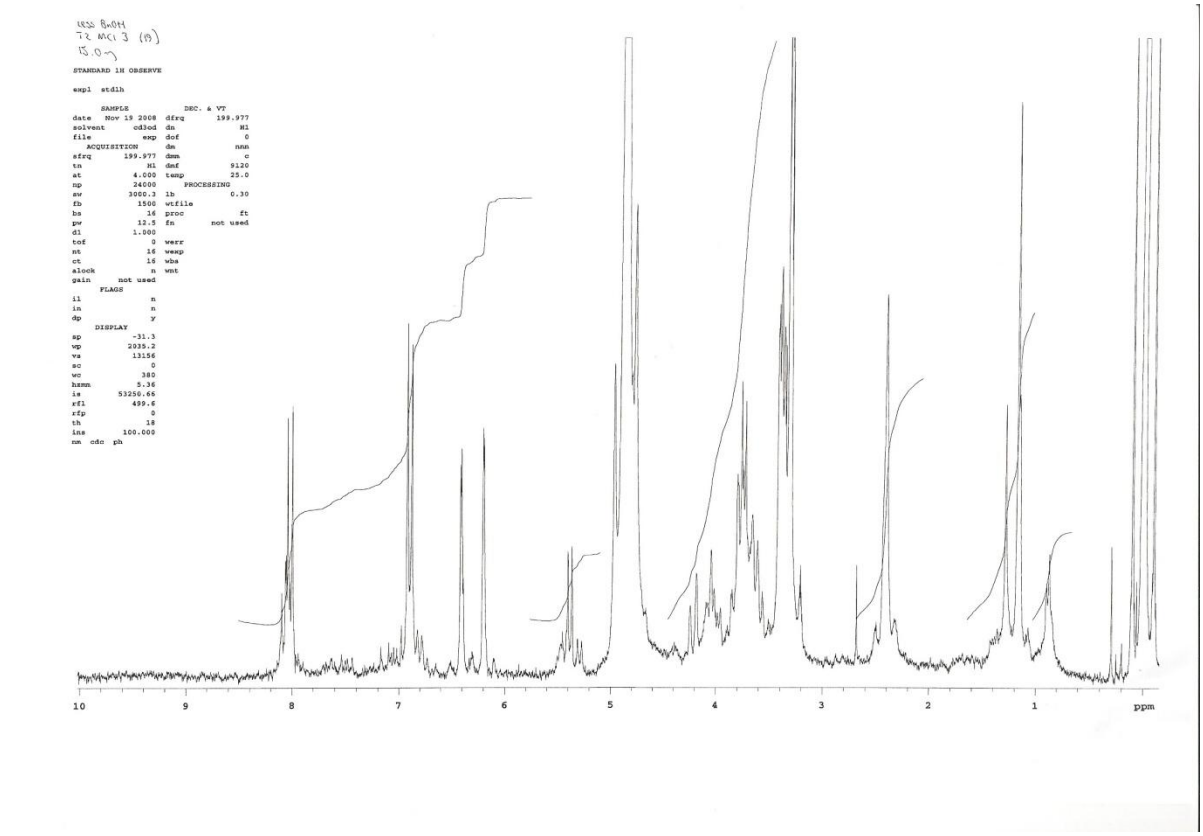
NMR spectra 14.87: 13C-NMR spectra of fraction B1-2 S.4-5 VRP T4.



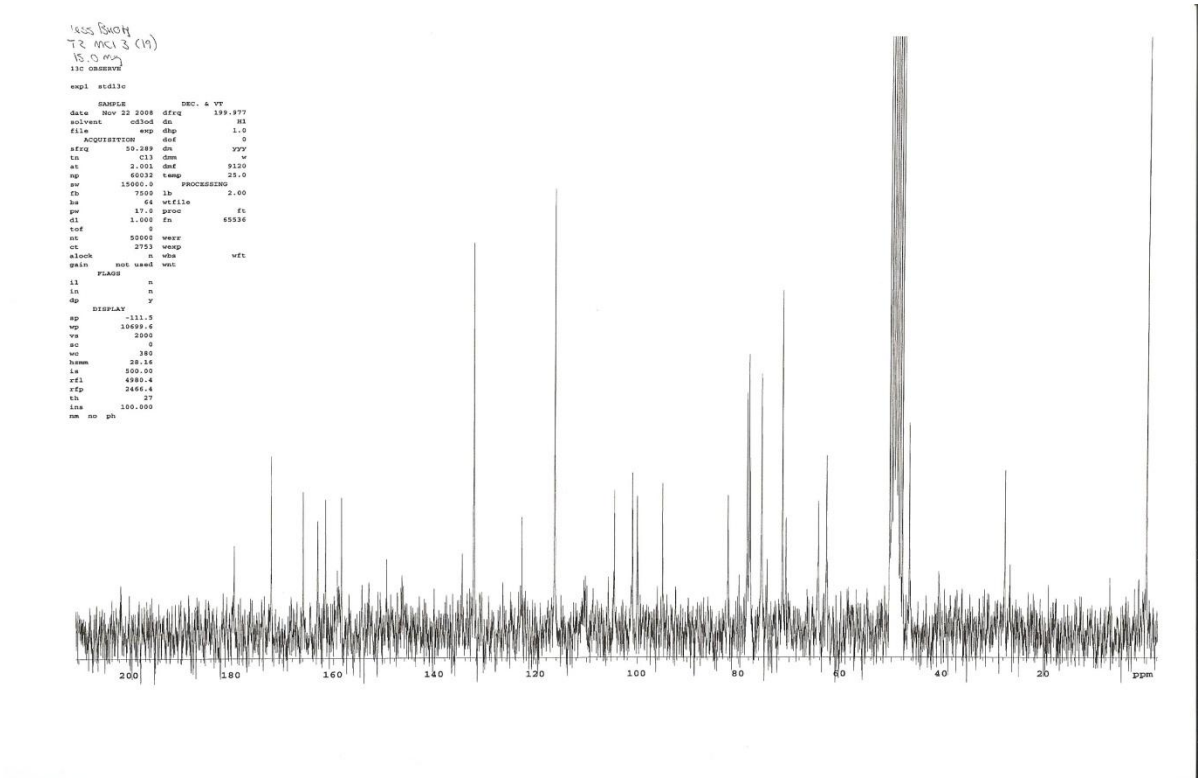
NMR spectra 14.88: 13C-NMR spectra of fraction B1-2 S.4-5 VRP. T2 M1.



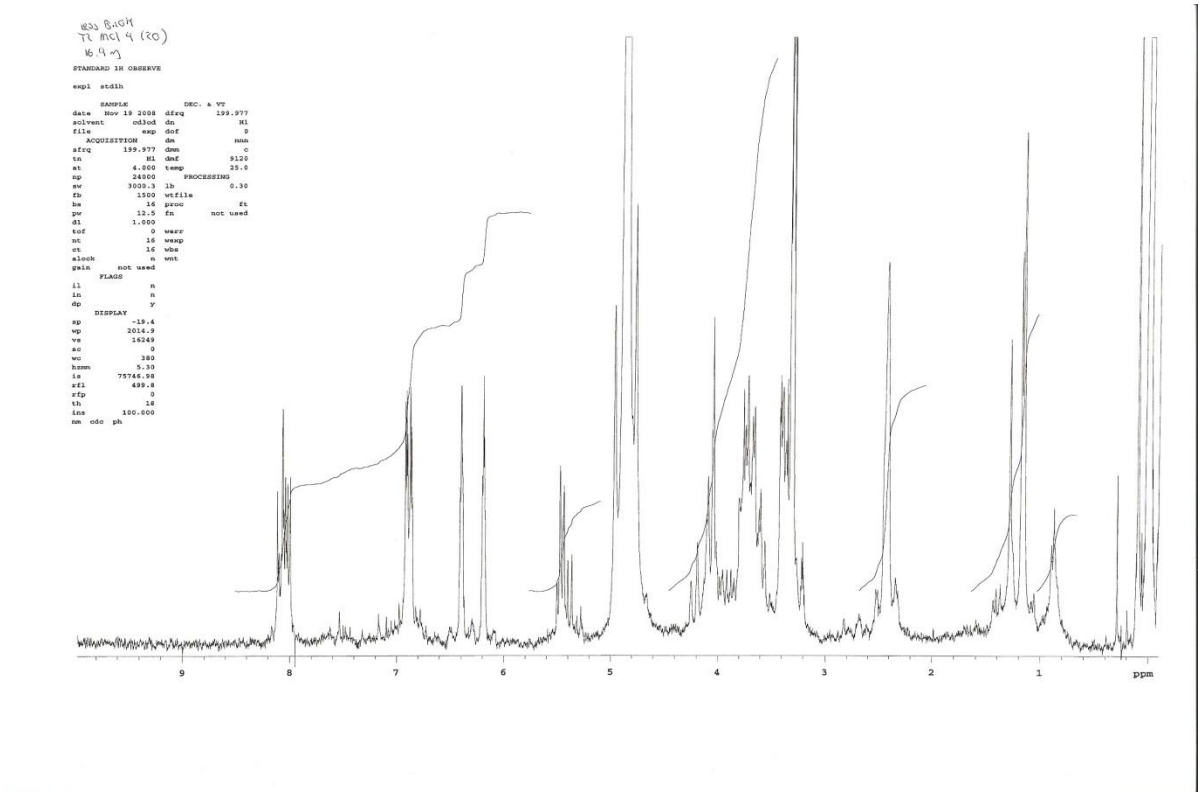
NMR spectra 14.89: 13C-NMR spectra of fraction B1-2 S.4-5 VRP. T2 M2.



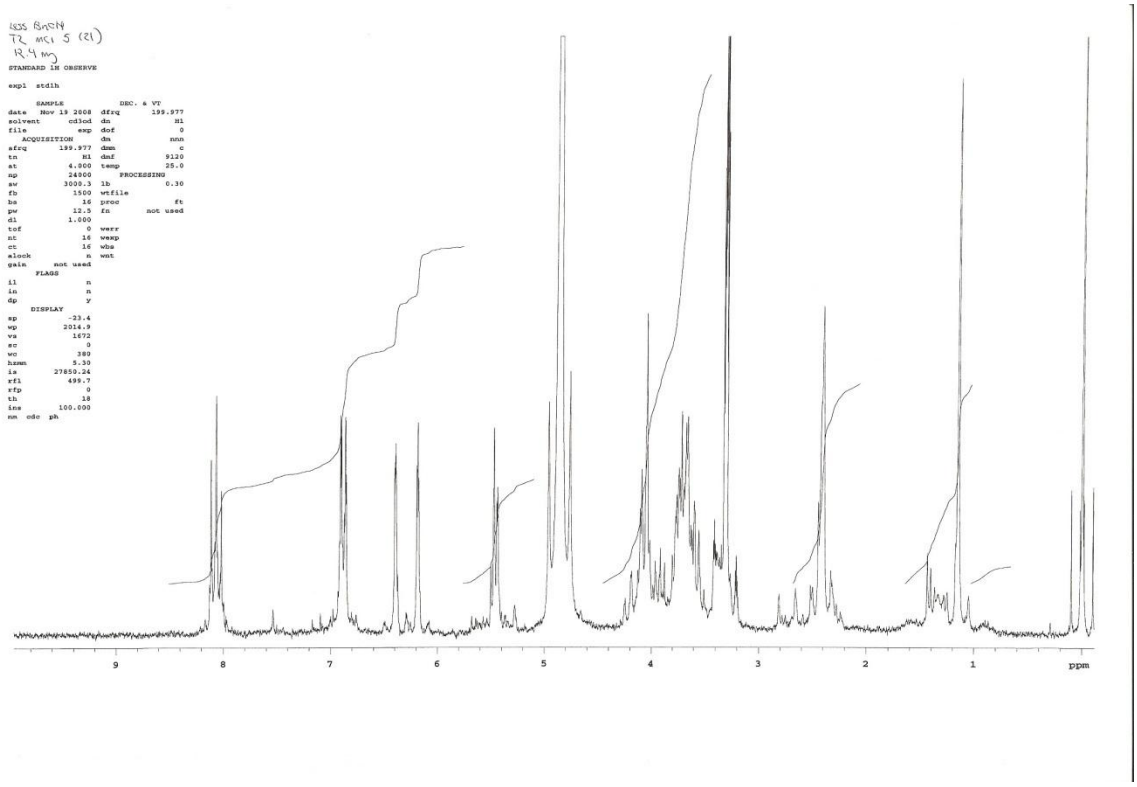
NMR spectra 14.90: 1H-NMR spectra of fraction B1-2 S.4-5 VRP. T2 M3.



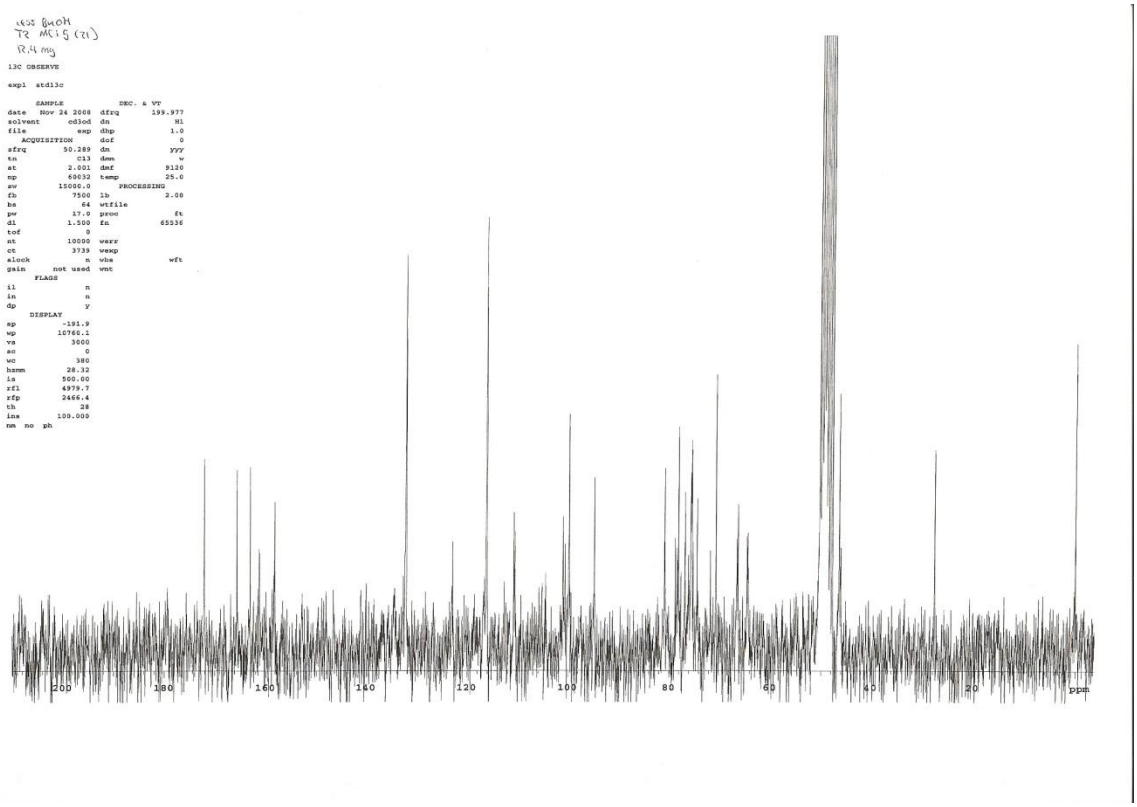
NMR spectra 14.91: 13C-NMR spectra of fraction B1-2 S.4-5 VRP. T2 M3.



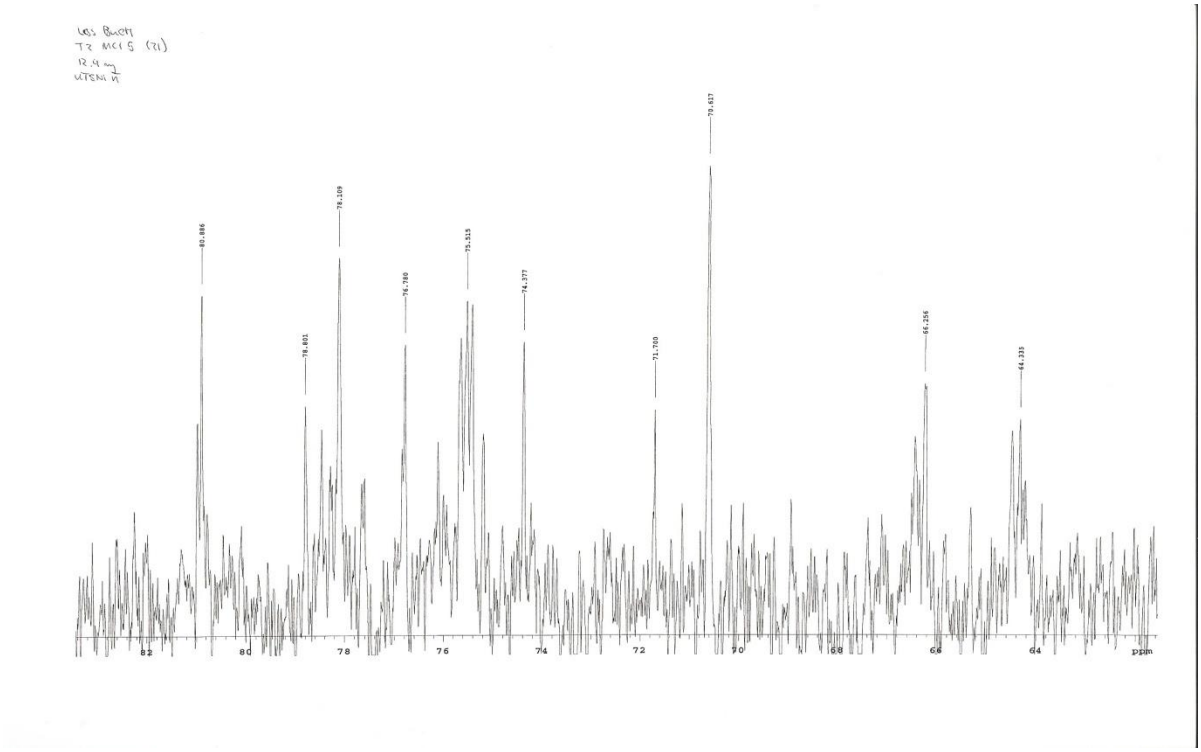
NMR spectra 14.92: 1H-NMR spectra of fraction B1-2 S.4-5 VRP. T2 M4.



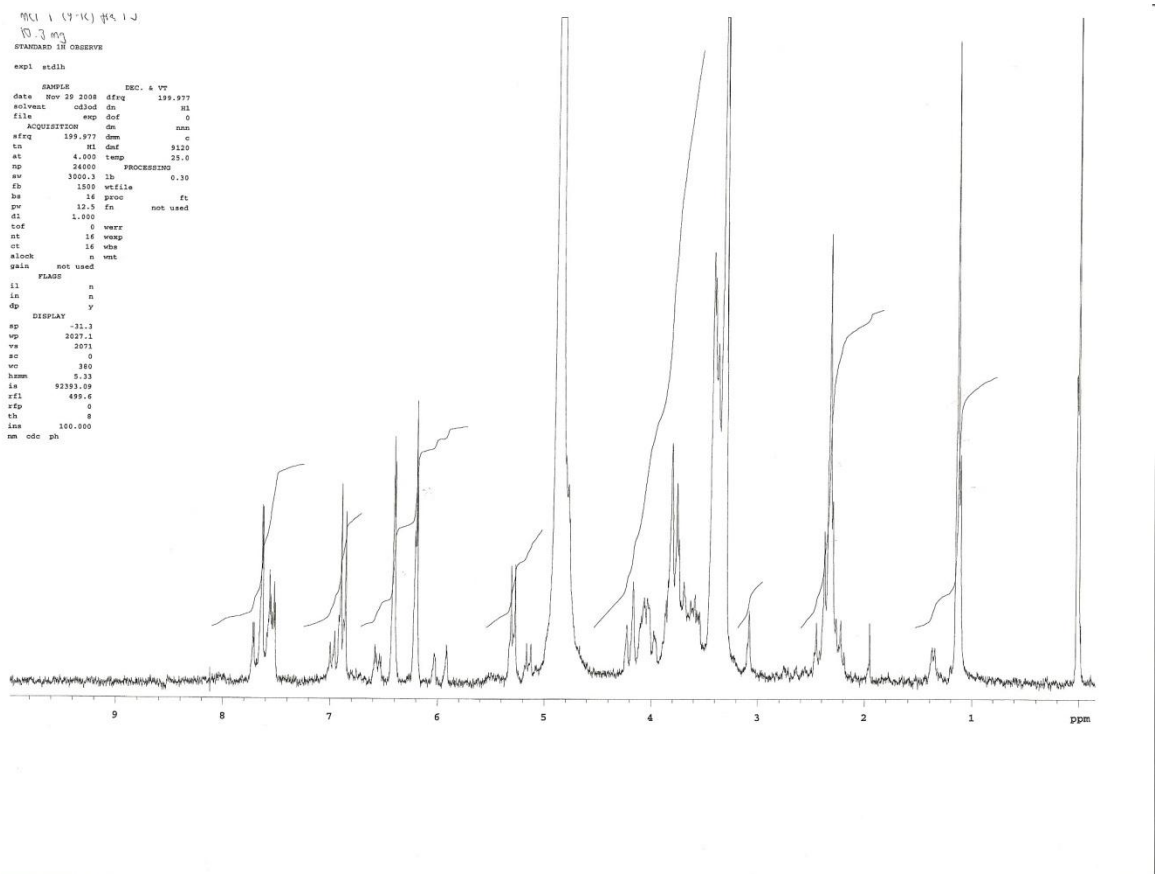
NMR spectra 14.93: 1H-NMR spectra of fraction B1-2 S.4-5 VRP. T2 M5.



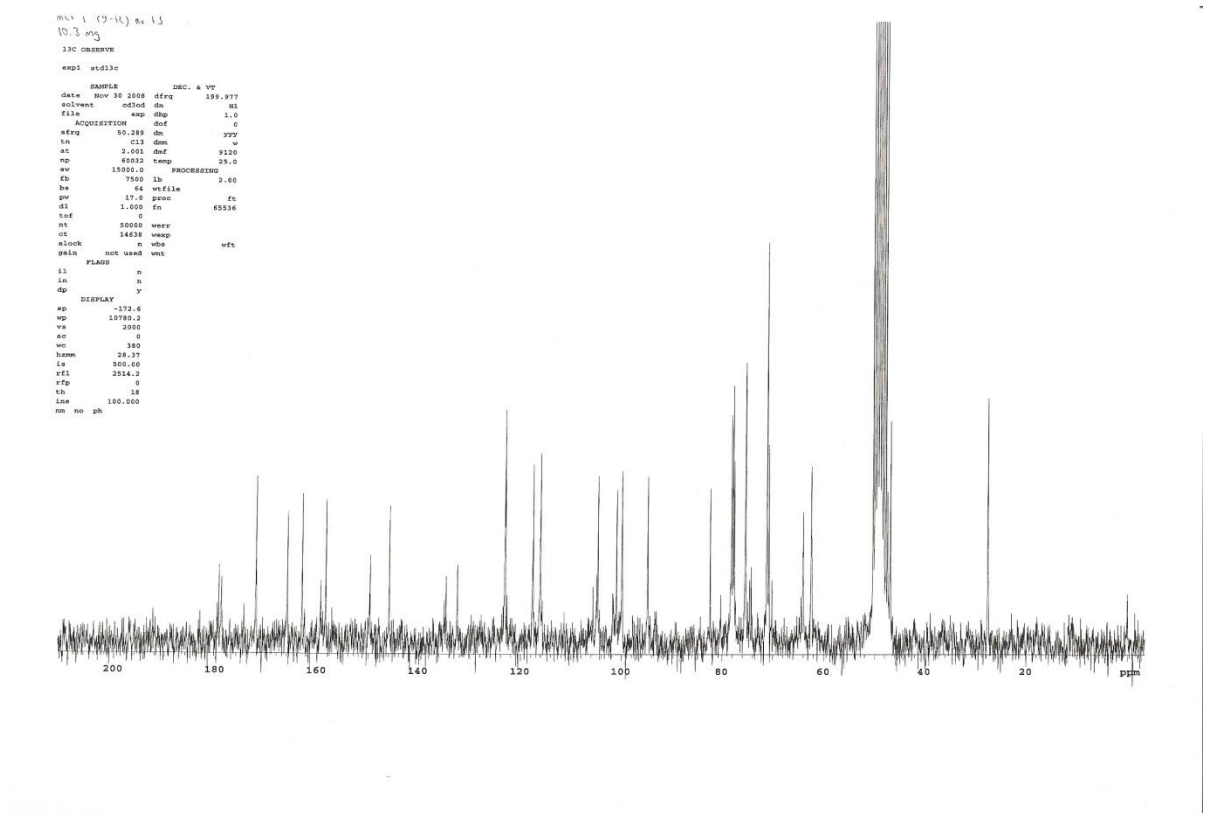
NMR spectra 14.94: 13C-NMR spectra of fraction B1-2 S.4-5 VRP. T2 M5.



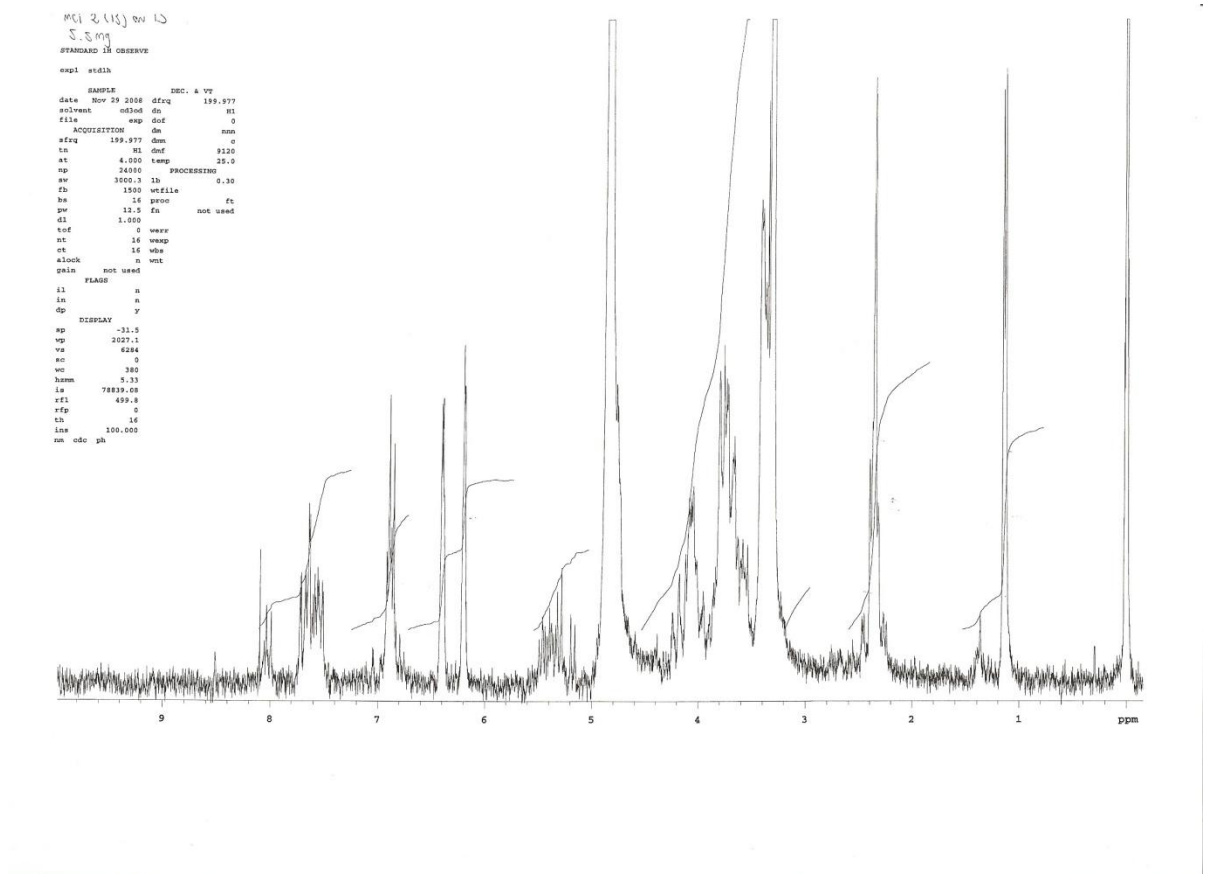
NMR spectra 14.95: partly from 13C-NMR spectra of fraction B1-2 S.4-5 VRP. T2 M5.



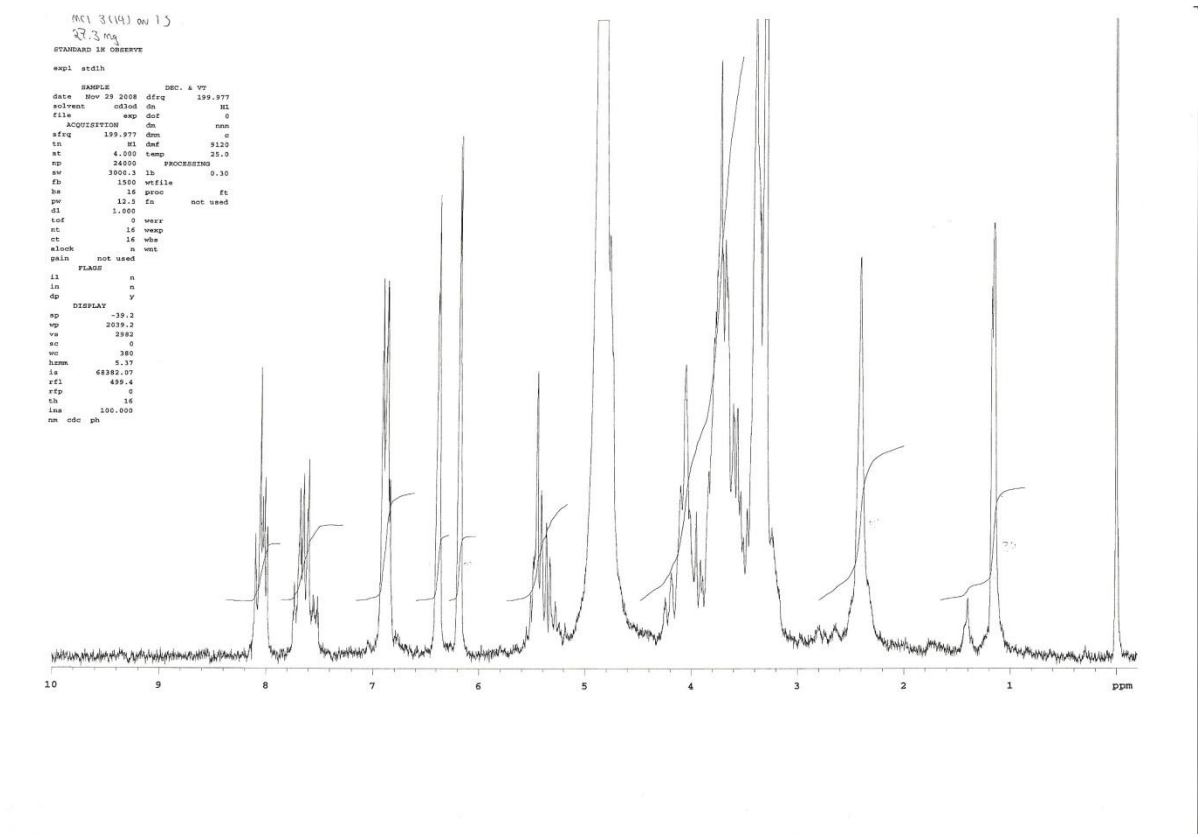
NMR spectra 14.96: 1H-NMR spectra of fraction B1-2 S.4-5 VRP. T3 M1.



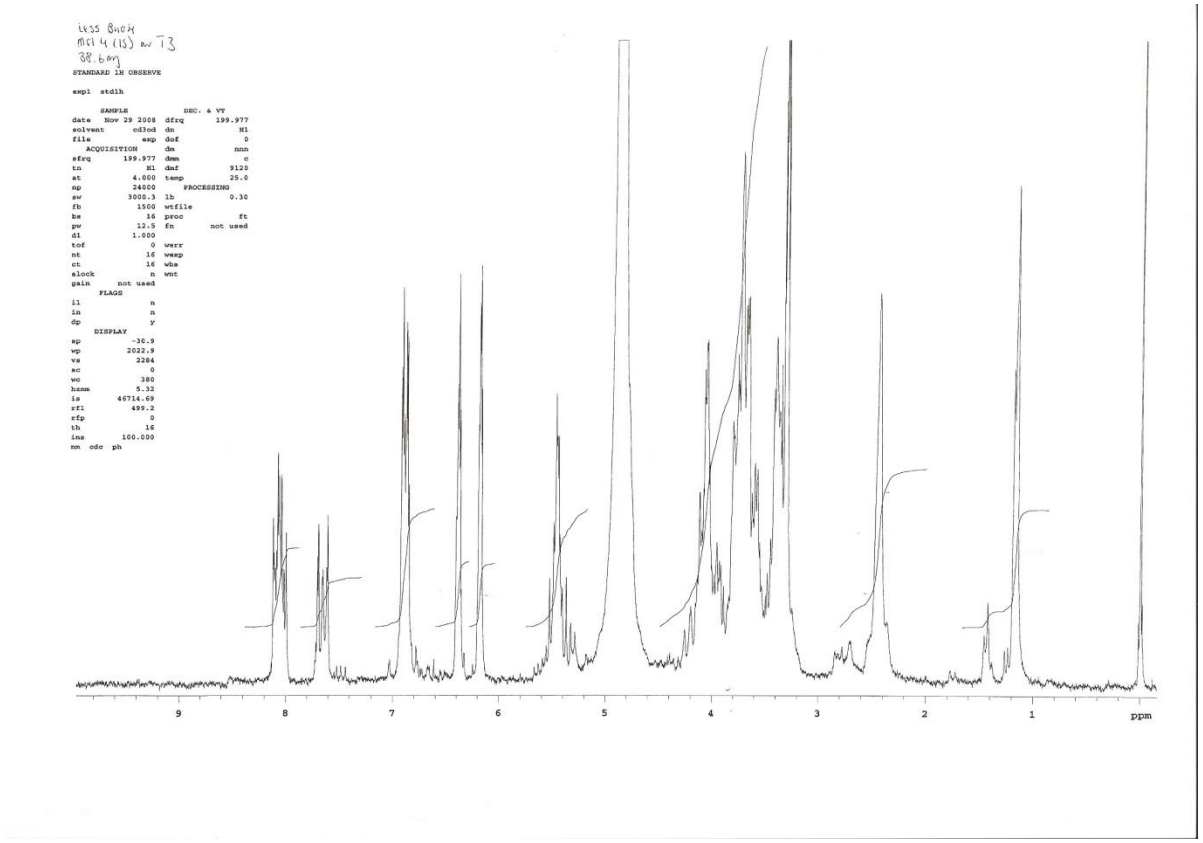
NMR spectra 14.97: partly from 13C-NMR spectra of fraction B1-2 S.4-5 VRP. T3 M1.



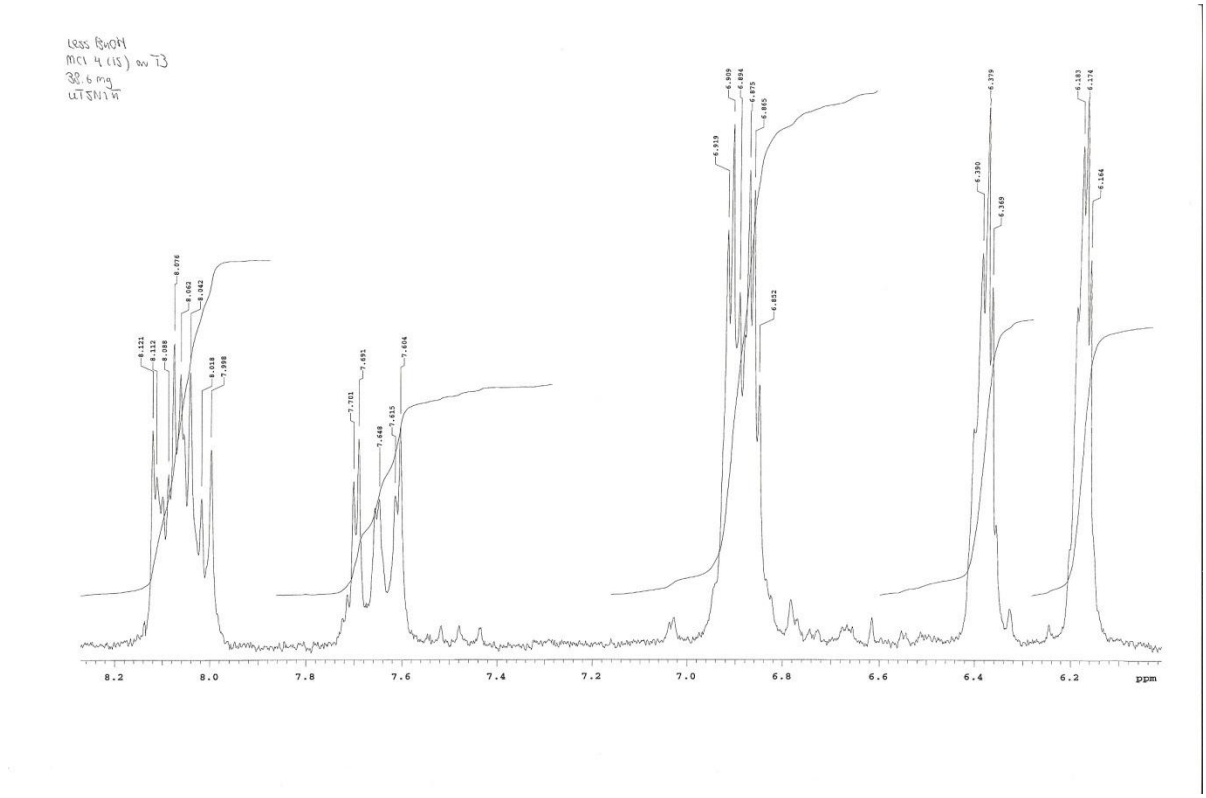
NMR spectra 14.98: 1H-NMR spectra of fraction B1-2 S.4-5 VRP. T3 M2.



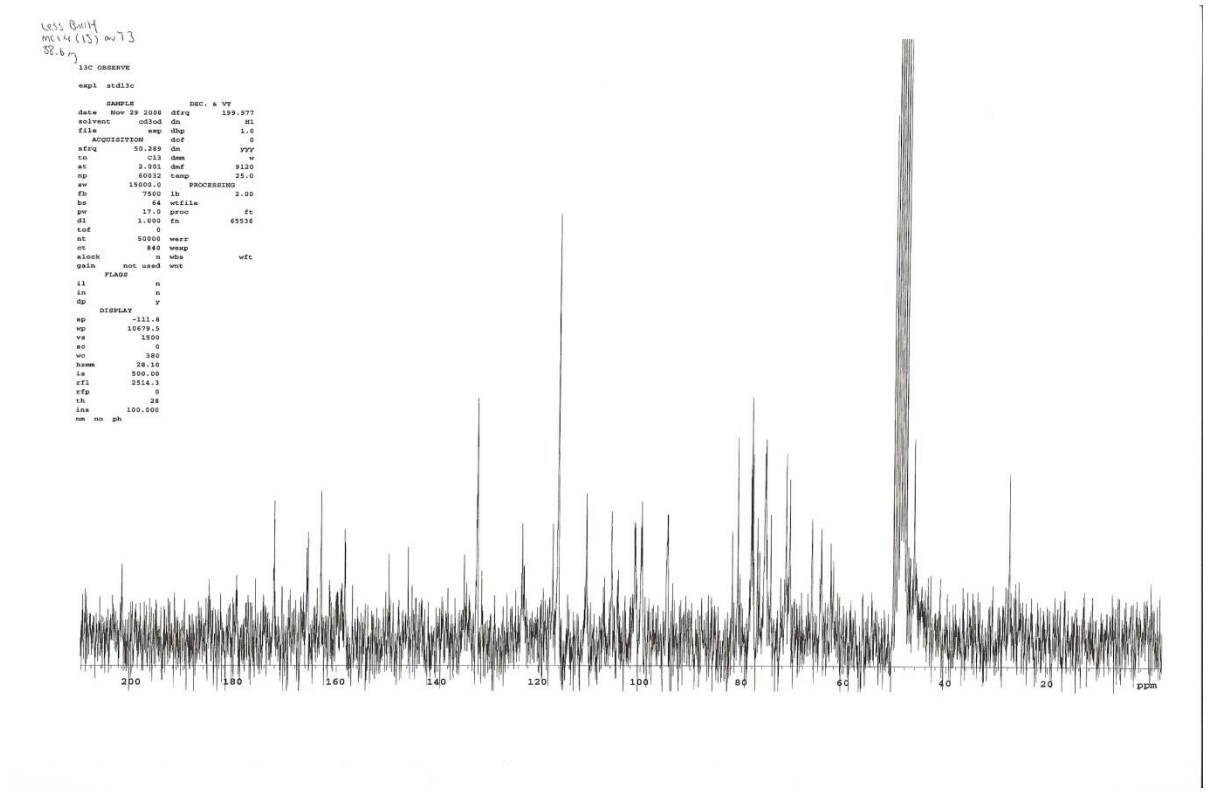
NMR spectra 14.99: 1H-NMR spectra of fraction B1-2 S.4-5 VRP. T3 M3.



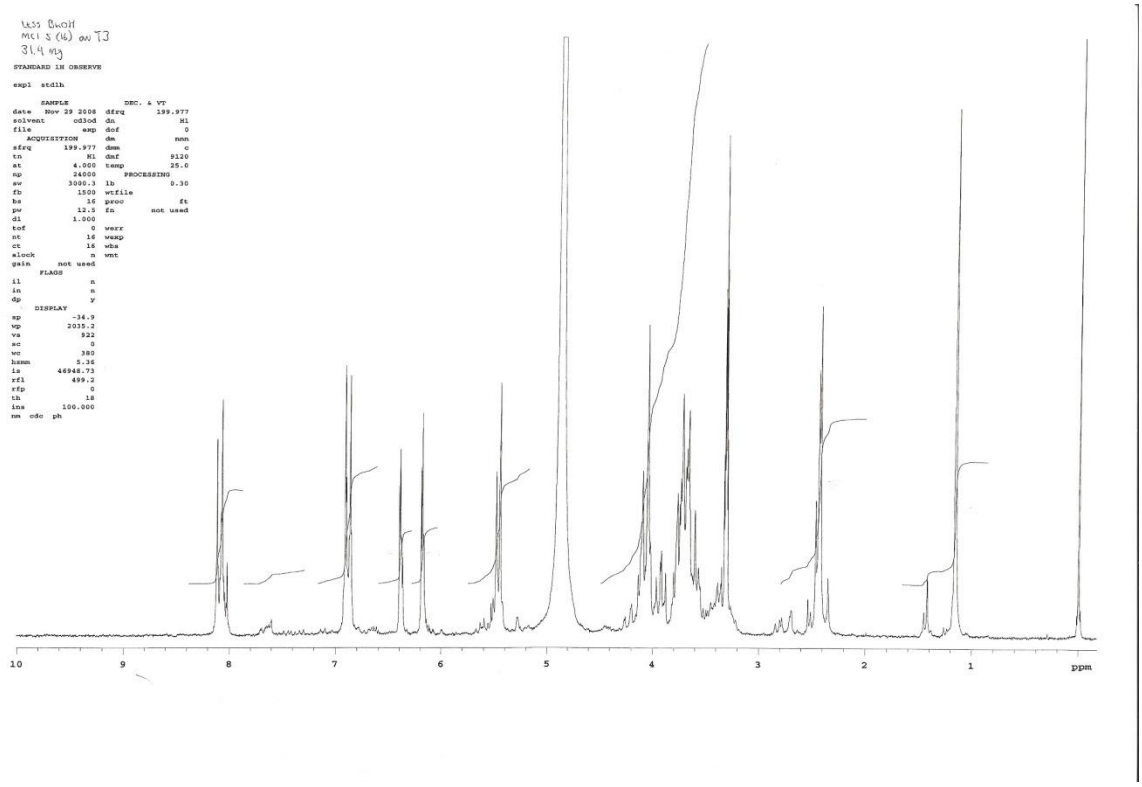
NMR spectra 14.100: 1H-NMR spectra of fraction B1-2 S.4-5 VRP. T3 M4.



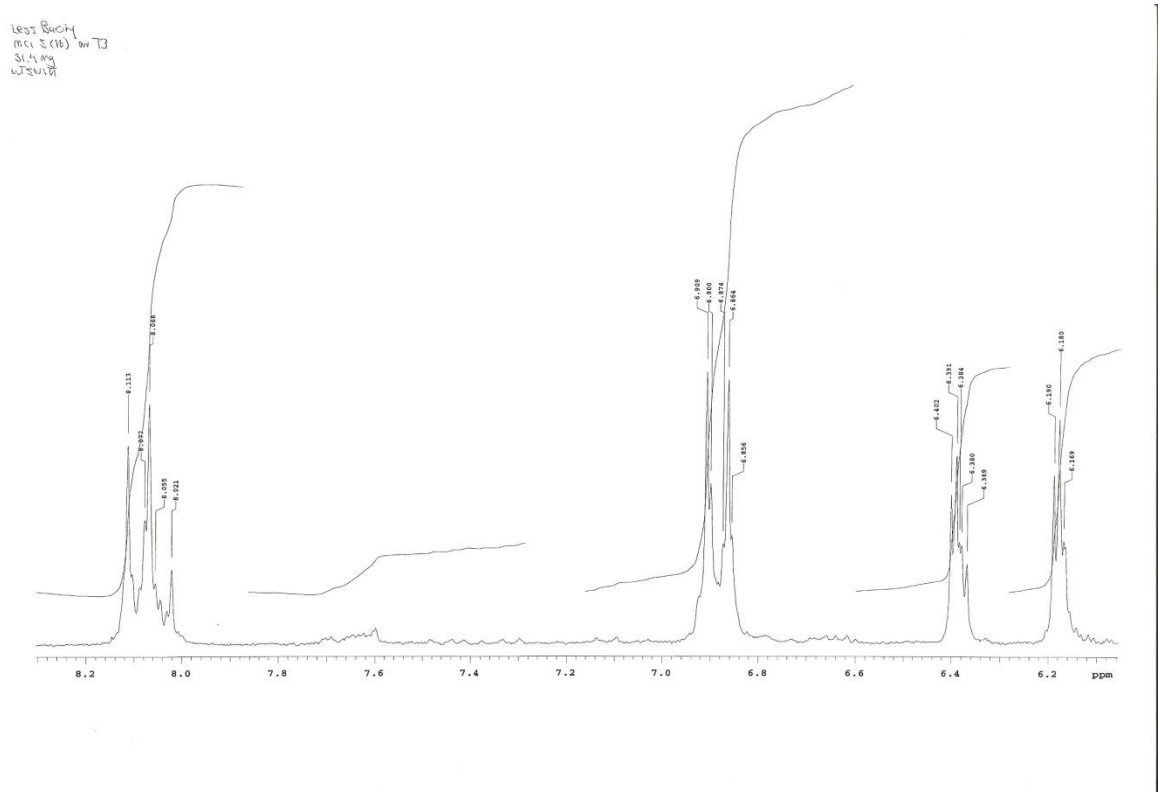
NMR spectra 14.101: partly from 1H-NMR spectra of fraction B1-2 S.4-5 VRP. T3 M4.



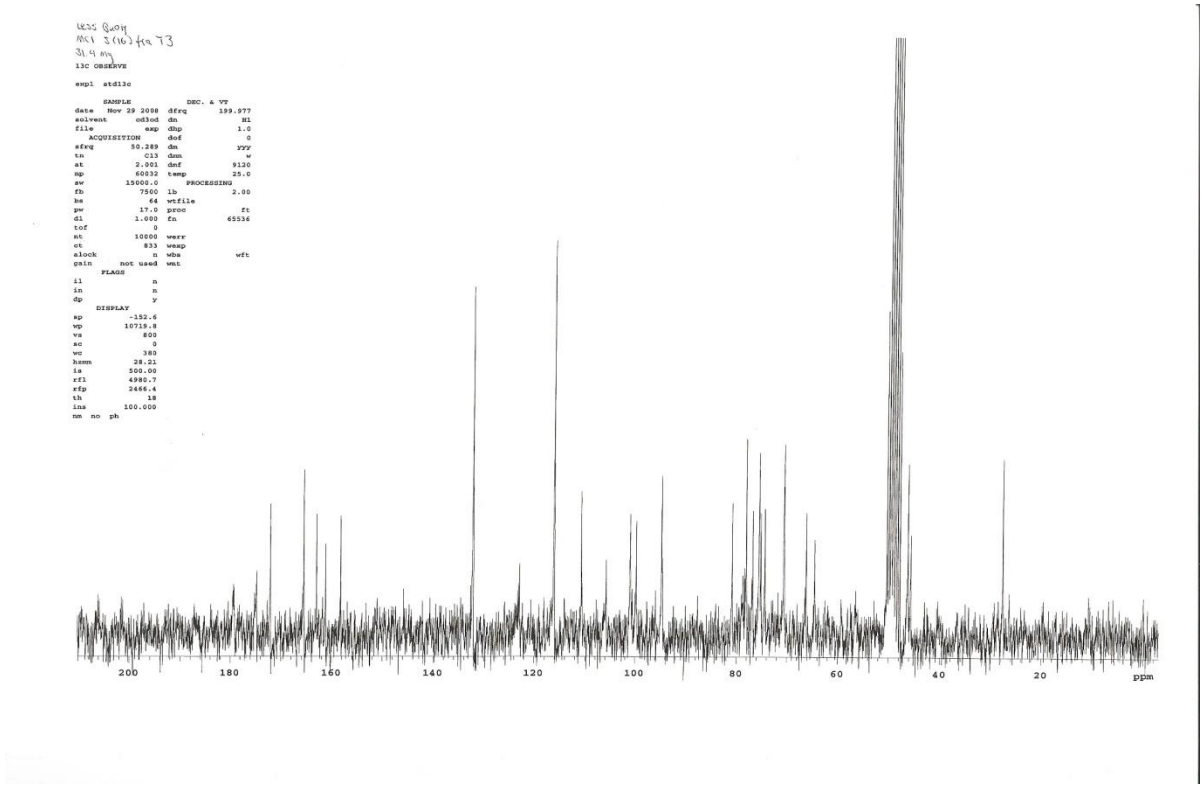
NMR spectra 14.102: 13C-NMR spectra of fraction B1-2 S.4-5 VRP. T3 M4.



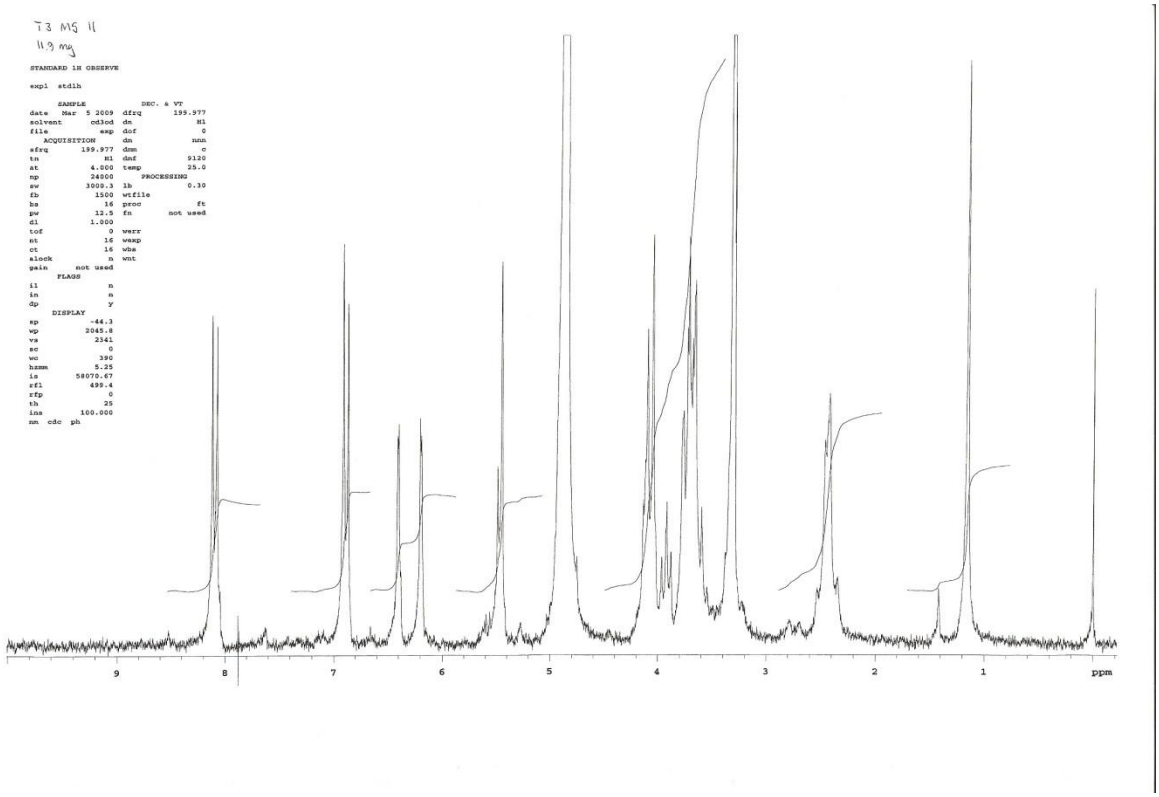
NMR spectra 14.103: 1H-NMR spectra of fraction B1-2 S.4-5 VRP. T3 M5.



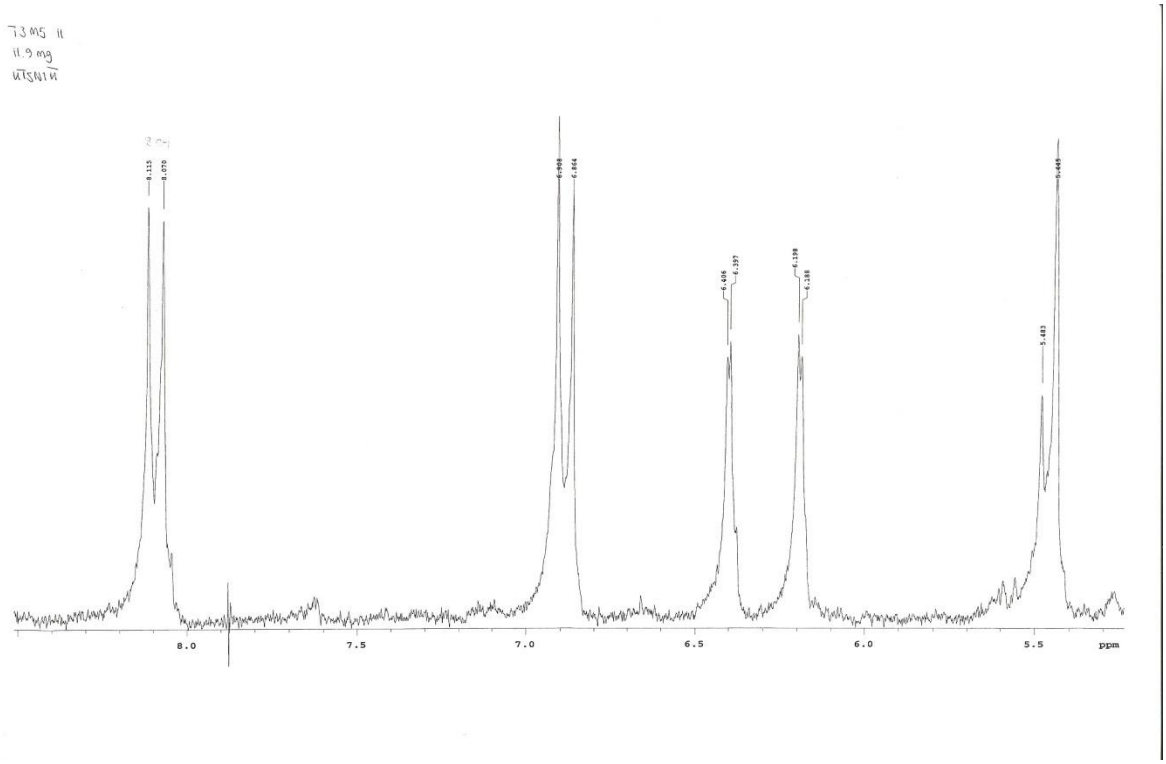
NMR spectra 14.104: partly from 1H-NMR spectra of fraction B1-2 S.4-5 VRP. T3 M5.



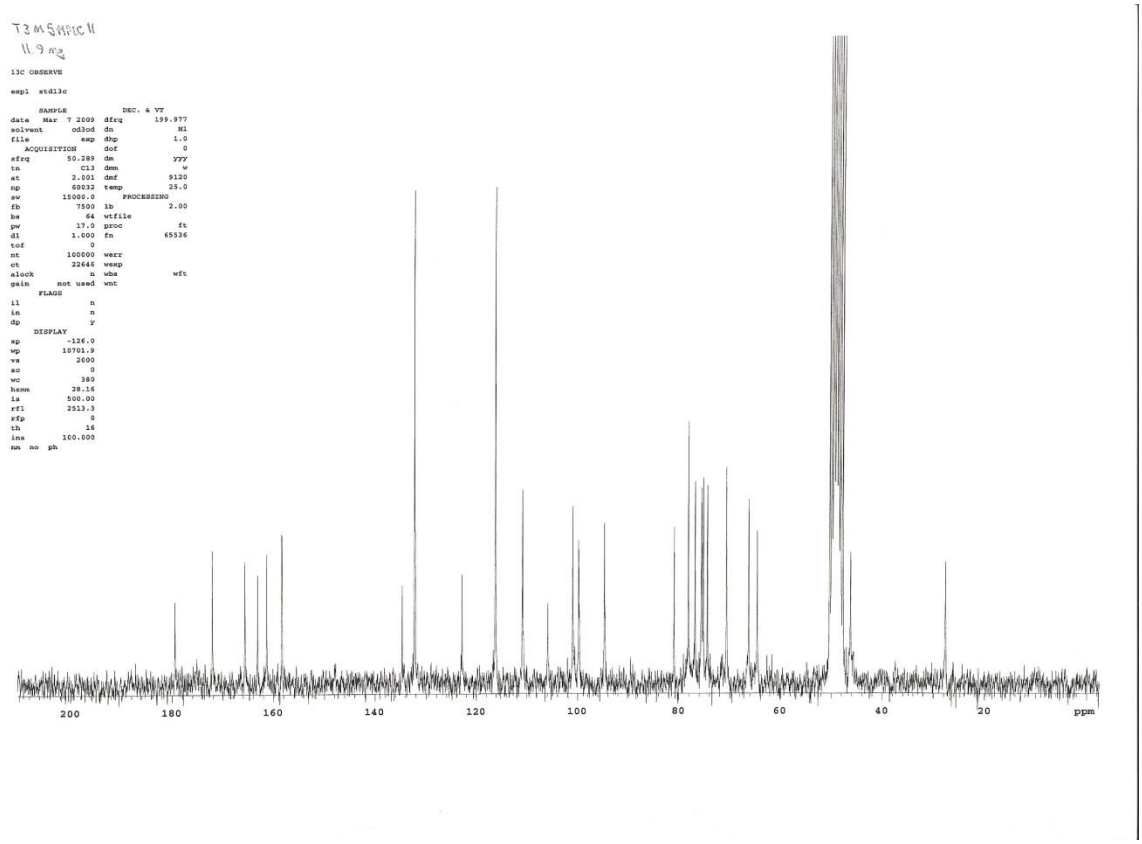
NMR spectra 14.105: 13C-NMR spectra of fraction B1-2 S.4-5 VRP. T3 M5.



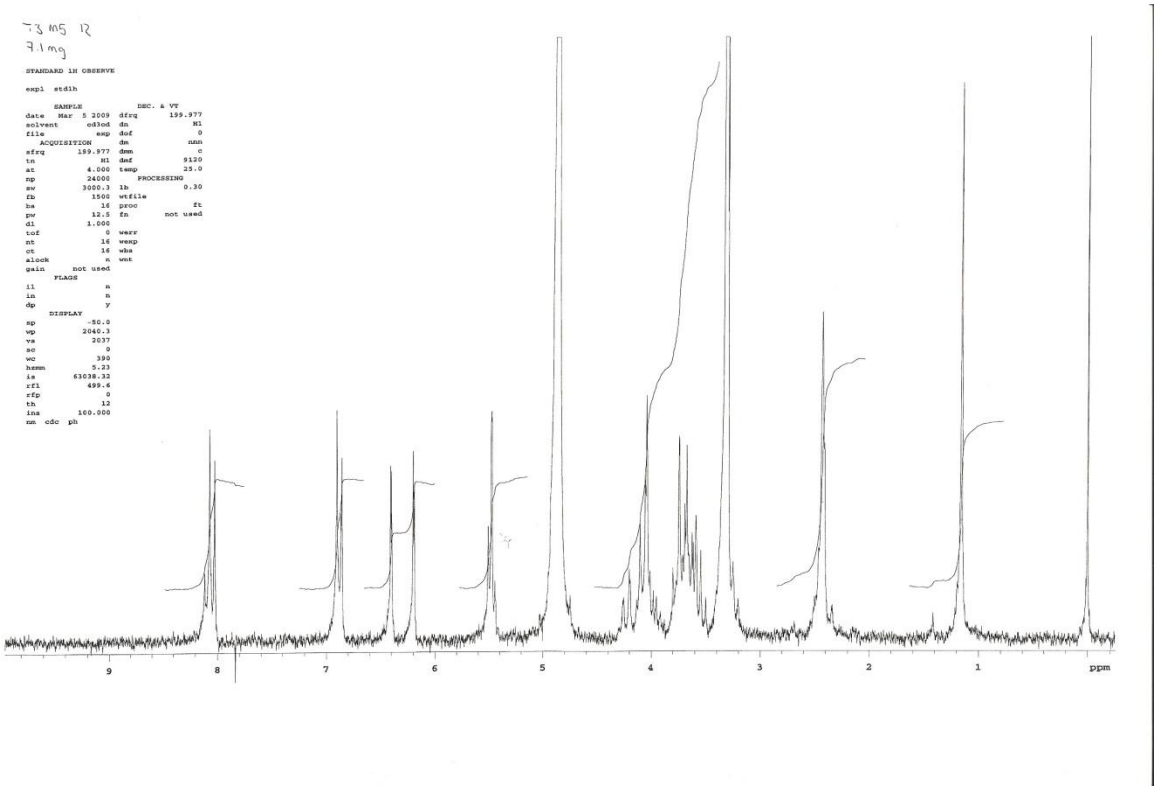
NMR spectra 14.106: 1H-NMR spectra of fraction B1-2 S.4-5 VRP. T3 M5 HPLC 11



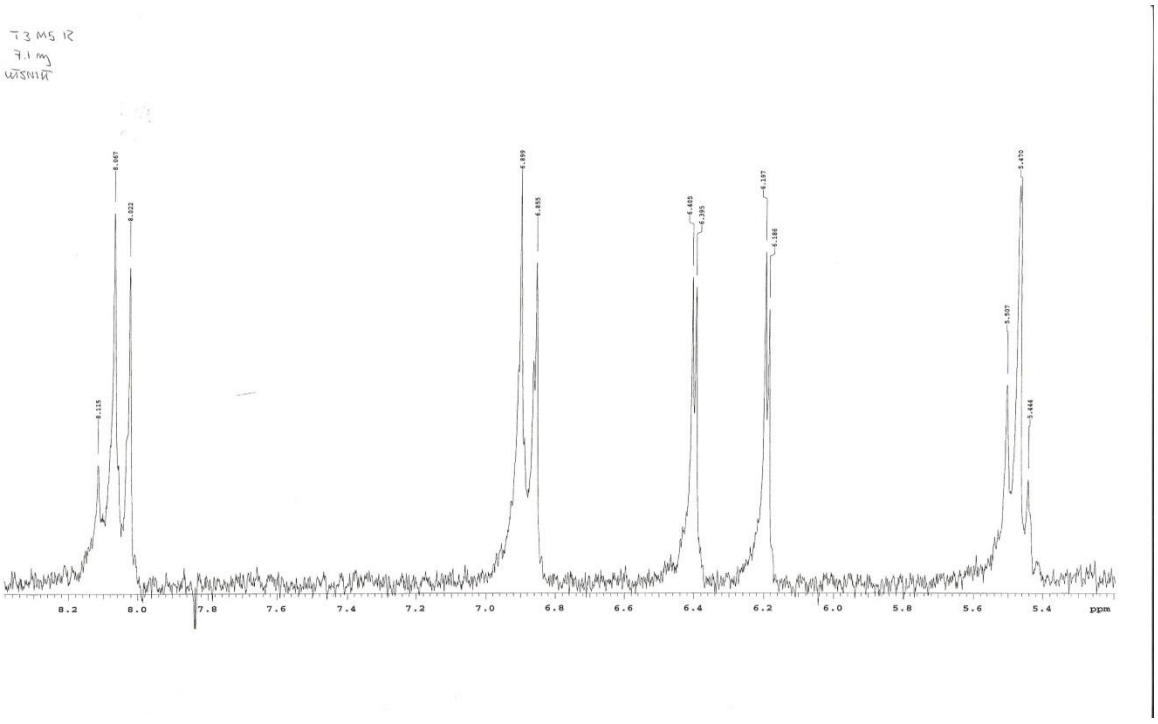
NMR spectra 14.107: partly from 1H-NMR spectra of fraction B1-2 S.4-5 VRP. T3 M5 HPLC 11.



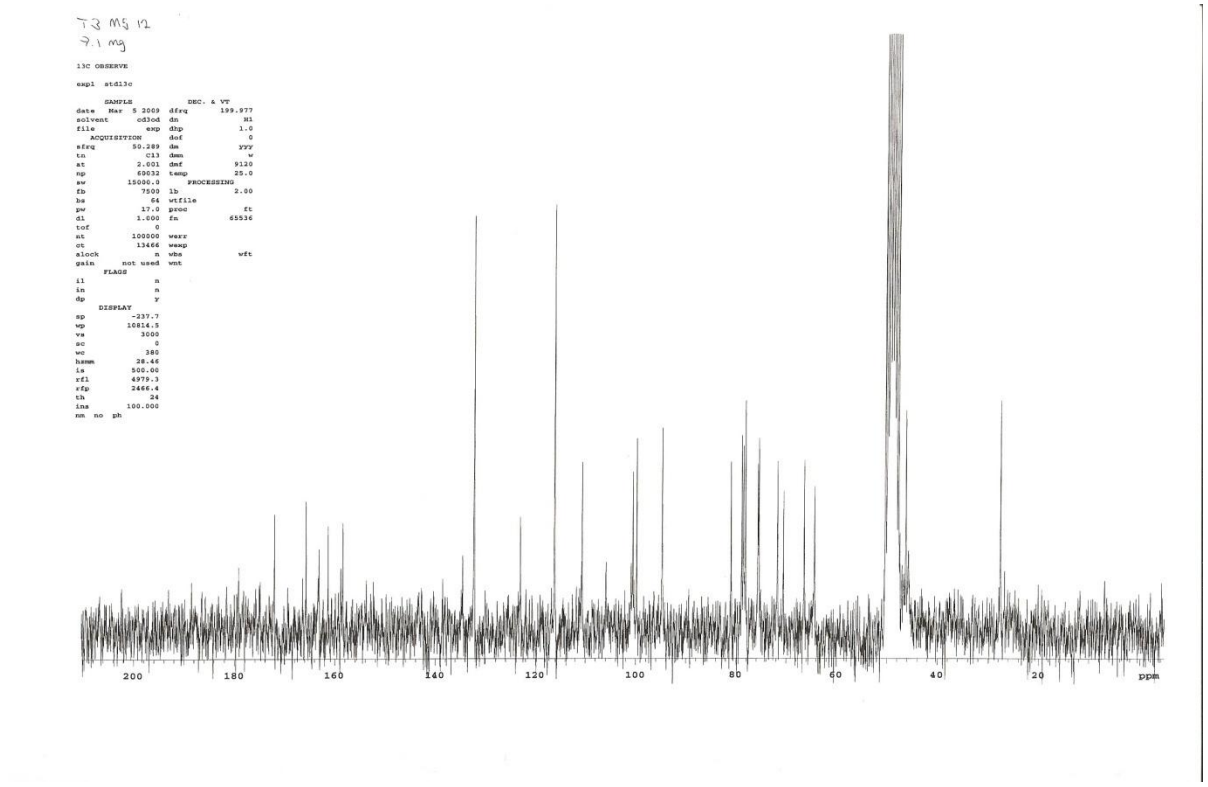
NMR spectra 14.108: 13C-NMR spectra of fraction B1-2 S.4-5 VRP. T3 M5 HPLC 11.



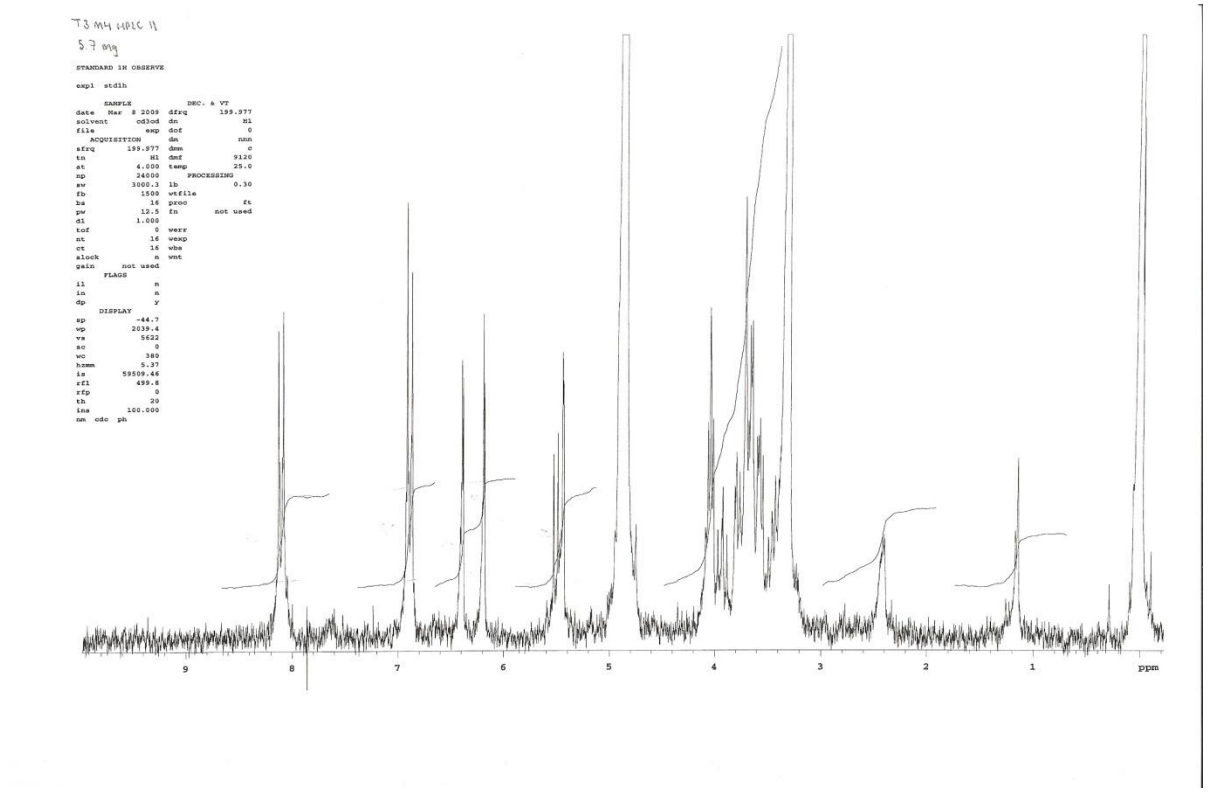
NMR spectra 14.109: 1H-NMR spectra of fraction B1-2 S.4-5 VRP. T3 M5 HPLC 12.



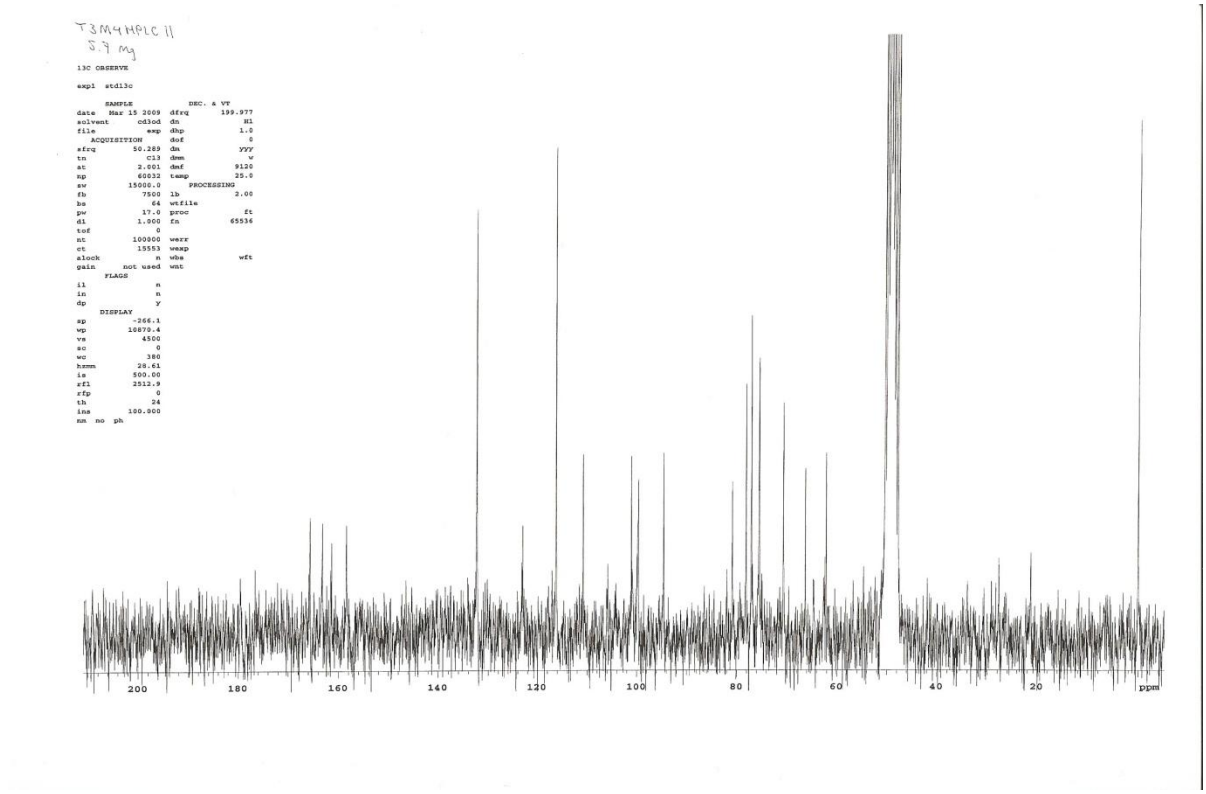
NMR spectra 14.110:partly from 1H-NMR spectra of fraction B1-2 S.4-5 VRP. T3 M5 HPLC 12.



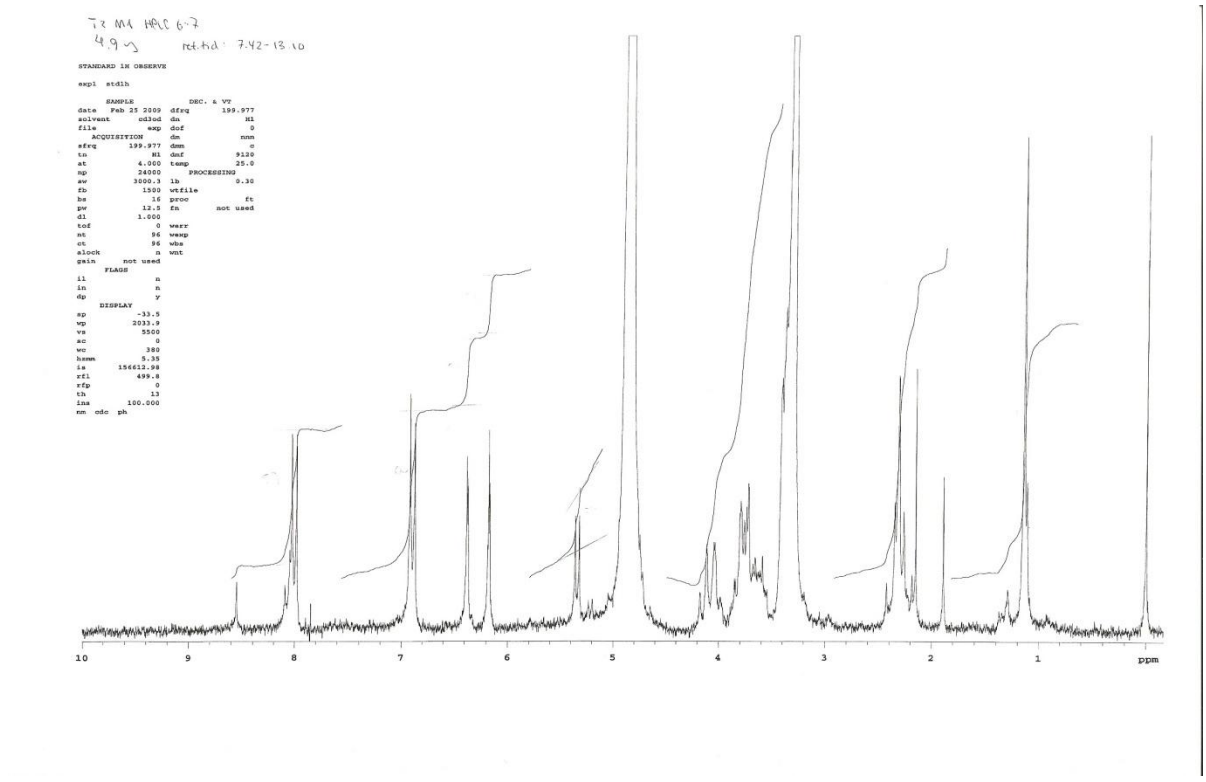
NMR spectra 14.111: 13C-NMR spectra of fraction B1-2 S.4-5 VRP. T3 M5 HPLC 12.



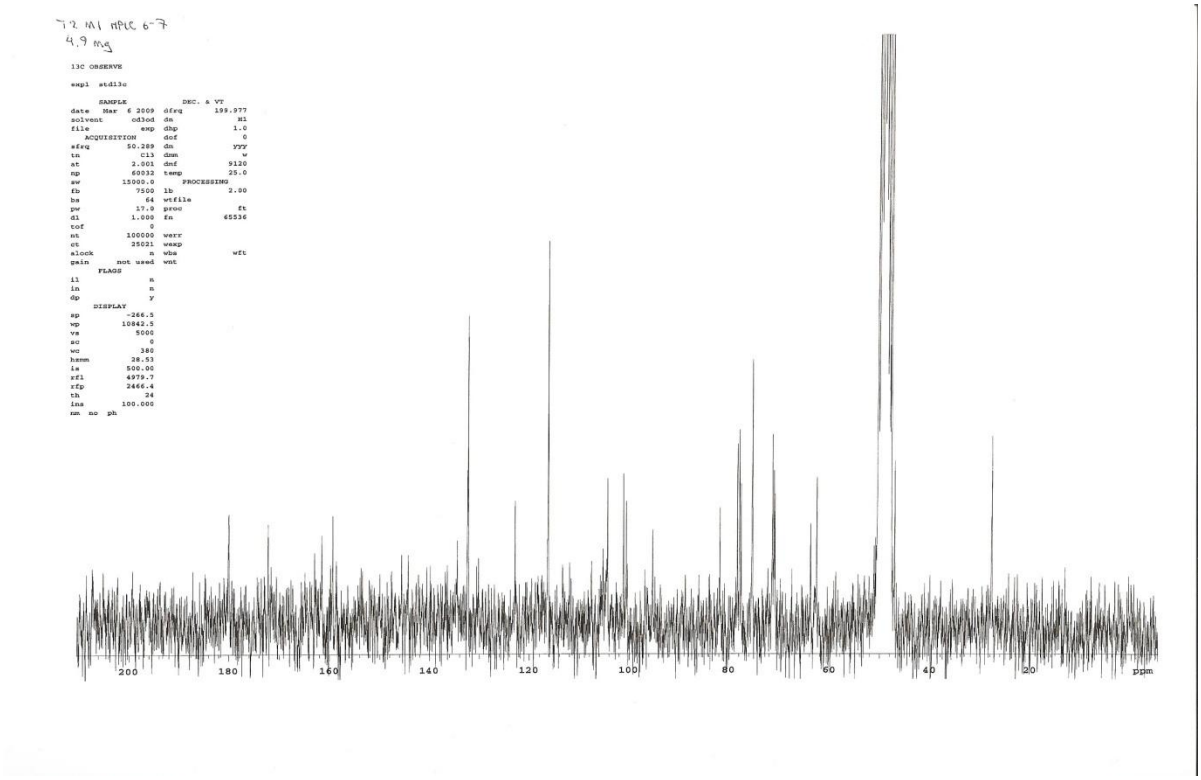
NMR spectra 14.111: 1H-NMR spectra of fraction B1-2 S.4-5 VRP. T3 M4 HPLC 11.



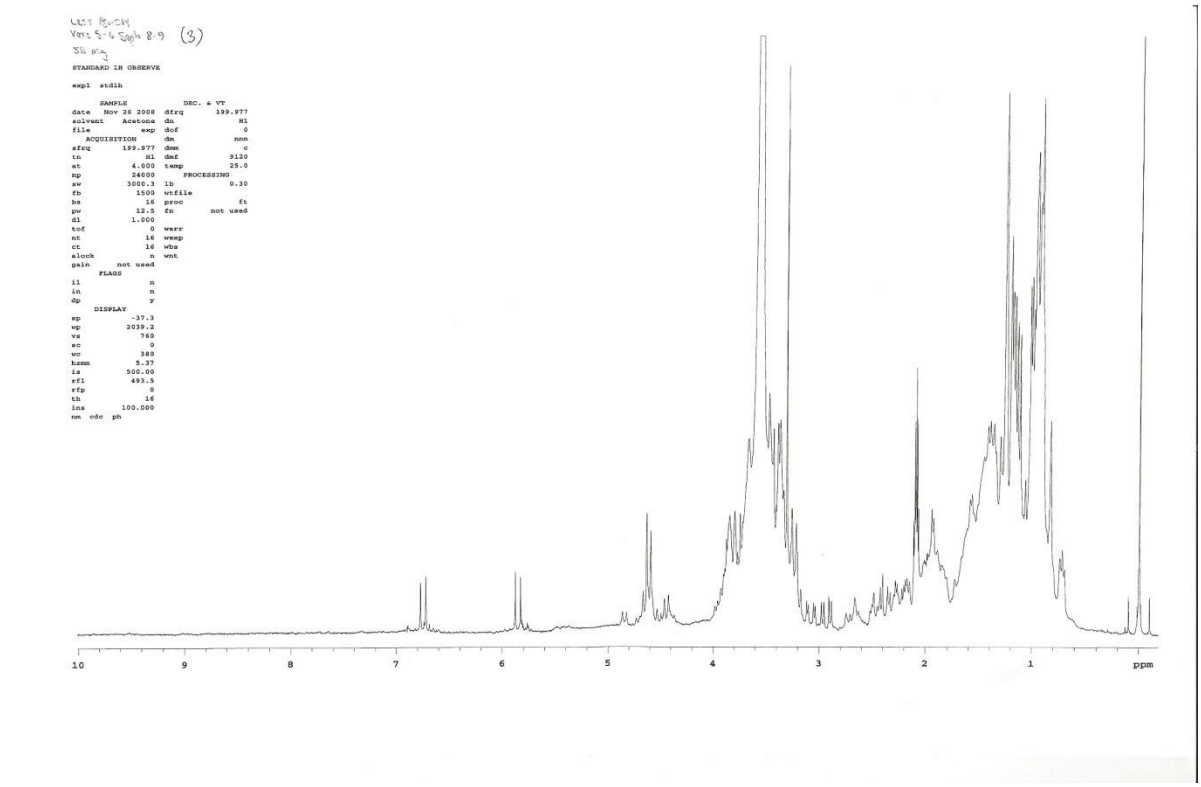
NMR spectra 14.112: 13C-NMR spectra of fraction B1-2 S.4-5 VRP. T3 M4 HPLC 11



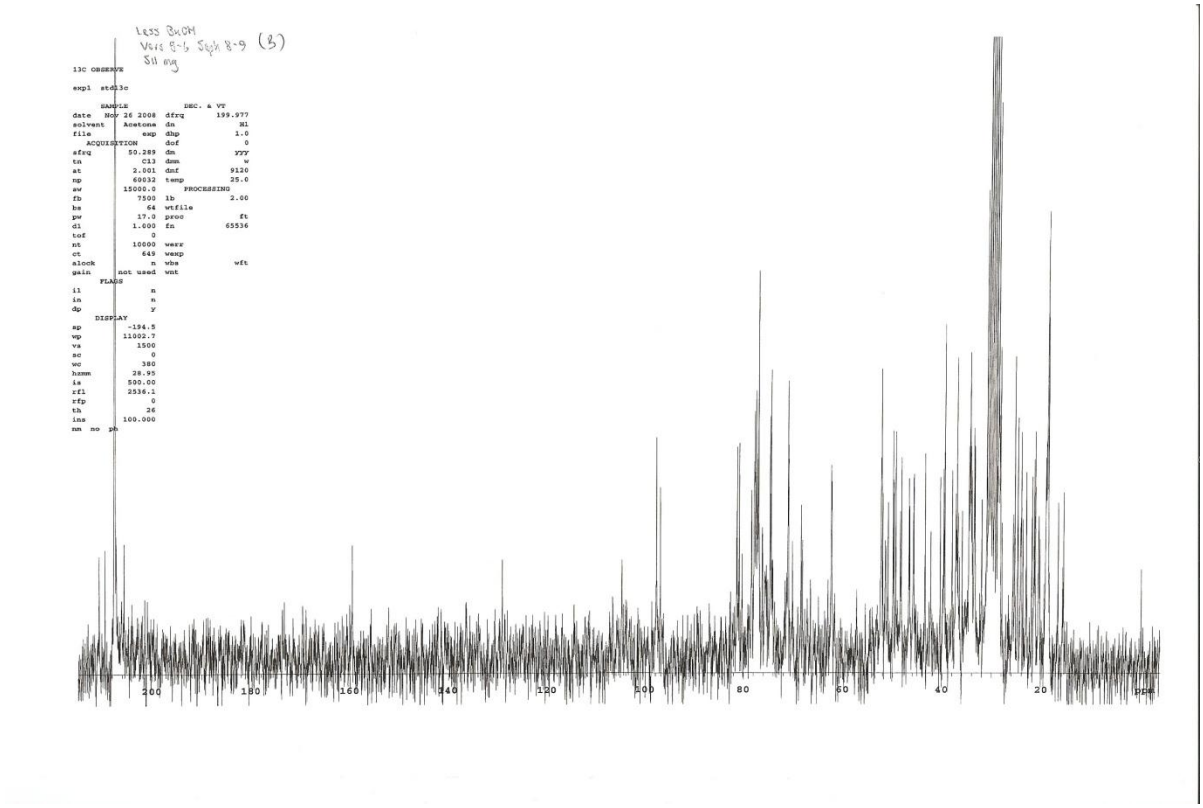
NMR spectra 14.113: 1H-NMR spectra of fraction B1-2 S.4-5 VRP. T2 M1 HPLC 6-7



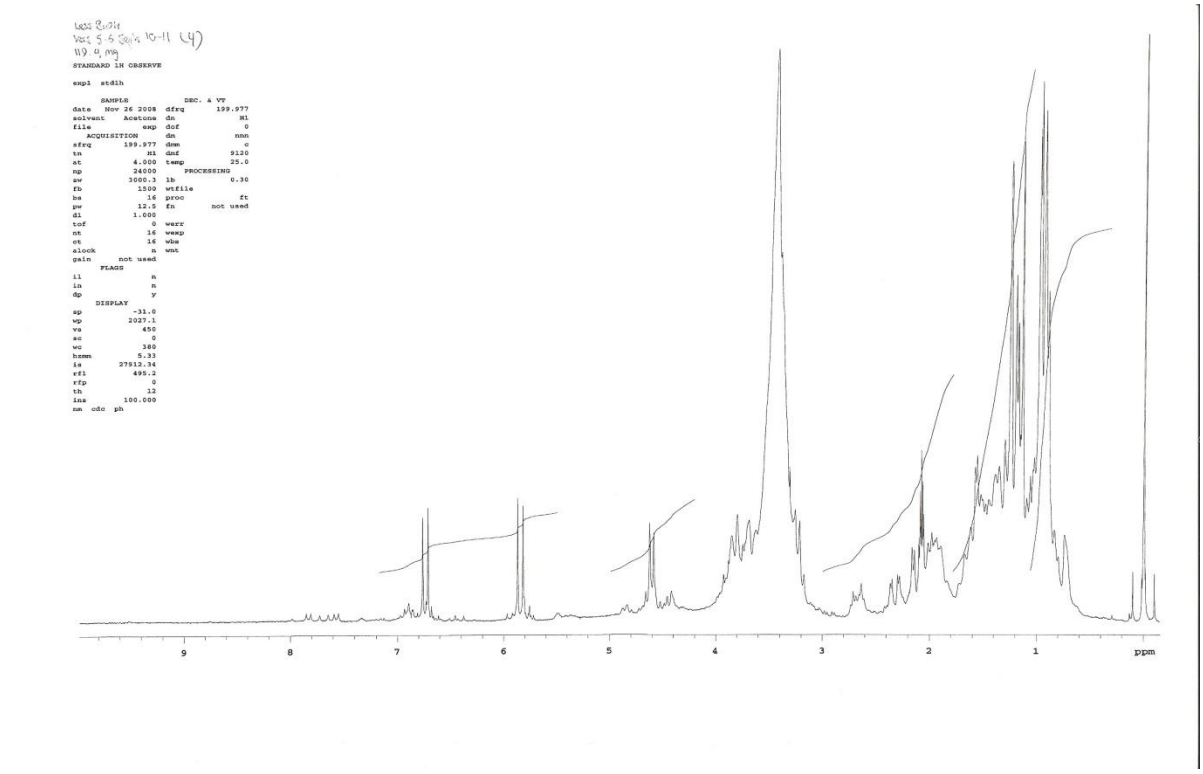
NMR spectra 14.114: 13C-NMR spectra of fraction B1-2 S.4-5 VRP. T2 M1 HPLC 6-7.



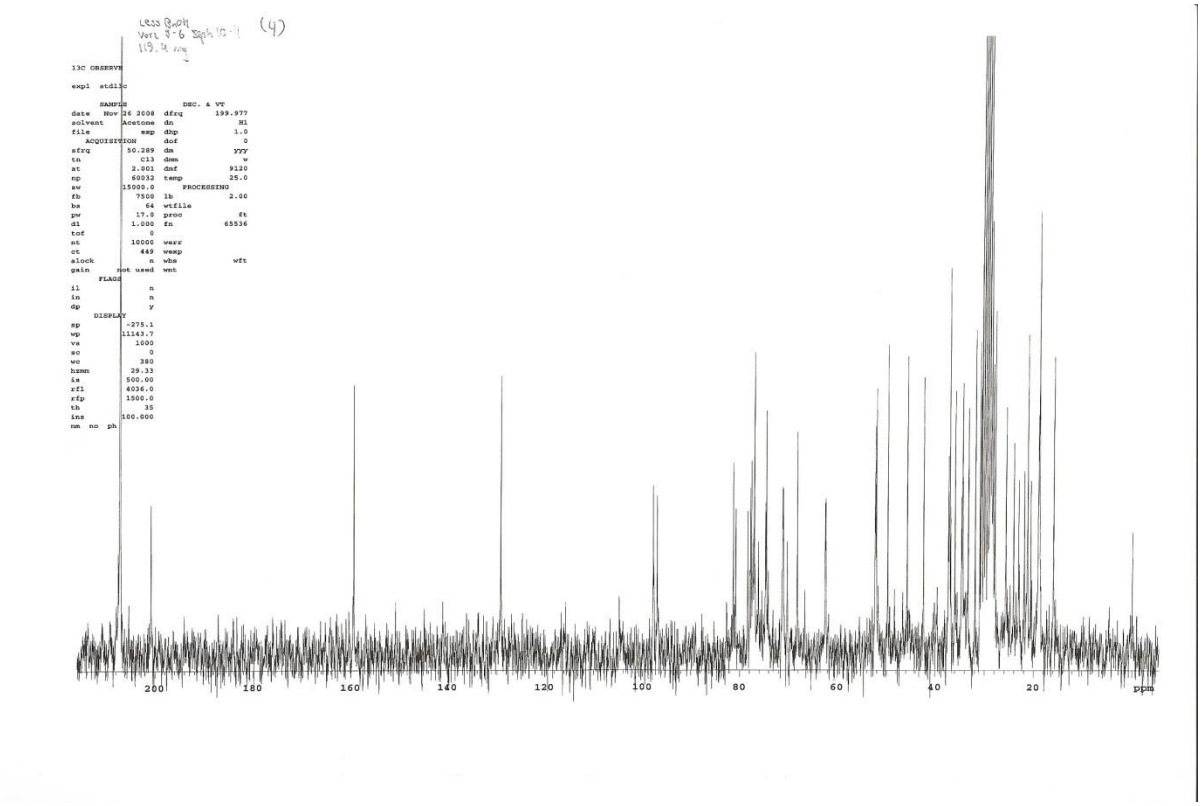
NMR spectra 14.115: 1H-NMR spectra of fraction B5-6 S.3.



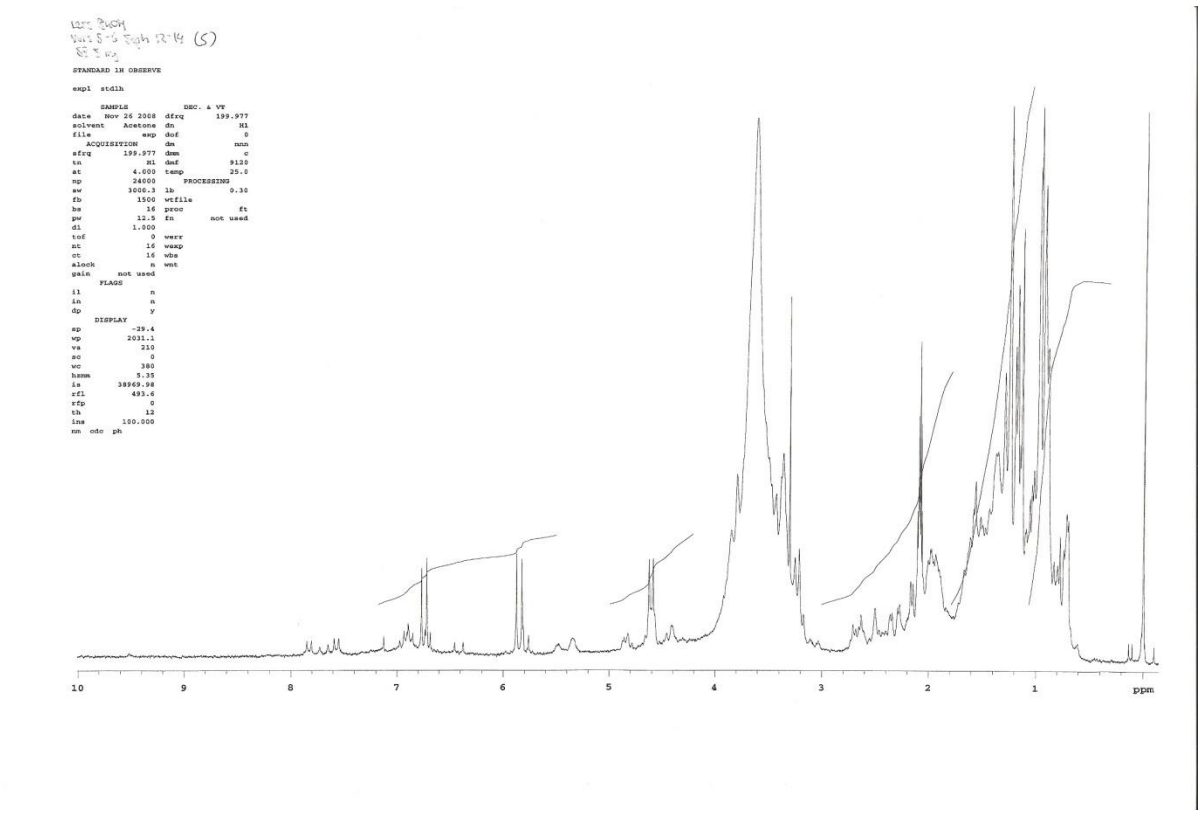
NMR spectra 14.116: 13C-NMR spectra of fraction B5-6 S.3.



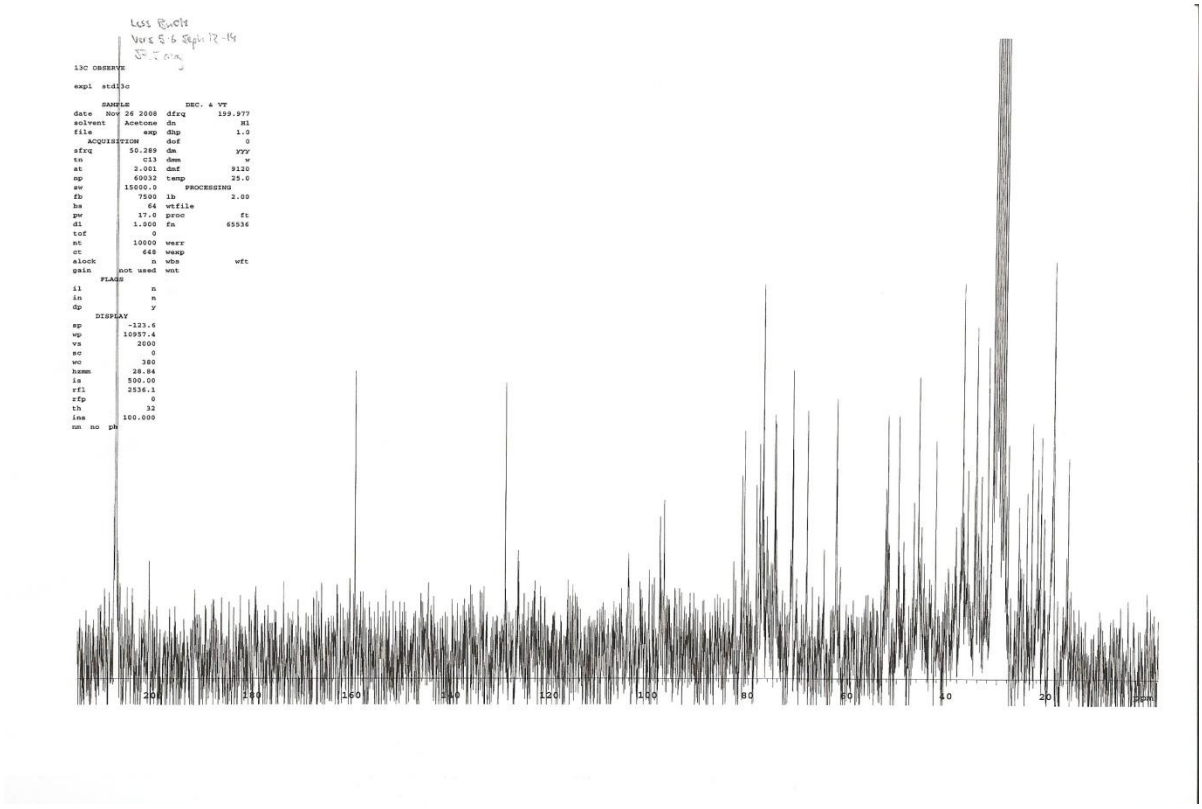
NMR spectra 14.117: 1H-NMR spectra of fraction B5-6 S.4.



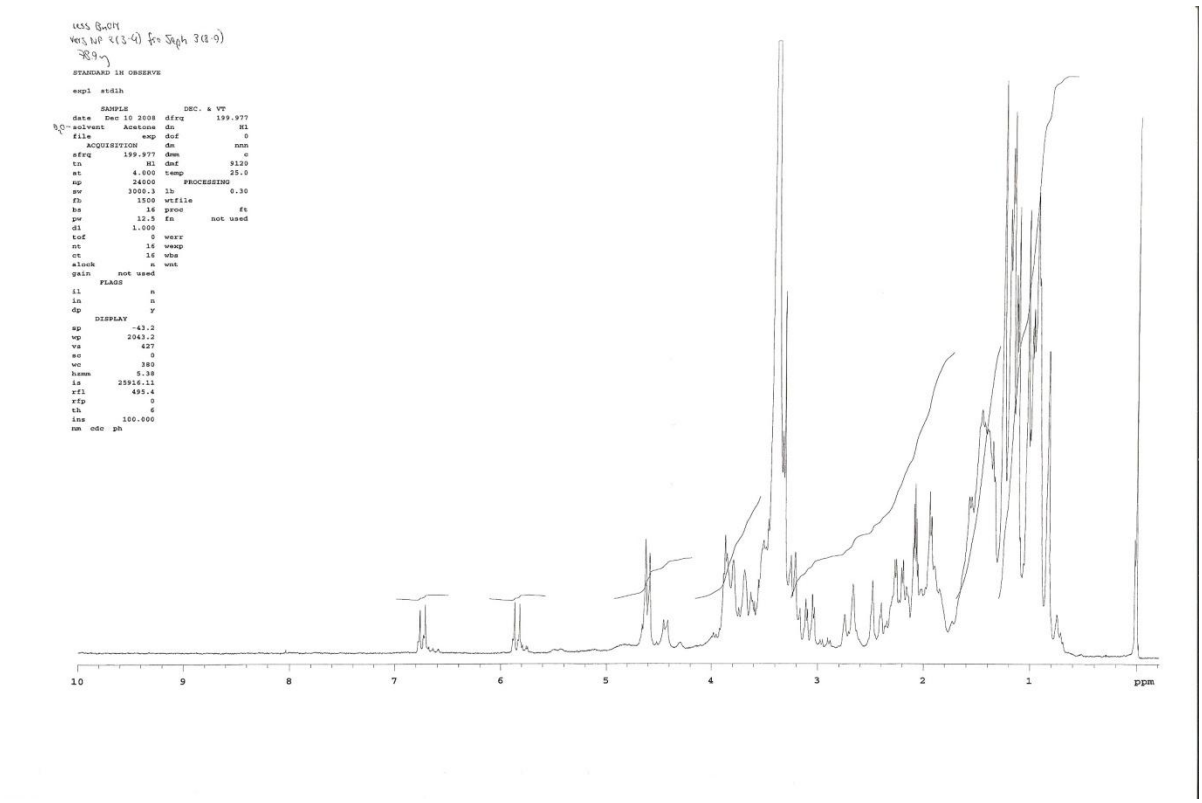
NMR spectra 14.118: 13C-NMR spectra of fraction B5-6 S.4.



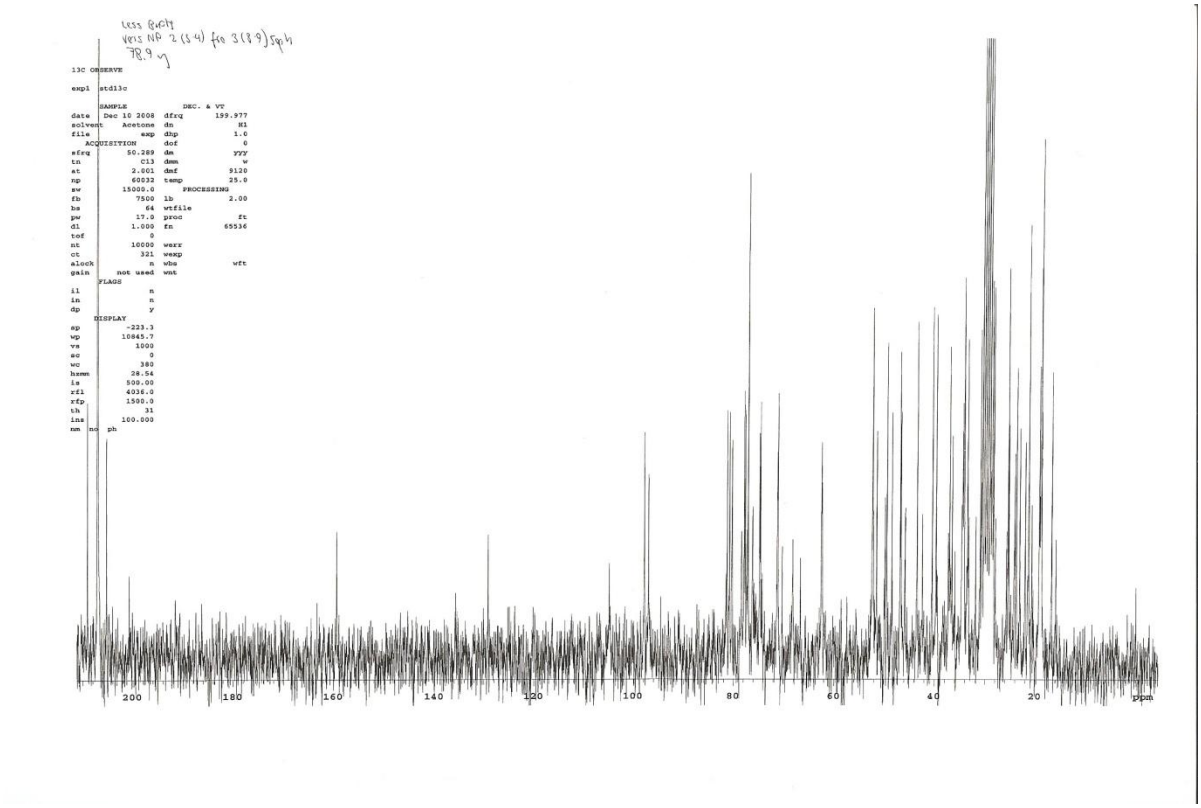
NMR spectra 14.119: 1H-NMR spectra of fraction B5-6 S.5.



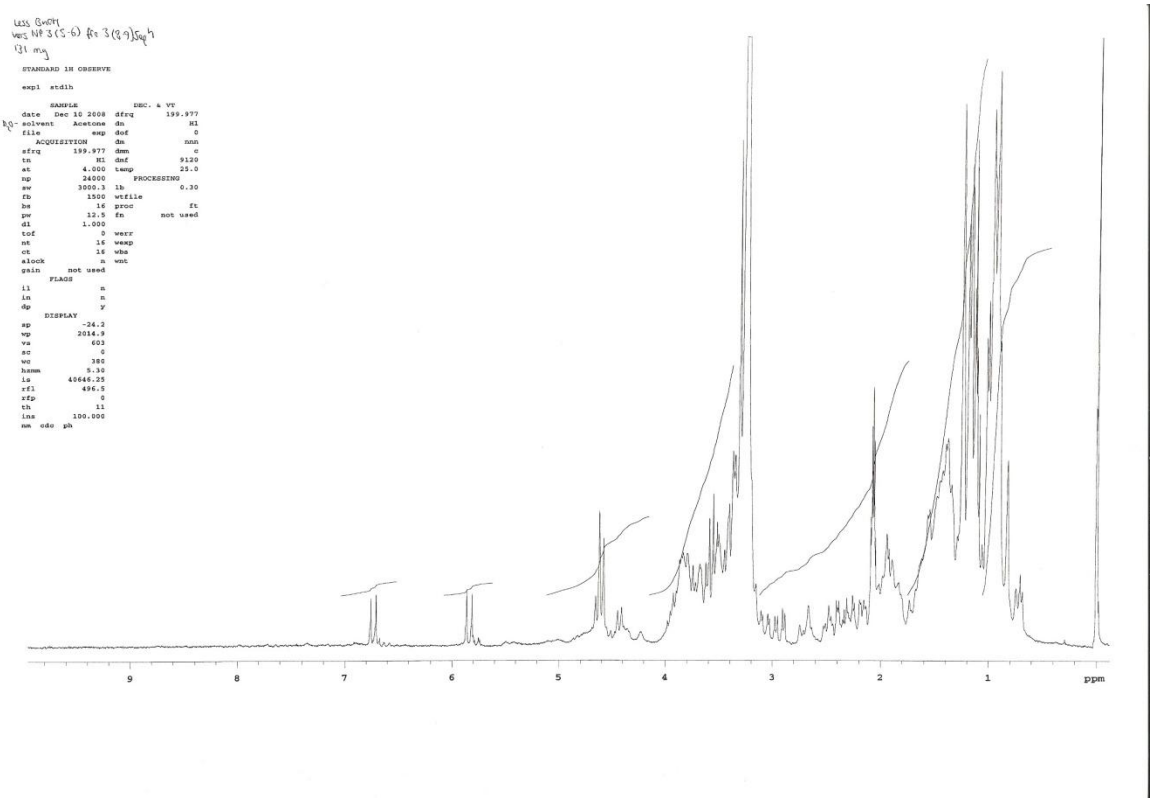
NMR spectra 14.120: 13C-NMR spectra of fraction B5-6 S.5.



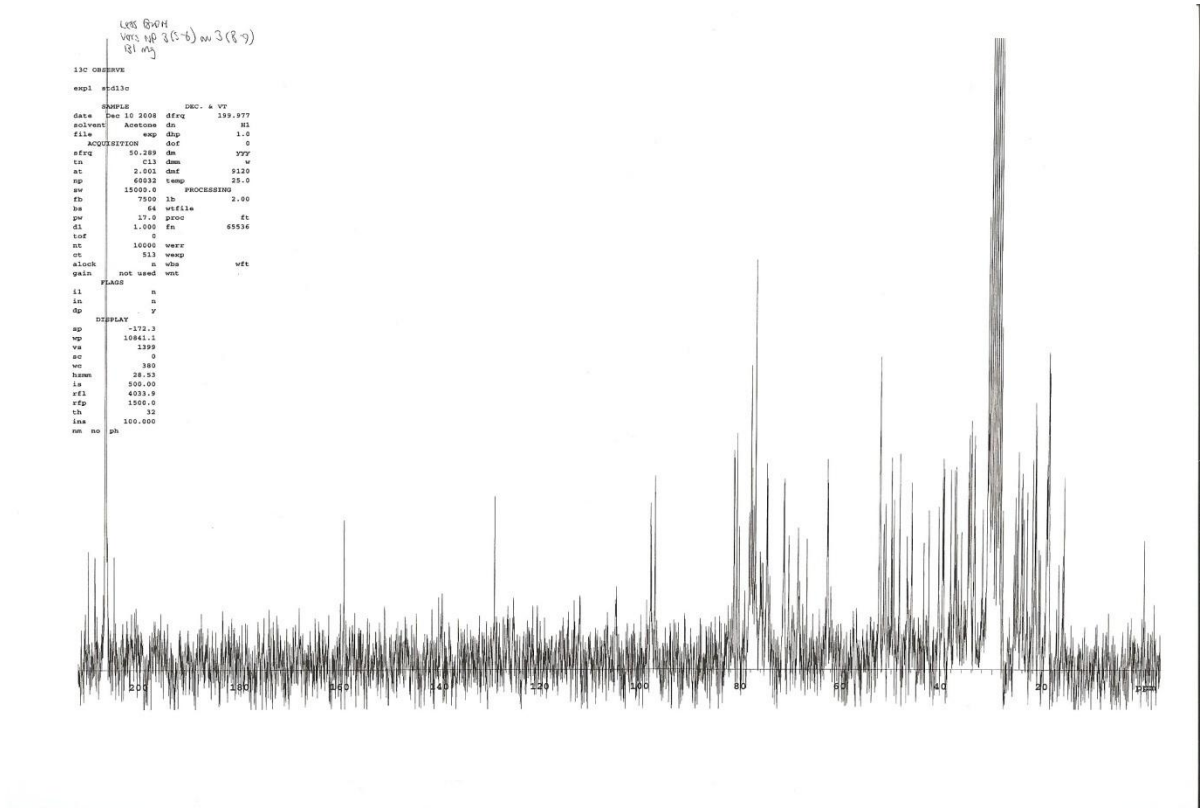
NMR spectra 14.121: 1H-NMR spectra of fraction B5-6 S.3 VNP 2.



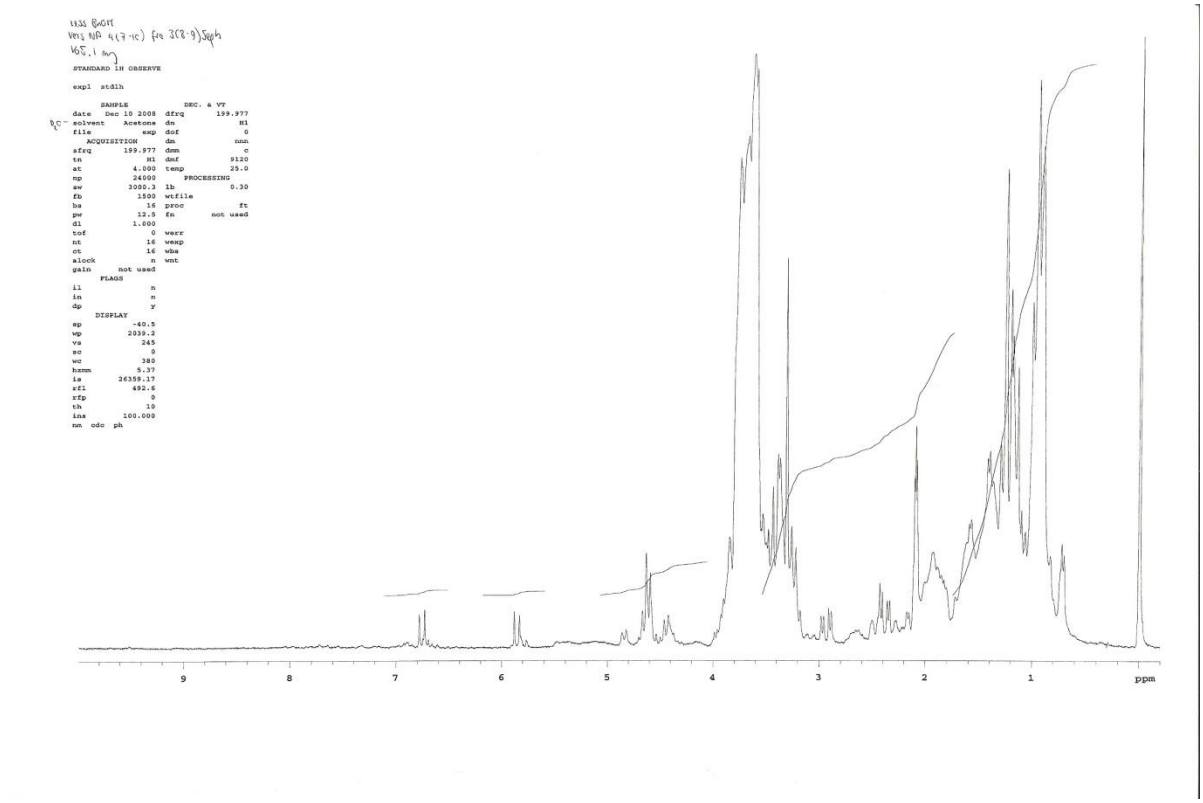
NMR spectra 14.122: 13C-NMR spectra of fraction B5-6 S.3 VNP 2.



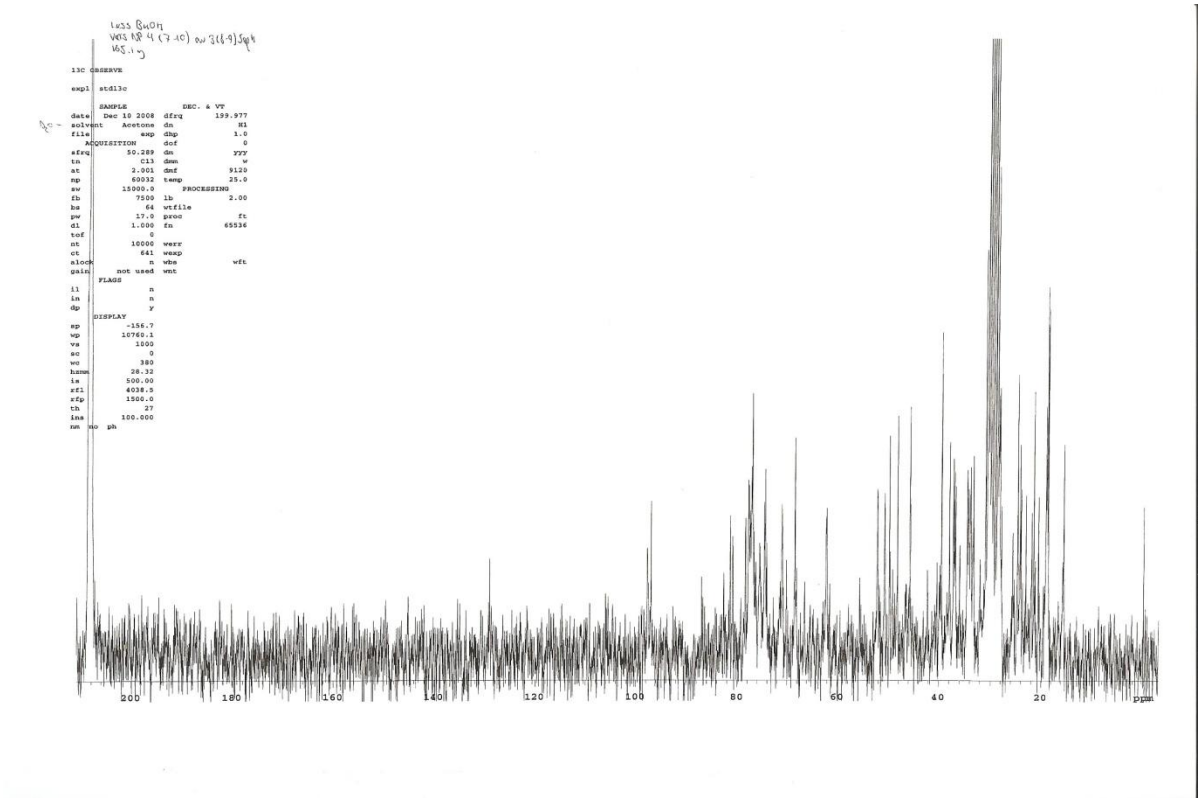
NMR spectra 14.123: 1H-NMR spectra of fraction B5-6 S.3 VNP 3.



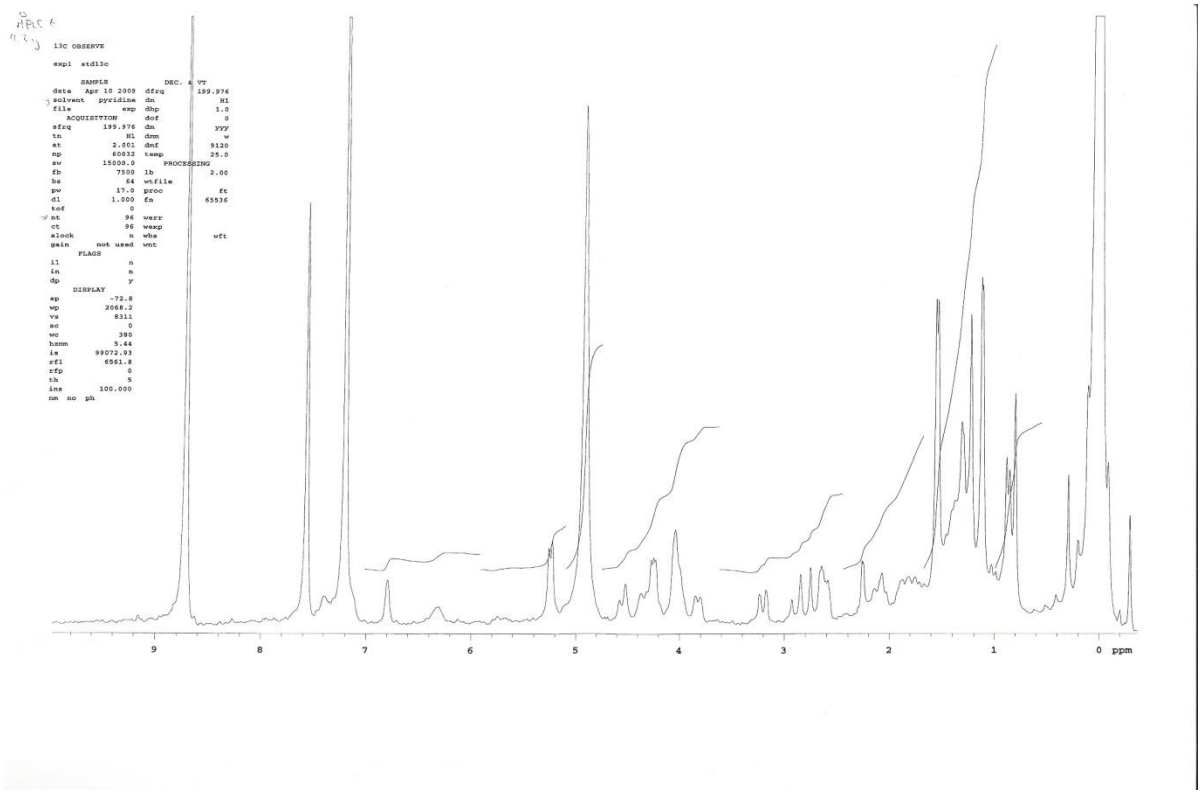
NMR spectra 14.124: 13C-NMR spectra of fraction B5-6 S.3 VNP 3.



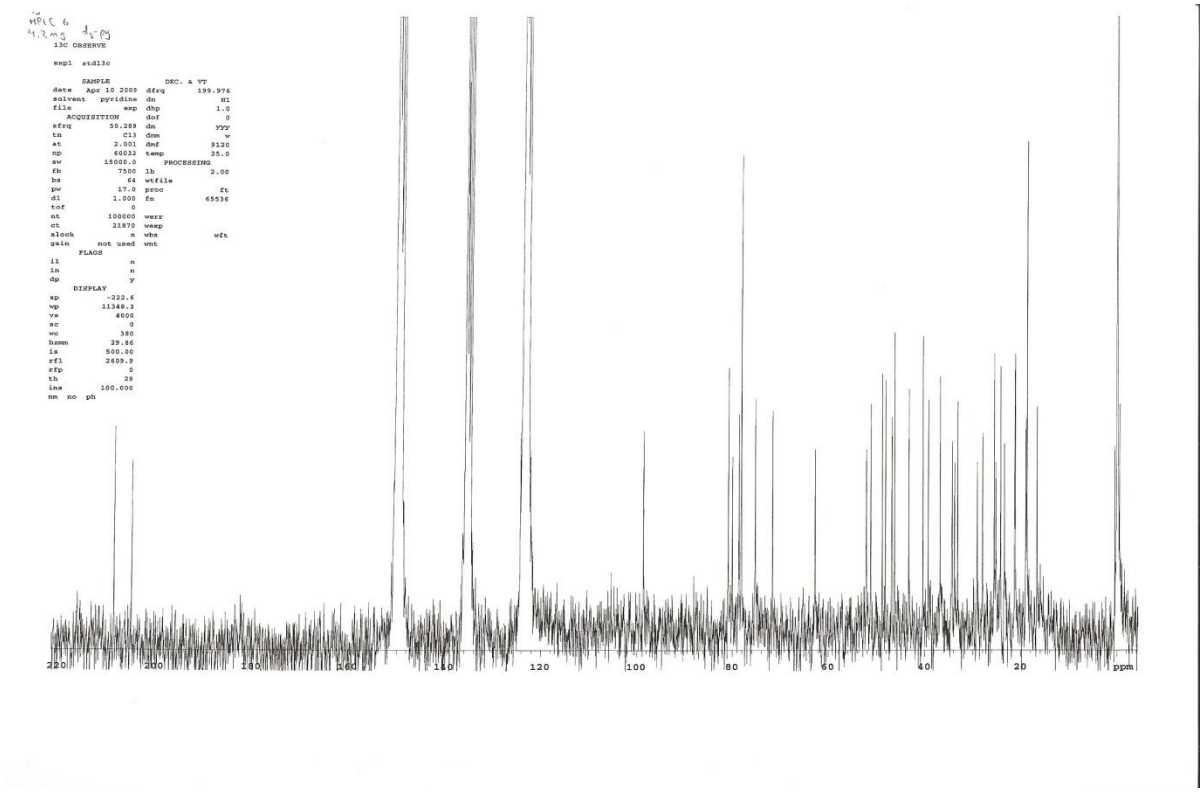
NMR spectra 14.125: 1H-NMR spectra of fraction B5-6 S.3 VNP 4.



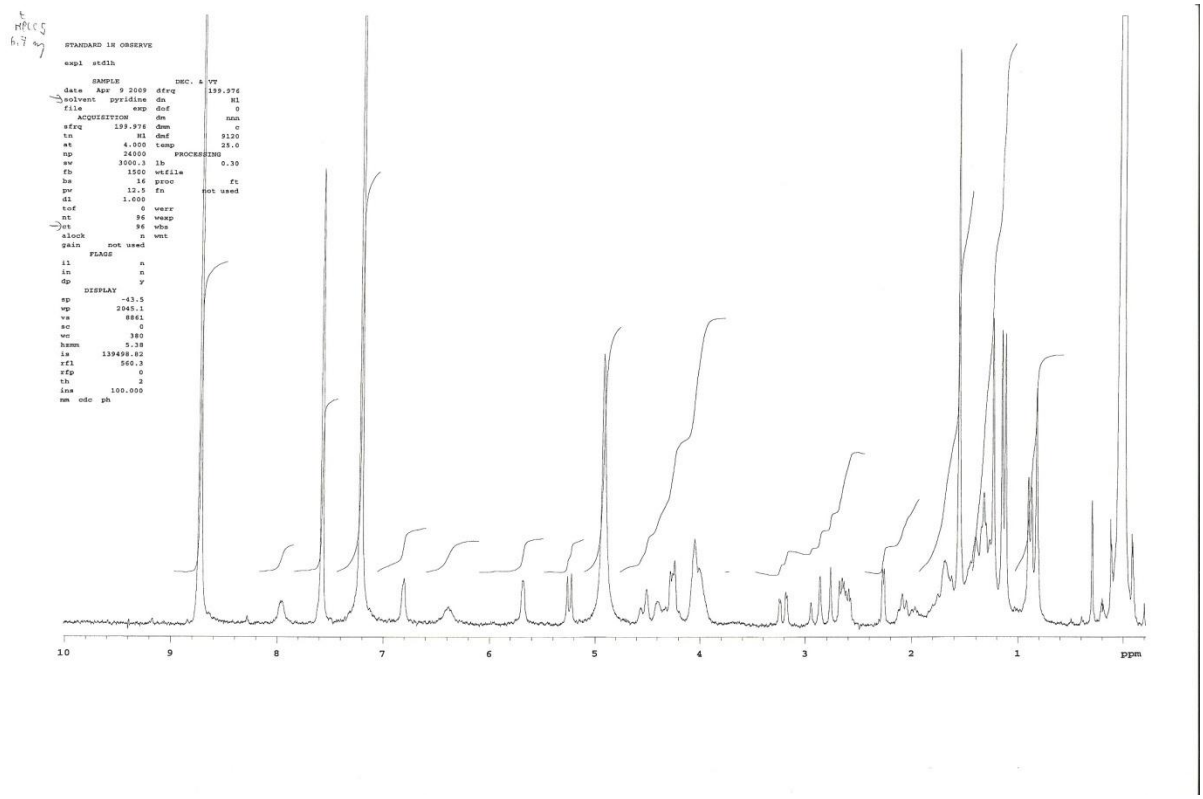
NMR spectra 14.126: 13C-NMR spectra of fraction B5-6 S.3 VNP 4.



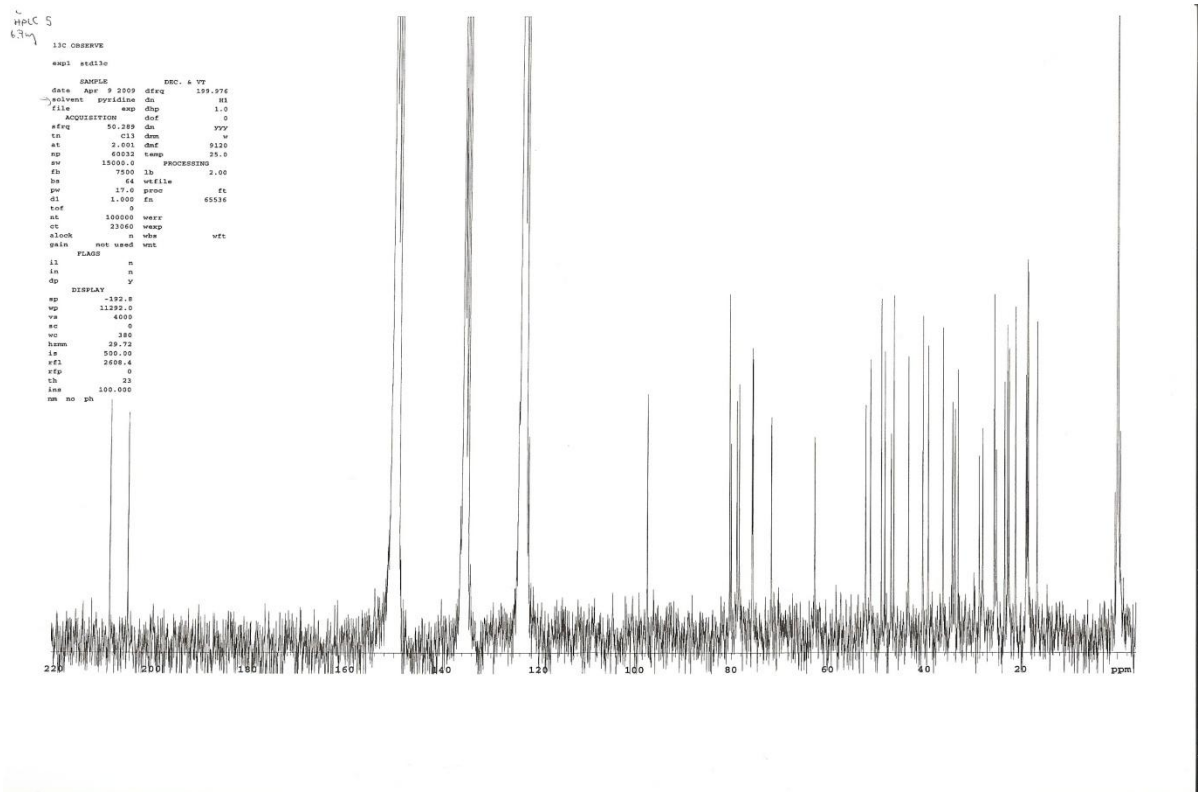
NMR spectra 14.127: 1H-NMR spectra of fraction B HPLC 6.



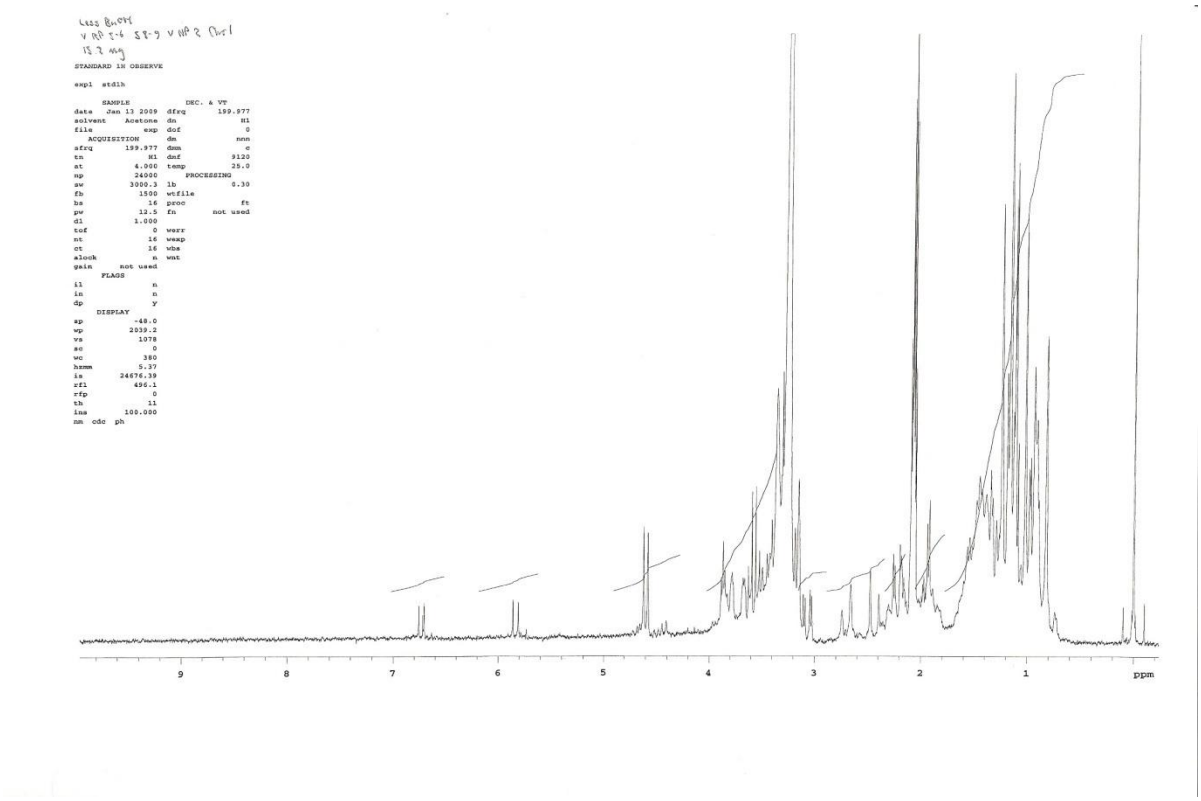
NMR spectra 14.128: 13C-NMR spectra of fraction B HPLC 6.



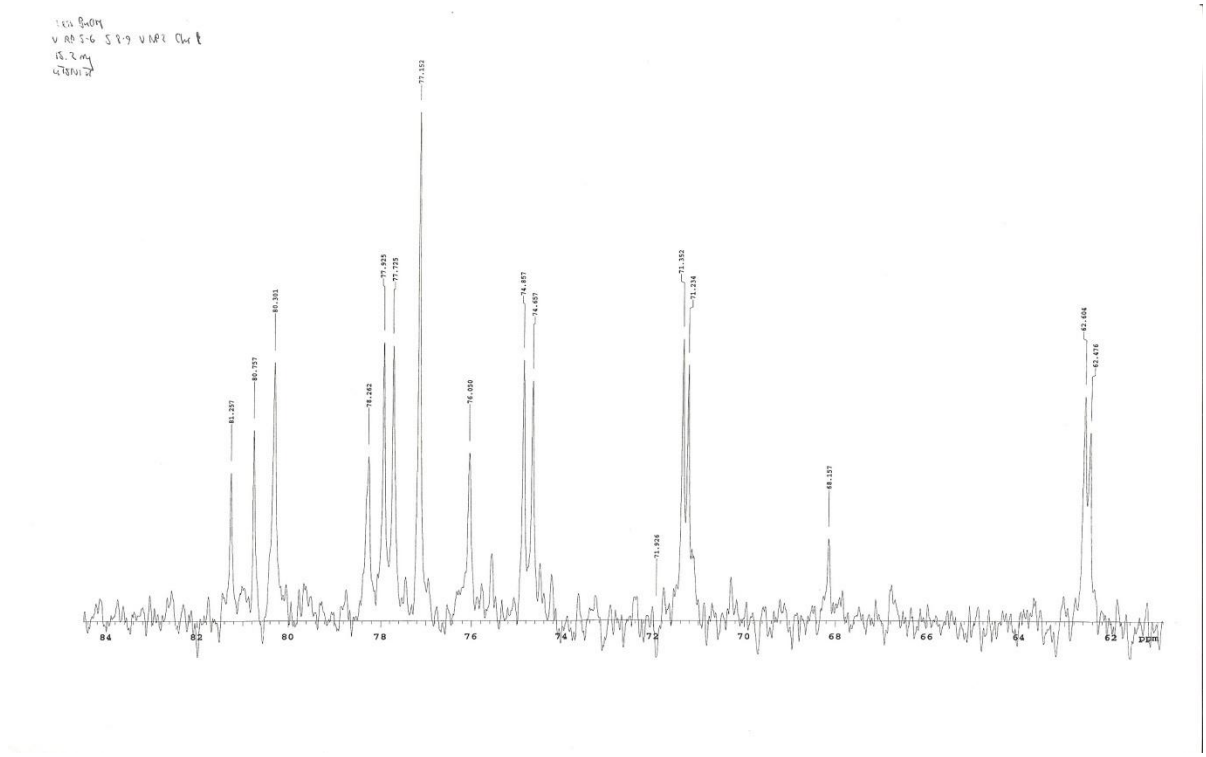
NMR spectra 14.129: 1H-NMR spectra of fraction E HPLC 5.



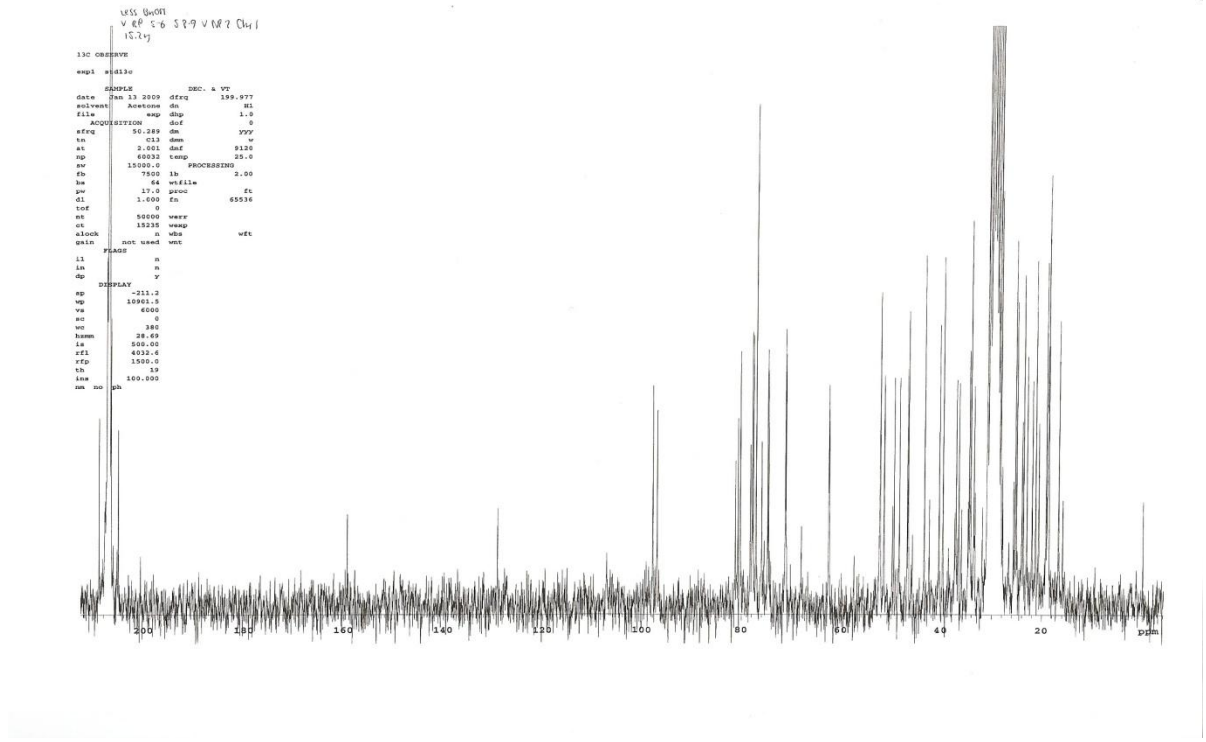
NMR spectra 14.130: 13C-NMR spectra of fraction E HPLC 5.



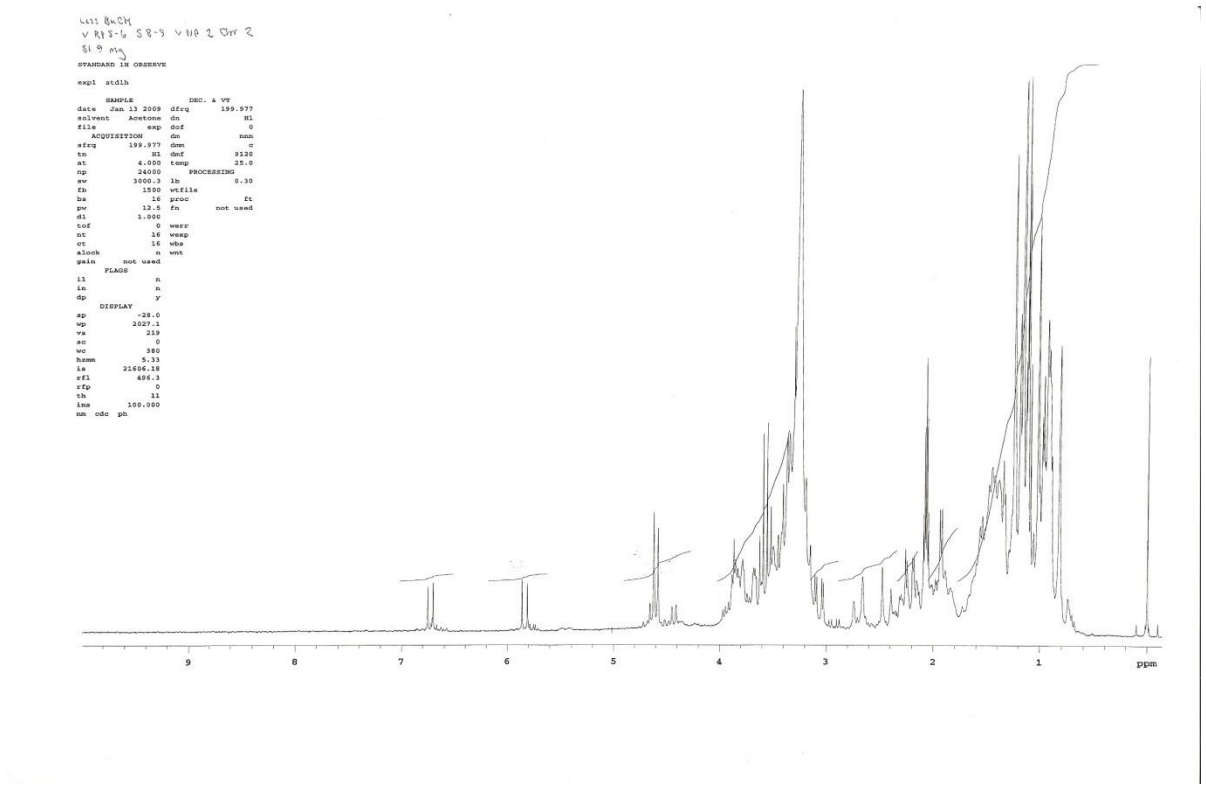
NMR spectra 14.131: 1H-NMR spectra of fraction B5-6 S.3 VNP 2 CHR 1.



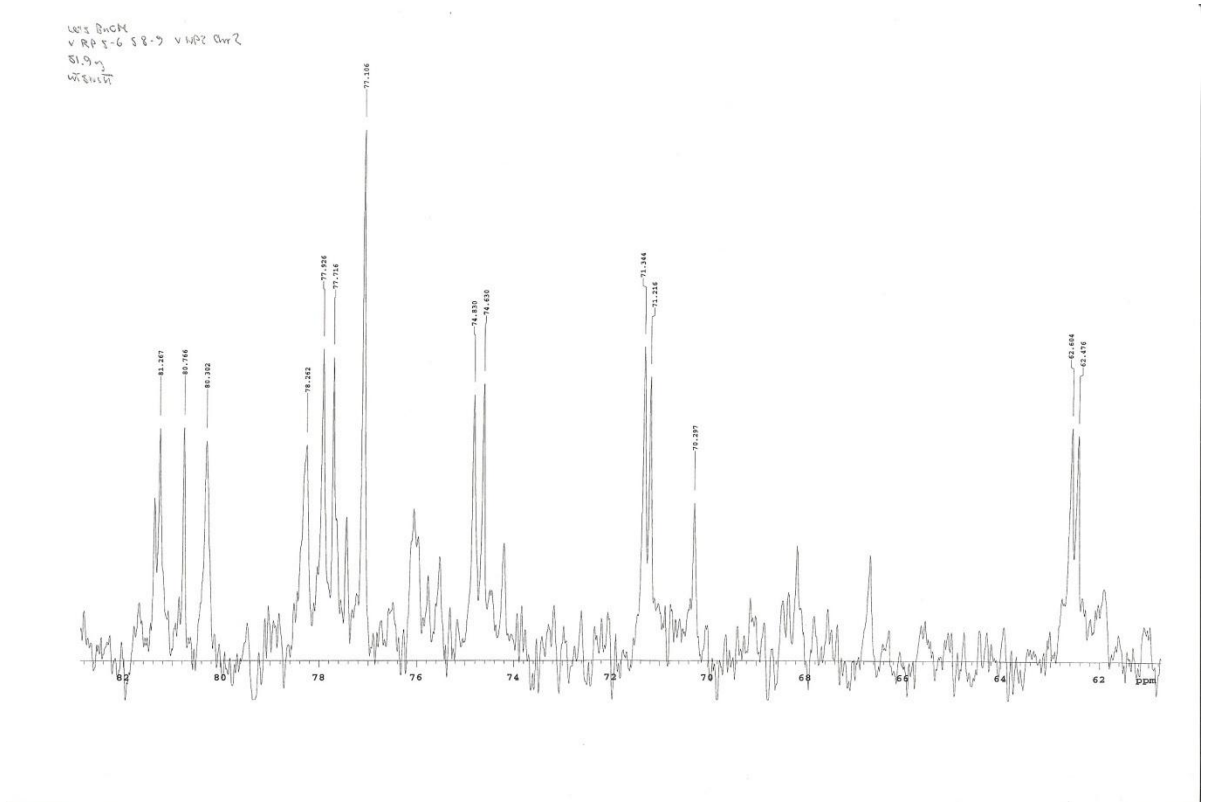
NMR spectra 14.132: partly from 1H-NMR spectra of fraction B5-6 S.3 VNP 2 CHR 1.



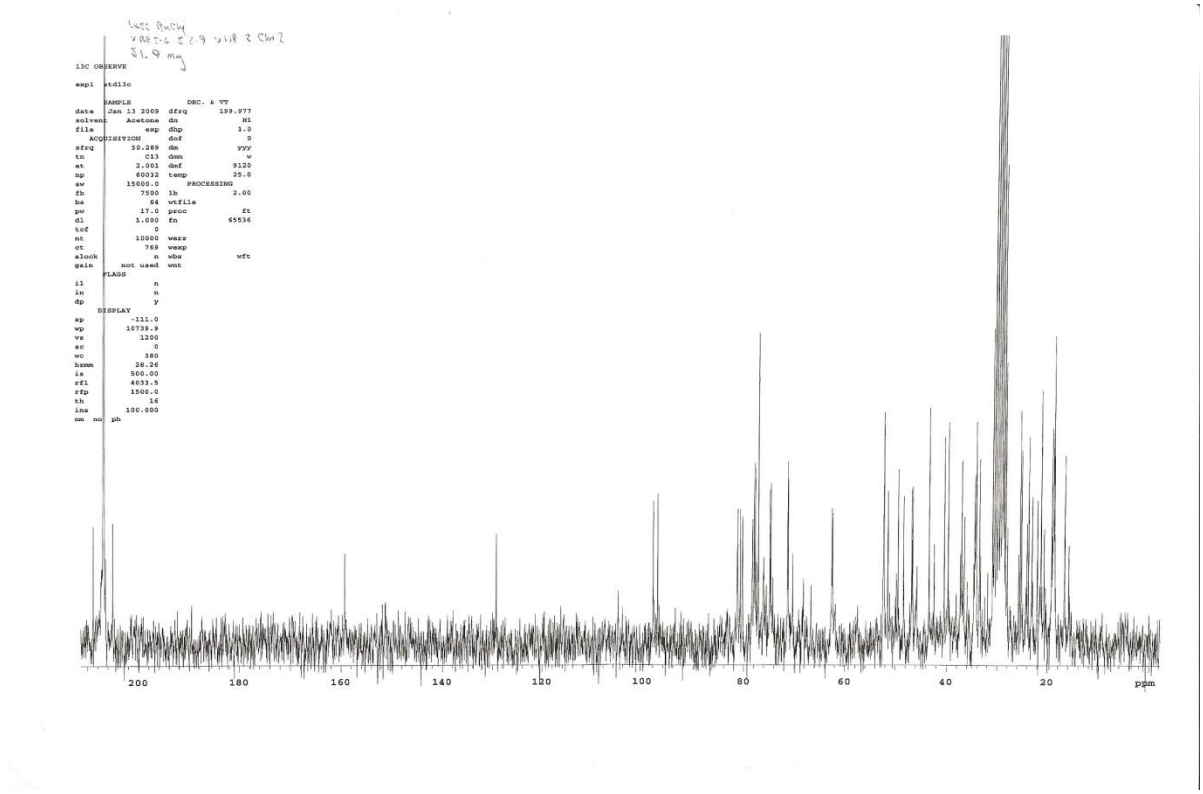
NMR spectra 14.133: 13C-NMR spectra of fraction B5-6 S.3 VNP 2 CHR 1.



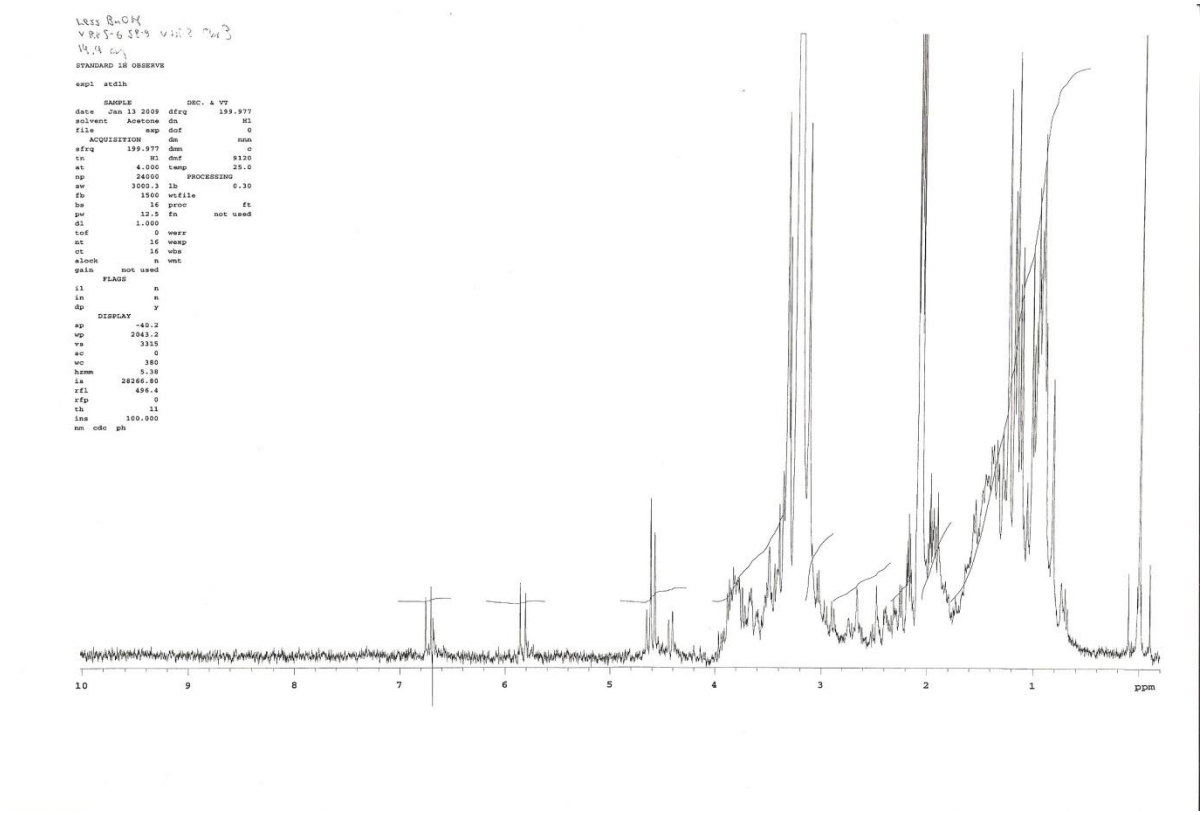
NMR spectra 14.134: 1H-NMR spectra of fraction B5-6 S.3 VNP 2 CHR 2.



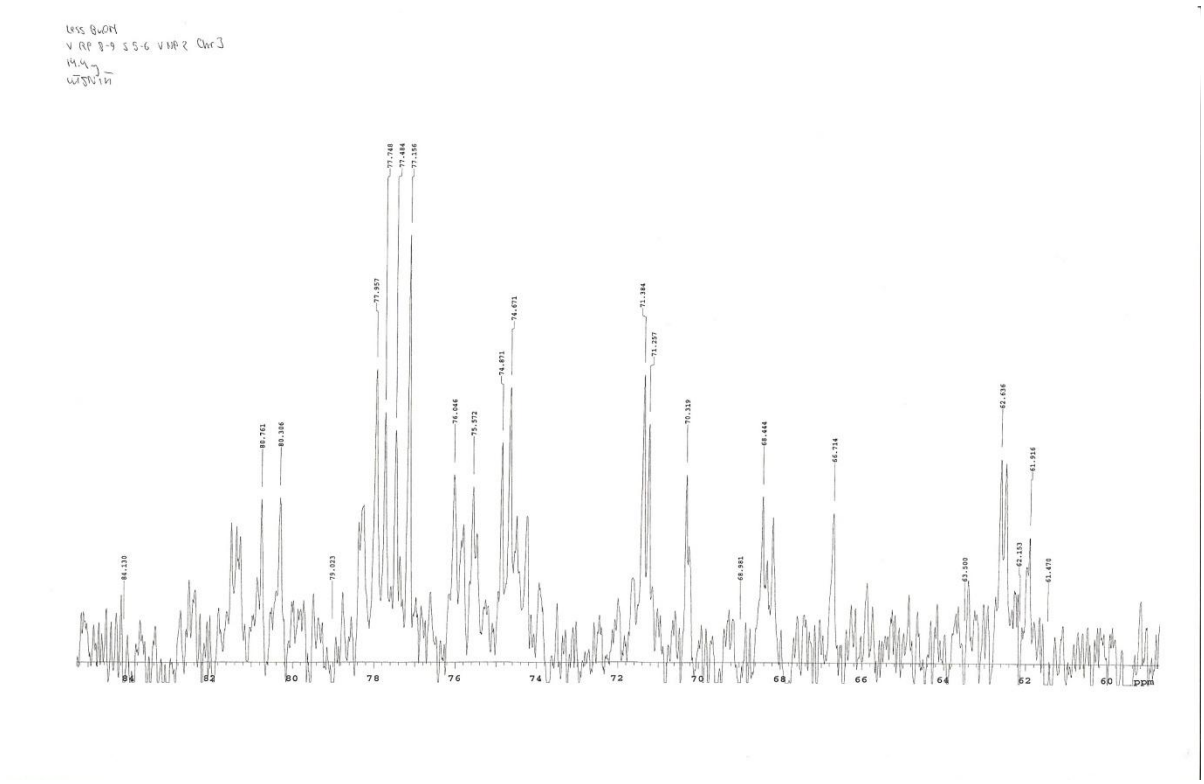
NMR spectra 14.135: partly from 1H-NMR spectra of fraction B5-6 S.3 VNP 2 CHR 2.



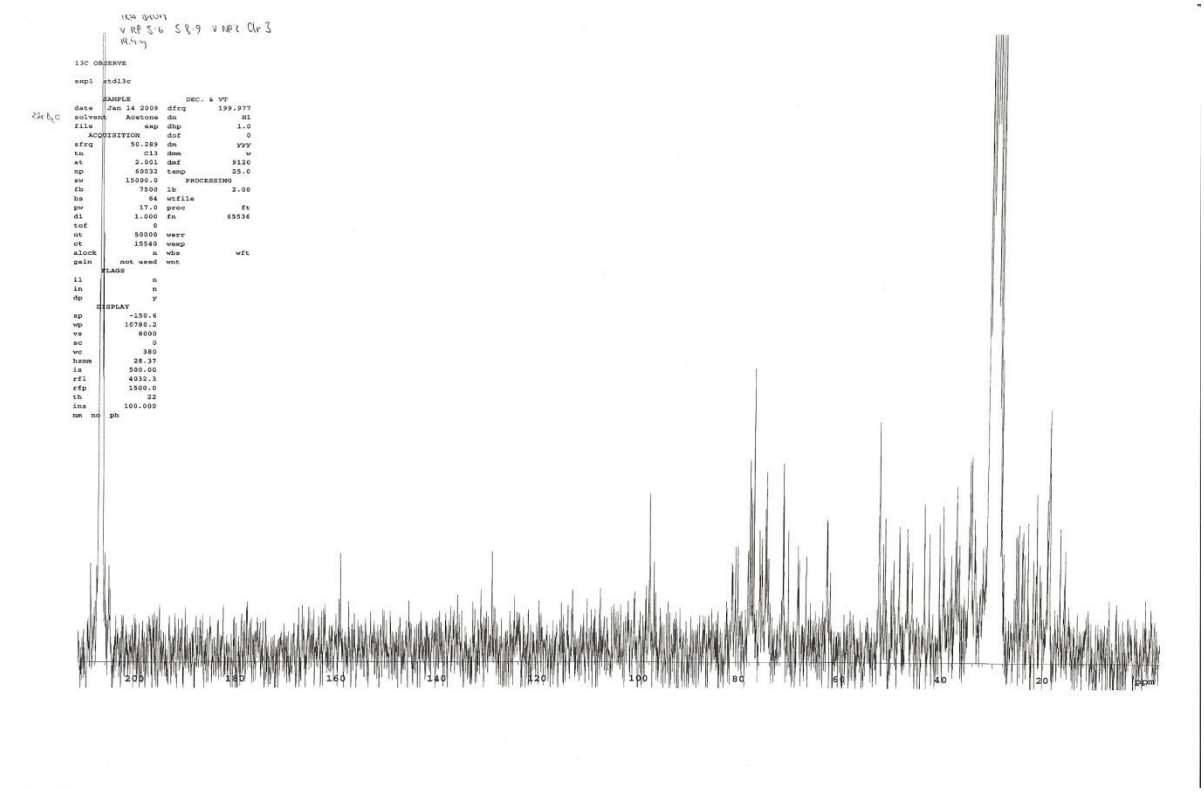
NMR spectra 14.136: 13C-NMR spectra of fraction B5-6 S.3 VNP 2 CHR 2.



NMR spectra 14.137: 1H-NMR spectra of fraction B5-6 S.3 VNP 2 CHR 3.

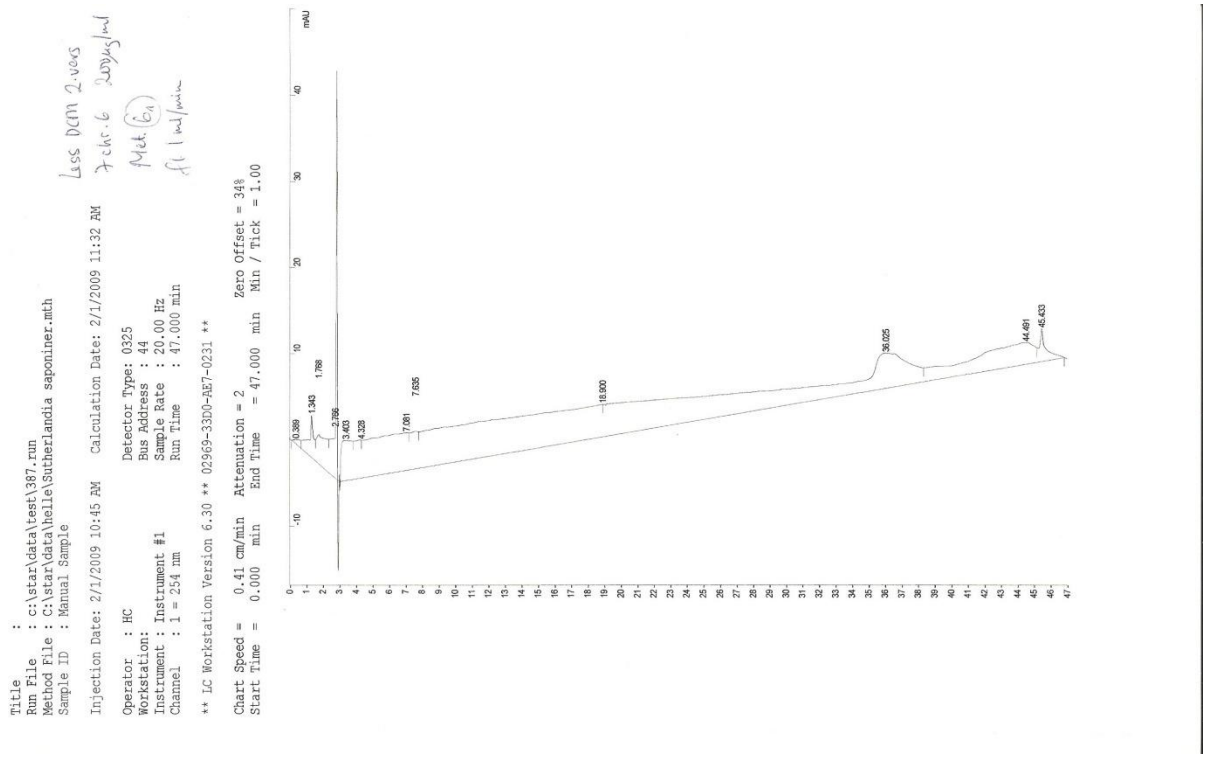


NMR spectra 14.138: partly from 1H-NMR spectra of fraction B5-6 S.3 VNP 2 CHR 3.

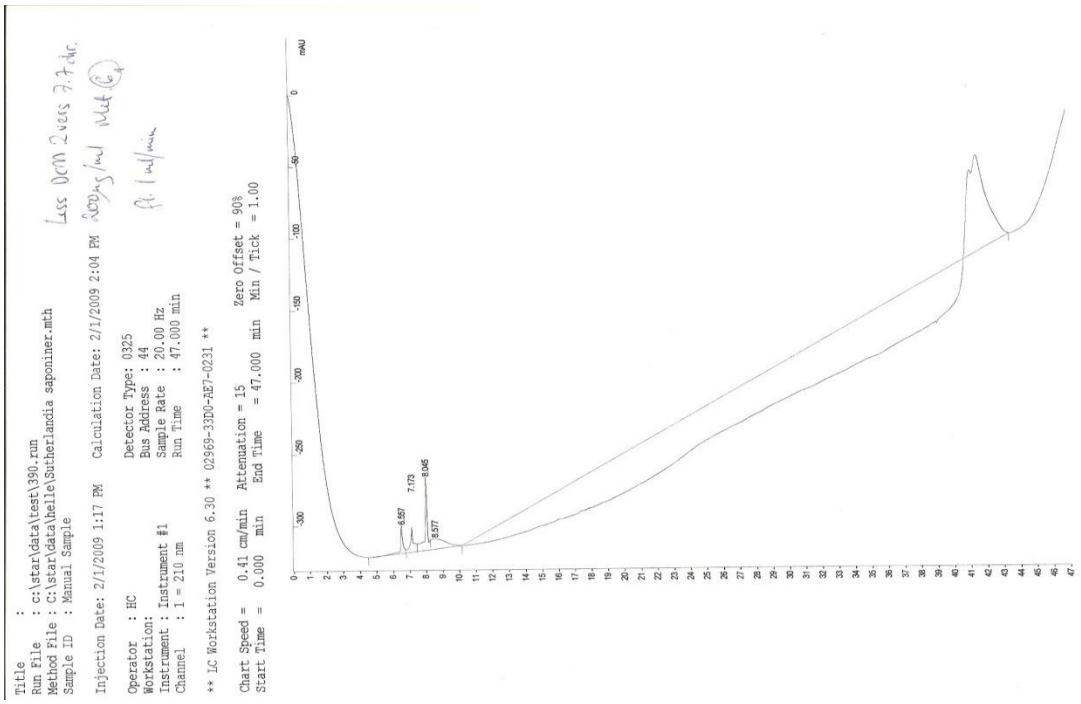


NMR spectra 14.139: 13C-NMR spectra of fraction B5-6 S.3 VNP 2 CHR 3.

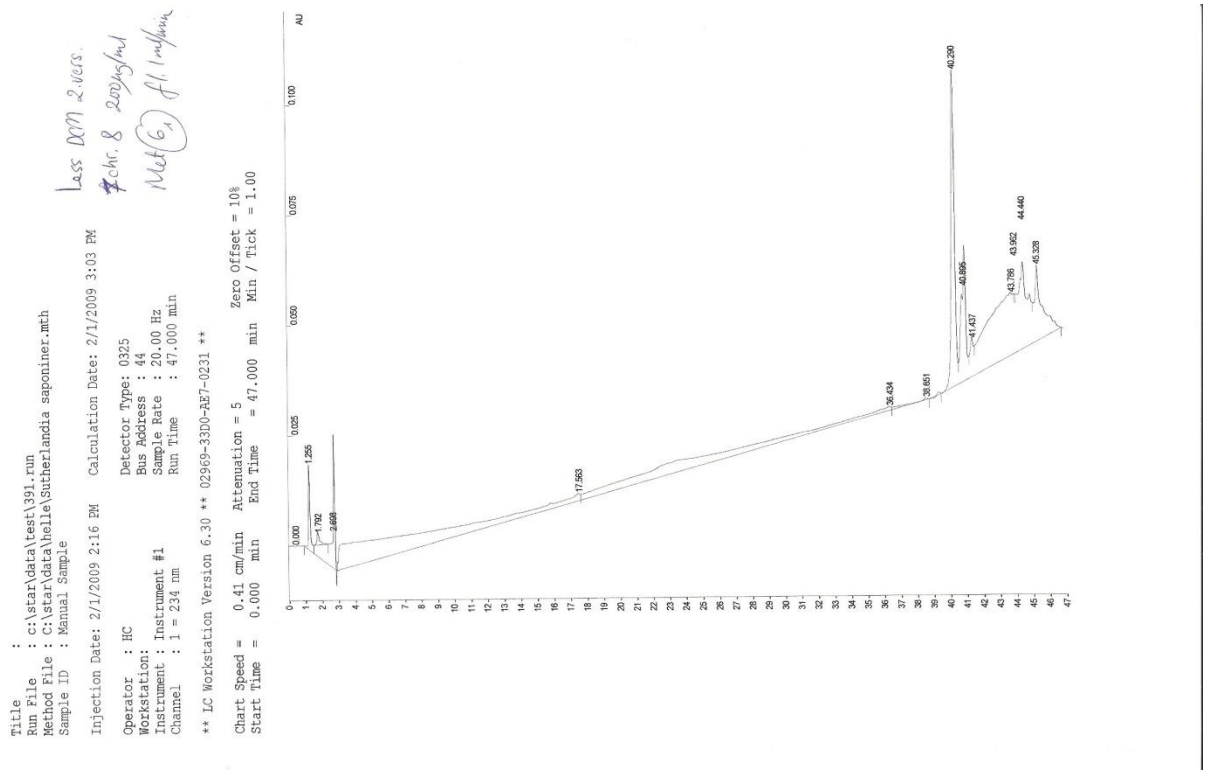
15. Chromatograms



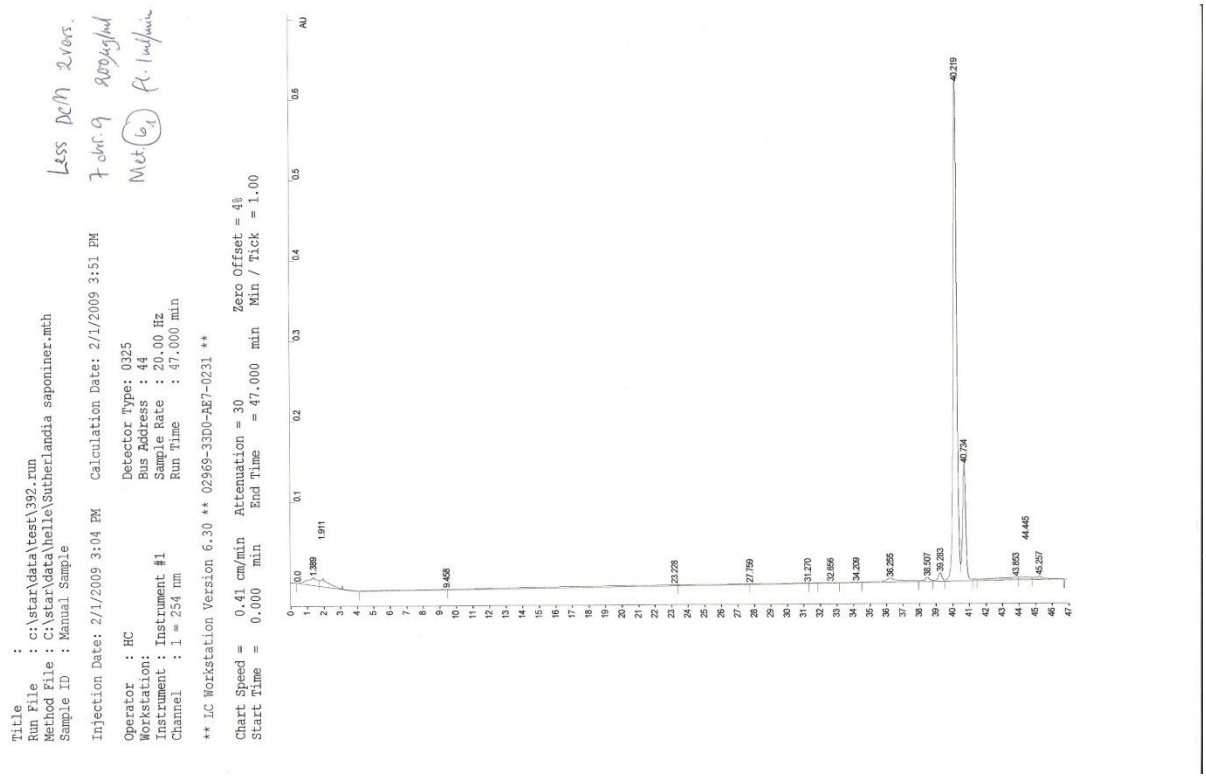
Chromatogram 15.1. Preparative HPLC chromatogram of fraction D2.7.6.



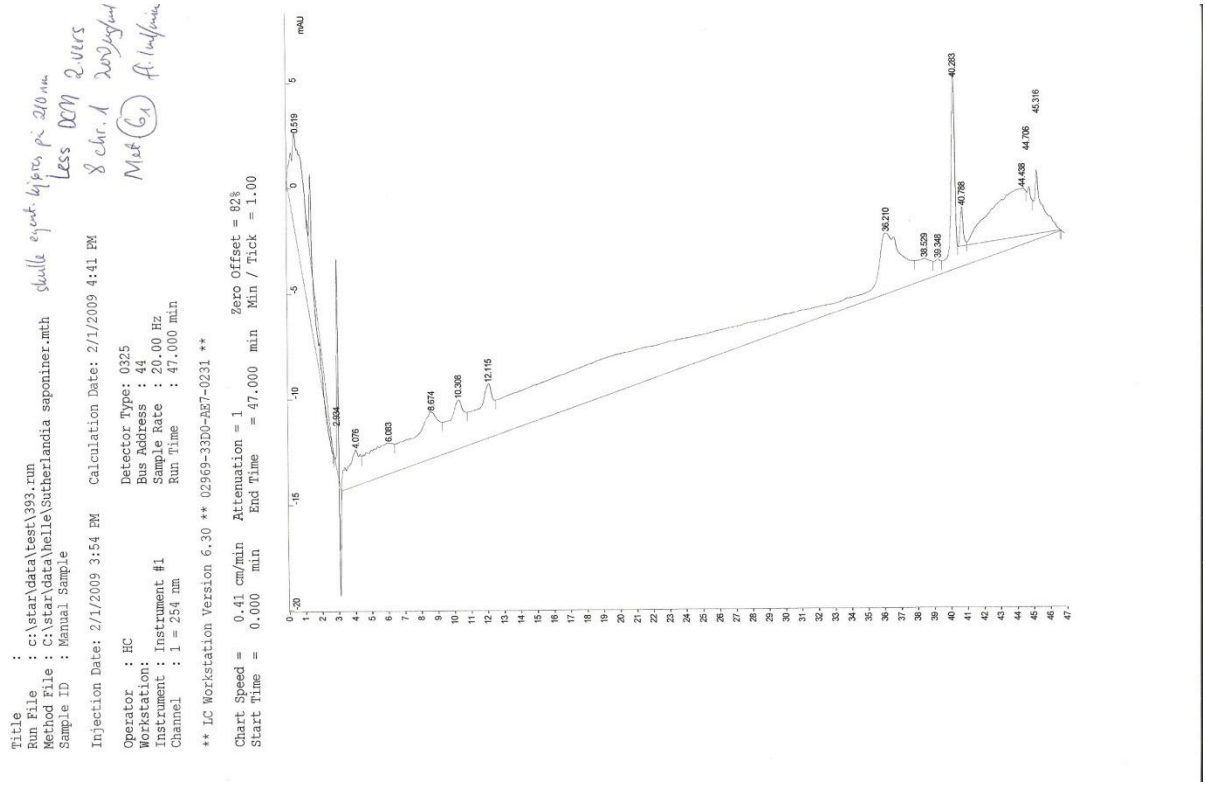
Chromatogram 15.2. Preparative HPLC chromatogram of fraction D2.7.7.



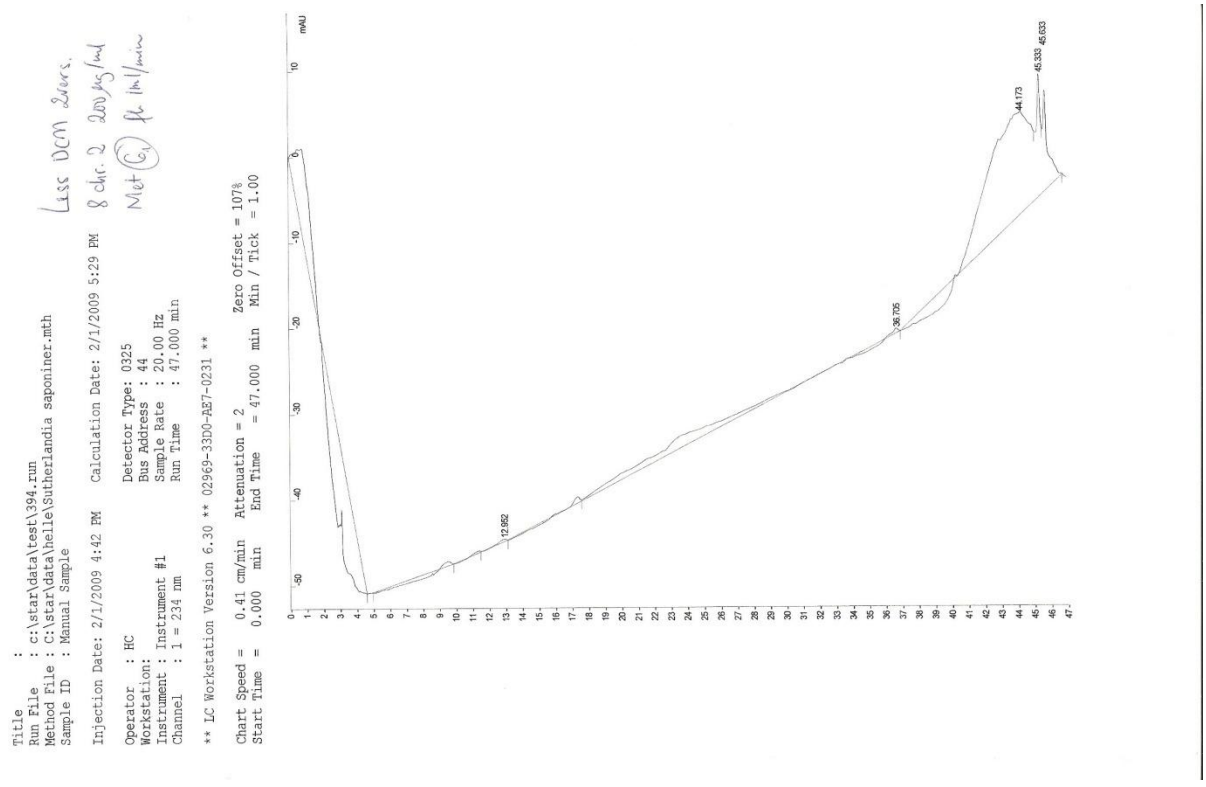
Chromatogram 15.3. Preparative HPLC chromatogram of fraction D2.7.8.



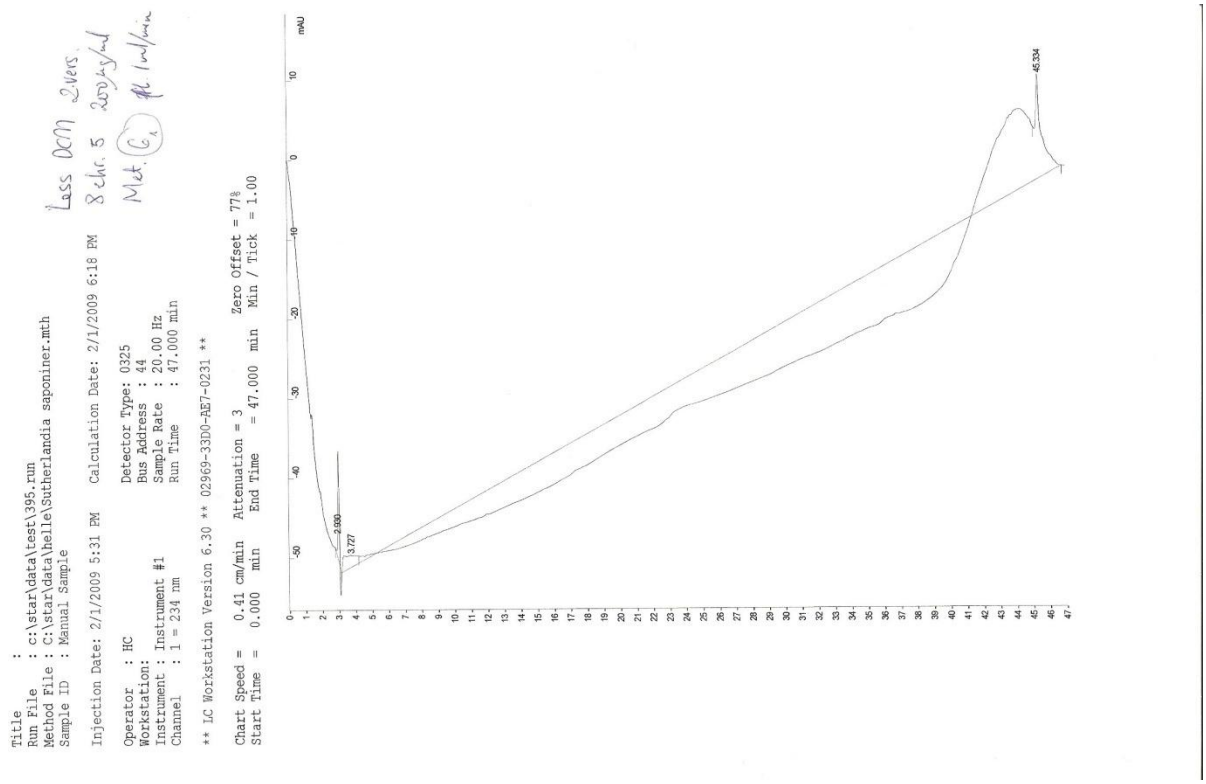
Chromatogram 15.4. Preparative HPLC chromatogram of fraction D2.7.9.



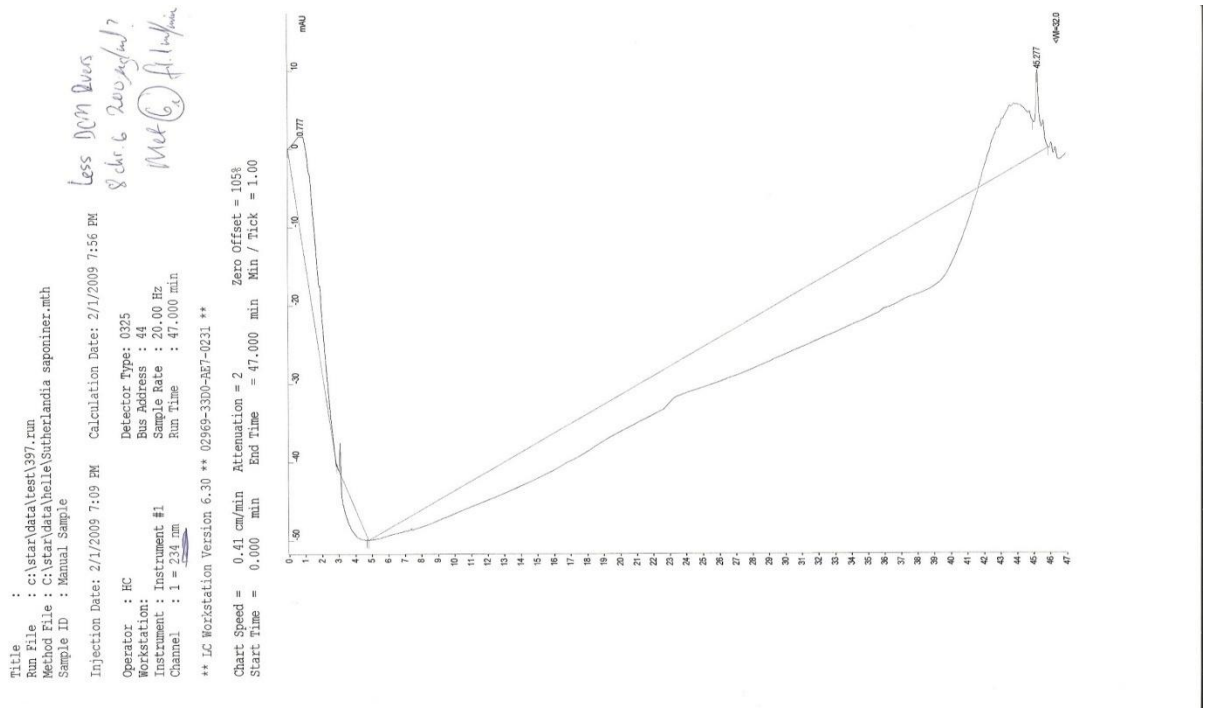
Chromatogram 15.5. Preparative HPLC chromatogram of fraction D2.8.1.



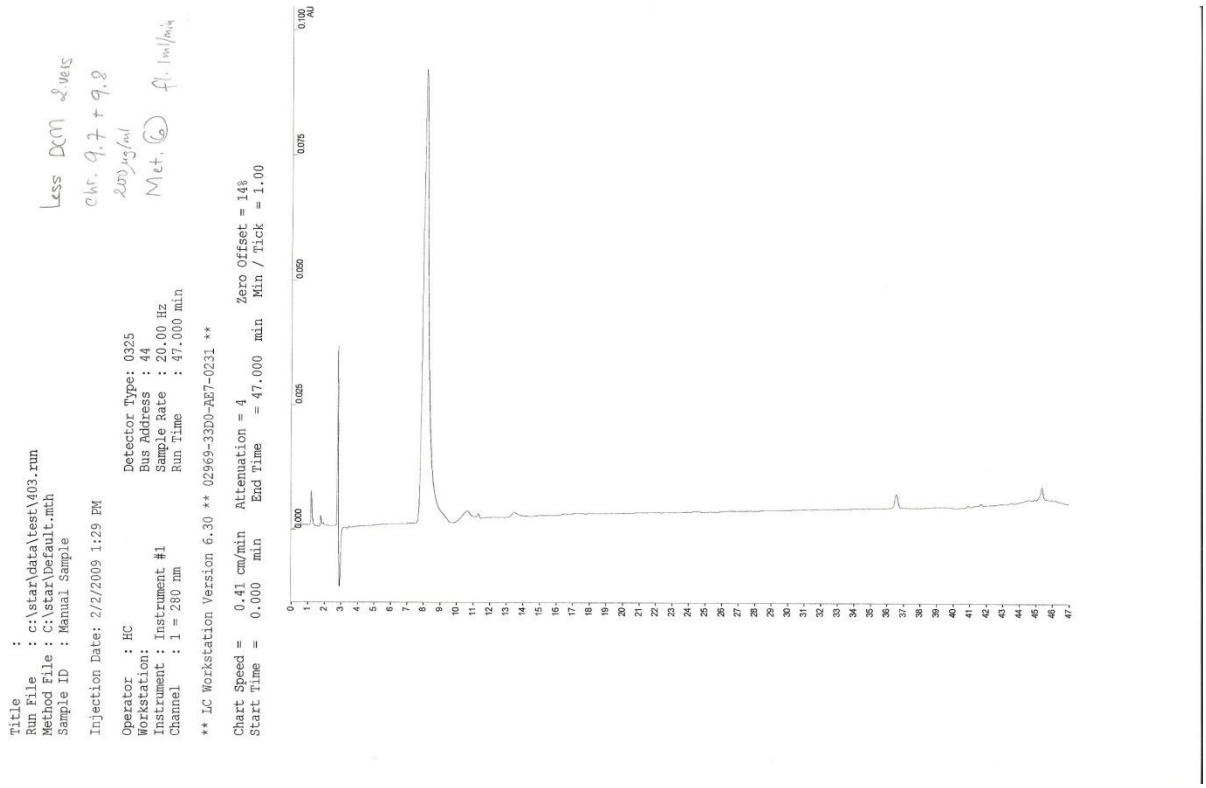
Chromatogram 15.6. Preparative HPLC chromatogram of fraction D2.8.2.



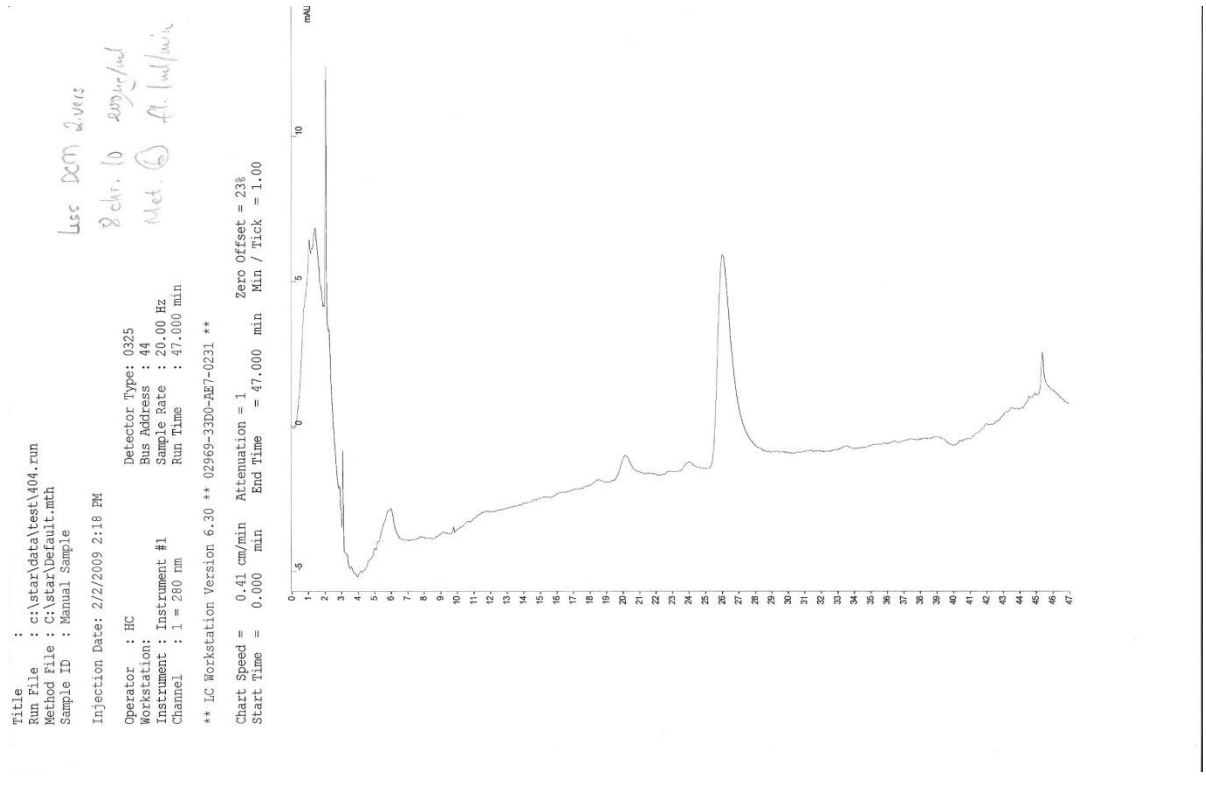
Chromatogram 15.7. Preparative HPLC chromatogram of fraction D2.8.5.



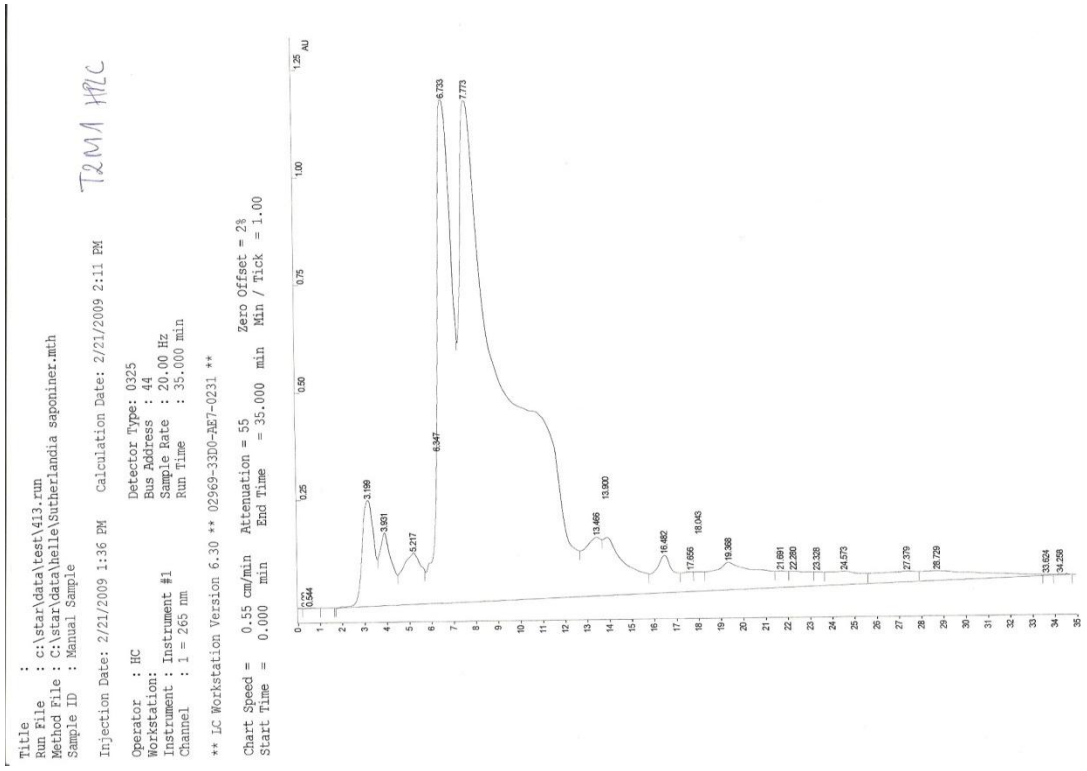
Chromatogram 15.8. Preparative HPLC chromatogram of fraction D2.8.6.



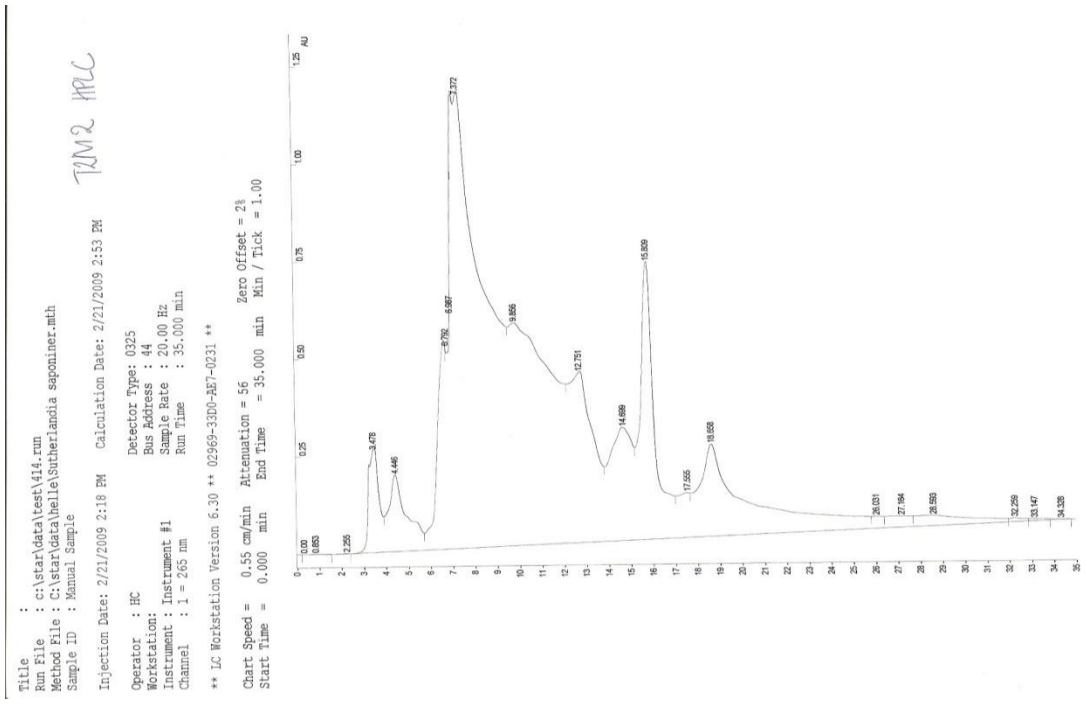
Chromatogram 15.9. Preparative HPLC chromatogram of fraction D2.9.7-8.



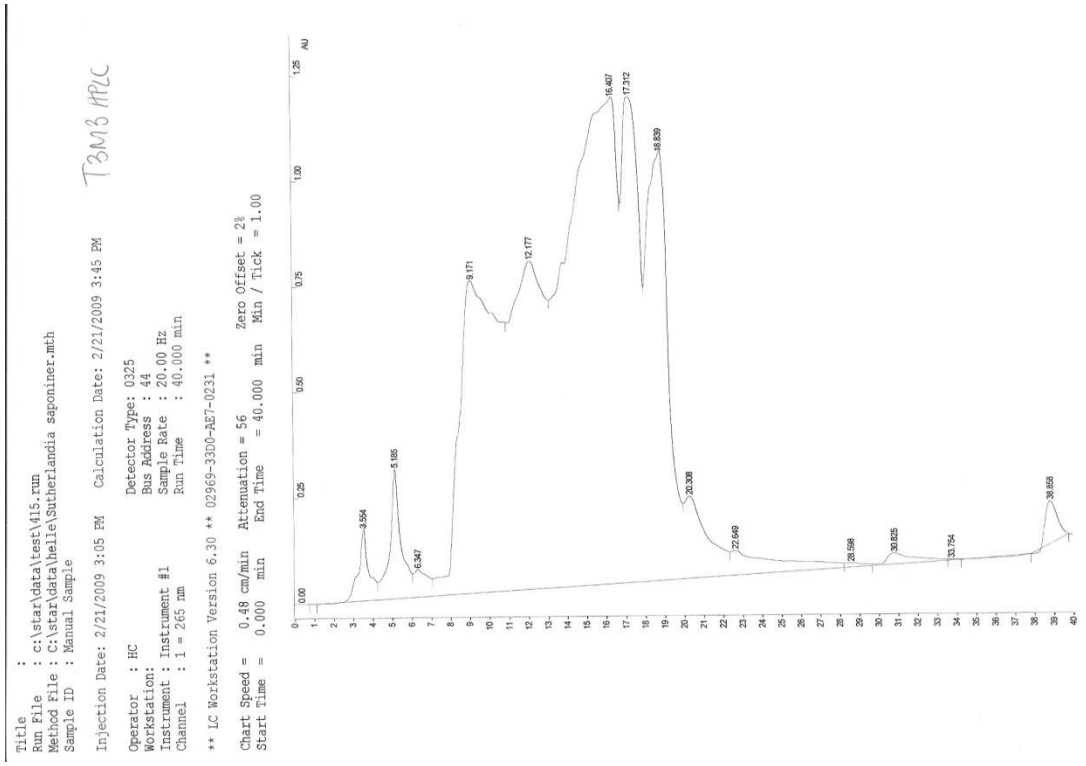
Chromatogram 15.10. Preparative HPLC chromatogram of fraction D2.8.10.



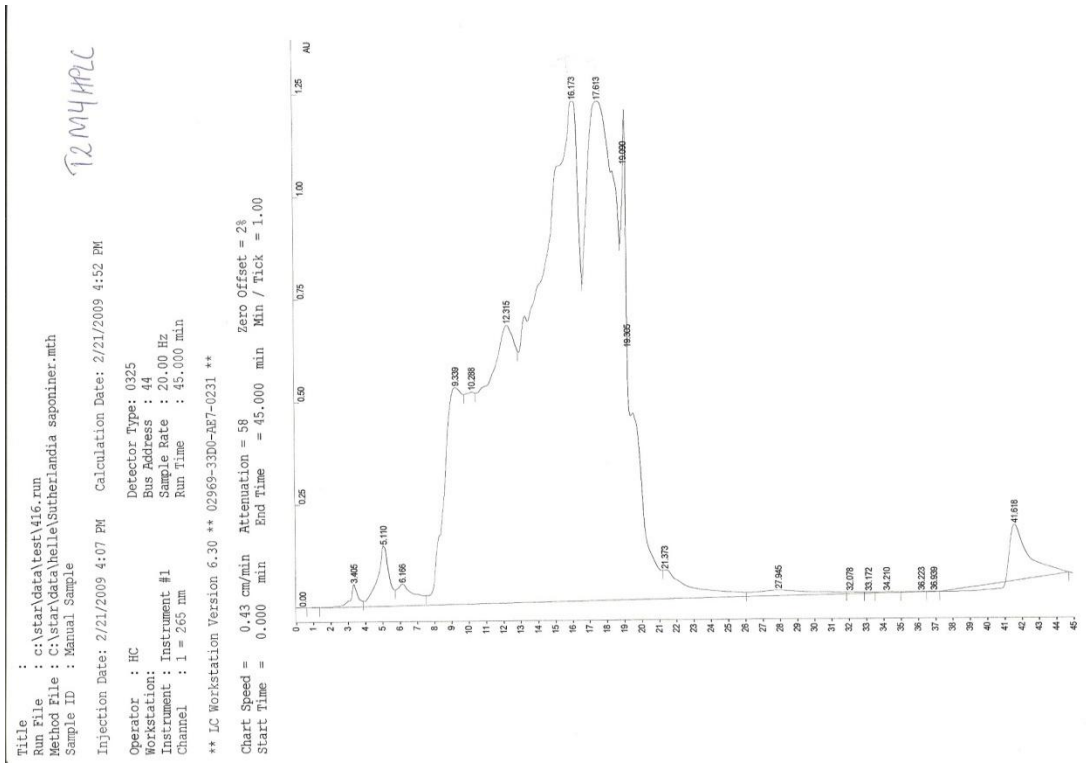
Chromatogram 15.11. Preparative HPLC chromatogram of fraction B1-2 S.4-5 VRP. T2 M1.



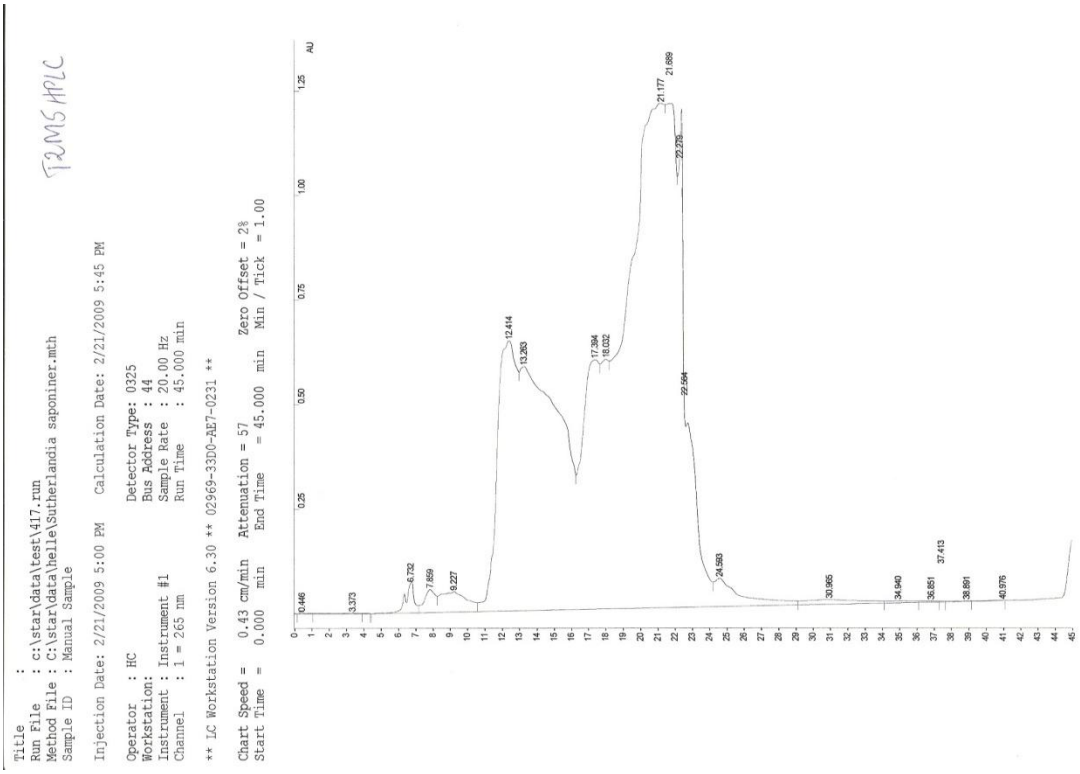
Chromatogram 15.12. Preparative HPLC chromatogram of fraction B1-2 S.4-5 VRP. T2 M2.



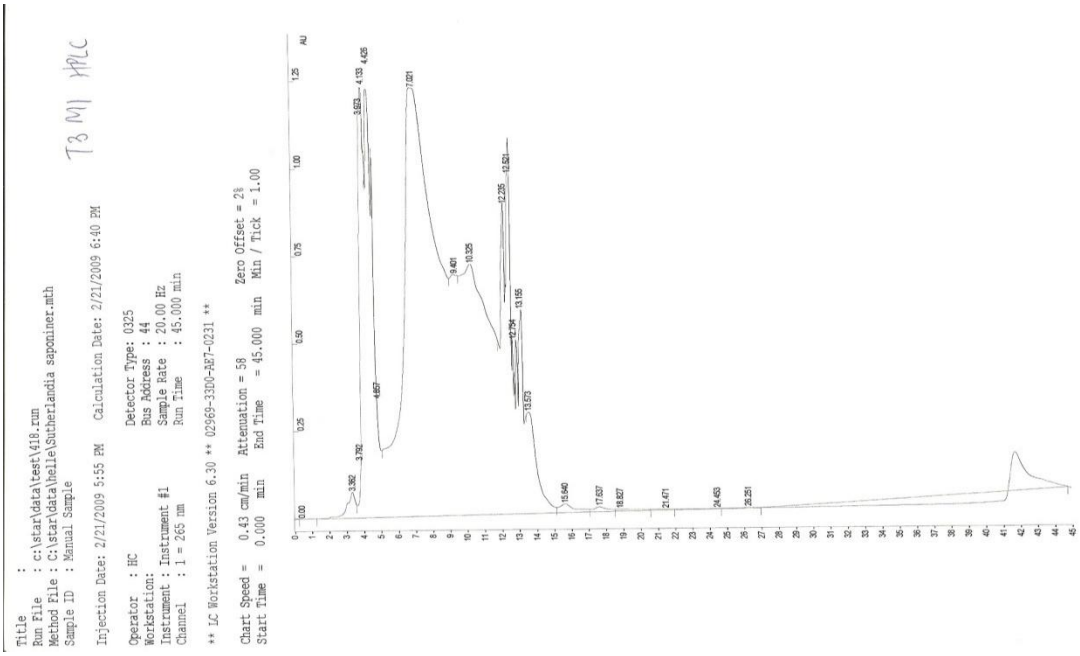
Chromatogram 15.13. Preparative HPLC chromatogram of fraction B1-2 S.4-5 VRP. T2 M3.



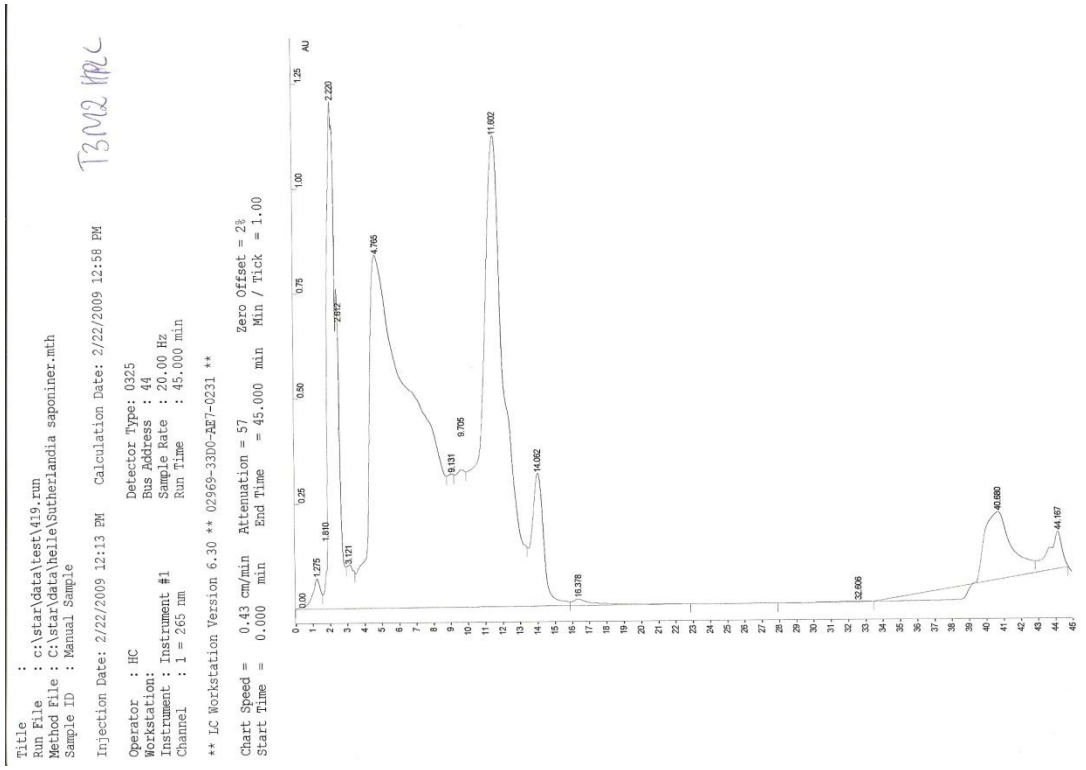
Chromatogram 15.14. Preparative HPLC chromatogram of fraction B1-2 S.4-5 VRP. T2 M4.



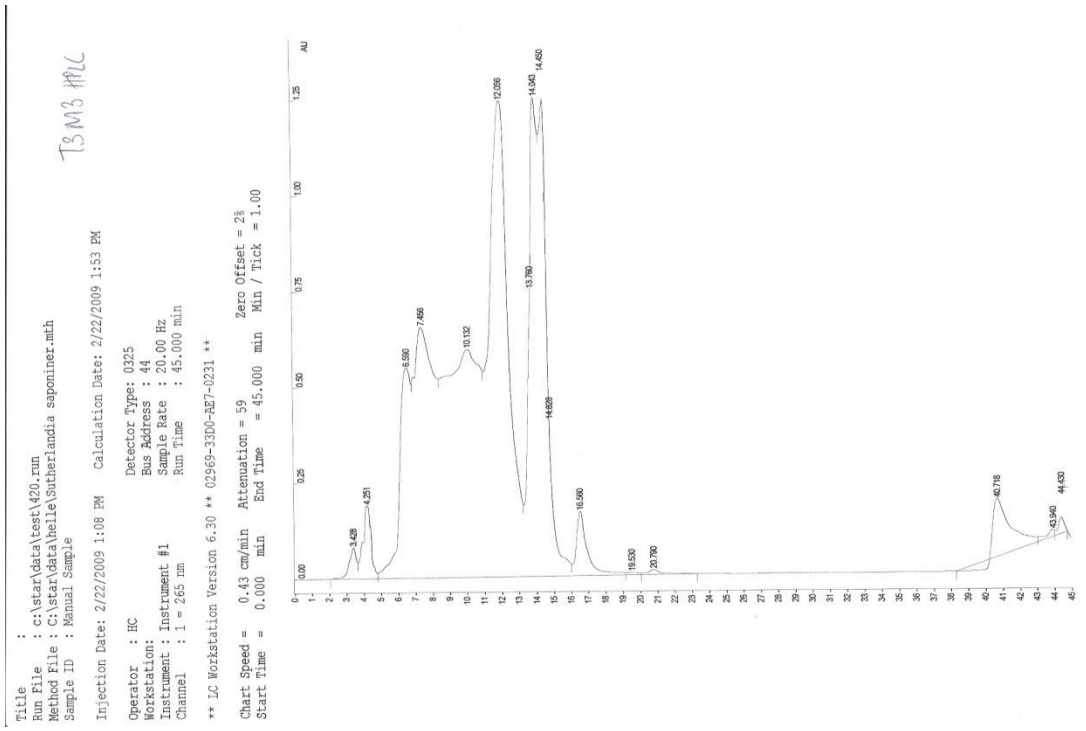
Chromatogram 15.15. Preparative HPLC chromatogram of fraction B1-2 S.4-5 VRP. T2 M5.



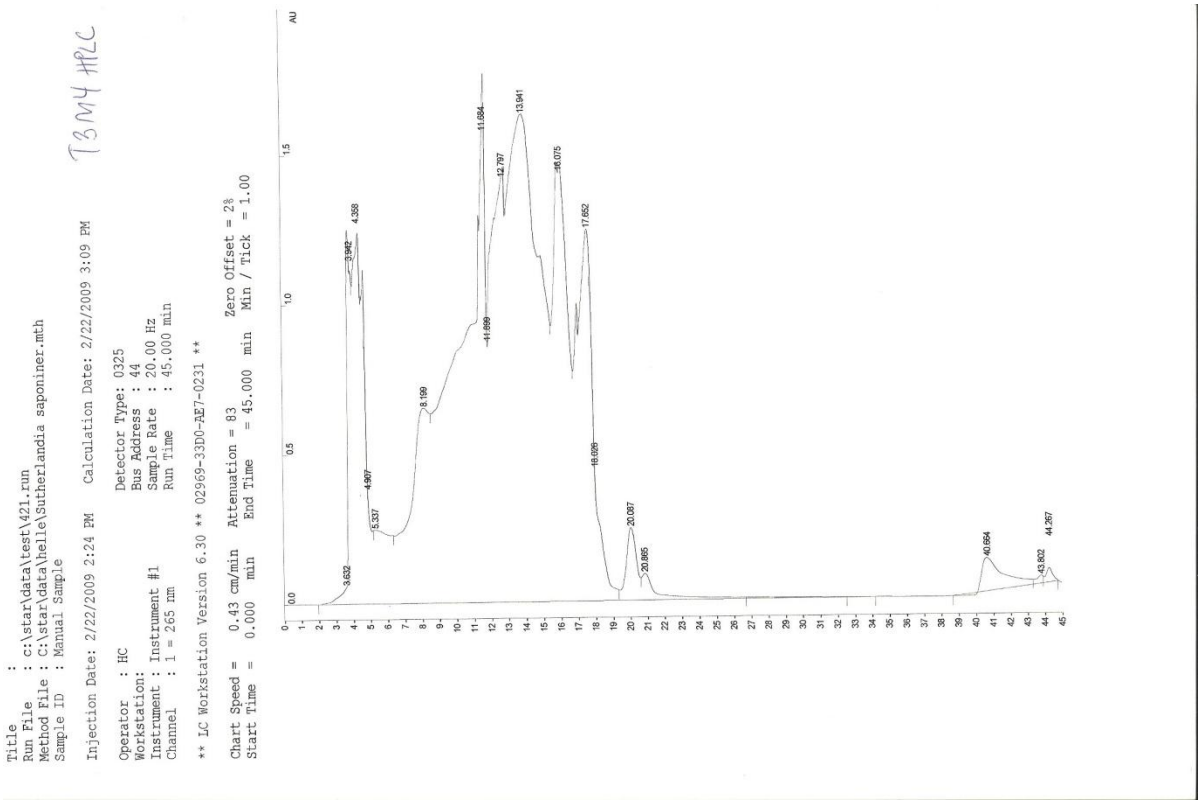
Chromatogram 15.16. Preparative HPLC chromatogram of fraction B1-2 S.4-5 VRP. T3 M1.



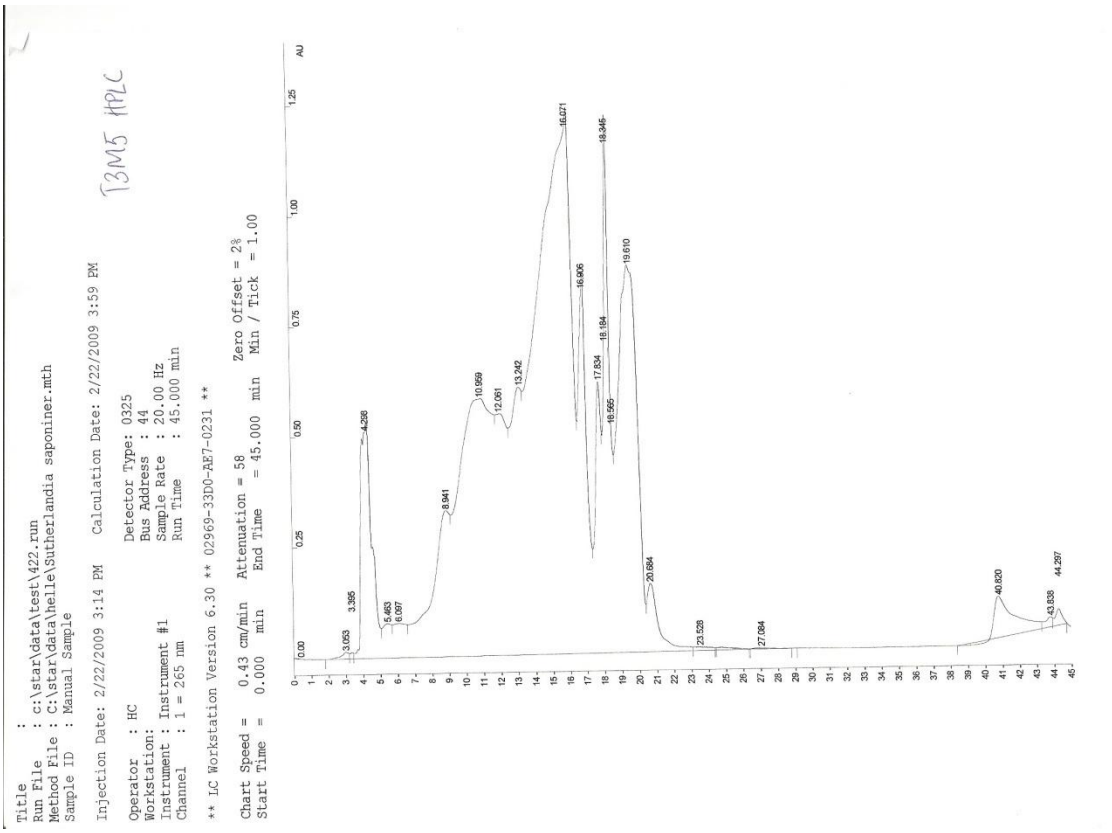
Chromatogram 15.17. Preparative HPLC chromatogram of fraction B1-2 S.4-5 VRP. T3 M2.



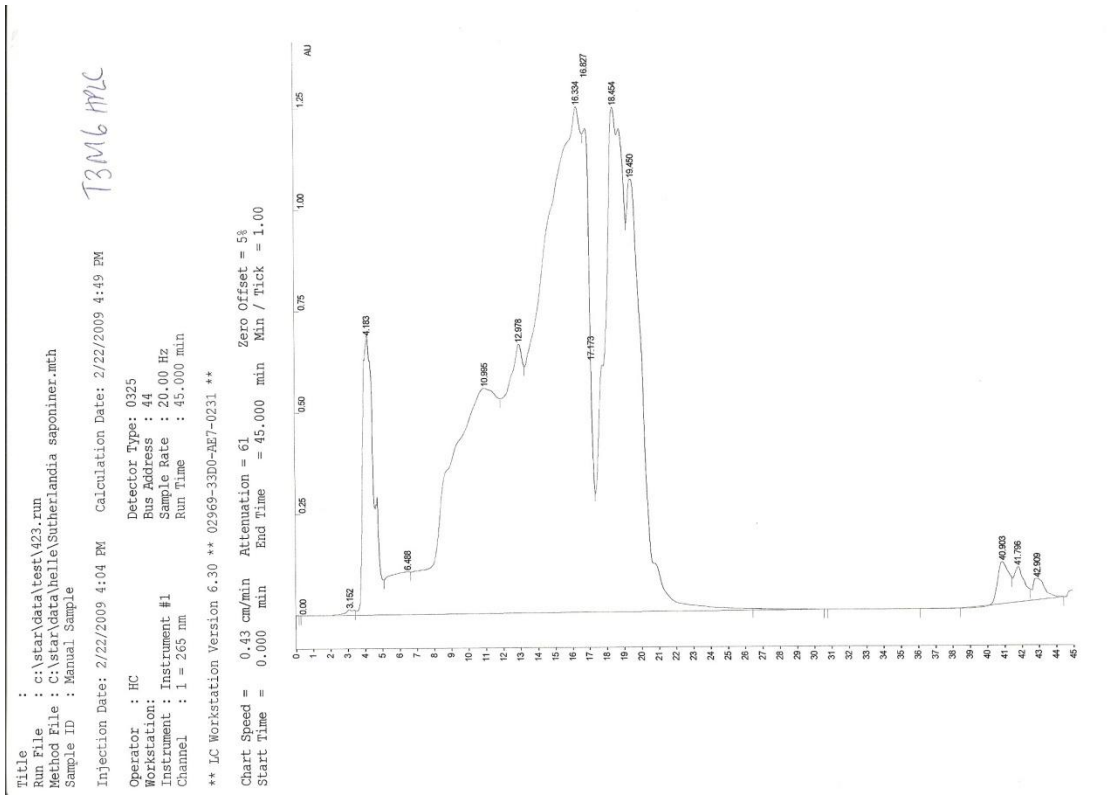
Chromatogram 15.18. Preparative HPLC chromatogram of fraction B1-2 S.4-5 VRP. T3 M3.



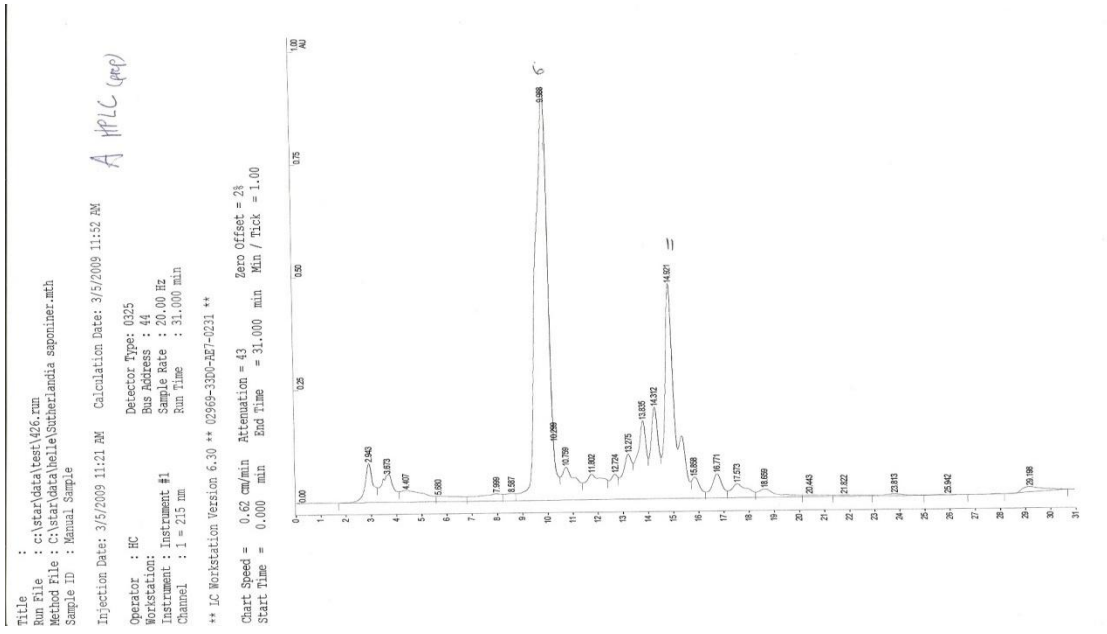
Chromatogram 15.19. Preparative HPLC chromatogram of fraction B1-2 S.4-5 VRP. T3 M4.



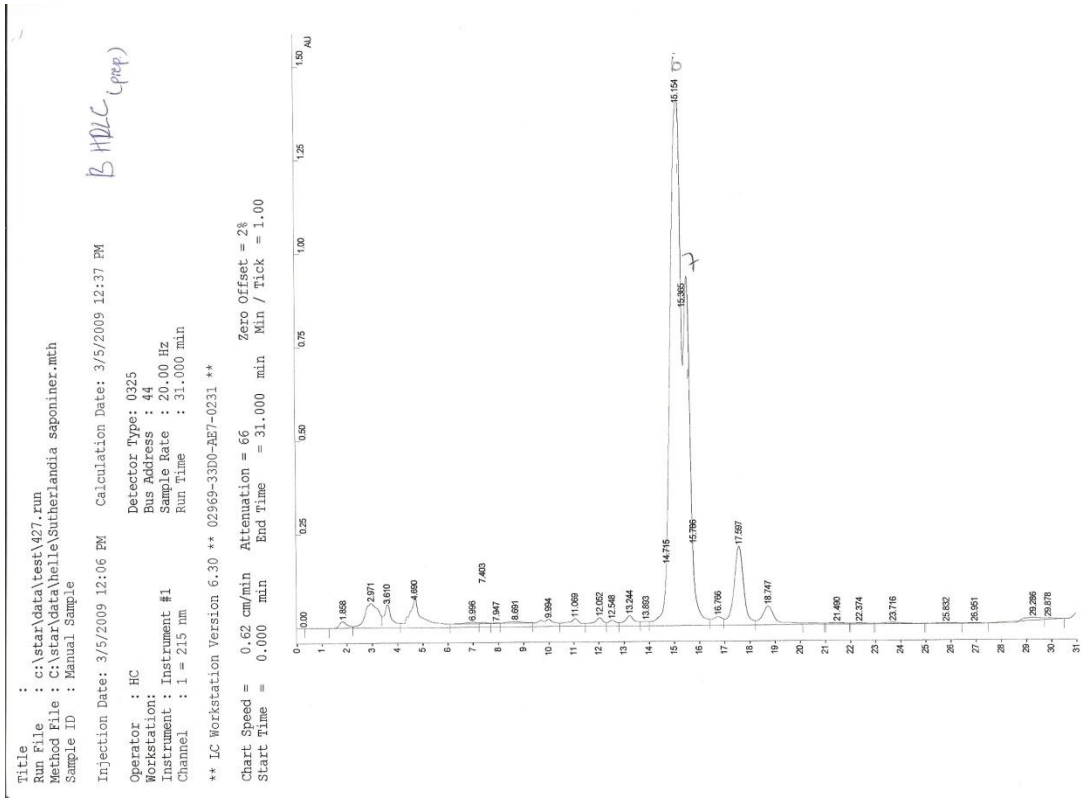
Chromatogram 15.20. Preparative HPLC chromatogram of fraction B1-2 S.4-5 VRP. T3 M5.



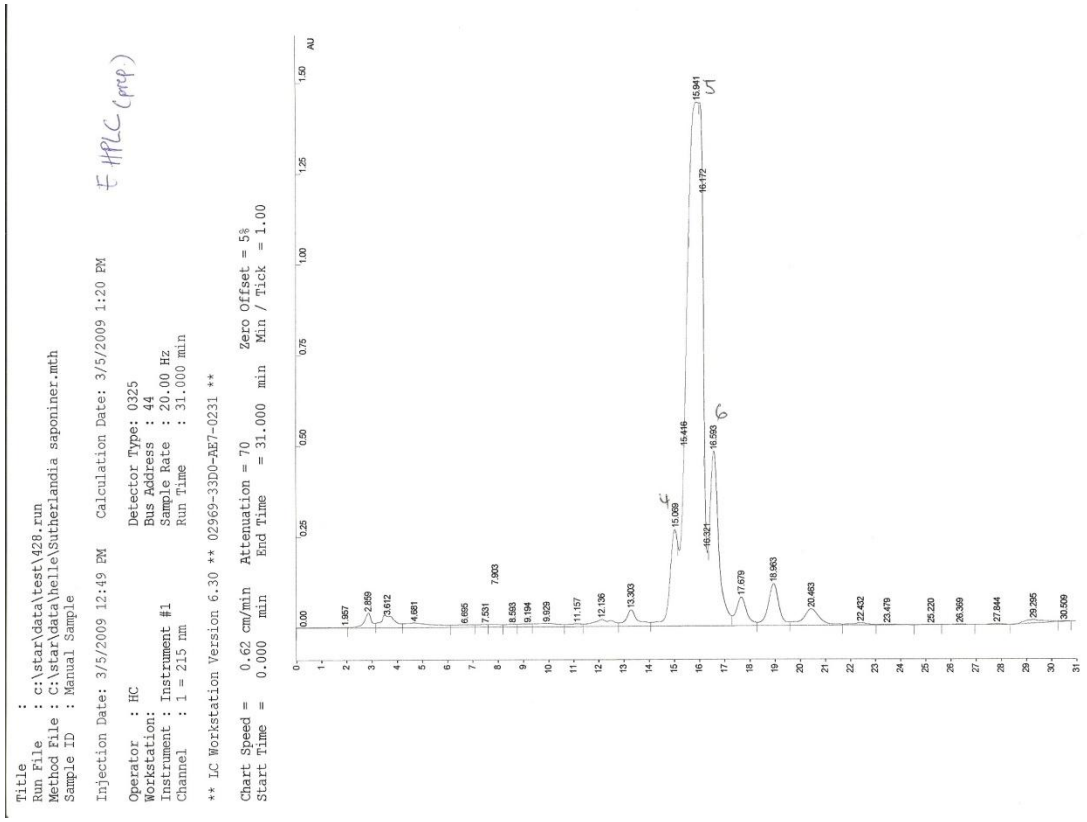
Chromatogram 15.21. Preparative HPLC chromatogram of fraction B1-2 S.4-5 VRP. T3 M6.



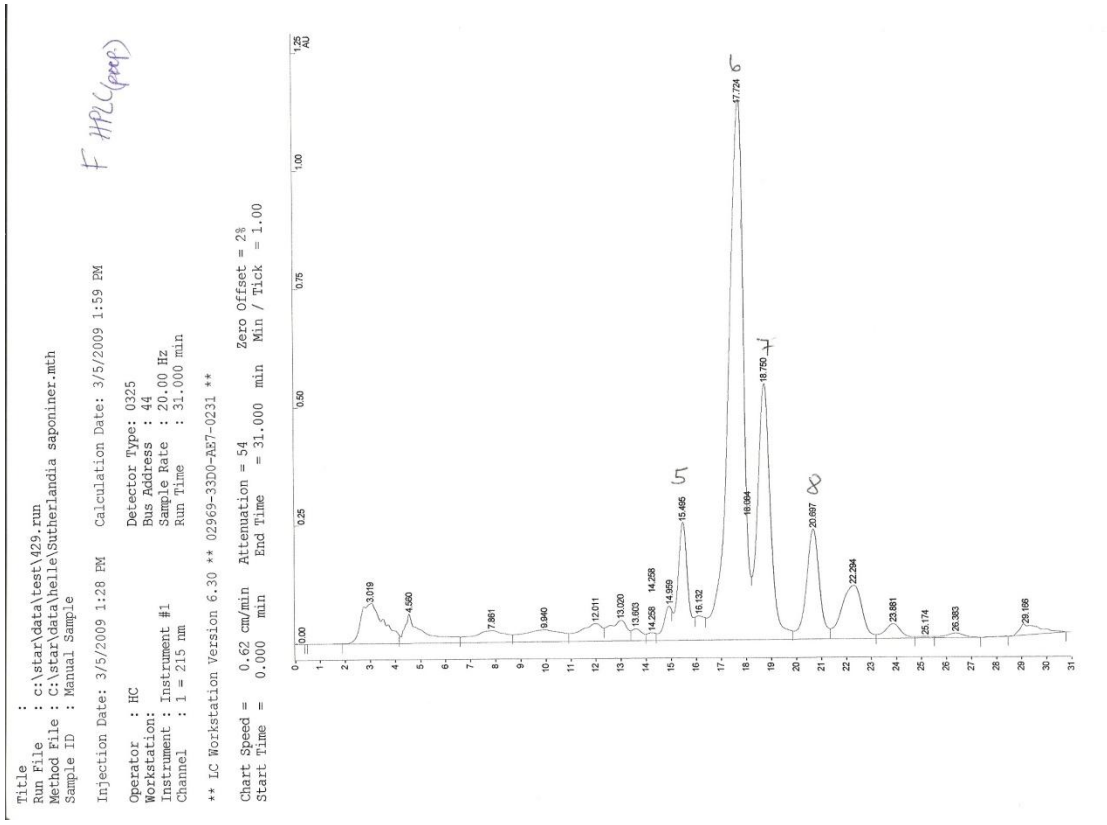
Chromatogram 15.22. Preparative HPLC chromatogram of fraction A.



Chromatogram 15.23. Preparative HPLC chromatogram of fraction B.



Chromatogram 15.24. Preparative HPLC chromatogram of fraction E.



Chromatogram 15.25. Preparative HPLC chromatogram of fraction F.

16. References

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