Phytochemical Observations on European Mistletoe (*Viscum album* L.)

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Thesis for the Master Degree in Pharmacy

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10.06.2009

Acknowledgments

I would like to express my gratitude to my supervisor, Prof. George Francis for all the guidance he has given me and for his experience and expertise. Throughout this experience he has always been patient and helpful.

I thank Terje Lygre for his assistance with DART, and Prof. Tanja Barth for her assistance with GCMS.

I would especially like to thank Prof. Saleh Rayyan for helping me with NMR. He was very generous with his time and made many good suggestions. I would also like to thank Prof. Torgils Fossen for his assistance in interpreting NMR spectra.

Finally I would like to thank my family and friends for being patient and supportive during this time. I would especially like to thank my classmate Ellen for her friendship and for keeping the spirits up in the student's hall. A special thanks goes also to Caroline for her friendship and support throughout this experience.

Summary

Non-polar constituents were extracted from *Viscum album* with hexane, by Soxhlet extraction. The extract was analysed by gas chromatography – mass spectrometry, and the following fatty acids, methyl esters and triterpenes were identified: methyl palmitate, palmitic acid, linoleic acid, linolenic acid, β – amyrin, lupenone, β – amyrin acetate and lupeol acetate.

The neutral fraction of Viscum album was extracted through several steps, and separated by column chromatography. At least two cyclic peptides were detected by TLC protosite reaction with ninhydrin. We identified the cyclic peptide viscumamide and found a possible molecular weight for an unidentified cyclic peptide, by DART mass spectrometry.

The fractions containing the unidentified cyclic peptide was purified by preparative TLC, and analysed by NMR spectroscopy. The analysis showed the presence of the amino leucine and an aromatic moiety. The complete structure of this compound could not be elucidated because of too many impurities in the sample.

Abbreviations

1D	One-dimensional
2D	Two-dimensional
APT	Adenosine triphosphate
ASTM	American Society for Testing and Materials
cAMP	Cyclic adenosine monophosphate
$CD4^+$ cells	Cluster of differentiation 4
COSY	Correlation Spectroscopy
DART	Direct Analysis in Real Time
ddd	Double double doublet
DMSO	Dimethyl sulfoxide
EI	Electron Impact
GC	Gas Chromatography
GCMS	Gas Chromatography Mass Spectrometry
GIT	Gastro Intestinal Tract
HCMV	Human cytomegalovirus
HMBC	Heteronuclear Multiple Bond Correlation
HSQC	Heteronuclear Single Quantum Coherence
Hz	Herz
LAK	Lymphokine Activated Killer
m	Multiplet /covered by impurities signals
M+	Molecular ion
min	Minute
ML	Mistletoe Lectin
MS	Mass Spectrometry
nm	Nanometer
NMR	Nuclear Magnetic Resonance
NOESY	Nuclear Overhauser Enhancement Spectroscopy
PDE	Phosphodiesterase
PMA	Phosphomolybdic Acid
ppm	Parts per million
Rf	Retention Factor
ROESY	Rotational nuclear Overhauser Effect Spectroscopy
SDBS	Spectral Database for Organic Compounds
TLC	Thin layer chromatography
TNF-alpha	Tumour necrosis factor-alpha,
TOCSY	Total Correlation Spectroscopy
UV	Ultraviolet
δ	Chemical shift in ppm

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1 Introduction

Aim of the Study

Mistletoe has been used in folk medicine since ancient times. Today it is applied as a remedy for a broad spectrum of different diseases, such as epilepsy, diabetes, hypertension, arthrosis and last but not least, cancer. Modern research clearly indicates that components in mistletoe may be valuable in medicine, but good clinical research is still lacking.

There are still doubts about which of the constituents contributes to the beneficial effects of mistletoe extracts. Research has for the most part focused on polar, water-soluble components of mistletoe, while non-polar and medium-polar substances have been mostly overlooked. We wanted to investigate non-polar constituents in European mistletoe (*Viscum album* L.) Previous research has claimed that there is an variation of these components in mistletoe.

An interesting cyclopeptide, viscumamide has been isolated from Korean mistletoe, *Viscum album* var. colaratum, but its pharmacological properties have not yet been fully investigated. We wanted to isolate this compound from European mistletoe, and also try to identify undiscovered cyclic peptides.

Mistletoe – the Historical Plant

Mistletoe belongs to the families Loranthaceae and Viscaceae, which both share the same order Santales, and are closely related. The Viscaceae family have several hundred species worldwide, which are divided into seven genera (Arceuthobium, Dendrophthora, Ginalloa, Korthalsella, Notothiox, Phodadendron and Viscum). This family of parasitic plants is mostly found in tropical areas.

The European (*Viscum album*) white berry mistletoe belongs to the family Loranthaceae. It is a small evergreen shrub with linear, lance - shaped leathery leaves, which lasts for many seasons. *Viscum album* is a semi-parasitic plant, growing on different host trees and never touching the ground. Birds, feeding of the berries, spread it from tree to tree. The plant grows yellowish-green flowers, which develop white translucent berries in the late fall and early winter (Büssing, 2000).

Figure 1. 2.1 The European white berry mistletoe (Viscum album)

There are three subspecies of *Viscum album* in Europe- album, abietis and austriacum. They can be found in temperate areas of Europe, Asia and Northern Africa. Another subspecies – colaratum grows in China and Japan (Büssing, 2000).
The Celtic Druids considered mistletoe sacred and representing everlasting life. Unlike other plants mistletoe doesn't follow a 12 months vegetation period, never touches the earth, and in the dead of winter when its host tree is bare, this "magic" plant is still green and flourishing. The Druids attributed miraculous powers to the plant and believed it was a cure for all things (Bowman, 1990)

Figure 1.2.2: Romantic picture of Celtic Druids chopping mistletoe of an oak, on which it rarely grows (http://www.smk.be/compendium/pdf/mistletoe.pdf)

Some of mistletoe's "magical" powers have endured and even today it is a symbol of fertility and good luck. In some European countries and North America the tradition of kissing under branches of mistletoe during Christmas, is still popular. The tradition is interpreted as a prediction of marriage and a long and happy life.

During the Middle ages people believed that hanging mistletoe from the ceilings would keep away evil spirits, and prevent witches and ghosts from entering the houses or stables. It has often been carried to battle as a symbol of strength and protection. The evergreen condition of the plant associates it with immortality, and the parasitism gives it powers to draw tribute from the host (Büssing, 2000).

Mistletoe also appears in the ancient Edda from Noridc mythology. This collection of ancient Viking poems tells about how the evil Loki kills Balder, the favourite son of Odin (the chief God in Norse paganism), with a spear made of mistletoe. Frigg, Odin's wife and the goddess of love and beauty, had put all objects and beings under an oath not to harm her son Balder, but neglected the mistletoe, which seemed unimportant to her. Loki then kills Balder with a twig of mistletoe shot by his blind brother Hödur, and Balder is sent to the realm of the dead. The Edda describes the hopes for Balder's return of those remaining behind, although this will not happen until the old world has come to an end. Then and a new world will rise, where all evil has vanished. This legend may have had

its origin from Asia or other parts of Europe, because *Viscum album* is rarely found in northern Scandinavia or Iceland, where the Edda was written (Büssing, 2000).

1.1 The Historical Uses of Mistletoe in Europe

The use of mistletoe in Europe can be traced all the way back to the ancient Greeks. It has been recorded that Hippocrates (460-377 BC) used mistletoe to treat diseases of the spleen and complaints associated with menstruation. The Platonist Celsus reported that mistletoe was used to treat swellings or tumours, around 150 AD. Later in the 1st century AD, the Druids used it, as a cure for every illness or "all-heal", as an antidote for poisons and to treat infertility (Büssing, 2000).

In the Middle Ages mistletoe was used for epilepsy. This use was based on the fact that mistletoe never falls to the ground. The plant has been used as a remedy for this condition up until the 18th century (Büssing, 2000).

During the 12th century the abbess and composer Hildegard von Bingen, described the use of mistletoe in treatment of diseases of the liver and spleen. In the 15th century rosaries made from mistletoe were popular, and it was believed that wearing them could prevent disease. During the 16th century mistletoe was applied for many conditions in Europe, including diseases of the kidney and spleen, ulcers, fractured bones and the relief of labour-pain. Mistletoe was commonly used as a helminthic drug, to treat children infected with intestinal parasites in the 17th century. During this time it was also used to treat gout, diseases of the lung and liver, hepatitis, leprosy and labour-pain. According to the homeopathic *materia medic*a, mistletoe was applied for "weakness of the heart", in the 18th century (Büssing, 2000).

Mistletoe was rejected as a folklore remedy by the end of the 19th century. Scientists only accepted a mistletoe containing ointment, Viscin, which has been reported to have effects on eczema, ulcers of the feet, burns and for granulating wounds (Büssing, 2000).

In the 20th century an interest for the use of mistletoe in cardiovascular medicine awoke, as Gaultier (1907,1910) investigated the effects of *Viscum album* extracts on blood

pressure in man and animals. In the 1920s modern interest for mistletoe began, as Rudolf Steiner introduced *Viscum album* as a possible treatment for cancer. Since the early 60s more than 30 clinical studies of mistletoe in cancer treatment have been published (Büssing, 2000).

The use of mistletoe is also reported in other parts of the world, like Northern America, Japan and Africa.

1.2 The Biological and Pharmacological Effects of Mistletoe

Viscum album is one of the most widely used remedies for the unconventional treatment of hypertension, arteriosclerosis, arthritis and most importantly cancer (Büssing, 2000).

About 80 randomized clinical studies have been identified, of which 58 have been excluded mostly for the lack of prospective trial design. Of the 21 studies included, 13 provided data on improved survival time, 7 on tumor regression, 16 on improved quality of life and 12 reports side effects of mistletoe treatment. Even though most of these studies are of poor quality many of them indicate beneficial effects, which should encourage further properly designed trials (Horneber et al., 2008).

1.2.1 Hypertension

Every year millions of people die of cardiovascular disease. Arterial hypertension is the major risk for developing arterial coronary disease, heart failure, cerebral vascular disease and renal damage. Established anti-hypertensive drugs may cause a number of side effects, and therefore the search for other anti-hypertensive agents continues.

European mistletoe was applied for "weakness of the heart" and oedema during the 18th century, and later for arteriosclerosis and hypertension. It is still (1990) listed as a vasodilator in Martindale's *Extra Pharmacopoeia*. This acceptance of mistletoe as an

antihypertensive substance is mainly due to the work of the French physician René Gaultier, published in 1907. He successfully treated a patient with pulmonary haemorrhage using a medicine containing mistletoe after other treatments had failed. After this experience he started a series of experiments. His research showed that mistletoe caused a rapid lowering of blood pressure in dogs. He was rewarded with a special prize from the Académie de Médecine in Paris for this work (Bowman, 1990).

However, this acceptance of mistletoe as an antihypertensive drug was not universally accepted. In 1911 the Belgian physician Felix Dossin stated that mistletoe was not useful clinically. He said that it was a dangerous treatment, which often leads to convulsions (Bowman, 1990).

Research on mistletoe's biological properties has mostly focused on its immunomodulating activity, and data on possible cardiovascular effects are scarce. In 2000 Deliorman and co-workers reported that *Viscum album* affected the diameter of coronary blood vessels (Deliorman et al., 2000).

This year M. Radenkovic and co-workers investigated the effects of *Viscum album* L. extracts on arterial blood pressure in rats. They found that the ethanol extract gave a significant reduction in the blood pressure at low concentrations. They also showed that the antihypertensive effect of the extract might be related to an action on muscarine receptors (Radenkovic, 2009). These results supplement other reports on mistletoe in antihypertensive treatment (Murray, 1995). It has been suggested that mistletoe flavonoids and/or gamma-aminobutyric acid (GABA) isolated from mistletoe is responsible for this action (Khwaja, 1990). Even though mistletoe is used in traditional phytotherapy for the treatment of hypertension, established medicine still has to confirm this activity and clinical trials must be conducted.

1.2.2 Diabetes

In Nigerian folk medicine extracts of mistletoe were used to treat diabetes mellitus (Büssing, 2000). Research showed that aqueous extracts from two African mistletoe

species decreased the serum glucose levels in both non-diabetic and diabetic rats (Obatomi, et al., 1994). Similarly, Swanson and Flat reported that an extract from *Viscum album*, supplied as 6,25% by weight of the diet, for 9 streptococin diabetic mice gave a relief of polydipsia, hyperphagia and loss of body weight, while glucose and insulin plasma levels remained unchanged (Swanston-Flatt et al., 1989). Further, in 1999 Gray and Flatt showed that clonal pancreatic B-cells was stimulated to secrete insulin by an aqueous extract of dried *Viscum album*. The treatments require further evaluation (Gray and Flatt, 1999).

1.2.3 Arthrosis

Plenosol, an aqueosus extract of *Viscum album* is being used as an alternative treatment for arthrosis and/or arthritis of several joints. Research showed that patients experienced release from severe pain, increased motility and improved endurance of the affected joints, after application of the drug. The side effects reported were inflammatory local reactions, slight increase of body temperature, swelling of local lymph nodes, night-sweating, headache, and exhaustion. The mode of action is still not clear. Most of the research done on Plenosol is of poor quality, and further studies are needed (Büssing, 2000).

Viscum album var. colatrum has been used in traditional Chinese medicine to treat inflammatory diseases. In 2006 Hwang and co-workers reported that elevation of cellular cAMP through inhibition of PDE (phosphodiesterase) is associated with an inhibition of inflammatory responses in human neutrophils. The pathogenesis of rheumatoid arthritis, chronic obstructive pulmonary disease and other inflammatory diseases, are closely connected to neutrophils. Tests have demonstrated that viscolin, which is isolated from *Viscum album* var. colatrum, is a potent inhibitor of PDE. This indicates that viscolin might be a useful treatment for these indications (Hwang et al., 2006).

1.2.4 Cancer

In the 1920's Rudolf Steiner introduced mistletoe as a possible treatment for cancer. Mistletoe is one of the oldest cancer treatments reported, and is still one of the most used in some European countries. Only the European white-berried mistletoe *Viscum album* is used in cancer therapy (Legnani, 2008).

Viscum album extracts for this purpose are marketed under different brand names, such as Iscador, Eurixor, Helixor, Isorel, Iscucin, Plenosol and Abnoba viscum. Some of them are sold and marketed under more than one name. In the USA none of them are available commercially. Mistletoe extracts are normally administered by subcutaneous injection. Other routes of administration like oral, intrapleural and intravenous have also been used. (Mistletoe Extracts - Health Proffesionals Version)

Extracts derived from mistletoe have been shown to kill cancer cells *in vitro,* and to stimulate immune system cells both *in vitro* and *in vivo*.(Mistletoe Extracts - Health Proffesionals Version)

Since the 1980s many laboratory and *in vitro* tests have been conducted on the active constituents in mistletoe. Especially viscotoxins, lectins and polysaccharides have been investigated. The following effects have been explained as a combined action of these constituents:

1) Cytotoxity in tumour cell cultures
2) Stimulation of lymphocytic proliferation
3) Increase in the number and activity of neutrophils
4) Increase in the number of $CD4^+$ cells in HIV-positive patients

The following therapeutic effects of mistletoe have been observed through clinical testing:

1) General improvement of clinical condition
2) Slowing of tumour growth
3) Reduction in cancer pain

4) Improvement of quality of life related to appetite, sleep and general wellbeing
5) Improvement of mood by anti-depressive action
6) Reduction of infection by stimulation of the immune system
7) Improved tolerance of radiotherapy and chemotherapy when applied as a therapeutic resource (Legnani, 2008)

The immune-stimulating and cytotoxic effects of mistletoe have both been investigated in laboratory and animal studies. The active components, viscotoxins and lectins, have been studied as active substances, but research on lectins has been more intensive. Purified mistletoe lectins (ML) have shown cytotoxic effects and stimulating effects on the immune system. Many of mistletoe's biological effects may be caused by the lectin ML-I, or *viscumin*. Until now four different lectins and a chitin-binding agglutinin have been isolated. When ML-I was removed from an extract there was a markedly reduced cytotoxic and immune-stimulating effect. However it should be noted that the fermented extracts of mistletoe contains a very small amount of ML-I.

A great number of clinical studies have investigated the value of mistletoe as a cancer treatment. All studies have been performed in Europe, mainly in Germany and Austria. These studies have for the most part been published in Germany alone. Iscador, Eurixor, Lekitinol and recombinant lectin ML-I are the mistletoe extracts/ products tested. About half of the reported studies were controlled and most were randomized clinical trials. The endpoint measured was mostly survival, but also other endpoints like tumour response, tumour recurrence and quality were used.

In most of the studies mistletoe was found to be therapeutically effective. However, the majority of these trials had one or more major weaknesses which reduced the validity of the findings. These errors included too small patient groups, large number of patients excluded or who could not be evaluated, mistletoe use concerning dosage or interruptions, mistletoe use not documented adequately, absence of control patients or randomization procedures not fulfilled. Another problem is that these studies are often lacking reports on clinical data about previous and current therapies received by the patients.

Although mistletoe is known to be a toxic plant the reports on side effects have been minimal and not life threatening. Soreness, inflammation at injection sites, headache, fever and chills are the most common side effects. Severe allergic reaction, including anaphylactic shock has occurred in a few cases. Seizures, vomiting and death have been reported following ingestion of untreated mistletoe plants and berries. The amount of these substances consumed and the species of mistletoe affects the seriousness of the toxic effect.

Among complementary and alternative anticancer medicines mistletoe is the most widely studied. Many clinical studies have shown that it improves survival and quality of life in cancer patients. Reports on mistletoe stimulation of the immune system have also been frequent. The major problem is that most of the studies have had one or more major weakness that make the validity of the finding questionable. There is also no proof to support the notion that the immune-stimulation activity of mistletoe can be connected to the improved survival in cancer patients. There are not yet reports on the use of mistletoe as a treatment for cancer in children, since all the clinical studies have been performed on adults. More evidence is needed before mistletoe can be recommended as a treatment for cancer outside the context of well-designed clinical trials(Mistletoe Extracts - Health Proffesionals Version).

1.3 The Chemical Constituents of Viscum album

Natural factors like sex, local climate, harvesting time, parts and age of plants used and host tree, affect the composition of different chemical constituents in mistletoe. Lectins, viscotoxins, flavonoids and many other constituents of mistletoe are being investigated in clinical studies in cancer treatments and other conditions (Pfüller, 2000). Pharmacological studies on *Viscum album* have focused on polar extracts and their water-soluble constituents, such as mistletoe lectins and viscotoxins. (Cebovic et al., 2008).

1.3.1 Mistletoe Lectins

Lectins have complex molecular structures made up of both proteins and carbohydrates. They are capable of binding to the surface of different cells (e.g. immune cells) and induce biochemical changes in them (Mistletoe Extracts - Health Proffesionals Version). They have at least two sugar-combining sites and show specificity for terminal and/or subterminal residues. Lectins will thus recognise specific carbohydrate residues and bind to them on the cell surface of cytoplasmic, nuclear structures and to components of extracellular matrix.

1.3.2 Viscotoxins

Viscotoxins, which are related to thionines, are a group of amphipathic strongly basic polypeptides. Samuelsson (1974) and Urech et al. (1995) isolated at least six isomers of viscotoxins mainly from *Viscum album*. They share a similar structure of 46 amino acids and are common in plants like corn and wheat among others, and are suggested to be protective substances against viruses, bacteria and fungi. Viscotoxins contain 3 to 4 disulphide bridges and a three-dimensional model of viscotoxin A3 showed that the polypeptide is amphipathic and may therefore interact with lipid bilayers. The viscotoxins are exceptionally stable substances and they can stay intact under denaturating conditions. They also tend to form protein-lipid-complexes and self-aggregate. Some of their pharmacological and biological properties may be explained by the physio-chemical properties of the membrane-permeabilising viscotoxins. However, it is not yet clear why amphipathic polypeptides from mistletoe and other plants, which are similar structurally, show different biological effects. It has been suggested that the cytotoxic potential could change by changing only a few amino acids. The viscotoxins are candidates for further investigations due to their outstanding properties and their occurrence in many mistletoe preparations (Pfüller, 2000).

1.3.3 Flavonoids

Flavonoids are most commonly known for their antioxidant activity. They are found in great variety and are widely distributed in nature. Because of their strong ability to modify the body's reaction to allergens, viruses and carcinogens, they have been referred to as "nature's biological response modifiers". In experiments flavonoids have shown anti-allergic, anti-innflammatory, anti-microbial and anti-cancer activity. Interest in their preventive role in cancer and cardiovascular disease has increased (Larmo et al., 2009, Yang et al., 2009).

Since the 1950s a great number of different flavanoids have been isolated from mistletoe (Pfüller, 2000). Among these quercetin has evoked most interest. Reports have shown that quercetin inhibits angiogenesis through many mechanisms. Results also indicate that the flavonoid enhances the anticancer effects of conventional treatment (tamoxifen) through anti-angiogenesis. More data are needed on dose-response, potential toxicities and appropriate combinations with other cancer therapies (Sagar et al., 2006).

1.3.4 Phenylpropanoids

Phenylpropanoids, commonly known as cinnamic acids are related to flavonoids by a common biogenetic pathway. The best known phenylpropanoids like caffeic acid, ferulic acid and sinapic acid, together with their degradation products, proptocatechuic acid, syringic acid, vanilic acid, anisic acid and genestic acid, have been isolated from *Viscum album* (Pfüller, 2000). Cinnamic acid is used in flavours, synthetic indigo, and certain pharmaceuticals, though its primary use is in the production of the methyl, ethyl, and benzyl esters for the perfume industry.

1.3.5 Phytosterols

Beta-sisterol, stigmasterol and their respective glycosides have been isolated from *Viscum album* (Pfüller, 2000). Phytosterols (also called plant sterols) are a group of steroid alcohols, phytochemicals naturally occurring in plants. They are white powders with mild, characteristic odor, insoluble in water and soluble in alcohols. They have many applications, for instance as food additive taken to lower cholesterol, as well as in medicine and cosmetics.

1.3.6 Triterpenes

Terpenes constitute a large and varied class of hydrocarbons, which are widespread in plants. They are classified according to the number of isoprene units $CH_2=C(CH_3)-CH-CH_3$, and have the general formula $(C_5H_8)n$. The isoprene units can be linked together as linear chains or rings.
Triterpenes consist of six isoprene units and have the molecular formula $C_{30}H_{48}$. Unlike mono- sequi- and diterpenes they join tail to tail.
There is a rich content of triterpenes such as β-amyrin, β-amyrin-acetat, betulinic acid, oleanolic acid and ursolic acid in *Viscum album* (Pfüller, 2000).

1.3.7 Alkaloids

Alkaloids are naturally occurring chemical compounds containing basic nitrogen atoms. They often have pharmacological effects and are used as medications and recreational drugs.

Viscum album L. does not contain typical alkaloids in contrast to *Viscum album* var. colaratum and other species. Hegnauer (1966) has summarised reports on "alkaloid like" substances, including phenylethylamine, choline and acetylcholine in *Viscum album* (Pfüller, 2000). Alkaloid-like substances in *Viscum album* L. have also been described by

Khawa and co-workers, but they have only been defined by the non-specific Dragendorff-alkaloid reagent and remain uncharacterized (Khwaja et al., 1986).

Kwaja and co-workers reported that alkaloids from Korean mistletoe inhibited the growth of cultured leukemia cells and increased the life span of leukemic mice. Because of the extreme lability of these alkaloids, their structures have not yet been defined. It has been proposed that the alkaloids are glycoconjugates with proteins and lectins from *Viscum album* (Pfüller, 2000).

1.3.8 Fatty acids

Saturated fatty acids isolated from *Viscum album* are mainly palmitic and arachidic acid. Unsaturated fatty acids isolated are mainly oleic and linoleic acids. Reports on the non-polar constituents of mistletoe are scarce (Deliorman and Orhan, 2006).

1.4.9 Other Biologically Active Components

A 5 kDa peptide with cytotoxic and tumour reducing qualities was isolated from *Viscum album* extract by Kuttan and Kuttan. An increased natural killer cell activity (NK-activity) was reported after administration of the peptide. It has been suggested that the immuno-stimulatory qualities of *Viscum album* extract (Iscador) is mainly attributed to this peptide (Kuttan and Kuttan, 1992).

Rhamnogalacturonan extracted from *Viscum album* showed NK (natural killer)- and lymphokine-activated killer (LAK)- activity against Human Cytomegalovirus (HCMV)-infected cells (Steinmassl and Anderer, 1996).

Induction of interferon-gamma from CD4+ cells and TNF-alpha from monocytes or macrophages was reported from an oligosaccharide extracted from *Viscum album* (Pfüller, 2000).

An undefined antigen from an extract of *Viscum album* from pine trees showed cytotxic as well as immuno-modulating qualities. The study suggests that the components of mistletoe extracts depend on the host tree and the manufacturing process (Stein and Berg, 1997).

In mistletoe the lectins amount to only 2% of the total polypeptide and protein content. Except for the viscotoxin type of polypeptide there is little information on structure and biological properties of other proteins present in the plant. A fraction of polypetides with immunomodulating activity was isolated by Kuttan and co-workers, but these polypetides have not yet been characterised (Pfüller, 2000).

In 1973 Okumura and Sakurai discovered viscumamide, a new amphiphilic cyclic pentapetide. They isolated it from *Viscum album* var. coloratum and performed chemical and structural studies on it (Okumura and Sakurai, 1972). In 1978 Okumura and Sakurai successfully synthesised viscumamide and three analogues, by the N-hydroxysuccinimide ester method, however at a low yield (Sakurai and Okumura, 1979). This cyclic peptide has not been described for the European mistletoe yet and until recently, there were no reports on biological activity. Two unpublished studies showed that viscumamide had pronounced membrane-binding qualities. By using different methods Becker and Pfüller observed an interaction between viscumamide and artificial membranes (Pfüller, 2000). In 2005 Poojary and Belagalib evaluated three cyclic peptides, viscumamide, yannin A and evolidine for antimicrobial and pharmacological activties. Viscumamide showed a moderate anti-inflammatory activity and weak anti-helminthic activity. Evolidine showed better growth inhibition against bacterial strains than yunnanin A and viscumamide (Poojary and Belagali, 2005).

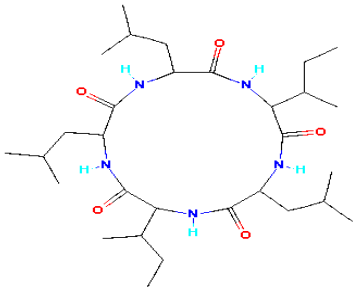

Figure 1.5.1: The structure of viscumamide, cyclic (L-isoleucyl-L-leucyl-L-isoluecyl-L-leucyl-L-leucyl)

As discussed earlier it is indicated that *Viscum album* extracts have beneficial effects in cancer treatment. There are also reports suggesting beneficial effects in the treatment of arthrosis. *Viscum album* extracts role in the treatment of hypertension and arteriosclerosis remains unclear due to the lack of convincing studies. Mistletoe lectins and viscotoxins are at present the components mainly attributed to be responsible for the beneficial effects of *Viscum album*. Because of the variety of biologically active components in mistletoe extracts, scientists have had difficulties in defining the component or components, responsible for beneficial effects (Büssing, 2000) . It is possible that other uninvestigated components such as *viscumamide,* could play an important part in this "puzzle". Research has shown that many cyclic peptides have unique qualities and a variety of biological effects. In the following chapters *viscumamide* and the interesting group of cyclopeptides will be discussed.

1.4 Plant Cyclopeptides

Cyclopeptides may be defined as cyclic compounds, mainly formed by peptide chains. The peptide chains consists of 2-37 protein or non-protein amino acids, mainly L(levorotatory)-amino acids.(Tan and Zhou, 2006)

The interest for cyclic peptides during the last few decades has led to intensified research in this field. In the early 70s it was noticed that women in Congo drank a

medicinal tea from a local plant to ease childbirth. 25 years later it was discovered that the cyclic protein kalata B1 with uterotonic activity, was responsible for this effect. Since then a number of cyclic peptides with promising biological effects have been identified from bacteria, plants and animals from Africa, South America, Australia and Europe. Often research has been done on plants that have been used traditionally in folk medicine. A variety of anticancer, antibacterial, anti-malaria, immunosuppressant and anti-HIV effects have been observed, among some of the different cyclopeptides (Tan and Zhou, 2006).

1.4.1 The Unique Qualities of Cyclopeptides

In addition to the wide range of biological activity cycloeptides also show an exceptional stability. Most proteins and peptides are synthesized as linear chains of amino acids with one free amino-terminus and one free carboxyl-terminus. In the cyclic peptides the two ends are linked together producing a circular topology. This property gives the cyclopeptides many advantages over linear peptides. When the two ends are joined together it makes it almost impossible for digestive enzymes to attack the peptide. This causes the cyclopeptides to stay intact through the gastric intestinal track (GIT). In medicine many protein-based drugs (e.g. insulin) must be given by injection to avoid degradation in the GIT. Now there is research on using cyclic proteins as a framework for linear peptides to keep them intact through the GIT. The cyclic peptide can be exposed to boiling and extremes of pH and still maintain structure and function. It has also been discovered that the cyclic backbone makes them more biologically active. This is probably due to lack of loose ends that makes it harder to strip them away from the target receptor. (Craik, 2006)

1.4.2 Classification

Plant cyclopeptides have been classified in many different ways, based on structural features, plant source and biological effects, size or amino acids sequence. In 2006 Tan

and Zhou classified 455 plant cyclopeptides in two classes, 5 sub-classes and 8 types, on the basis of structural features and distribution in plant families (Tan and Zhou, 2006) .The following passages follow closely their review.

The two main classes divide them into heterocyclic and homocyclic peptides. The heterocyclic structures are constituted by both protein- and non-protein amino acids, whilst the homocyclic peptides are constituted by protein-amino acids alone. The cyclopeptides are divided into 5 sub-classes on the basis of number of rings: heteromono-, heterodi-,homomono-, homodi- or heteropolycyclopetides. Finally they are grouped into 7 types according to structural features or distribution in plants. The two largest types are type I (Cyclopeptide alkaloids) and type VI (Caryophyllaceae-type cyclopeptides). Cyclopetides have mainly been found in the following plant families: Annonaceae, Caryophyllaceae, Rhamnaceae and Rubiaceae (Tan and Zhou, 2006).

Viscumamide is the only cyclopeptide discovered in the Loranthaceae family.

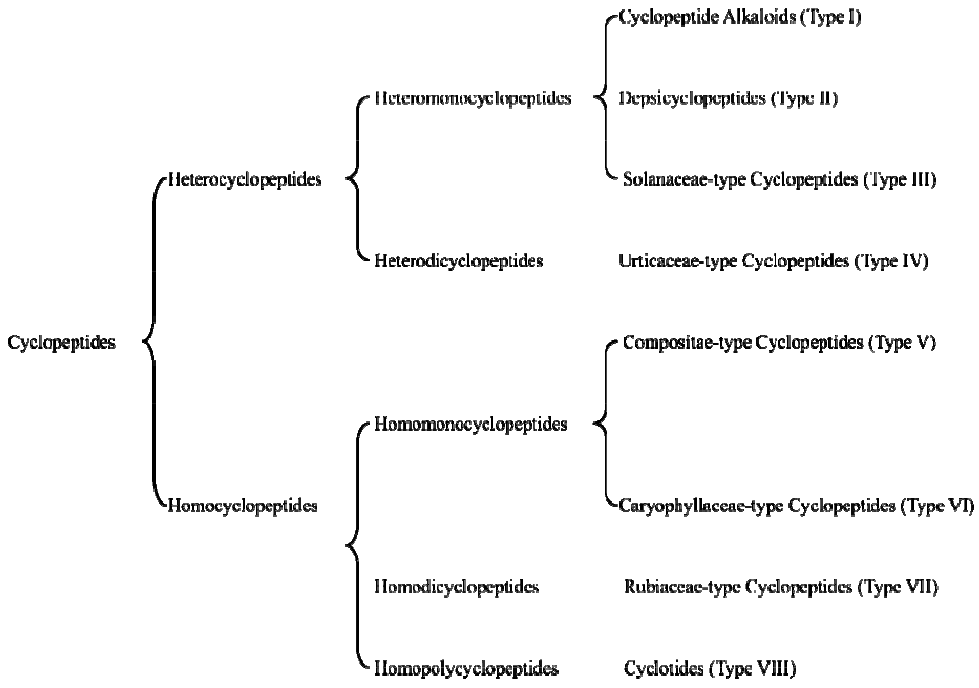

Figure 1.6.1 Classification of Cyclopeptides in Plants (Tan and Zhou, 2006)

Viscumamide has not yet been classified according to this system, probably because of the little amount of research available. The substance has a homocyclic structure,

constituted by protein – amino acids connected together in one single ring. This kind of structure would best fit in with the homomonocyclopeptides.

The homomonocyclopeptides are divided into two types: Compositate – type cyclopeptides (Type V) and Caryophyllaceae – type cyclopeptides (Type VI). Type V – cyclopeptides are halogenated cyclic pentapeptides. There have been isolated 9 Type V cyclopeptides, mainly from the Compositae family, where 3 have shown antitumour activity (Tan and Zhou, 2006).

Caryophyllaceae-type cyclopeptides are a large group, which include cyclic di-, penta-, hexa-, hepta-, octa-, nona-, deca-, undeca-, and dodecapep- tides. They are mainly found in the Caryophyllaceae family, but have also been isolated from other plant families. During the past half century around 168 Type VI cyclopeptides have been isolated and many of them show biological activities, such as cytotoxic, antiplatelet, antimalarial and immunomodulating activities (Tan and Zhou, 2006).

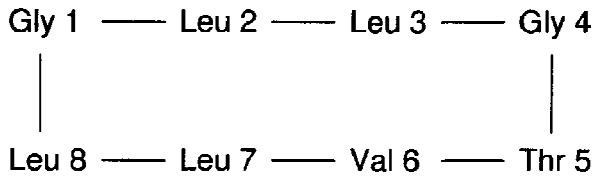

Figure 1.6.2: The structure of an immunomodulating Caryophyllaceae – type cyclopeptide, Curacycline A,

Because viscumamide does not have any halogenated groups, such as Type V (Compositae –type cyclopeptides), it would be safe to say that is most similar to the Type's VI (Caryophyllaceae-type cyclopeptides). Viscumamide has the elemental composition of $C_{30}H_{55}N_5O_5$ and a molecular weight of 565,8. It is a cyclic pentapeptide constituted of leucin and isoleucin units. Viscumamdie and three analogs have been synthesised by Sakurai and Okumura (Okumura and Sakurai, 1972).

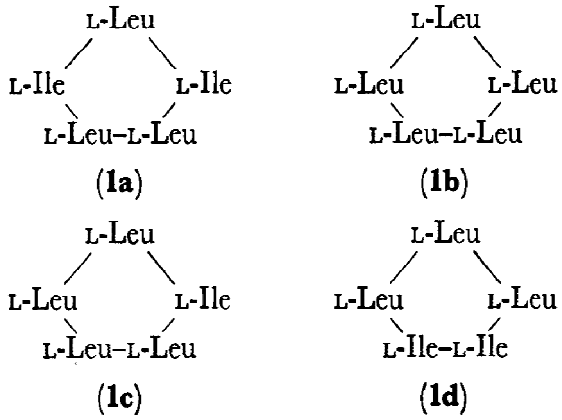

Figure 1.6.3: Viscumamide (1a) and three synthetic analogs (1b, 1c and 1d)

1.6.3 Chemical and Physical Properties

Caryophyllaceae-type cyclopeptides generally have melting points around 200 ˚C and are mostly crystalline structures or powders. They can vary from di- to dodecapeptides and mainly consist of protein amino acids. These types of cyclopeptides are soluble in water, DMSO, C_5H_5N, methanol, $CHCl_3$ and some other organic solvents (Tan and Zhou, 2006).

1.4.3 Chemical Detection

Cyclopeptide alkaloids are often detected by Dragendorff's reagent. This reagent reacts with heterocyclic amines to give an orange precipitate. This method however is often not specific enough to detect other types of cyclic peptides (Tan and Zhou, 2006). Because there has been no special chemical method for detection, cyclopeptides have been investigated phytochemically less often. In 2000 a new specific method for detecting cyclopeptides in plants was developed by Zhou and Tan. The method induces a new thin layer chromatography (TLC) protosite reaction with ninhydrin reagent. Based on their behaviour in TLC, cyclopeptides can also be distinguished from peptidic amides. Additionally it is a useful method to guide cyclopeptide separation and purification.

Since this new method was developed, many new cyclopeptides have been identified (Zhou and Tan, 2000).

1.4.4 Extraction and Isolation

Caryophyllaceae-type cyclopetides. Plant material is dried, ground and often treated directly with methanol or ethanol. The extract is then partitioned with chloroform, ethyl acetate or butanol. The fractions are then chromatographed on a silica gel column repeatedly (Tan and Zhou, 2006).

1.4.5 Structural Elucidation

Chemical, enzymatic and spectral methods have been used to determine structures of Caryophyllaceae-type cyclopeptides. Chemical methods normally involve analysis of amino acids after hydrolysis. Enzymatic methods involve sequence determination after enzymatic hydrolysis. IR, NMR and MS are some of the spectral methods used for structure determination (Tan and Zhou, 2006).

2 Methods and Materials

2.1 General Methods

2.1.1 Preparation of Plant Material

Plant material: dried whole plants (without berries) from *Viscum album* L.

Equipment: electrical blender, Waring

Procedure:
Plant material was weighed and ground into fine a fine powder

2.1.2 Removal of Water and Solvents

Vacuum evaporation:
Vacuum evaporation is a method used for efficient and gentle removal of solvents from samples. A rotary evaporator, which is often used for this purpose, reduces the pressure in a closed system in order to lower the boiling point of the solvent. This allows removal of the liquid solvent without excessive heating, which may damage the sample. The rotary evaporator consists of a heated rotating flask, which is kept under vacuum through a tube connected to a condenser. Vapour from the solvent goes through the connecting tube and condenses in the condenser section. In this study all mixtures were evaporated under reduced pressure at 40-45° C.

Apparatus: Rotavapor RE 120, Büchi.

Small amounts were evaporated with nitrogen (g)

2.1.3 pH Measurement

In this study all pH-values were measured with pH-indicator strips from Merck.

2.1.4 Thin Layer Chromatography

Thin layer chromatography (TLC) is a chromatography technique used to separate mixtures of different compounds. The TLC plate, which can be made of glass, plastic or aluminium foil, is coated with a thin layer of adsorbent material. This layer, which is also known as the stationary phase, is usually made of silica gel, aluminium oxide or cellulose.

After application of the sample onto the plate, a solvent, also known as the mobile phase, is drawn up the plate via capillary action. Different compounds in the sample travel different distances on the plate, depending on the their attraction to the stationary phase and the difference in solubility in the mobile phase. The polarity of the compounds can then be calculated on the basis of an Rf-value (distance travelled by the mobile phase/distance travelled by the compound)

In this study TLC was used to evaluate the separation and purity of the extracted components, as well as their polarity.

Procedure:
TLC plates are spotted at the bottom of the plates with capillary tubes. The plates are then put in a closed container, containing a small amount of the solvent – system. The plates are removed when the mobile phase reaches approximately one cm from the top. Then they are dried and viewed under UV-light before they are sprayed. To activate spots the plates are heated in a heating cabinet.

Detection methods:

UV-light

A UV-light (254 nm) is used to visualize the colourless components in the different extracts. Aromatics, alpha- and beta-unsaturated carbonyls and conjugated systems are some of the typical groups, which are visualized by using UV-light. When the TLC plates are exposed to UV-light the silica gel will fluoresce and organic compounds, which absorb UV light, will appear as dark blue spots.

PMA – spray

PMA (Phosphomolybdic Acid) spray is a good "universal" spray, which is fairly sensitive to solutions with low concentrations. Most functional groups will stain, but it will not distinguish between differences between them based upon the colour of the spots. Most often compounds of interest will appear as a dark green colour, while spots of less interest will appear as a light green colour. To activate the stains for visualization it is necessary to heat the TLC plate after treatment.

2.1.5 Column Chromatography

Column chromatography is a method used to purify individual compounds from mixtures of compounds. The column, a glass tube with a tap at the bottom, is filled with a slurry, prepared from an eluent and a stationary phase powder, to give an even filling without air bubbles. The organic material is put on top of the stationary phase, and finally it is topped with a small layer of sand. The sand prevents the organic material from being disturbed by the addition of fresh eluent. The eluent slowly passes through the stationary phase to advance the organic material. The different components in the organic material are retained by the stationary phase differently, thus they travel at different speeds through the column. This makes it possible to collect the different compounds or fractions. During the process it is normal to change the polarity of the eluents gradually, so that the least polar components elute first and are then followed by components of increasing polarity. Coloured substances can be seen through the glass wall as they travel downwards as moving bands.

Procedure:

A slurry made from the first mobile phase and alumina, is added to the column. The large- scale column contains a glass-wool plug at the bottom to prevent bleeding. The sample is then added to the column, and finally a layer of sand on top. The column is eluted with the mobile phases and the fractions are collected.

Mobile phases:

A: hexane (dead volume)
B: hexane-benzene (2:1)
C: hexane-benzene (1:2)
D: benzene
E: benzene-ether (2:1)
F: benzene-ether (1:2)
G: ether
H: ether-ethyl acetate (1:1)
I: ethyl acetate-ethanol (2:1)

J: ethyl acetate-ethanol (1:2)
K: ethanol (x2)

Stationary phase: Merck aluminium oxide (Al_2O_3) 90 active basic
Particle size: 0,063 – 0,200 mm (70 – 230 mesh ASTM)
Activity level I (= 0% water)

Sand: Merck extra pure sea sand

2.1.6 Extraction Methods

2.1.6.1 Liquid-solid Extraction

Liquid-solid extraction is the process where a solvent with a specific polarity is added to a solid. Any compound in the solid, which has similar polarity to the liquid, is extracted into the liquid phase and may be separated from the solid by gravity or vacuum filtration. A sequence of solvents with different polarity can be used to separate complex mixtures into different fractions.

2.1.6.2 Soxhlet Extraction

Soxhlet extraction is a purification or extraction technique developed by Franz von Soxhlet in 1879. It is particularly used when the compound is partially soluble in a solvent but the impurity is not or vice versa. Soxhlet extraction has many advantages to other extraction methods. For one the material to be extracted is continuously exposed to clean solvent. It is also possible to adjust the extraction temperature, which gives a more efficient extraction process. Additionally, the continuous extraction allows rapid solvent recovery and thus markedly reduces the amount of solvent required

The Soxhlet extractor is placed onto a flask containing the solvent, and condenser is

attached to the Soxhlet. The solid, or in this case, the plant material is put into a paper "tube" placed into the main chamber of the apparatus. The solvent is warmed up to reflux and goes up through the distillation arm, before it floods into the main chamber with the paper tube. As the solvent slowly fills up the chamber the compound will start to dissolve. When the chamber is full it empties through the side arm and the cycle repeats. In this way the compound is extracted a little at a time, and the solid impurity stays in the thimble.

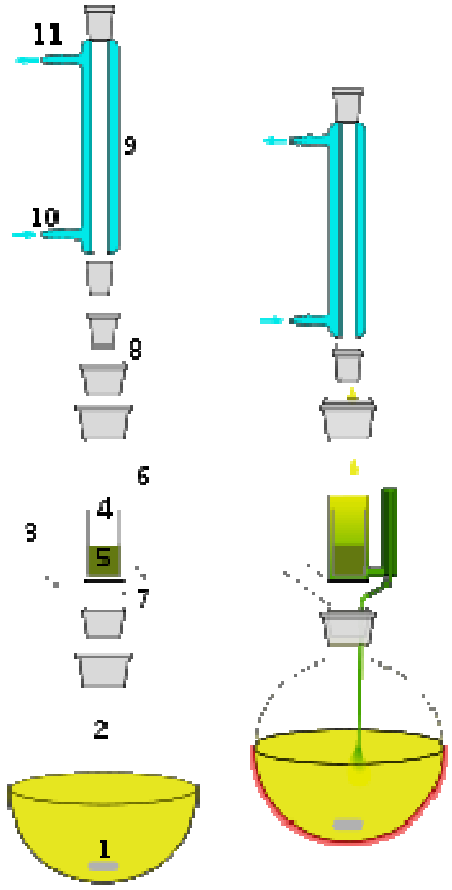

Figure 2.1.1: Schematic representation of a Soxhlet extractor
***1:** Stirrer bar **2:** Still pot (the still pot should not be overfilled and the volume of solvent in the still pot should be 3 to 4 times the volume of the soxhlet chamber) **3:** Distillation path **4:** Thimble **5:** Solid **6:** Siphon top **7:** Siphon exit **8:** Expansion adapter **9:** Condenser **10:** Cooling water in **11:** Cooling water out*
(http://en.wikipedia.org/wiki/File:Soxhlet_extractor.svg)

2.1.6.3 Liquid-liquid Extraction

Liquid-liquid extraction is a method to separate compounds based on their relative solubility in two different immiscible liquids. Water and an organic solvent are usually used. The two solvents create two separate phases, and the substances are extracted from one phase to another depending on their polarity. The two phases can then be separated using a separating funnel.

2.1.6.4 Acid-base Extraction

Acid-base extraction is a method using sequential liquid-liquid extractions to purify acids and bases from mixtures based on the their chemical properties. The procedure is commonly used to free crude extracts from neutral, acidic or basic impurities. The mixture or compound is dissolved and poured into a separating funnel. The pH of the aqueous phase is adjusted to bring the substances of interest into its required form and then added to the separating funnel. The mixture is then shaken and allowed to separate before the phase containing the substance or substances of interest is collected. The procedure is repeated with this phase adjusted to a different pH-value.

2.1.6.5 Digestion

Digestion, or precipitate ageing is a process where a freshly formed precipitate is left after boiling a mixture. The impurities are left in the solution, and the digested precipitate is purer and easier to wash and filter. This physio-chemical process follows the principle of Ostwald ripening. The Ostwald ripening phenomenon describes how solids or liquid solutions become inhomogenous structures over time. Energetic factors will cause large precipitates to draw material from smaller precipitates, which shrink.

2.1.7 Gas Chromatography – Mass Spectrometry

Gas chromatography-mass spectrometry (GCMS) is a method that combines the advantages of two techniques: gas chromatography and mass spectrometry. It is a good way for analyzing mixtures of compounds. The individual components in the mixture are separated by gas chromatography, and characterized by mass spectroscopy. This enables both a qualitative and quantitative evaluation of a mixture containing a number of components.

Instrumental details and settings:

Hewlett Packard 5890 II Gas Chromatograph with dual column and flame ionization detection (FID) in GC unit and a Hewlett Packard 5972 series, mass sensitive detector (MSD) HP S971, run from computer with a delicate HP-Chem laboratory data system.

GC column: Agilent Technologies INC Catalof 190918-105, HP- Ultra 2.

50m x 200mm, 0,33 Micron, -60 to 325/3500SN, US6698711H

The samples were run with an initial temperature 50 °C and initial time 1 min.

Mass range: 50 m/z - 100 m/z

Gas Chromatography:

Gas chromatography (GC) is a chromatography technique used for separating and analyzing compounds that can be vaporized without decomposing. GC is used to separate the different components of a mixture, or to test the purity of a substance. GC can also in some cases be used to identify a certain compound. A carrier gas is used as a mobile phase and the stationary phase is a microscopic layer of liquid or polymer, which coats the inside of a glass or metal tube, also called the column. In principle gas chromatography is similar to column chromatography. The analytes in their gaseous form interact with different stationary phases in the column, and each compound elutes at a different retention time. By comparing the different retention times each component can be analyzed.

Mass Spectroscopy:

Mass spectrometry (MS) is an analytical technique for identifying unknown compounds, determining molecular weight, isotopic composition of elements in a molecule, and the structure of a compound by analyzing its fragmentation. MS can also be used quantitatively to find the concentration of a compound in a sample.

As the individual compounds elute from the GC column, they enter an electron ionization source. In this case an EI (electron impact) source was used. The EI source produces ions by the interaction with energetic electrons and gas phase atoms. It is considered a classical method of analyte ionization, especially when coupled with a gas chromatograph. It is routinely used to analyze volatile organic compounds with low mass and thermal stability. An EI source gives high levels of fragmentation and is considered to represent a "hard" method of ionization.

When the compounds are ionized they break into large or small fragments with a certain mass and charge. The mass to charge ratio (m/z) ratio usually represents the molecular mass of the fragment, because most fragments have a charge of + 1.

According to their m/z ratio the ions are sorted and separated in a mass analyzer, in this case a quadrapole analyzer was used. The ions are then detected and tallied in a detector. Finally the results are displayed on a chart or spectrum, where the x-axis represents the m/z ratios, and the y-axis represents the signal intensity (abundance) for each of the detected fragments. This graph is referred to as a mass spectrum.

As mentioned earlier the EI Source is a "hard" ionizing technique, and due to this the mass spectra will be dominated by fragmentation. The molecular ion M+, which represent the molecular weight of the analyte will often undergo fragmentation, and sometimes not show up in the mass spectrum. However, because many compounds have characteristic fragmentation patterns, this could be a very helpful tool to identify them.

2.1.8 DART Mass Spectrometry

Direct analysis in Real Time (DART) Mass Spectrometry uses a new atmospheric pressure ion source for the direct analysis of molecules from the surface. It instantaneously ionizes gases, liquids and solids at atmospheric pressure, by simply placing the sample between the DART ionizing source and the mass spectrometer. There is no need for solvent or sample preparation and ionization can take place directly on surfaces, such as, tablets, bodily fluids, glass, plant leaves, fruits and vegetables. Contradictory to mass spectrometry with an EI Source, DART Mass Spectrometry uses a so-called "soft" ionizing technique, which gives a relatively simple mass spectra dominated by $[M+H]^+$ -, [M-H] - or M^+ - molecules. DART can be used to identify substances by giving exact mass measurement and possible elemental compositions.

DART settings:
Helium velocity: 9,0 1/min
Gas temperature: 250 C°

MS settings:
Orifice temperature: 120 C°
Ion Guide Peak voltage 1500 V
Detector voltage 2400 V
Mass range 100 – 1000 m/z

2.1.9 NMR Spectroscopy

Nuclear magnetic resonance (NMR) is a physical resonance phenomenon, which occurs when the nuclei of certain atoms are immersed in a static magnetic field and exposed to a second external magnetic field. Only nuclei that possess a property called spin, experience this phenomenon. These nucleons have odd numbers of neutrons.

NMR spectroscopy is a frequently used method to study simple chemical structures by one-dimensional techniques. One-dimensional NMR gives information about the number and types of functional groups or chemical entities in a molecule. To determine the structure of more complicated molecules two-dimensional methods are used.
Many types of information can be obtained from an NMR spectrum.

NMR spectroscopy takes advantage of the qualities of nuclei with spin properties. The ^{1}H- isotope and the ^{13}C – isotope are most commonly studied.

Chemical shift:

When a nucleus with an odd number of neutrons is placed in a magnetic field it absorb energy at a frequency characteristic of the isotope. For instance different ^{1}H - or ^{13}C - atoms absorb energy at slightly different frequencies. Chemical shift is the frequency of the absorption for a nucleus of interest relative to the frequency of the absorption of the standard.

The chemical shift of a nucleus is reported in ppm and given the symbol delta (δ):

$$\delta = (n - n_{REF}) \times 10^{6} / n_{REF}$$

Proton NMR:

Proton NMR is the application of nuclear magnetic resonance with respect to the ^{1}H – nuclei in the molecule being investigated. It is used to identify hydrogen atoms in organic molecules and determine their structures. It is important that hydrogen in the solvent does not interfere. Therefore, deuterated solvents are mostly used. Chemical shifts range between +12 to -4 ppm for most proton NMR spectra of organic compounds.

^{13}C NMR:

^{13}C NMR is the application of nuclear magnetic resonance with respect to the ^{13}C – nuclei in the molecule being investigated. It is used to identify carbon atoms in organic molecules and determine their structure.

This method is used when C-H bonds are lacking, and when distinguishing between isomers is difficult based on proton NMR alone. Even though the abundance of ^{13}C is only 1,1% it is possible to conduct a NMR experiment.

The combination of ^{13}C NMR and proton NMR is a powerful tool to in the elucidation of molecular structures.

2.2 Investigation of Non-polar Constituents in Viscum album

Research has mainly been focused on the polar, water-soluble constituents in mistletoe and their biological activities. In this section we wanted to investigate the constituents in a non-polar *Viscum album* extract, by GCMS.

2.2.1 Soxhlet Extraction

Non-polar constituents in *Viscum album* were extracted with hexane, by Soxhlet extraction.

Equipment:
Heating mantle
Stirrer with magnet
Flask (500ml)
Soxhlet column connected to a condenser
Paper thimble for plant material

Procedure:
Dried and ground plant material (14g) was placed in a thimble inside the Soxhlet column. Hexane (300 ml) was heated until boiling in the flask while stirring. The plant material was extracted for approximately four hours. Then the yellow extract was removed from the flask, and fresh hexane (300ml) added. The process continued until the extract from the plant material was almost colourless. The plant residue after extraction was weighed to 12,3 g. Hexane was removed from the extract by vacuum evaporation. The yellow oily concentrate was dissolved in a small amount of hexane and a sample was prepared for GCMS.

2.3 Isolation of Viscumamide from Viscum album

In this section we attempted to extract and isolate the cyclic peptide viscumamide from the neutral fraction of *Viscum album*. The extraction-process has many steps and was performed on a large scale, because of an expected low yield of viscumamide. The cyclic peptide has previously been isolated from Korean mistletoe, *Viscum album* var. coloratum. The methods of Sakurai and Okumura were followed. (Okumura and Sakurai, 1972)

2.3.1 Extraction with Methanol

Polar- and medium – polar constituents were extracted from *Viscum album* with methanol by, Solid-liquid extraction. This was the fraction we were interested in working with since viscumamide is a medium-polar constituent.

Equipment and material:
Dried and ground plant material
Erlenmeyer flask (1000 ml)
Shaker
Filter paper and filter funnel
Solvent: methanol

Procedure:
Ground plant material (1kg) was extracted with ethanol (3L) for approximately 24 hours. The methanol fraction was filtered with filter paper and a fresh portion of methanol (3L) was added to the plant residue. The plant residue was extracted for another 24 hours, before it was filtered through filter paper. The extract was shaken the whole time to ensured controlled and complete mixing. The plant residue was weighed to 718,7 g. The bright green methanol fraction was concentrated under reduced pressure. A dark green oily concentrate was left after removal of the solvent.

2.3.2 Extraction with Ethyl acetate

Medium-polar constituents were separated from the most polar constituents in the methanol extract with ethyl acetate by, liquid-liquid extraction.

Equipment and material:
Erlenmeyer flask (1000 ml)
Shaker
Filter paper and filter funnel
Solvent: ethyl acetate

Procedure:
The oily concentrate from the methanol fraction was extracted with ethyl acetate for approximately 24 hours. A sticky brown residue formed. The bright green ethyl acetate fraction was filtered through filter paper, a fresh portion of ethyl acetate was added to the residue, and shaken for another 24 hours. This process was repeated three times. Using a shaker ensured controlled and complete mixing of the extract. The sticky brown residue was weighed to 160,5 g. Ethyl acetate was removed from the fraction by evaporation under reduced pressure. A dark green oily concentrate was left after removal of the solvent.

2.3.3 Neutral Fraction

To remove organic acids and bases from the neutral compounds in the ethyl acetate fraction, acid-base extraction was performed.

Equipment and material:

pH-strips

Concentrated HCl (l)

Na_2CO_3(s)

Separating funnel

Solvents: Ethyl acetate and water

Procedure:

The oily concentrate from the ethyl acetate fraction was re -dissolved in ethyl acetate (35 ml) and added to a separating funnel. Concentrated HCl (l) was diluted with water until pH=3, and an aliquot of 50 ml added to the ethyl acetate fraction. The mixture was thoroughly shaken and left to separate in the separating funnel. After several days the light green acid fraction was removed from the separating funnel.

Na_2CO_3(s) was diluted with water until pH=9, and an aliquot of 50 ml were added to the separating funnel with the dark green ethyl acetate fraction. The mixture was shaken thoroughly and left to separate. After several days the light green basic fraction was removed from the separating funnel, leaving the neutral ethyl acetate fraction behind. The neutral fraction was dark green and contained some lumps. Ethyl acetate was removed form the neutral fraction by evaporation under reduced pressure.

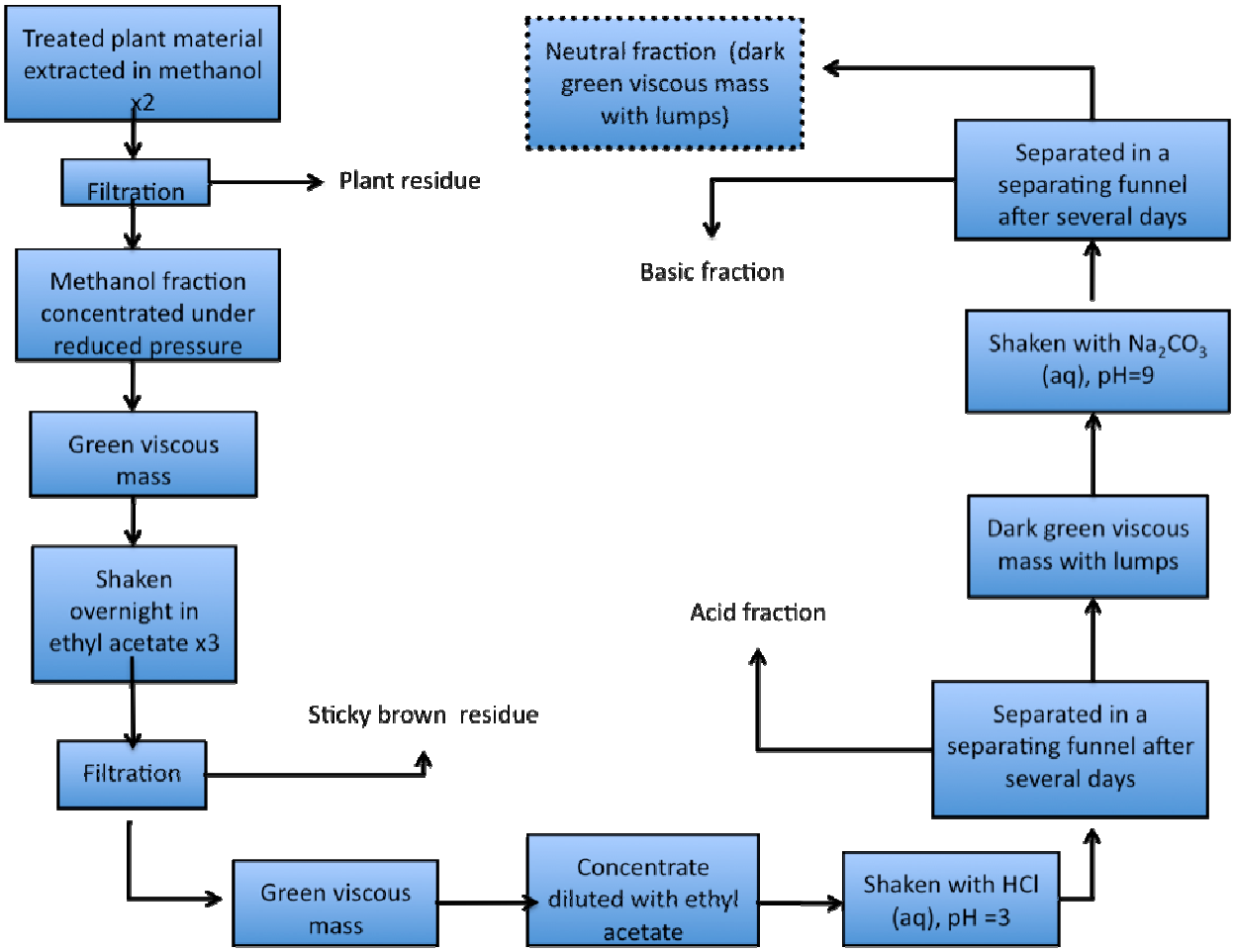

Figure 2.3.1: Flowchart, extraction of neutral fraction

2.3.4 Digestion

Digestion with boiling hexane was performed to remove remaining non-polar impurities from the neutral fraction.

Equipment and material:

Flask (500ml)

Heating mantle

Stirrer with magnet

Condenser

Filter paper and filter funnel
Solvent: hexane

Procedure:
Hexane (150ml) was heated until boiling and allowed to cool a little before it was added to the neutral fraction. The mixture was heated again and allowed to boil for approximately 30 minutes, while stirring. A dark green residue precipitated from the mixture. The residue (17,3g) was filtered with filter paper, washed with hexane and left to dry overnight.

2.3.5 Column Chromatography of Neutral Fraction

Column chromatography was performed to separate different constituents in the neutral fraction. A sample was prepared from the neutral fraction and eluted with solvents of increasing polarity. It was expected that viscumamide would elute in one of the medium – polar fractions.

Equipment and material:
Flasks (1000 ml) for the individual fractions
Glass column with a diameter of 7 cm
Alumina (25g) for sample
Sand
Glass wool
Alumina (700 g) fro column

Preparation of sample:
The dark green residue from the digestion with hexane was dissolved in hot methanol (100 ml). Alumina was added to the mixture and it was evaporated until dryness under reduced pressure.

Preparation of column:

The column was prepared as described in section 2.1.5. The sample prepared from the neutral fraction was eluted with 500 ml of each of the mobile phases A-K. The constituents in the sample were separated and collected into 11 fractions, and vaporised under reduced pressure.

2.3.6 TLC Analysis of Fractions 1-11

The 11 fractions from the column chromatography were run on TLC plates with β - amyrin as reference substance. This was done to identify which of the fractions contained medium polar constituents.

Materials:

Stationary phase**:** Merck silica gel on glass plate (20 x 20 cm) with fluorescence indicator F_{254}. Layer thickness: 0,25 mm

Mobile phase**:** Petroleum ether – acetone (8:2)

Procedure:

Samples from each of the fractions were run on TLC plates, following the procedure described in section 2.1.4. The plate was viewed under UV – light and sprayed with PMA, before heating.

2.3.7 Digestion of Fractions 9 -11

The polar to medium-polar fractions (9-11) were pooled and digested with boiling hexane to remove remaining non-polar constituents. The procedure was followed according to section 2.3.4.

2.3.8 Column Chromatography of Polar to Medium-polar Fractions (Precipitate)

A sample from the precipitate was prepared for column chromatography, according to section 2.3.5.

Equipment and material:

Mobile phases: G-K

Glass column with a diameter of 3,5 cm

Flasks (500 ml) for the fractions

Alumina (10 g) for sample

Alumina (70g) for column

Sand

Procedure:

The column was prepared as described in section 2.1.5. The sample was eluted with 200 ml of each of the mobile phases G-K. The constituents in the sample were separated and collected into 14 fractions, and vaporised under reduced pressure.

2.3.9 TLC Analysis of Fractions 1-14

The 14 fractions were run on TLC plates with β -amyrin as reference substance. This was done to investigate their polarity and purity

Materials:

Stationary phase**:** Merck silica gel on glass plate (20 x 20 cm) with fluorescence F_{254}.

Layer thickness 0,25 mm

Mobile phase**:** Petroleum ether – acetone (8:2)

Procedure:

Samples from each of the fractions were run on TLC plates, following the procedure described in section 2.1.4. The plate was viewed under UV – light and sprayed with PMA, before heating.

2.3.10 TLC Protosite Reaction with Ninhydrin

TLC protosite reaction is a specific method for detecting cyclopeptides in plants. Ninhydrin, which is a common reagent for amino acids, reacts with free amino groups (NH or NH_2) to produce a pink or purple compound. Most cyclopeptides cannot react with ninhydrin, because there are usually no free amino groups present. The TLC protosite reaction solves this problem by hydrolysing the peptide links in the cyclopeptide to produce free amino groups. The fractions containing medium-polar constituents (5 – 12) were analysed by this method.

Equipment and Material:

Stationary phase: Fluka silica gel on glass plate (5 x 10 cm).
Layer thickness: 0,25 mm
Mobile phase**:** chloroform-methanol (8,5:1,5)
Concentrated HCl (l)
Sealed glass vessel

Procedure:

This procedure follows the methods of Zhou and Tan. (2000). Two identical TLC plates (plate 1 and 2) are spotted with samples from fraction 5 -12 (3 samples on each plate), and developed with the chloroform-methanol system, following the procedure described in section 2.1.4.

After the plates had dried, plate 2 was placed and hung in a sealed glass vessel, containing concentrated HCL (1ml). The vessel was placed in an oven (110˚ C) for 1-2 hours. Plate 2 was then cooled for a few minutes and the HCl volatilized.

Plate 1(non-hydrolyzed) and plate 2 (hydrolyzed) were sprayed with 0,2% ninhydrin-acetone reagent, and heated in a drier for several minutes. This final step was repeated.

2.3.11 DART Mass Spectrometry

The fractions giving positive results for the TLC protosite reaction with ninhydrin, were analysed by DART mass spectrometry.

Preparation of samples:
Samples were dissolved in methanol-ethyl acetate (1:1) at 100 micrograms/ml.

2.4 Investigation of an Unidentified Cyclic Peptide in Viscum album

In this section we wanted to investigate further fraction 8 and 9 from section 2.3.10, which indicated the presence of an unidentified cyclic peptide.

2.4.1 Preparative TLC

Fraction 8 and 9 were pooled together with fraction 7 and 10. This was done because traces of the unidentified compound could also be present in these fractions, and to prevent the loss of this material. The constituents in the sample were separated by preparative TLC, which is a standard analytical separation method.

Equipment and material:

Stationary phase: Merck preparative silica gel on glass plates (20 x 20 cm) with fluorescent. Layer thickness 60,2 mm
Mobile phase: Chloroform - methanol (9:1)

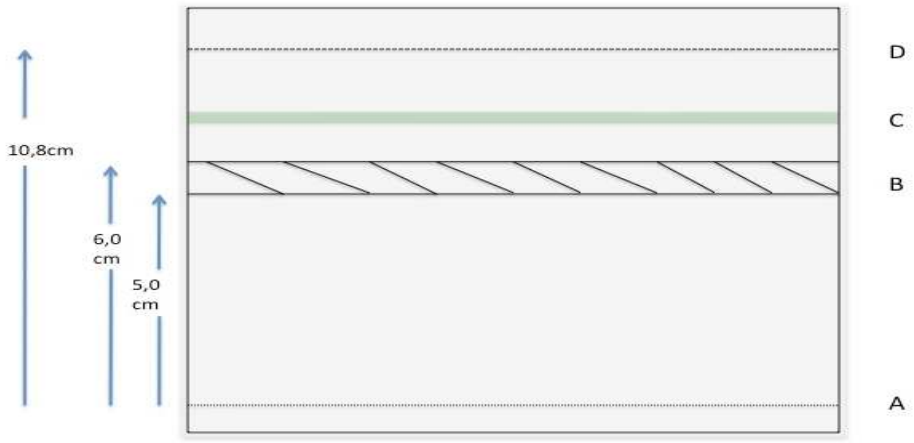

Figure 3.4.1:Schematic figure of preparative TLC plate, showing the site of the sample application (A), the zone recovered by scraping (B), a band of green material (C), and the distance travelled by the mobile phase (D).

Procedure:

The sample was applied as a long streak approximately one cm above the edge of the plate, and then developed with the chloroform – methanol system, following the procedure described in section 2.1.4. Two preparative TLC plates were used to separate the sample. The plate was divided into zones, which were scraped off. The scraped zones were separately eluted with warm ethanol, and the silica gel was filtered under reduced pressure. Finally the fractions were vaporized under reduced pressure.

2.4.2 NMR Spectroscopy

One of the fractions from the preparative TLC showed some crystallisation, indicating a better purity than the other fractions. This fraction was also in the expected area of the Rf-values of the cyclic peptide, according to the TLC – protosite reaction with ninhydrin (marked zone in figure 2.4.1). A sample was prepared and analysed by NMR spectroscopy.

There are no standard protocols to determine the structure of cyclic peptides by NMR. Yang and Zou has proposed a strategy for structural elucidation by NMR, which can be used as a guide. This involves ^{1}H NMR and ^{13}C NMR in C_5D_5N, DMSO-d, or CD_3OD to determine the planar structure of the compound. If one set of sharp ^{1}H and ^{13}C signals under suitable conditions can be obtained 2D NMR techniques can be used to determine the molecular composition (Tan and Zhou, 2006).

Instrumental settings:

The NMR experiments (^{1}H, ^{1}H-^{13}C HMBC, ^{1}H-^{13}C HSQC, ^{1}H-^{1}H COSY, ^{1}H-^{1}H TOCSY, ^{1}H-^{1}H ROESY, ^{1}H-^{1}H NOESY and ^{13}C (C) APT were obtained at 600.13 and 150.90 MHz for ^{1}H and ^{13}C, respectively, on a User Biospin AV-600 MHz instrument equipped with a TCI ^{1}H-$^{13}C/^{15}N$ CryoProbe. All experiments were recorded at 298K. Chemical shift values were set relative to the deuterio-methyl ^{13}C signal and the residual ^{1}H signal of the solvent at δ 49.0 and δ 3.34 for CD_3OD.

3 Results and Discussion

3.1 Investigation of Non-polar Constituents in Viscum album

The chromatographic profile of the hexane extract evidenced the presence of 11 compounds. We attempted to identify them by their fragmentation patterns, and searches in a mass spectral library and SBD (Spectral Database for Organic Compounds). The 6 first peaks (I-VI) in the gas chromatogram represented fatty acids and fatty acid esters, and the last peaks (VIII-XI) represented triterpenoids. Most of these have already been found in *Viscum album*.

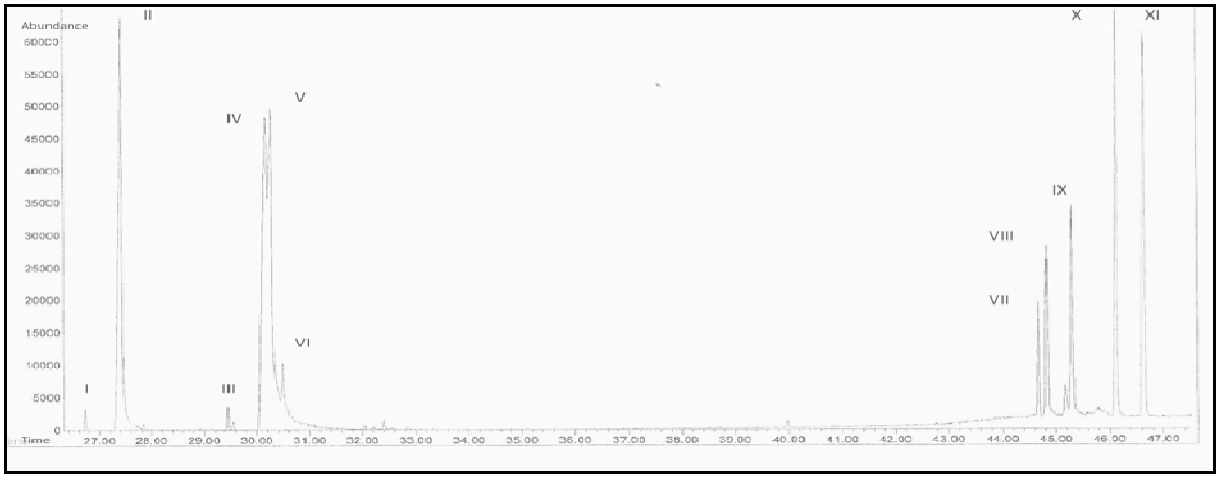

Figure 3.1.1 Gas chromatogram of hexane extract, where the peaks are numbered I – XI

3.1.1 Results from Gas Chromatography Mass Specrometry

Compound I

Abundant peaks appear in the mass spectra (appendix 2) at m/z 55, 74 (base peak), 87 and 143. The base peak, m/z 74 indicates that a methyl ester is present. The base peak m/z 74 is a McLafferty rearrangement ion, which is used to identify most methyl ester derivates of fatty acids. The ions at m/z 87 and 143 of the general formula $[(CH_2nCOOCH_3]^+$, where 87 is the most abundant., are formed by the losses of neutral aliphatic radicals .The molecular ion is missing, but methyl esters usually show weak M+ ions. A library search showed that this most likely is methyl palmitate.

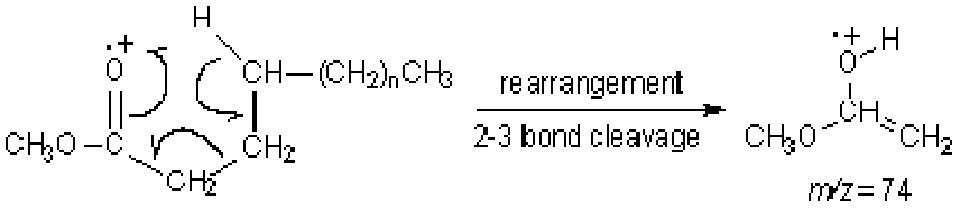

Figure 3.1.2: Mclafferty rearrangement in methtyl esters (http://w ww.lipidlibrary.co.uk/ms/ms03/index.htm)

Compound II

Abundant peaks appear in the mass spectra (appendix 3) at m/z 60, 73,129, and 256(M^+). The m/z 60 is a typical Mclafferty rearrangement ion for aliphatic carboxylic acids. The spectra shows the regular series of fragment ions separated by 14 amu, which indicates that no other functional groups are present.
A library search showed a striking similarity with a standard spectrum of palmitic acid, which is one of the most common saturated fatty acids found in animals and plants.

Compound III and IV

The mass spectra of compound III and IV (appendix 4 and 5) were similar, indicating that they are related to each other. The mass spectrum of compound III gave little information, but the abundant peak at m/z 67 ($C_5H_7^+$) as a result of double bond transfer, is characteristic for biunsaturated fatty acids (Wu et al., 2006).

A library search suggested that compound number IV might be linoleic acid. The ions 55, 67, 81,95,109,123 as a result of cleavage of alpha series, are characteristic for linoleic acid. Compound III is probably an isomer, with double bonds located in different positions.

Compound V

The mass spectrum of compound V (appendix 6) shows similarities to the mass spectrum IV, with abundant peaks at m/z 55 and 67, which suggests they are related. However, the spectrum of compound IV has 79 m/z (C_6H_7+) as the most abundant ion. According to Wu Hui-Qin and coworkers, the base peak can determine the degree of unsaturation in fatty acids, where m/z 74, 55, 67 and 79 is characteristic for saturated, monounsaturated, biunsaturated and polyunsaturated fatty acids respectively (Wu et al., 2006). Spectrum V also showed a strikingly similarity to a standard spectrum of the polyunsaturated fatty acid, linolenic acid (SDBS)

Compound VI

The mass spectrum of compound VI (appendix 7) was poor and gave very little information. A search in a library and SDBS gave no results. The base peak is at m/z 55 it is possible that this is a biunsaturated fatty acid. It is otherwise difficult to say anything more about this compound.

Compound VII

The mass spectrum of compound VII (appendix 8) shows a typical fragmentation pattern of triterpenoids. A library search gave no result. However, Kolhe and coworkers isolated a triterpene, *butyrospermenone*, which showed a similar fragmentation pattern, with a base peak at 69 m/z, in addition to abundant fragments at 409, 207, 311. Butyrospermenone *has the* molecular formula $C_{30}H_{48}O$, the molecular weight 424, and is related to oxo triterpenes, such as β-amyrenone, α-amyrenone and lupenone. It was difficult to find more information about this compound (Kolhe et al., 1981).

Compound VIII

A library search gave no results for this compound (appendix 9), and a standard spectrum was not found in SDBS. However, the abundant ions at m/z 218, 203, 207 and 189 are typical for the fragmentation of β-amyrin. The spectra also had striking similarities with a mass spectrum of a standard sample of β-amyrin, by Liu and co-workers (figure 3.1.4) (Liu et al., 2009).

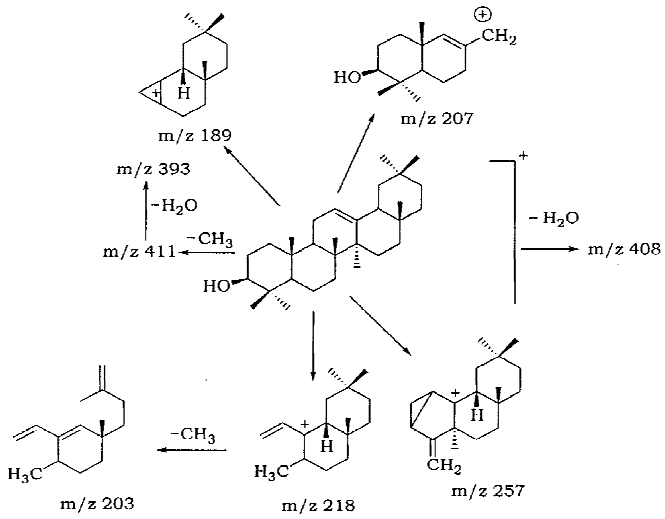

Figure 3.1.3: Mass fragmentation of β – amyrin (http://prr.hec.gov.pk/Chapters/426-9.pdf)

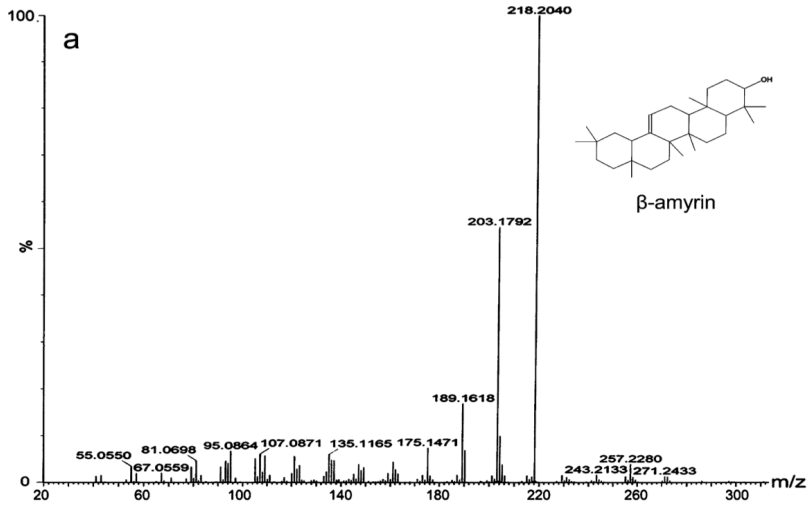

Figure 3.1.4: Mass spectra of standard sample of β-amyrin (Liu et al., 2009)

Compound IX:

A library search gave no results for the mass spectrum of compound IX (appendix 10), and a standard spectrum was not found in SDBS. However, the abundant peaks at m/z 424 (M^+), 205, 218 and 189 are typical for the fragmentation of lupenone.

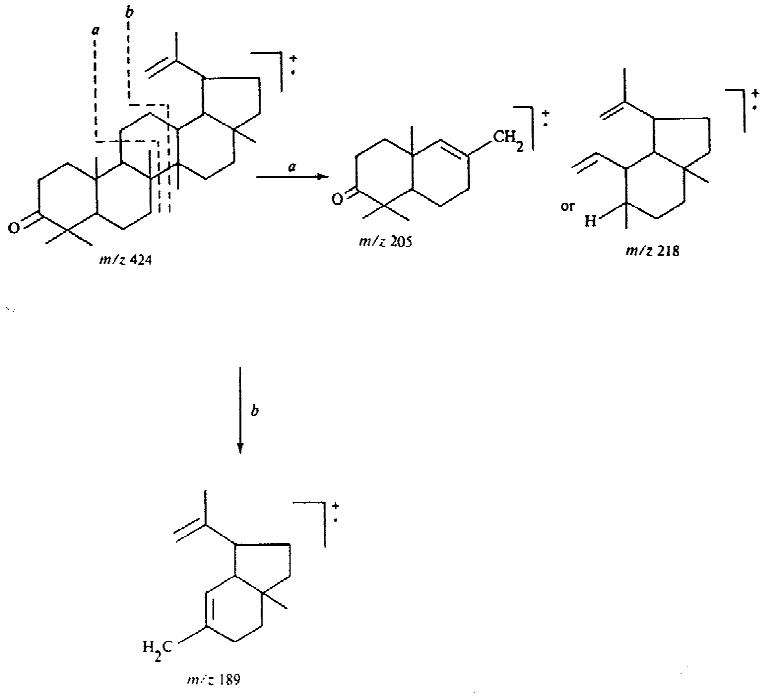

Figure 3.1.5: Mass fragmentation of lupenon (Plumejai and Saifah, 1986)

Compound X:

The mass spectrum of compound X (appendix 11) was strikingly similar to a standard spectrum of β-amiryn aceate (SDBS). Both the unknown and the standard spectra showed abundant peaks at m/z 218 (base peak), 203, 189 and 69. The molecular ion (468 m/z) is not visible in the unknown or the standard spectrum.

Compound XI

It was difficult to find a standard mass spectra that matched this fragmentation pattern well in a library or a database. Judging from the abundant peak at m/z 189 and the molecular ion at m/z 468 this is most likely lupeol acetate, or an isomer (appendix 12).

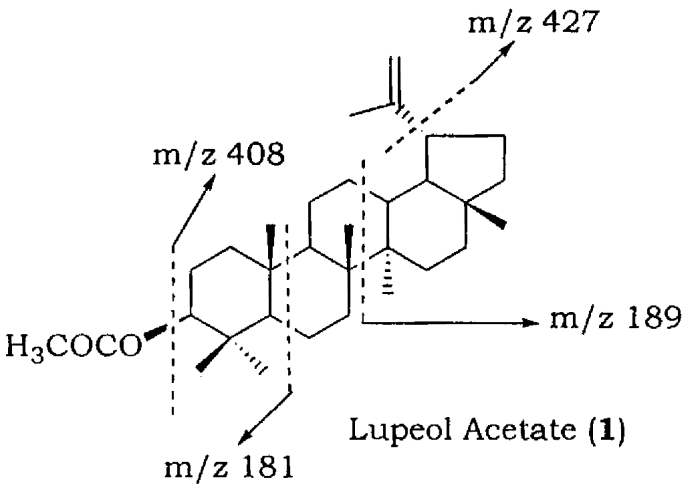

Figure 3.1.6: Mass fragmentation of lupeol acetate (http://prr.hec.gov.pk/Chapters/426-9.pdf)

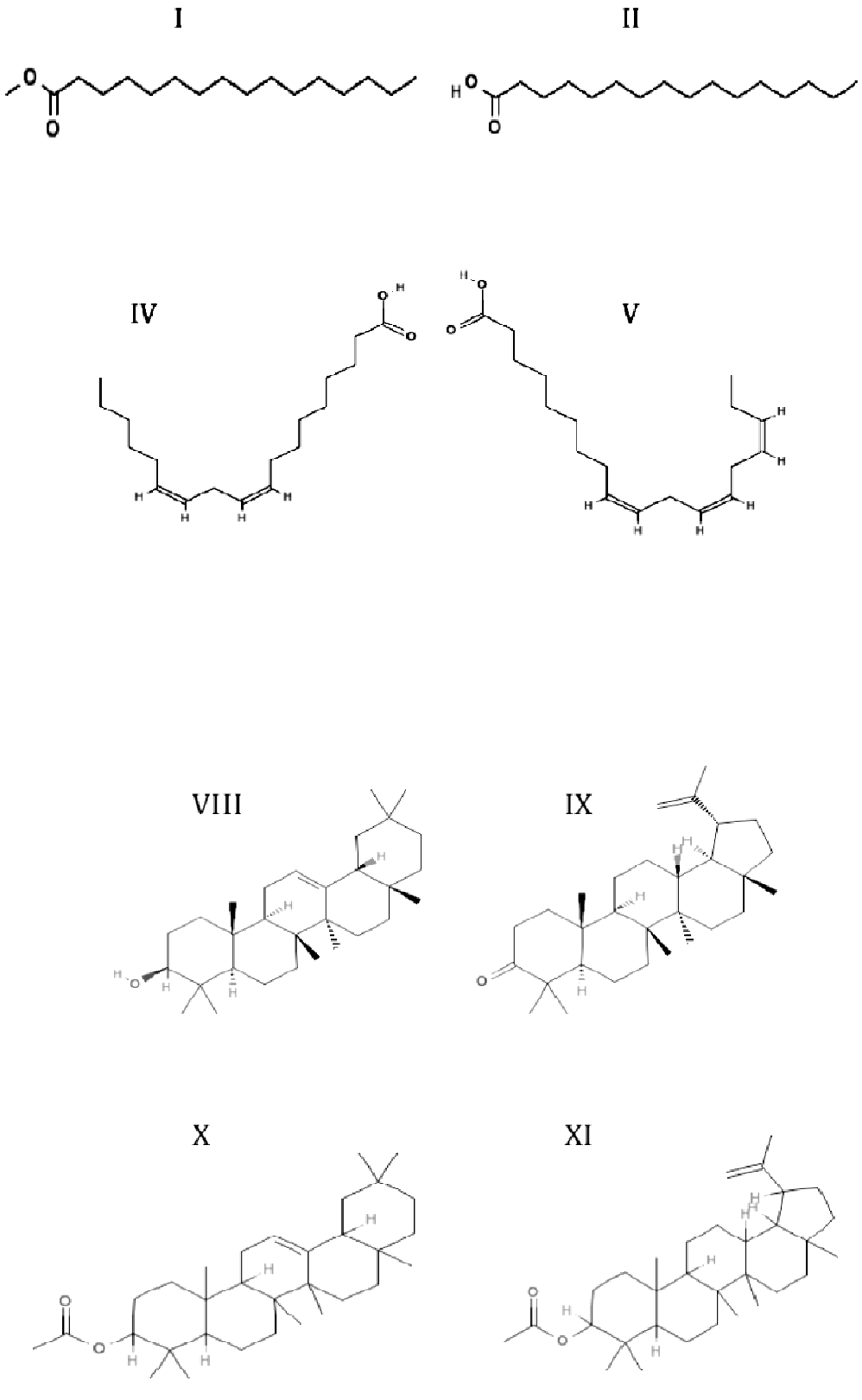

Figure 3.1.7: Identified compounds in hexane extract

Compound		***Ret. time*** *(min.)*	***Ions (m/z)*** *(descending intensity)*	***Mw***	***Mf***
I	Methyl palmitate	26,728	74, 87, 55, 143	270.5	$C_{17}H_{34}O_2$
II	Palmititc acid	27,38	73, 60, 129, 256	256.4	$C_{16}H_{32}O_2$
III	Linoleic acid (isomer)	29,45	67, 55, 81, 95	280.4	$C_{18}H_{32}O_2$
IV	Linoleic acid	30,16	67, 55, 81, 95, 280	280.4	$C_{18}H_{32}O_2$
V	Linolenic acid	30,28	79, 67, 55, 95, 108	278.4	$C_{18}H_{30}O_2$
VI	Unidentified fatty acid	30,5	55, 73, 95, 129	-	-
VII	Unidentified triterpene	44,66	69, 55, 207, 311, 409	408	-
VIII	β -amyrin	44,83	218, 203, 55, 69, 189, 281	426.7	$C_{30}H_{50}O$
IX	Lupenone	45,3	55, 95, 109, 205, 189, 424	424.7	$C_{30}H_{48}O$
X	β - amyrin acetate	46,132	218, 203, 55, 69, 189	468.8	$C_{32}H_{52}O_2$
XI	Lupeol acetate	46,65	69, 95, 189, 207, 468	468.8	$C_{32}H_{52}O_2$

Table 3.1.1 Identified and unidentified compounds detected in hexane extract

3.2 Isolation of Viscumamide from Viscum album

The neutral fraction of *Viscum album* was extracted through several steps. Column chromatography was performed to isolate the cyclic peptide from other constituents in the neutral fraction.

3.2.1 Column Chromatography of Neutral Fraction

Fraction	Eluent	Volume (ml)	Fraction colour	Solvent	Crystals/ Precipitation
1	A	500	-	Hexane	-
2	B	500	-	Hexane	-
3	C	500	-	Hexane	++
4	D	500	LY	Hexane	-
5	E	500	LY	Ethyl acetate	-
6	F	500	LY	Ethyl acetate	-
7	G	500	Y	Ethyl acetate	-
8	H	500	Y	Ethyl acetate	-
9	I	500	BY/G*	Methanol	+
10	J	500	BG	Methanol	-
11	K	500	LG	Methanol	-

Table: 3.2.1: Column chromatography of neutral fraction
L= light colour, B = bright colour, Y = yellow, G = green

It was expected that viscumamide would elute in one of the medium polar fractions. Fraction 9, which showed some crystallisation, was analysed by DART spectroscopy. However, the MS spectra showed no significant peak in the excepted area for Viscumamide. This was most likely because too many other compounds were present in the fraction.

* *Was presented as a strong coloured band in the column*

3.2.3 TLC Analysis of Fractions 1-11

TLC analysis of fraction 1-11, from the column chromatography, was performed to get an indication of the polarity and purity of the individual fractions. β-amyrin is a non-polar compound, so we were interested in the fraction below the Rf-value for this compound (fraction 9-11).

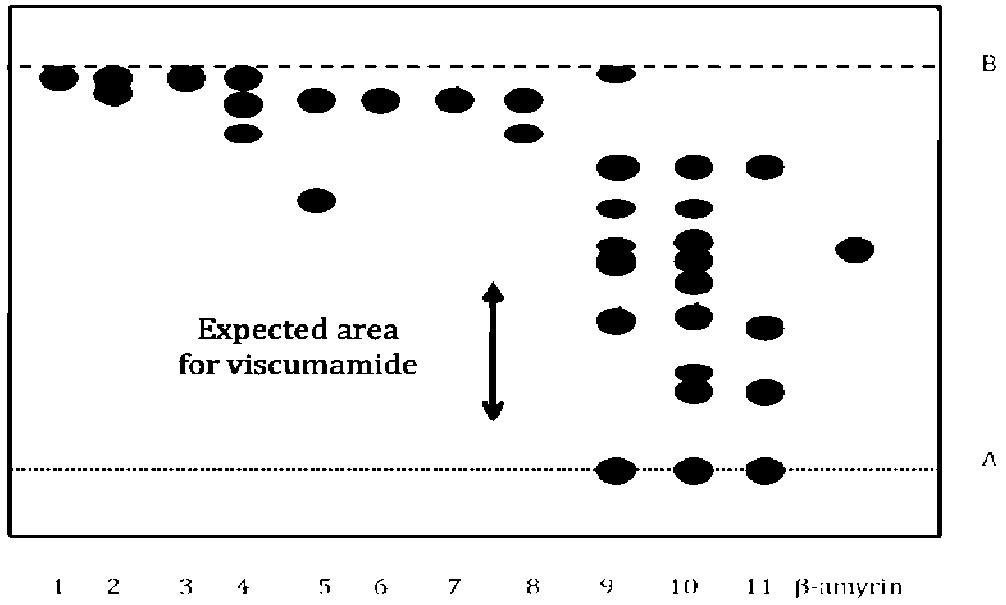

Figure 3.2.1 Schematic figure of TLC plate with samples from fraction 1-11 and β - amyrin, after spraying with PMA.
A = application site, B = distance travelled by mobile phase

3.2.4 Column Chromatography of Polar to Medium-polar Fractions (Precipitate)

To improve the purity of fraction 9 -11, we repeated digestion with boiling hexane (section 2.3.7). A sample was prepared from the precipitate, and separated by column chromatography into 14 fractions.

Fraction	Eluent	Volume (ml)	Fraction-colour	Solvent	Crystals /Precipitation
1	G	200	LY	Ethyl acetate	-
2	G	50	-	Ethyl acetate	-
3	G + H	50	BY	Ethyl acetate	-
4	H	150	BY	Ethyl acetate	+
5	H	50	Y	Ethyl acetate	-
6	H + I	50	Y	Ethyl acetate-ethanol (1:1)	-
7	I	50	BY/G *	Ethyl acetate-methanol (1:1)	++
8	I	50	BG/Y	Ethyl acetate-methanol (1:1)	-
9	I	50	BG	Ethyl acetate-methanol (1:1)	-
10	I + J	50	BG	Ethyl acetate-methanol (1:1)	-
11	J	50	LG	Methanol	-
12	J	100	LG	Methanol	-
13	J + K	50	LG	Methanol	-
14	K	150	LG	Methanol	-

Table 3.2.2: Column chromatography of the medium - polar and polar fractions (precipitate)
L= light colour, B = bright colour, Y = yellow, G = green,

* *Was presented as a strong coloured band in the column*

3.2.5 TLC Analysis of Fractions 1-14

TLC analysis of fraction 1-14 was performed to get an indication of the polarity and purity of the individual fractions. The analysis showed that fraction 5 -12 contained the medium polar constituents we wanted to investigate further.

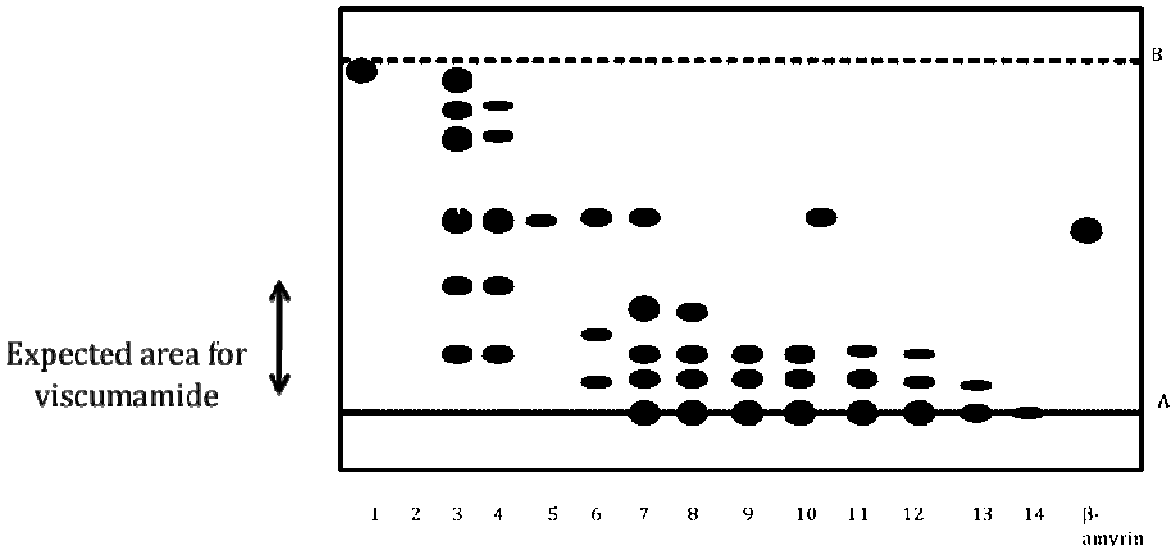

Figure 3.2.2: Schematic figure of TLC plate with samples from fraction 1-14 and β-amyrin, after spraying with PMA.
A = application site, B = distance travelled by mobile phase

3.2.6 TLC Protosite Reaction with Ninhydrin

Fraction 5-12 was investigated by TLC protosite reaction with ninhydrin, to determine if there are cyclic peptides present.

Clear pink/purple spots appeared in the plates with samples from fraction 6,7,8 and 9, on the hydrolysed plates. The corresponding non-hydrolysed plates gave no positive reaction after spraying with ninhydrin. This gives a strong indication that there are cyclic peptides present. Fraction 7, 8 and 9 showed two clearly separated spots on the

hydrolysed plate, indicating that there might be more than one cyclic peptide present in these fractions.

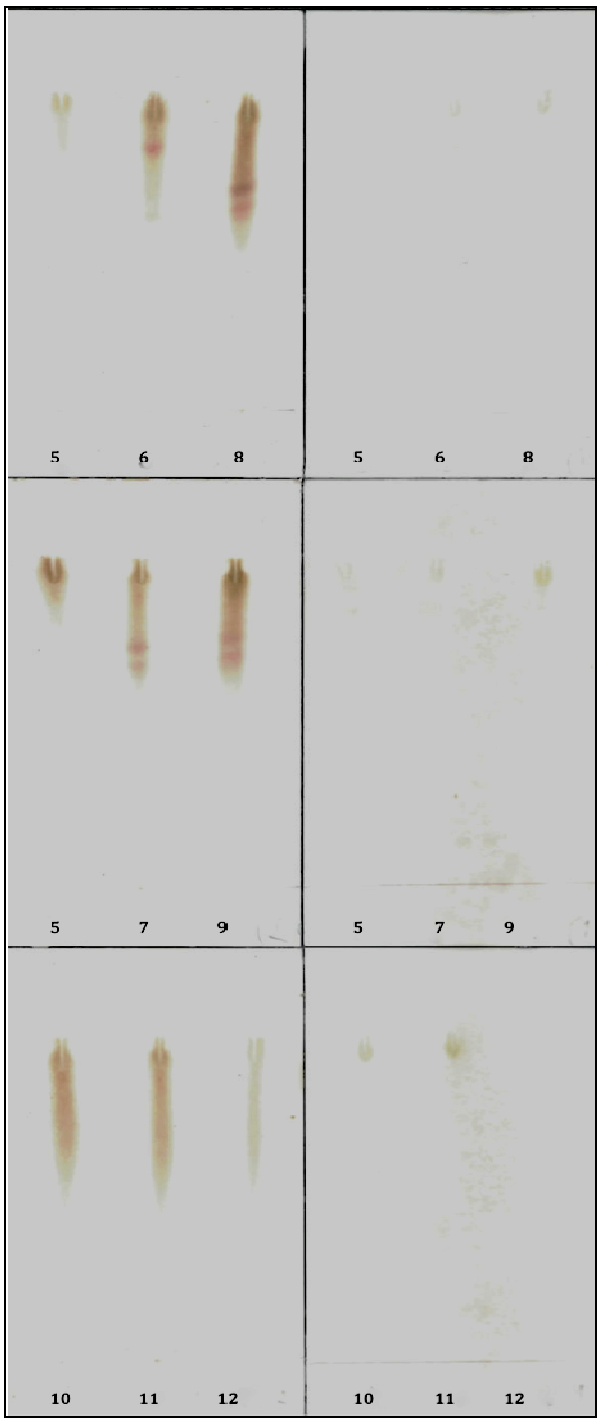

Figure 3.2.3: Hydrolyzed (left side) and non-hydrolyzed (right side) TLC plates with samples from fraction 5-12 after spraying with ninhydrin.

Fraction	Non-hydrolyzed plate	Hydrolyzed plate	Rf -value
6	-	Pink spot	0,80
7	-	Pink spot	0,66
		Purple spot	0,72
8	-	Pink spot	0,61
		Purple spot	0,67
9	-	Pink spot	0,68
		Purple spot	0,73

Table 3.2.3: Results from the TLC protosite reaction with ninhydrin, showing the presence of pink/purple spots and their corresponding Rf-values.

3.2.7 Results from DART Spectroscopy

Fraction 6:

The mass spectrum showed a relatively small peak at m/z 566, which corresponds to the molecular mass of viscumamide. There is an excess of other components in this sample, judging from the large range of peaks in the mass spectrum,

Fraction 7:

The mass spectrum showed a relatively strong peak at m/z 566 and had the clearest spectrum (figure 3.2.4) A computer program calculated the possible elemental compositions of m/z 566 (appendix 14). Among these was the molecular formula $C_{30}H_{56}N_5O_5$, which corresponds well to the molecular formula of viscumamide, $C_{30}H_{55}N_5O_5$. The m/z 566 probably represents an M+ H ion.

Fraction 7 eluted with a strong bright yellow/green band (section 3.2.4), and it seems that most of the viscumamide eluted with this bright coloured material. However, there are other compounds in this sample, judging from the mass spectra.

We attempted to purify this fraction by re-crystallisation with ethanol, as performed by Okumara and Sakurai, but it was unsuccessful. The fraction contained some crystals but they were brown and not needle shaped as described in literature (Okumura and Sakurai, 1972).

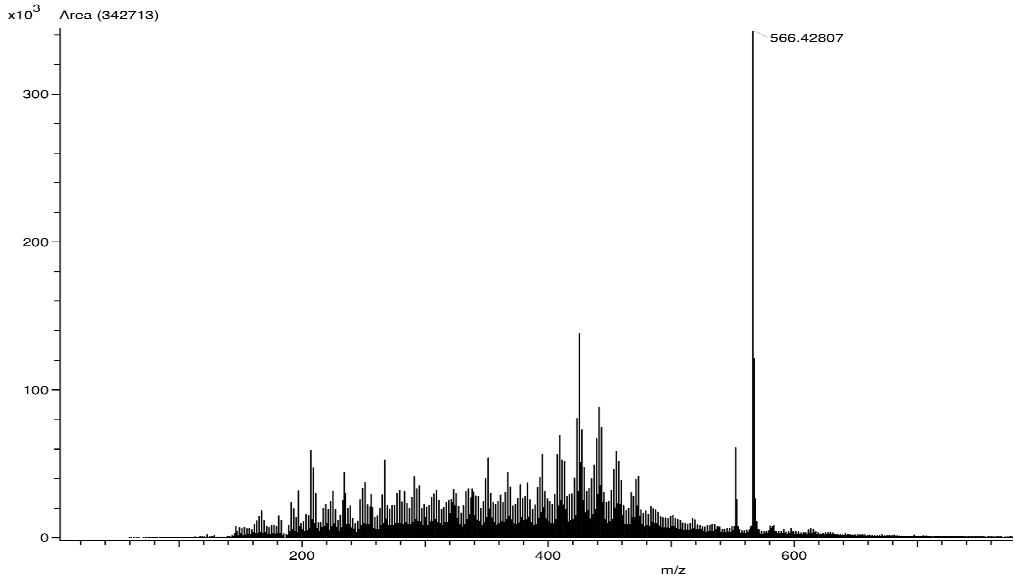

Figure 3.2.4: MS spectrum from fraction 7 at the time of maximum for m/z 566.

Fraction 8:

The mass spectrum showed a small 566 peak, and an excess of other components. A small but interesting peak at m/z 717 m/z was observed in this fraction. It is possible that the peak corresponds to an unidentified cyclic peptide. Results from the TLC protosite reaction indicated that there were two individual cyclic peptides present in fraction 7, 8 and 9 or possibly two different isomers.

Fraction 9:

The mass spectrum showed a very small peak at m/z 566. The m/z 717 peak is most abundant in this fraction and gives the clearest spectrum (appendix 16).

As we can see from table 3.2.4 m/z 566 is found in all four fractions, but mainly in fraction 7. The intensity decreases from fraction 8 to 9.

Sample	Time of maximum for 566 m/z (min)	Intensity x10^4
6	1,2	520
7	2,3	840
8	3,2	180
9	4,2	80

Table 3.2.4: The distribution of m/z 566 in sample 6,7,8 and 9 at the time of maximum

Traces of the m/z 717 is also found in all four fraction but mainly in fraction 9, and with an increasing intensity from fraction 6 to 9 (table 3.2.5)

Sample	Time of maximum for 717 m/z (min)	Intensity x10^4
6	1,3	10
7	2,4	20
8	3,4	36
9	4,5	92

Table 3.2.5: The distribution of m/z 717 in sample 6,7,8 and 9 at time of maximum

3.3 Investigation of an Unidentified Cyclic Peptide in Viscum album

Results from the TLC protosite reaction with ninhydrin (section 3.2.6) and DART mass spectrometry (section 3.2.7) indicated the presence of an unidentified cyclic peptide in fraction 8 and 9. The fractions were purified by preparative TLC and analysed by NMR spectroscopy.

3.2.1 Results from NMR Spectroscopy

The 1D NMR spectrum showed signals in the aliphatic region and the aromatic region. The aliphatic part is most likely leucine, because of characteristic chemical shift values (table 3.3.1). Signals in the aromatic region indicate an aromatic moiety, which is symmetrically substituted at C1 and C4 with carbonyl (table 3.3.1). We tried to find out how the aromatic part and the aliphatic part are connected together by 2D NMR techniques, but it was difficult because of impurities in the sample.

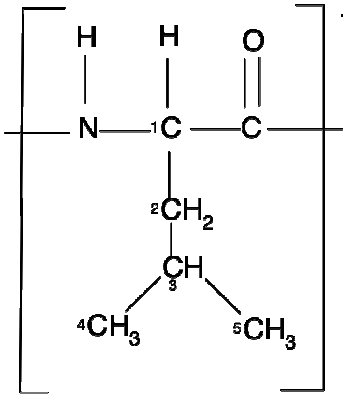

Figure 3.3.1: Leucine- part

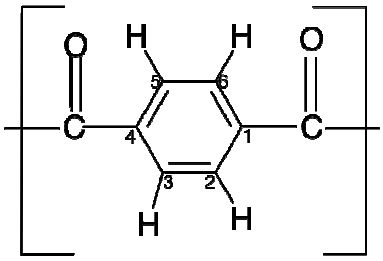

Figure 3.3.2 Aromatic part

Aromatic part		
	^{1}H (ppm)	**^{13}C (ppm)**
C=O		169.2
1		133.5
2	7.75 *ddd* 0.7, 3.3, 5.6	129.8
3	7.66 *ddd* 0.7, 3.3, 5.6	132.4
4		132,4
5	7.66 *ddd* 0.7, 3.3, 5.6	132.4
6	7.75 *ddd* 0.7, 3.3, 5.6	129.8
C=O		169,2
Leucine -part		
	^{1}H	**^{13}C**
C=O		169.2
1	4.25 t 5.1	68.85
2	1.71 m*	40.1
3	1.46 m	24.9
4	0.97 m	14,3
5	0.97 m	14.3

Table 3.3.1 NMR results from 1D ^{1}H, ^{13}C, COSEY 2D HMBC and 2D HSQC

* Due to impurities we could not report the coupling constant

Conclusion:

The non-polar constituents found in *Viscum album* correspond well with previous investigations of mistletoe species, especially concerning the triterpenoids. It could indicate that the composition of substances doesn't vary as much from species to species or from host to host as previously suggested. However, there were no records found on the detection of linolenic acid and lupenone in *Viscum album*, so it is possible that there are some variations. To determine some of the structures in the non-polar extract standard samples could have been used. By comparing mass fragmentation and retention time we could have established the identity of these compounds. To investigate the full profile of non-polar constituents *in Viscum album,* extracts with different polarities could be used. In this study we only investigated the volatile non-polar components in *Viscum album*.

The isolation of the cyclic peptide viscumamide from the neutral fraction of *Viscum album* was attempted. The high content of polysaccharides and other polar substances made this very difficult. Even though the extract was thoroughly washed with acidic and basic solutions, and repeatedly separated by column chromatography, the cyclic peptide was not successfully isolated. The generally low content of cyclic peptides in plants is a familiar problem when it comes to isolation and purification. However, we managed to detect viscumamide by DART mass spectrometry in some of the neutral fractions. This cyclic peptide has previously only been isolated from Korean mistletoe. To isolate viscumamide or other cyclic peptides a large amount of plant material must be used and good separation techniques.

Additionally an unknown cyclic peptide was detected in the neutral fraction of *Viscum album.* The TLC protosite reaction with ninhydrin showed that more than one cyclic peptide was present in the neutral fraction and results from DART mass spectrometry indicated the molecular weight to be 717 g/mol. NMR spectroscopy indicated the presence of leucine and aromatic moieties. We could not find out how they are connected because of impurities in the sample. With a pure sample the structures of cyclic peptides can be determined by NMR, MS and amino acid analysis. It could be useful to purify this cyclic peptide with an Amberlite XAD-7 column. This column

reversibly adsorbs aromatic compounds, whereas free sugars and other non-aromatic compounds are removed by washing with distilled water (Andersen and Francic, 2004)

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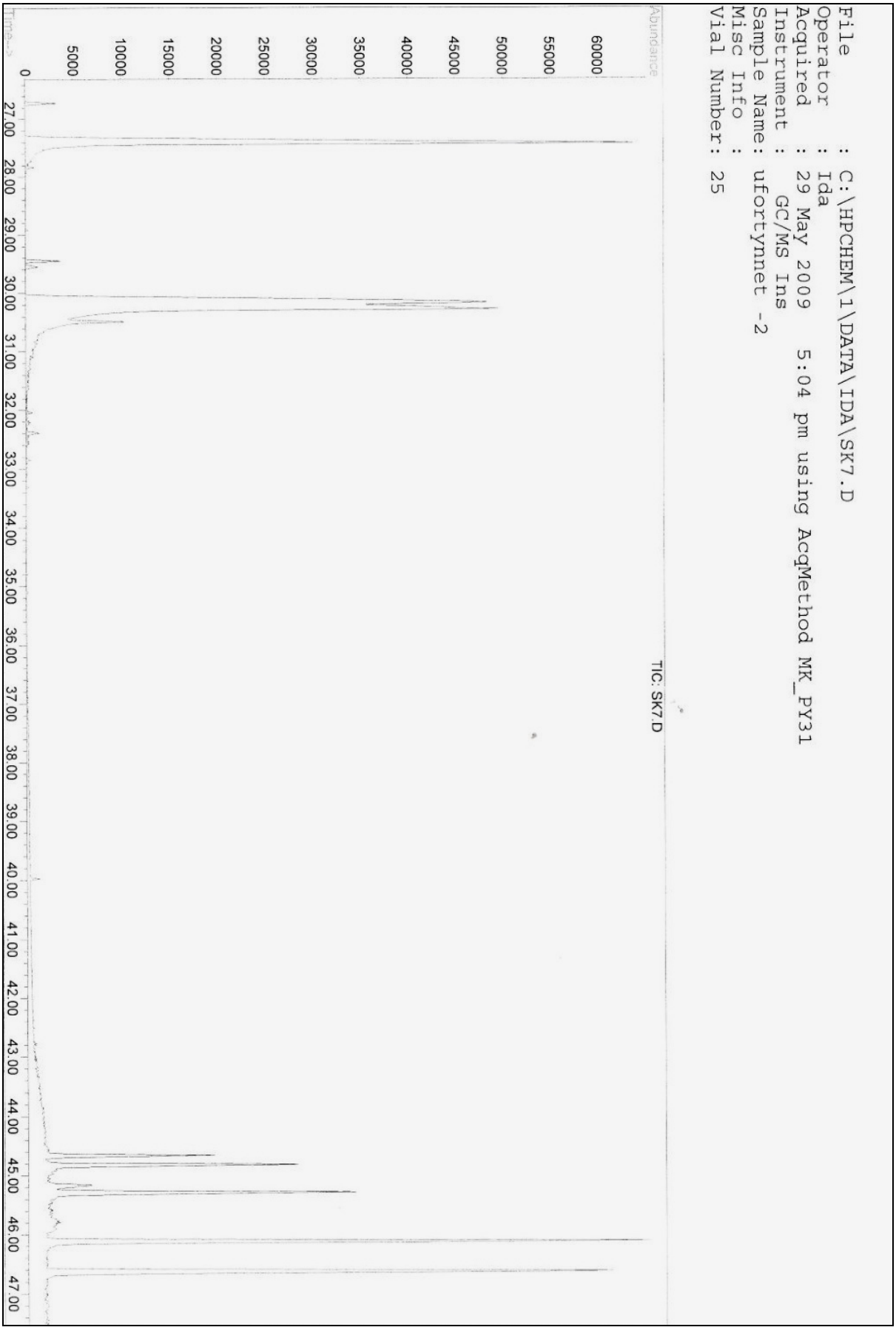
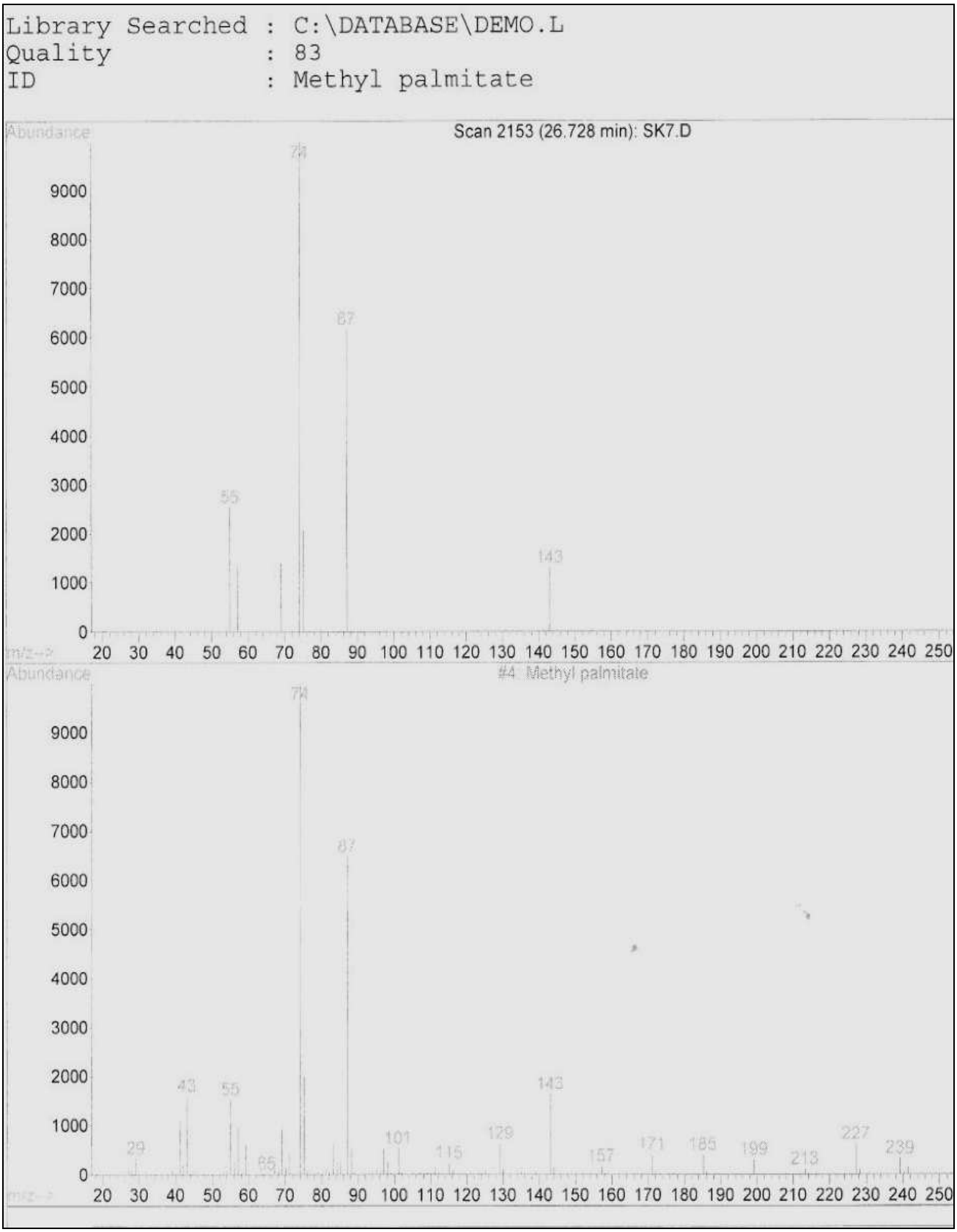
List of Chemicals

Substance	***Formula***	***Purity (%)***	***Brand***
Acetone	$CH_3CH_2CH_2OH$	>99,5	Sigma -Aldrich
β – amyrin	$C_{30}H_{50}O$	Not given	Koch-Light
Chloroform	$CHCl_3$	99,0 – 99,4	Ridel-de Häen
Diethyl ether	$(CH_3CH_2)_2O$	>99,5	Sigma -Aldrich
Ethyl acetate	$C_4H_8O_2$	>99,5	Sigma- Aldrich
Ethanol	CH_3CH_2OH	Not given	Arcus Kjemi
Hexane	C_6H_{14}	>99,0	Sigma-Aldrich
Hydrochloride Acid	HCl	Minimum 37	Ridel-de Häden
Methanol	CH_3OH	>99,9	Sigma-Aldrich
Sodium Carbonate	Na_2CO_3	p.a	JT Baker
Ninhydrin	$C_6H_4COC(OH)_2CO$	Puriss	Fluka
Petroleum ether	Not applicable	>95	Riedel-de Häden
Phosphomolybdic Acid (PMA)	$H_3PMo_{12}O_{40}$	A.C.S reagent	Sigma-Aldrich

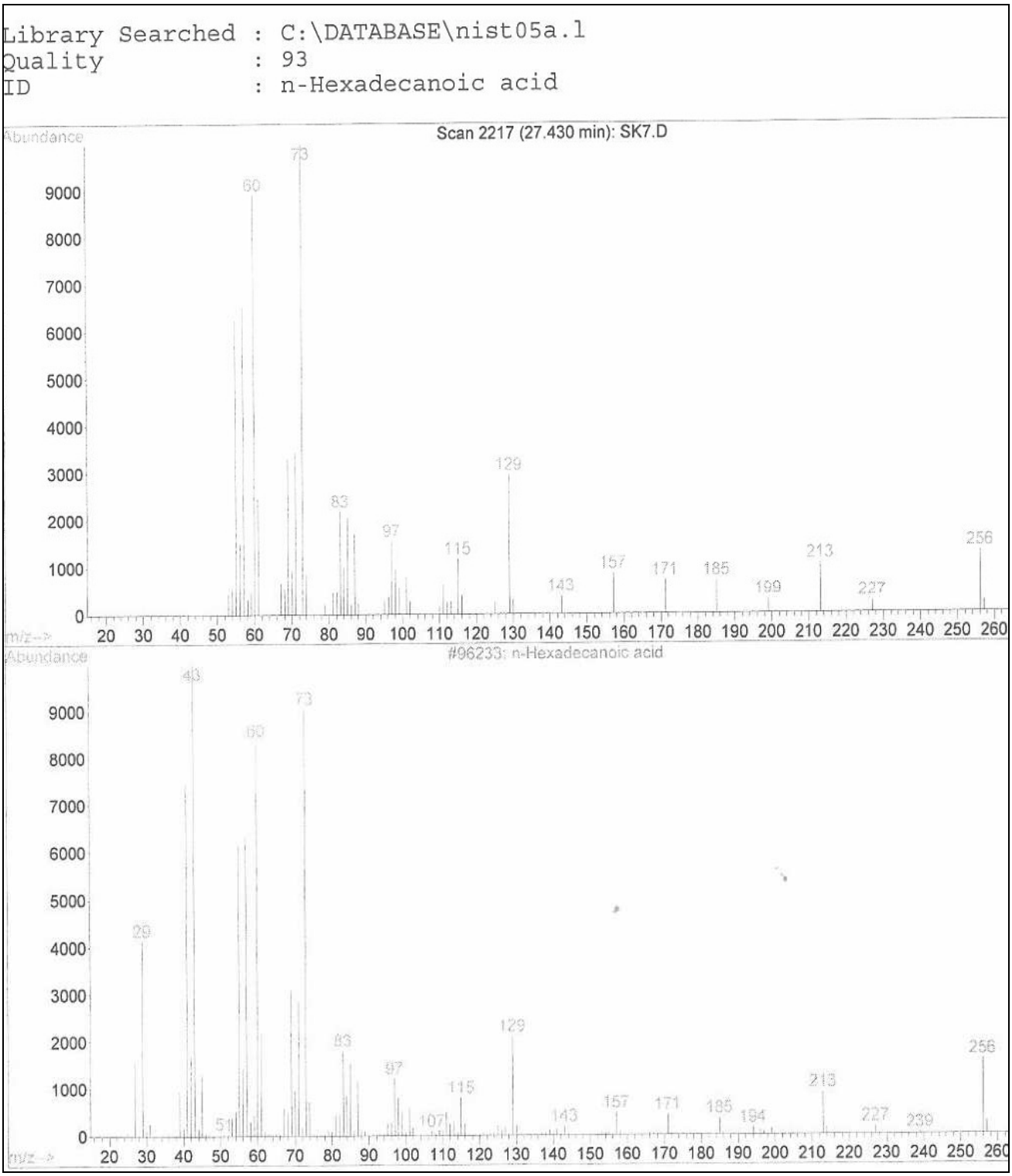
Appendix 1


File : C:\HPCHEM\1\DATA\IDA\SK7.D
Operator : Ida
Acquired : 29 May 2009 5:04 pm using AcqMethod MK_PY31
Instrument : GC/MS Ins
Sample Name: ufortynnet -2
Misc Info :
Vial Number: 25
Abundance
TIC: SK7.D
Time
60000
55000
50000
45000
40000
35000
30000
25000
20000
15000
10000
5000
0
27.00
28.00
29.00
30.00
31.00
32.00
33.00
34.00
35.00
36.00
37.00
38.00
39.00
40.00
41.00
42.00
43.00
44.00
45.00
46.00
47.00

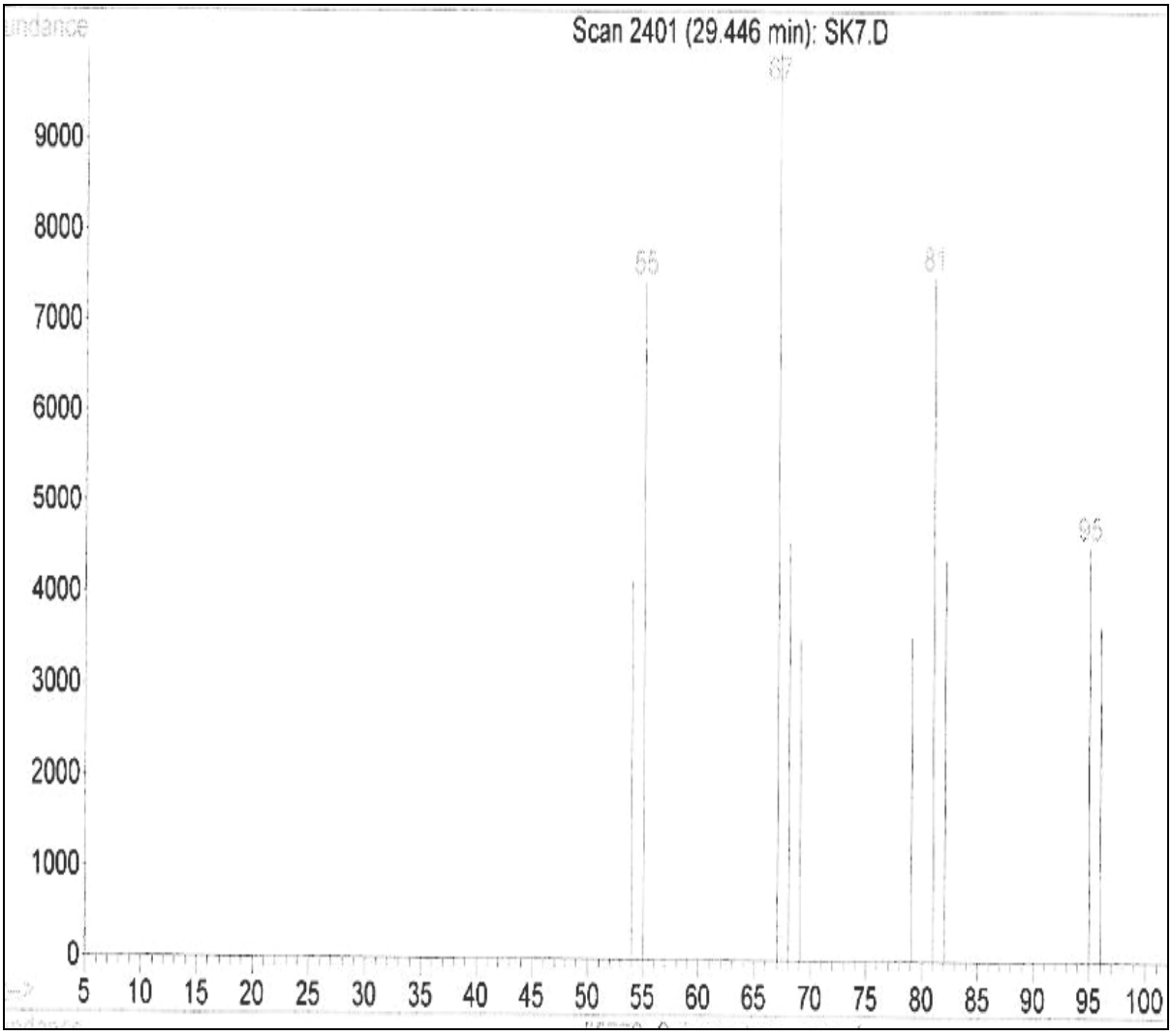
Appendix 2


Library Searched : C:\DATABASE\DEMO.L
Quality : 83
ID : Methyl palmitate
Abundance
Scan 2153 (26.728 min): SK7.D
74
87
55
143
m/z-->
#4 Methyl palmitate
43
29
101
115
129
157
171
185
199
213
227
239

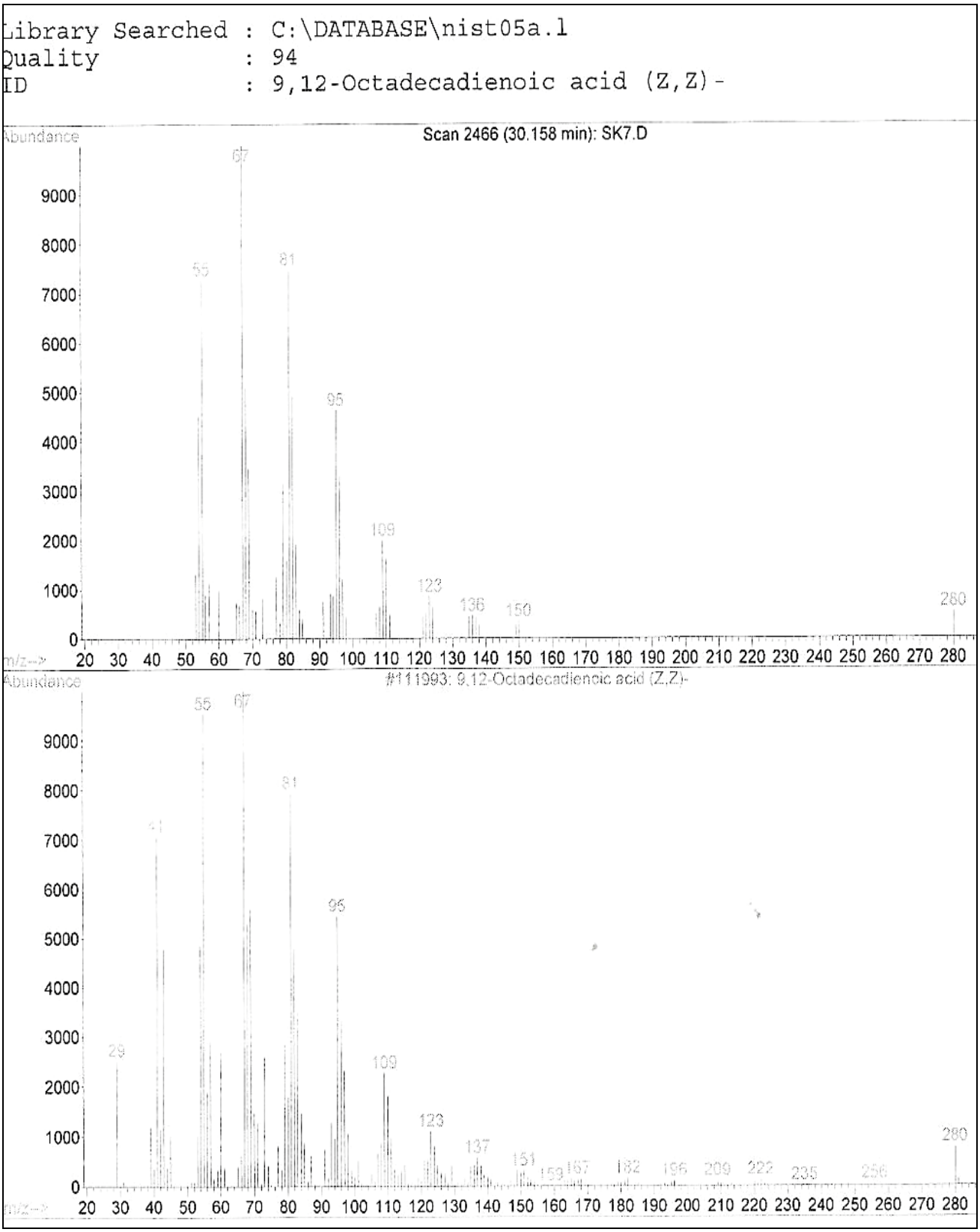
Appendix 3


Library Searched : C:\DATABASE\nist05a.l
Quality : 93
ID : n-Hexadecanoic acid
Scan 2217 (27.430 min): SK7.D
Abundance
m/z-->
#96233: n-Hexadecanoic acid

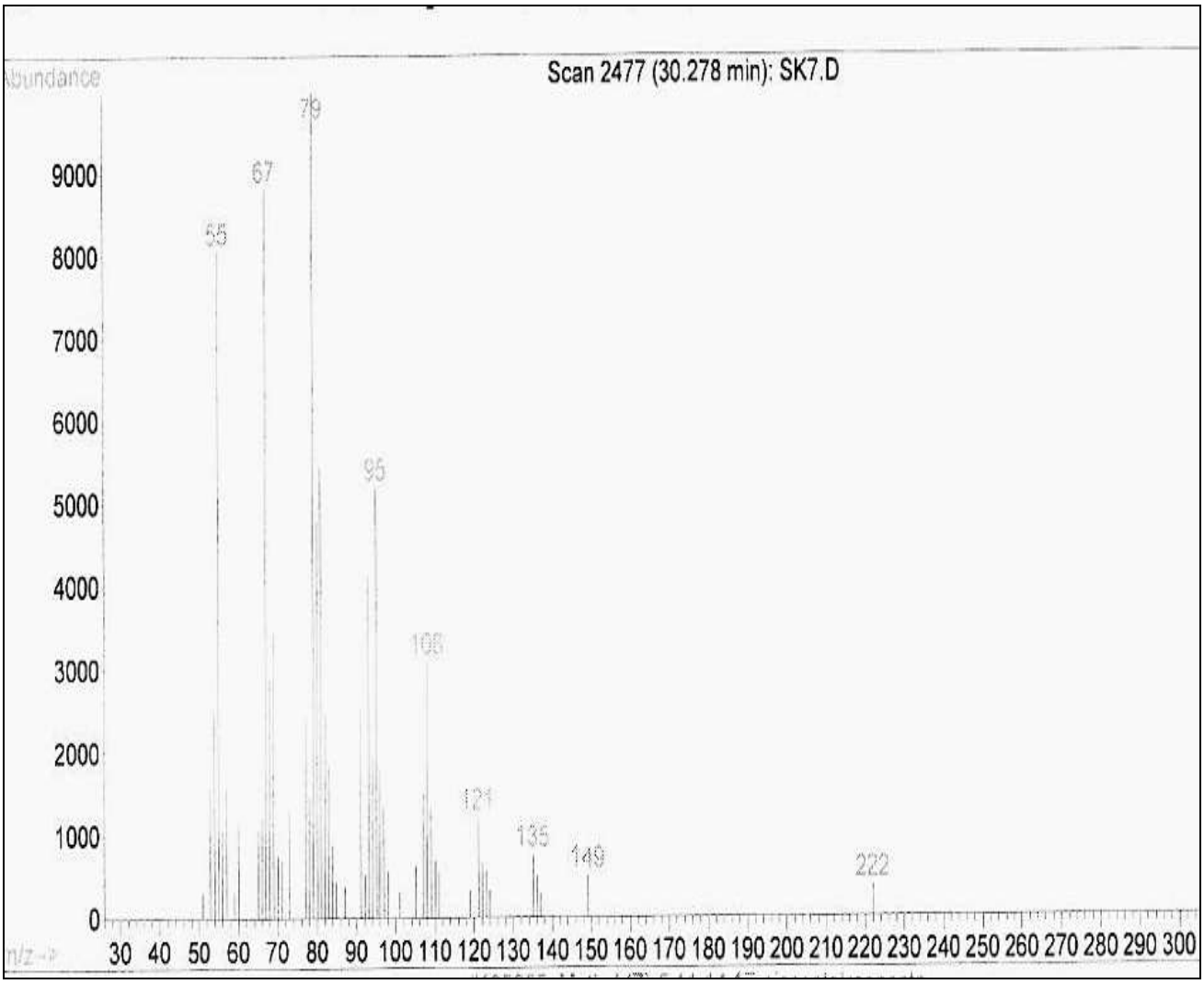
Appendix 4


Scan 2401 (29.446 min): SK7.D
67
55
81
95
9000
8000
7000
6000
5000
4000
3000
2000
1000
0
5 10 15 20 25 30 35 40 45 50 55 60 65 70 75 80 85 90 95 100

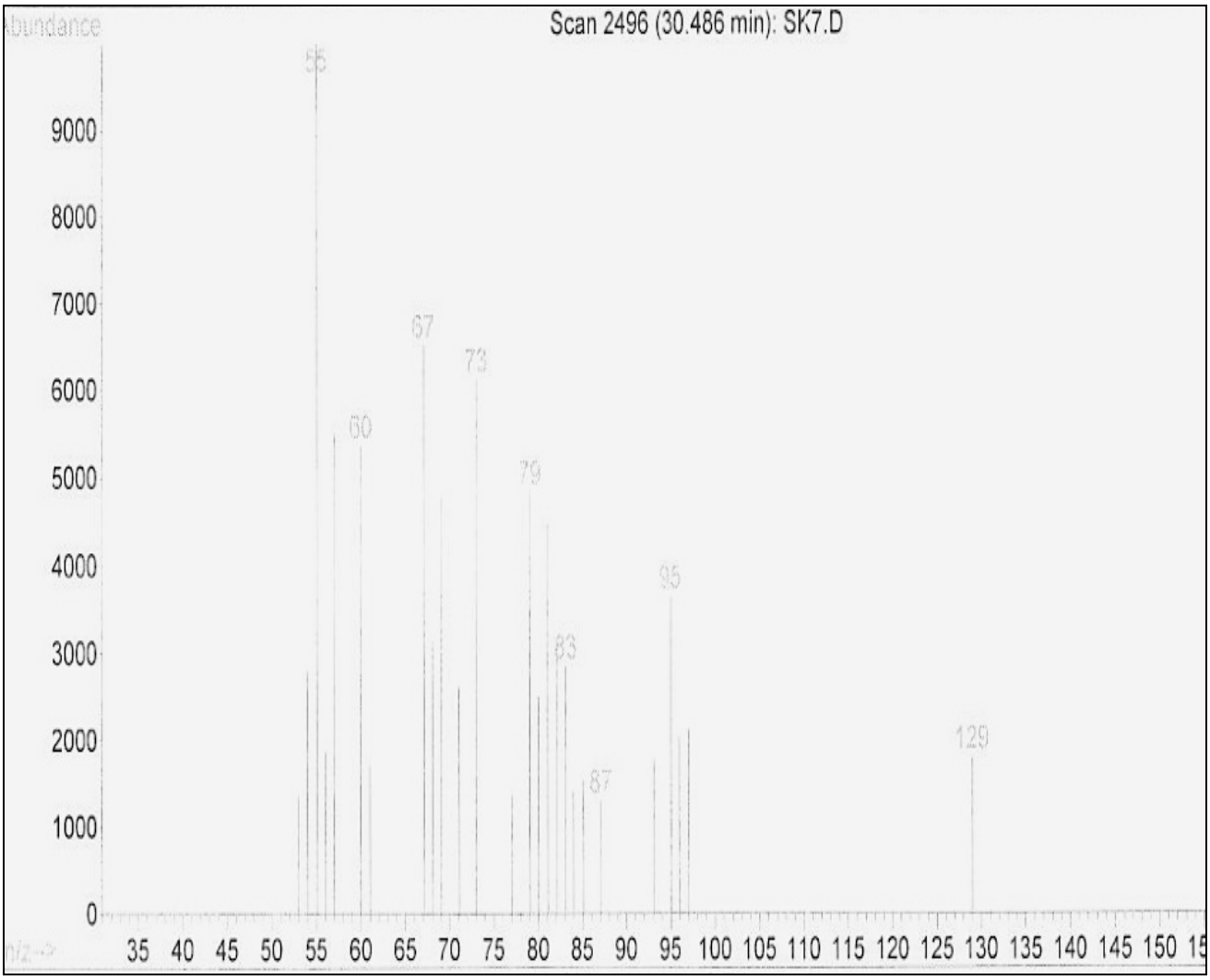
Appendix 5


Library Searched : C:\DATABASE\nist05a.l
Quality : 94
ID : 9,12-Octadecadienoic acid (Z,Z)-
Scan 2466 (30.158 min): SK7.D
Abundance
m/z-->
#111993: 9,12-Octadecadienoic acid (Z,Z)-

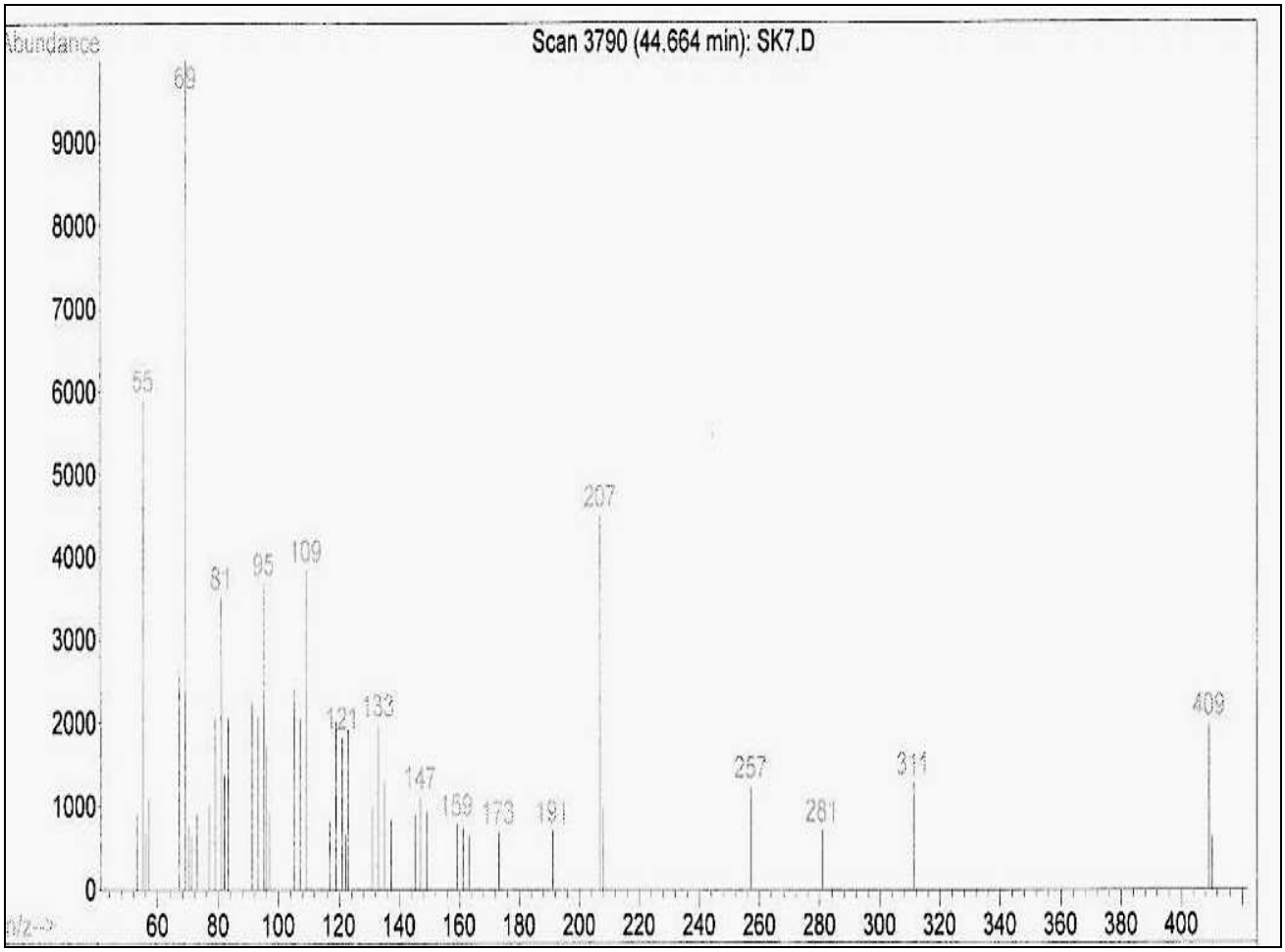
Appendix 6


Scan 2477 (30.278 min): SK7.D
Abundance
m/z→
79
67
55
95
108
121
135
149
222
9000
8000
7000
6000
5000
4000
3000
2000
1000
0
30 40 50 60 70 80 90 100 110 120 130 140 150 160 170 180 190 200 210 220 230 240 250 260 270 280 290 300

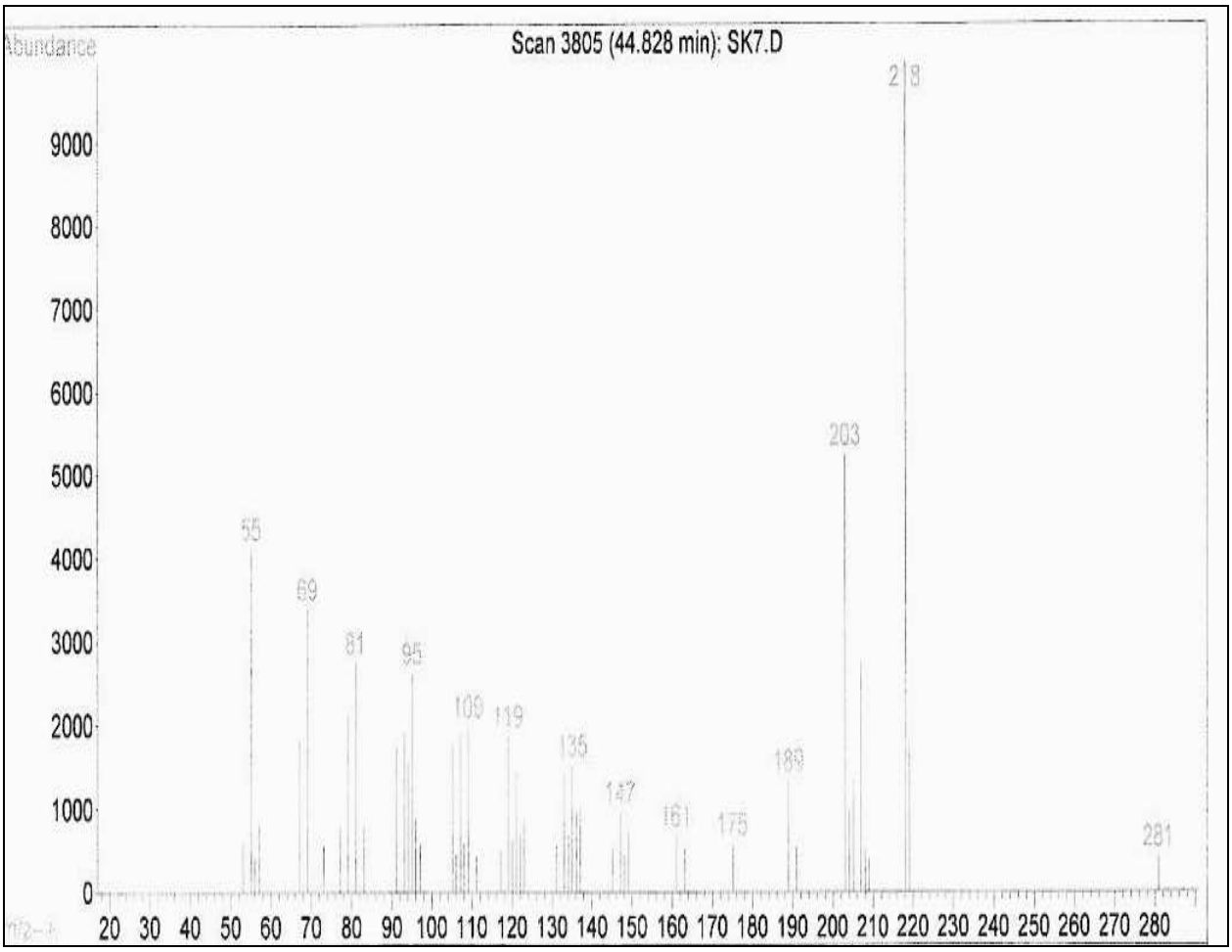
Appendix 7


Abundance
Scan 2496 (30.486 min): SK7.D
55
67
73
60
79
95
83
87
129
9000
8000
7000
6000
5000
4000
3000
2000
1000
0
m/z-->
35 40 45 50 55 60 65 70 75 80 85 90 95 100 105 110 115 120 125 130 135 140 145 150

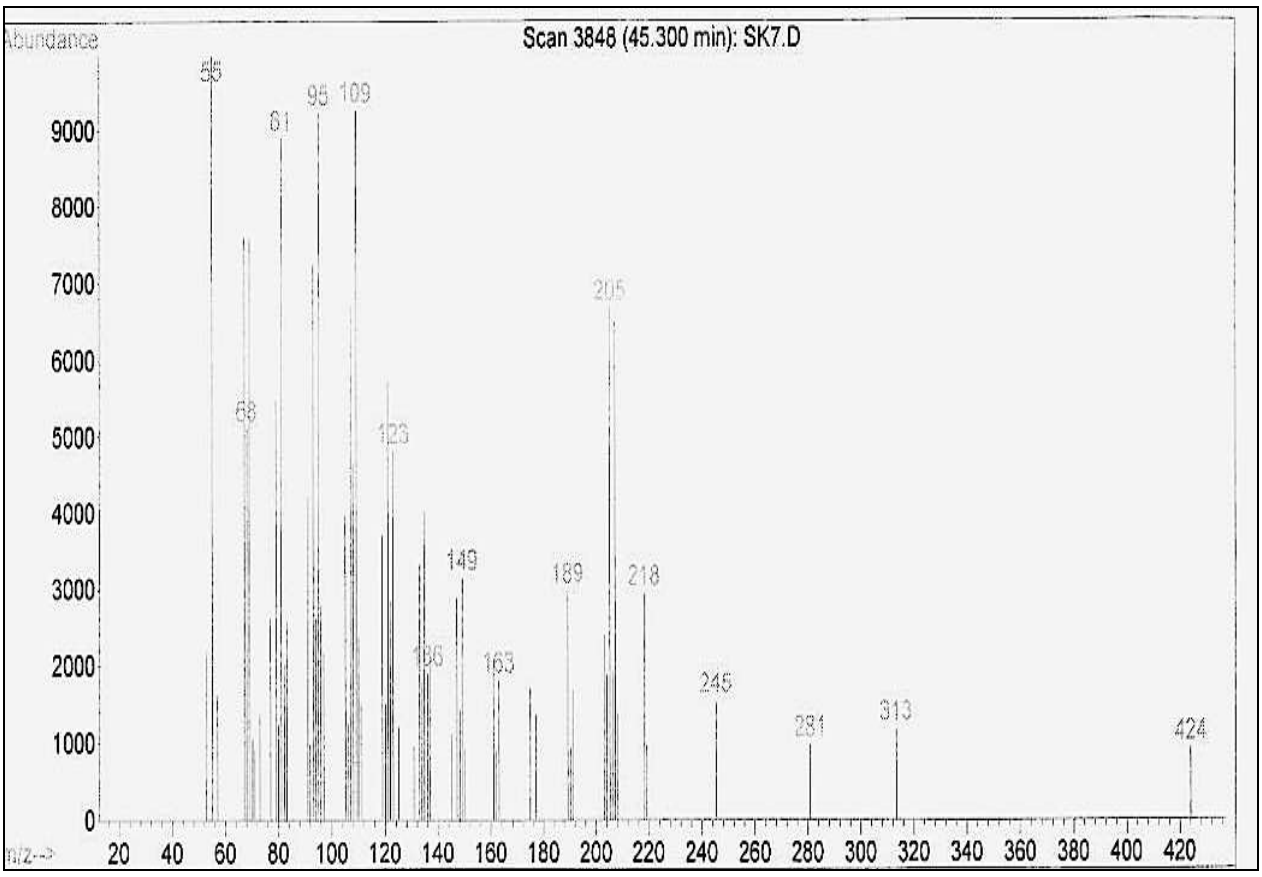
Appendix 8


Abundance
Scan 3790 (44.664 min): SK7.D
69
55
81
95
109
121
133
147
159
173
191
207
257
281
311
409
m/z-->
9000
8000
7000
6000
5000
4000
3000
2000
1000
0
60
80
100
120
140
160
180
200
220
240
260
280
300
320
340
360
380
400

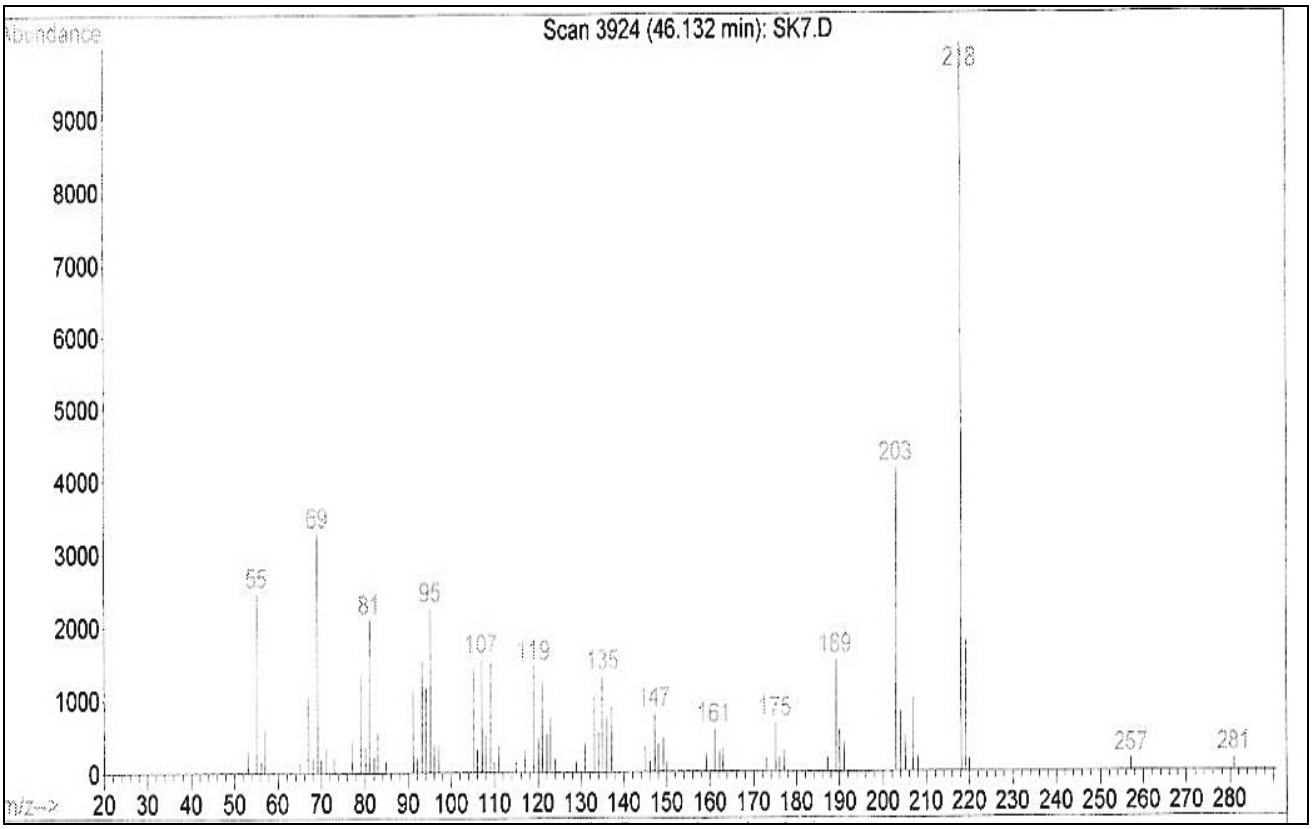
Appendix 9


Abundance
Scan 3805 (44.828 min): SK7.D
9000
8000
7000
6000
5000
4000
3000
2000
1000
0
218
203
55
69
81
95
109
119
135
147
161
175
189
281
20 30 40 50 60 70 80 90 100 110 120 130 140 150 160 170 180 190 200 210 220 230 240 250 260 270 280

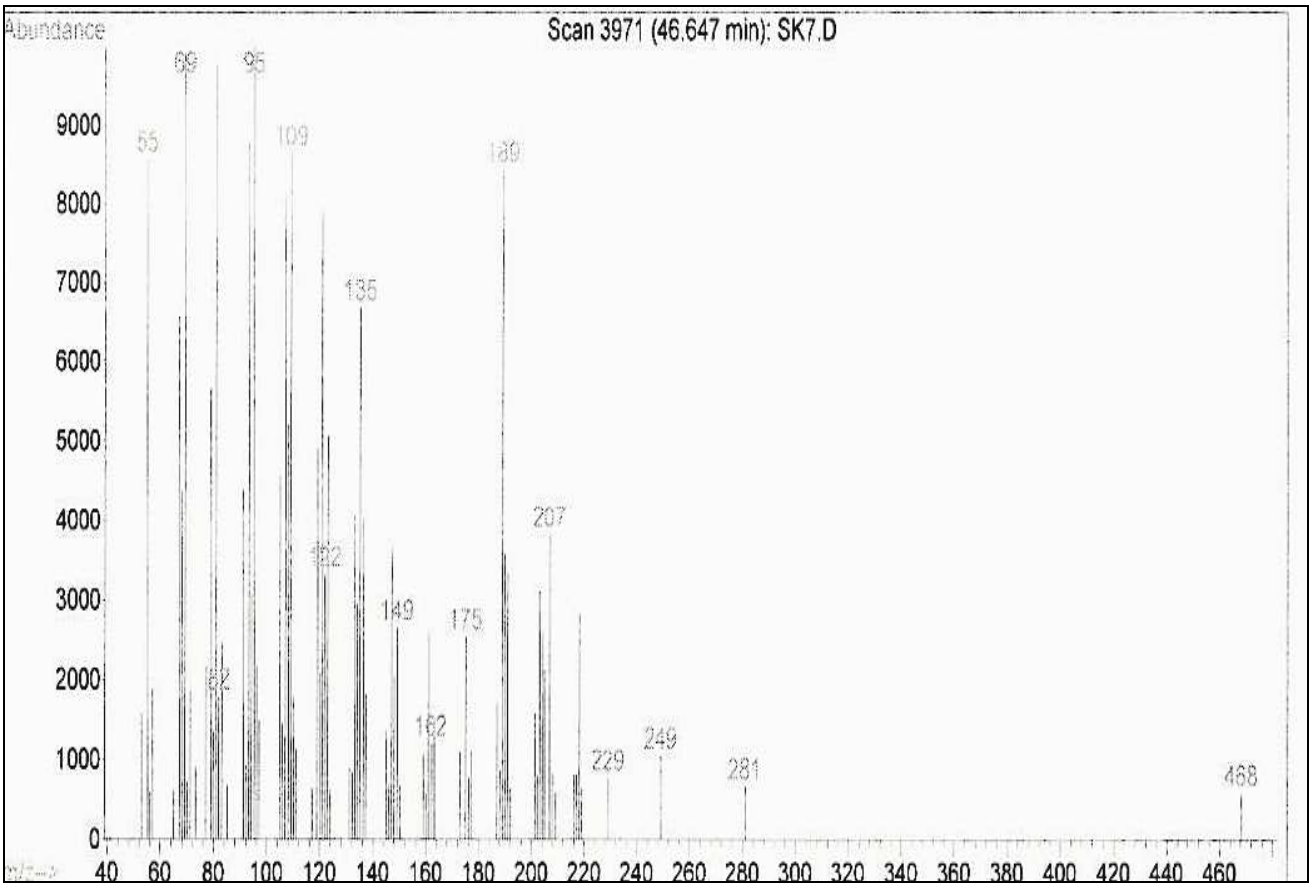
Appendix 10


Abundance
Scan 3848 (45.300 min): SK7.D
55
95
109
81
68
123
205
149
189
218
166
163
245
281
313
424
m/z-->
0
1000
2000
3000
4000
5000
6000
7000
8000
9000
20 40 60 80 100 120 140 160 180 200 220 240 260 280 300 320 340 360 380 400 420

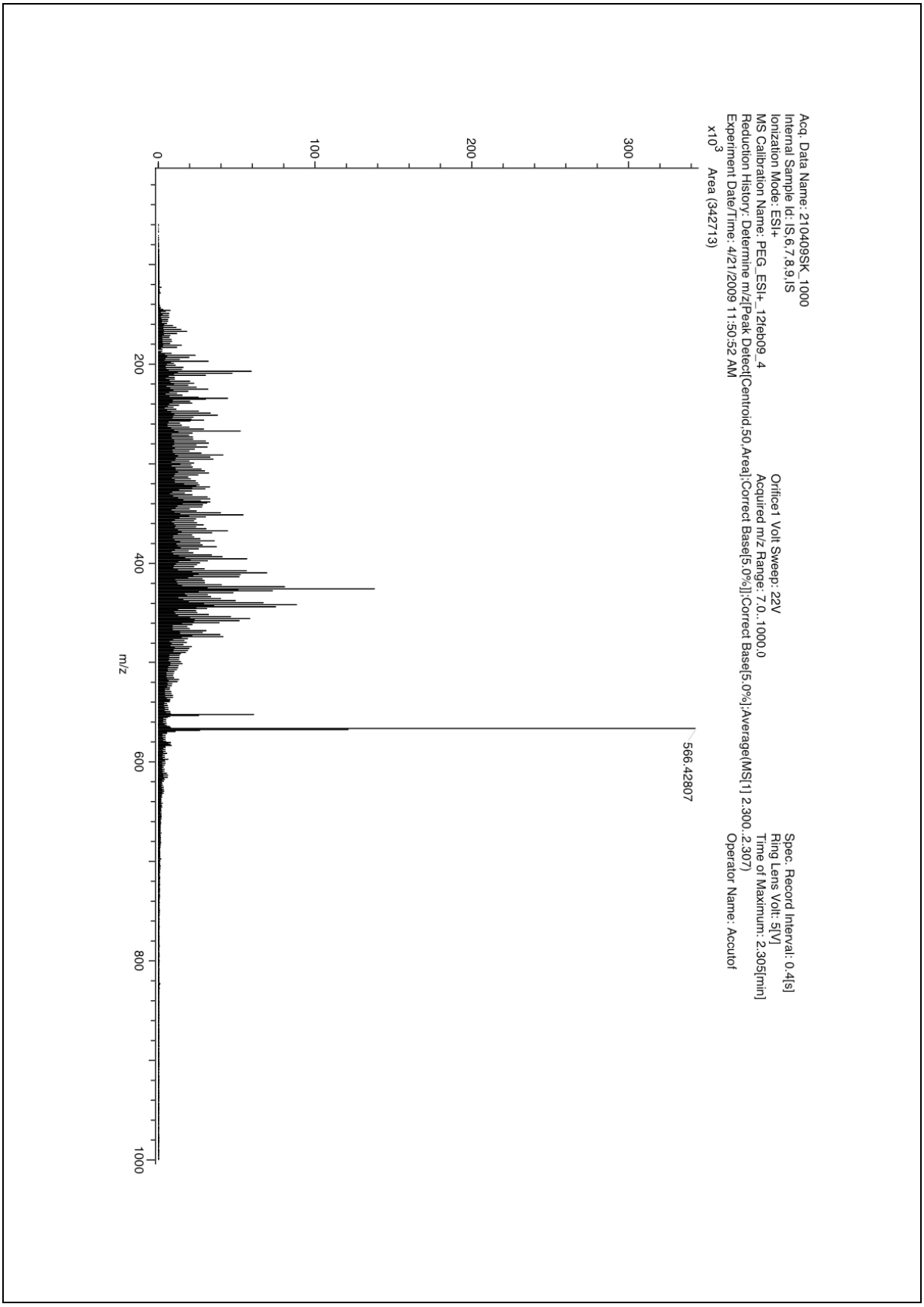
Appendix 11


Abundance
Scan 3924 (46.132 min): SK7.D
218
203
69
55
81
95
107
119
135
147
161
175
189
257
281
9000
8000
7000
6000
5000
4000
3000
2000
1000
0
m/z-->
20 30 40 50 60 70 80 90 100 110 120 130 140 150 160 170 180 190 200 210 220 230 240 250 260 270 280

Appendix 12


Abundance
Scan 3971 (46.647 min): SK7.D
9000
8000
7000
6000
5000
4000
3000
2000
1000
0
55
69
82
95
109
135
149
162
175
189
207
229
249
281
468
40 60 80 100 120 140 160 180 200 220 240 260 280 300 320 340 360 380 400 420 440 460

Appendix 13


Acq. Data Name: 210409SK_1000
Internal Sample Id: IS,6,7,8,9,IS
Ionization Mode: ESI+
MS Calibration Name: PEG_ESI+_12feb09_4
Reduction History: Determine m/z[Peak Detect[Centroid,50,Area];Correct Base[5.0%]];Correct Base[5.0%];Average(MS[1] 2.300..2.307)
Experiment Date/Time: 4/21/2009 11:50:52 AM
Orifice1 Volt Sweep: 22V
Acquired m/z Range: 7.0..1000.0
Spec. Record Interval: 0.4[s]
Ring Lens Volt: 5[V]
Time of Maximum: 2.305[min]
Operator Name: Acculof
x10^3
Area (342713)
300
200
100
0
200
400
600
800
1000
m/z
566.42807

Appendix 14

Data:260209sk
Sample Name:
Description:
Ionization Mode:ESI+
History:Determine m/z[Peak Detect[Centroid,50,Area];Correct Base[5.0%]];Correct Base[5.0%];Average(MS[1] 4.176..4.177)

Acquired:2/26/2009 11:06:35 AM
Operator:Accutof
Mass Calibration data:PEG_ESI+_12feb09_4
Created:4/28/2009 6:10:09 PM
Created by:chromuser

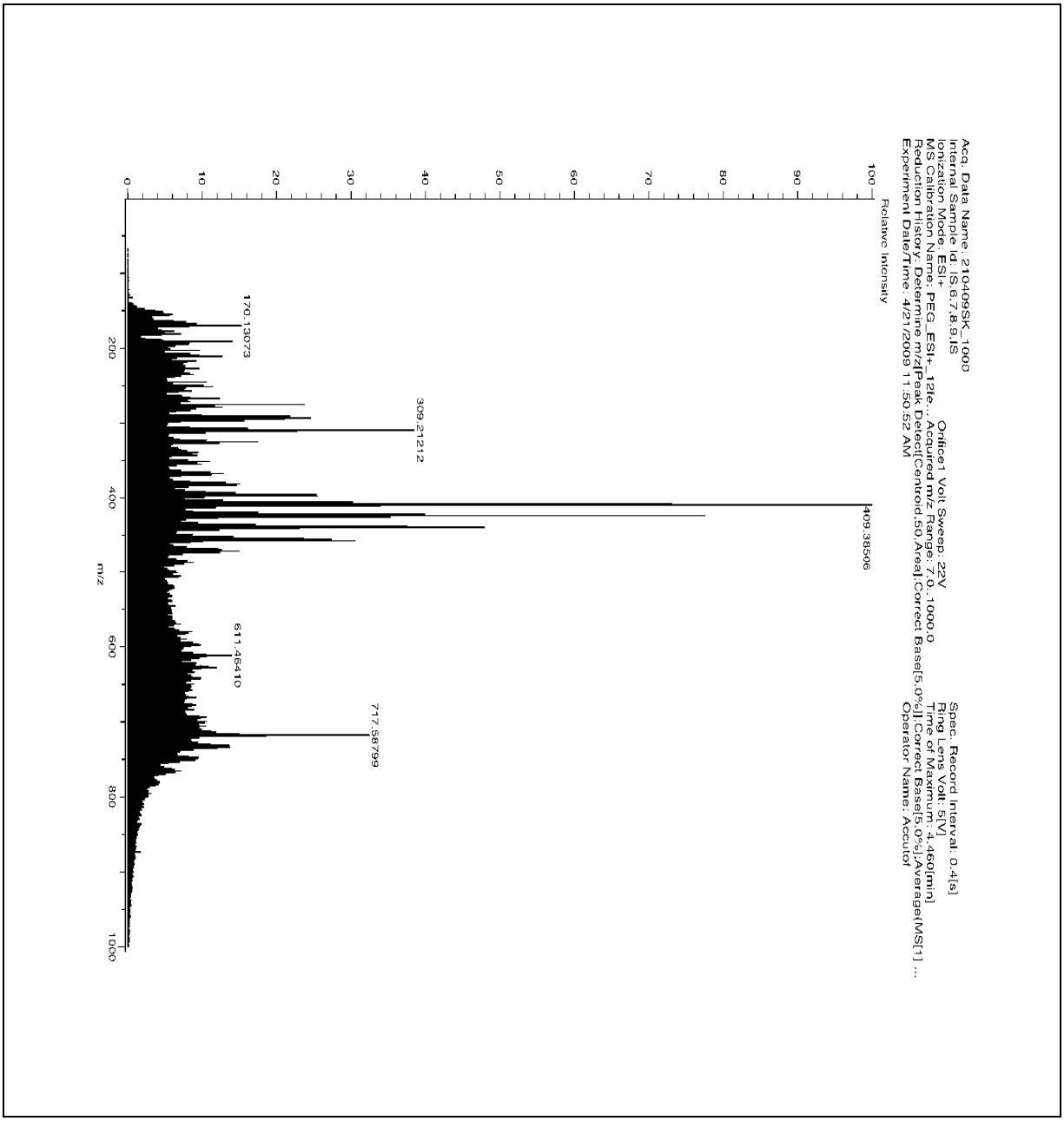
Charge number:1
Element:^{12}C:0 .. 100, ^{1}H:0 .. 200, ^{14}N:0 .. 10, ^{16}O:0 .. 10

Tolerance:5.00(mmu)

Unsaturation Number:-1.5 .. 20.0 (Fraction:Both)

Mass	Intensity	Calc. Mass	Mass Difference (mmu)	Mass Difference (ppm)	Possible Formula	Unsaturation Number
566.42607	5047.06	566.42546	0.61	1.08	$^{12}C_{27}{}^{1}H_{58}{}^{14}N_{4}{}^{16}O_{8}$	1.0
		566.42680	-0.73	-1.28	$^{12}C_{28}{}^{1}H_{54}{}^{14}N_{8}{}^{16}O_{4}$	6.0
		566.42681	-0.73	-1.29	$^{12}C_{29}{}^{1}H_{60}{}^{14}N_{1}{}^{16}O_{9}$	0.5
		566.42412	1.95	3.45	$^{12}C_{25}{}^{1}H_{56}{}^{14}N_{7}{}^{16}O_{7}$	1.5
		566.42814	-2.07	-3.66	$^{12}C_{30}{}^{1}H_{56}{}^{14}N_{5}{}^{16}O_{5}$	5.5
		566.42361	2.46	4.34	$^{12}C_{39}{}^{1}H_{54}{}^{14}N_{2}{}^{16}O_{1}$	14.0
		566.42278	3.29	5.82	$^{12}C_{23}{}^{1}H_{54}{}^{14}N_{10}{}^{16}O_{6}$	2.0
		566.42948	-3.41	-6.02	$^{12}C_{31}{}^{1}H_{52}{}^{14}N_{9}{}^{16}O_{1}$	10.5
		566.42949	-3.41	-6.03	$^{12}C_{32}{}^{1}H_{58}{}^{14}N_{2}{}^{16}O_{6}$	5.0
		566.42227	3.80	6.71	$^{12}C_{37}{}^{1}H_{52}{}^{14}N_{5}$	14.5
		566.43082	-4.75	-8.39	$^{12}C_{33}{}^{1}H_{54}{}^{14}N_{6}{}^{16}O_{2}$	10.0

Appendix 15


Acq. Data Name: 210409SK_1000
Internal Sample Id: IS,6,7,8,9,IS
Ionization Mode: ESI+
MS Calibration Name: PEG_ESI+_12le...
Reduction History: Determine m/z[Peak Detect[Centroid,50,Area],Correct Base[5.0%]]
Experiment Date/Time: 4/21/2009 11:50:52 AM
Orifice1 Volt Sweep: 22V
Acquired m/z Range: 7.0..1000.0
Spec. Record Interval: 0.4[s]
Ring Lens Volt: 5[V]
Time of Maximum: 4.460[min]
Correct Base[5.0%],Average(MS[1] ...
Operator Name: Accutof
Relative Intensity
m/z
170.13079
309.21212
409.38506
611.46410
717.58799

Appendix 16

Data:210409SK_1000 Acquired:4/21/2009 11:50:52 AM
Sample Name:IS,6,7,8,9,IS Operator:Accutof
Description: Mass Calibration data:PEG_ESI+_12...
Ionization Mode:ESI+ Created:4/27/2009 3:37:43 PM
History:Determine m/z[Peak Detect[Centroid,50,Area];Correct Base[5.0%]];Cor... Created by:chromuser

Charge number:1 Tolerance:5.00(mmu) Unsaturation Number:-1.5 .. 20.0 (Fra..
Element:^{12}C:0 .. 100, ^{1}H:0 .. 200, ^{15}N:0 .. 10, ^{16}O:0 .. 10

Mass	Intensity	Calc. Mass	Mass Difference (mmu)	Mass Difference (ppm)	Possible Formula
717.58799	48960.75	717.58797	0.02	0.03	$^{12}C_{41}{}^{1}H_{75}{}^{15}N_{10}$
		717.58806	-0.07	-0.10	$^{12}C_{41}{}^{1}H_{81}{}^{16}O_{9}$
		717.58753	0.46	0.64	$^{12}C_{46}{}^{1}H_{75}{}^{15}N_{6}$
		717.58849	-0.51	-0.70	$^{12}C_{36}{}^{1}H_{81}{}^{15}N_{4}{}^{16}O_{9}$
		717.58709	0.89	1.25	$^{12}C_{51}{}^{1}H_{75}{}^{15}N_{2}$
		717.58529	2.70	3.76	$^{12}C_{42}{}^{1}H_{76}{}^{15}N_{7}{}^{16}O_{2}$
		717.59073	-2.74	-3.82	$^{12}C_{40}{}^{1}H_{80}{}^{15}N_{3}{}^{16}O_{7}$
		717.58486	3.13	4.36	$^{12}C_{47}{}^{1}H_{76}{}^{15}N_{3}{}^{16}O_{2}$
		717.59117	-3.18	-4.43	$^{12}C_{35}{}^{1}H_{80}{}^{15}N_{7}{}^{16}O_{7}$
		717.58306	4.93	6.87	$^{12}C_{38}{}^{1}H_{77}{}^{15}N_{8}{}^{16}O_{4}$
		717.59297	-4.98	-6.94	$^{12}C_{44}{}^{1}H_{79}{}^{15}N_{2}{}^{16}O_{5}$