Phytochemicals as a Source of Novel Drugs Against Prostate Cancer – Preparation of Animal Experiments and Isolation and Identification of Flavonoid Glycosides from *Abies pindrow*

Master's thesis in Pharmacy

By

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The TCMCANCER network

This Master's thesis serves as part of a larger project called "Traditional Chinese Medicine in the Post-Genomic Era: Identifying Lead Therapeutic Compounds Against Cancer" (TCMCANCER). The TCMCANCER project is an international collaboration between four different partners: Centre de Recherche Public de la Santé (Luxembourg), the Institute of Medicinal Plant Development (Beijing), the Modern Research Center for Traditional Chinese Medicine (Shanghai) and the Gade Institute at the University of Bergen (Norway). Aims of the TCMCANCER project are to isolate and identify novel lead therapeutic compounds with antitumor activities from medicinal plants utilized in Traditional Chinese Medicine (TCM).

Compounds from selected plants are first isolated and characterized at the Modern Research Center for Traditional Chinese Medicine. Centre de Recherche Public de la Santé perform *in vitro* tests by the use of high-throughput screening when large enough quantities of materials have been purified. Potential lead compounds are subsequently tested in preclinical animal models at the Institute of Medicinal Plant Development. The Gade Institute provides both phenotypic and genomic screening systems for the evaluation of selected compounds.

A staff exchange program is incorporated to the TCMCANCER project. This program is supported by the European Council through FP7-PEOPLE-IRSES-2008, Marie Curies Actions-International Research Staff Exchange Scheme 2009-2013, project number 230232. Staff exchange allows for specialized knowledge to be gained and spread among collaborating institutions. European staff at the MRCTCM study and practice the science of isolation and structure determination of natural products.

The European Council funded my entire stay in Shanghai from August to December of 2011.

Aims of the study

The main aim of this Master's thesis is to contribute to the development of novel lead therapeutic compounds against prostate cancer. This is closely related to the aims of the TCMCANCER project. European exchange students at the MRCTCM are obliged to follow the "Description of Work" annex stated in the TCMCANCER project. The main aim of this study is divided into two intermediate aims (A. and B.). Intermediate aim A. entails the theoretical part while intermediate aim B. entails the practical part.

A. Theoretical part:

The first intermediate aim of this study is to present a literature review of general aspects involved in preclinical trials for the evaluation of efficacy and toxicity of novel lead therapeutic compounds against prostate cancer. Thus, this part encompasses sections about natural sources of medicinal drugs, available animal models, human and animal pharmacology, project design, monitoring methods and ethical aspects.

B. Practical part:

The second intermediate aim of this study is to present the isolation and characterization of secondary metabolites from the leaves and branches of *Abies pindrow*. Thus, this part encompasses sections about isolation and identification theory, materials, methods, results and discussion and conclusion. The TCMCANCER project states that European exchange students at the MRCTCM will isolate compounds from plants used in TCM to learn about the purification of natural products. The TCMCANCER project states further that knowledge about structure elucidation will also be acquired. Hopefully, further work with these isolated compounds will generate novel lead therapeutic compounds against prostate cancer.

Summary

Introduction:

Plants and herbs have been utilized for centuries by traditional medicinal systems (e.g. TCM, Ayurveda) to alleviate minor illnesses and major diseases. In recent decades, interest in traditional medicine has increased significantly among drug development institutions. Currently, several research groups worldwide are conducting isolation, characterization and bioassay evaluation of secondary metabolites from plants and herbs utilized in traditional medicine. Such research aims to produce potential lead therapeutic compounds for future evaluation in preclinical animal models. Also terrestrial and marine organisms have been shown to produce biologically active secondary metabolites. In this Master's thesis, key elements in the preparation of animal experiments for the evaluation of lead therapeutic compounds against adenocarcinoma of the prostate are presented. Thus, potential medicinal sources, prostate cancer animal models, pharmacology of humans and animals, study design, metastasis, monitoring techniques and ethical aspects are described. The practical part of this Master's thesis is focused on the isolation and characterization of flavonoids from the branches and leaves of *Abies pindrow*. Previous purification of this plant species has been reported in the literature.

Methodological considerations:

Low-pressure column chromatography is dependent on four mechanisms of separation: adsorption, size-exclusion, partition and ion-exchange. Silica gel column chromatography is a form of adsorption chromatography. Sephadex LH-20 separates compounds mainly through size-exclusion, but adsorption can also be involved. Planar liquid chromatography comprises popular techniques such as TLC and PTLC, which are based on adsorption chromatography. MS and NMR are commonly employed in structure elucidation. MS is used to investigate molecular masses, while NMR describes the chemical environment of NMR active nuclei.

Materials:

A range of organic solvents were utilized in the isolation process. MeOH and $CHCl_3$ were most often employed. Much of the work involved both TLC and PTLC plates. Sephadex LH-20 particles were employed more frequent than silica gel particles. The isolation process required equipment such as TLC and UV lamps, rotary evaporator and automatic fraction collector. MS and NMR analysis was performed to enable structure elucidation.

Methods:

Only simple chromatographic techniques were used. Analytical silica adsorbent TLC was employed throughout the isolation process. Silica adsorbent PTLC and polysaccharide size-exclusion chromatography were frequently employed. Silica gel column chromatography was only performed twice.

Results and discussion:

Nine compounds were initially isolated and purified. Two purified compounds were found to be the same prior to MS and NMR analysis, thus, only eight compounds were analyzed by MS and NMR. Out of these eight compounds, three were not pure enough to enable structure characterization and two were identified as the same compound on the basis of resulting spectra. Therefore, the isolation and identification of flavonoids from *Abies pindrow* yielded a total of four structurally dissimilar compounds.

Conclusion:

Four structurally dissimilar compounds were isolated through the use of simple chromatographic techniques and identified by MS and NMR analysis. All of the identified compounds belong to a class of plant secondary metabolites called flavonoids, or more specifically; flavonoid glycosides. They are known as: (1) kaempferol-3-O-(2″, 4″-di-E-p-coumaroyl)-α-L-rhamnopyranoside, (2) kaempferol-3-O-α-L-(4″-E-p-coumaroyl)-rhamnoside, (3) quercitrin and (4) afzelin. Hopefully, investigation of these compounds on a molecular and cellular level will result in novel lead therapeutic compounds against cancer for future evaluation in animal models.

Introduction

1. Plants, herbs, fungi, bacteria and marine organisms as a potential source of medicinal drugs

1.1 Nature as a source of bioactive compounds

Nature has long been exploited by humans as a source of biologically active chemicals utilized to maintain good health and restore imbalances among the ill. A generic term for such medicinal systems is Traditional Herbal Medicine (THM). Long before Western medicine was established, nature was considered holy, and thus, it was believed to possess divine healing and re-vitalizing powers. People used plants, animals and minerals to treat imbalances and restore inner energy. Approximately 80 % of people living in developing countries still hold on to these medicinal systems. Use of remedies from THM in industrialized countries has increased significantly in the last decade. Currently, many healthcare systems around the world have incorporated THM as part of their healthcare services. Researchers worldwide are devoting their work to the isolation and identification of chemical constituents and potential biological effects of compounds isolated from sources utilized in THM. Plants utilized in traditional medicinal systems, such as Ayurveda, Traditional Chinese Medicine (TCM), Traditional Unani Medicine, Traditional Western Herbal Medicine and Traditional Japanese Kampo Medicine, are all regarded as potential sources of bioactive compounds. [1]

1.2 Panels of natural compounds

International collaboration between research institutions have enabled construction of large panels consisting of isolated phytochemicals (i.e. chemical derived from plants). A project called “Natural Inhibitor of Carcinogenesis”, funded by the National Cancer Institute, has led to the collection of over 5000 plant extracts, wherefrom more than 250 compounds have displayed anti-tumour activity. Research groups in India, Pakistan and Brazil are conducting similar research. The project developed by the TCMCANCER network called ”Traditional Chinese Medicine in the Post-Genomic Era: Identifying Lead Therapeutic Compounds Against Cancer” has led to the construction of the largest panel of natural compounds that currently exists in the People’s Republic of China. [1]

Numerous preclinical and clinical trials in the literature seek to describe the molecular mechanisms underlying the anti-tumour activities of potential lead therapeutic compounds. Vast majority of evaluated compounds originate from natural sources in their initial structural forms, as derivatives or as semi-synthetic molecules. As a result, chemotherapeutics currently in use stem from different panels of natural compounds. Phytochemicals and natural compounds from both marine and terrestrial organisms have all been shown to display potential anti-tumour activities in various reports. Much work has been done in the last decade to increase our knowledge about TCM through evidence-based methodology in order to reveal biological activity of plant secondary metabolites from TCM in the search of lead therapeutic compounds against cancer. [2]

1.3 Aims of the TCMCANCER network

The main goal of the TCMCANCER network is to identify lead therapeutic compounds against cancer and test them in preclinical animal models, which may subsequently lead to the development of new chemotherapeutics. This Master's thesis serves as a supplement to the on-going scientific work performed by a research group at the Gade Institute in the field of oncology. This research group is particularly interested in adenocarcinoma of the prostate, which has resulted in the development of an *in vitro* cell model that enables screening of novel chemotherapeutic agents. The screening model developed at the Gade Institute is specifically utilized for the evaluation of activity against cancer stem cells. As a contribution to the aims of the TCMCANCER network and the work of the research group at the Gade Institute, this Master's thesis is intended to provide an overview of how the panel of natural compounds at the MRCTCM is generated and expanded, and to describe key elements in preparation of animal experiments.

The MRCTCM holds, at the present time, one of the largest collections in the People's Republic of China of extracts and compounds isolated from plant material. To this date, as much as 65 % of traditional medicinal plants in TCM have been investigated in the laboratories of the MRCTCM. Plants have been collected from many different parts of the country. Over 6000 compounds have been isolated and characterized, wherefrom 454 had not been reported in the literature prior to their isolation at the MRCTCM. As many as 8000 extracts from 342 plant families traditionally utilized as Chinese herbal medicines have been investigated. This vast amount of work has been performed by students, scientists and professors at the MRCTCM under the leadership of Professor Zhang Wei-Dong. In addition to building up their own Natural Product Library at the MRCTCM, information about each identified compound, such as CAS, name, category, structure, molecular formula, molecular weight, bioactivity and references, is all gathered in a database called Chinese Nature Products Database (CNPD).

1.4 Plant secondary metabolites

Although a lot of effort and money has been provided by governments all over the world to the field of cancer research, cancer still remains a leading cause of death worldwide. Much knowledge has been brought about in terms of cancer initiation and progression on a molecular level due to major advances in molecular and biological technology. Consequently, massive amount of research has been conducted to evaluate potential anti-tumour effects of plant secondary metabolites. Plants and herbs are traditionally utilized in TCM to prevent and alleviate minor ailments and major diseases. Research groups around the world are currently isolating, characterizing and evaluating potential therapeutic activities of plant secondary metabolites from TCM. Curcumin, berberine and genistein are just a few of the many plant secondary metabolites that have been shown to display chemotherapeutic properties. [3]

1.4.1 Curcumin

Curcumin is a conjugated, di-phenolic molecule that can be isolated from the plant species called *Curcuma longa*. Utilization of this plant as a medicinal source originates from Ayurveda (traditional medicine native to India) and TCM. Preclinical trials have demonstrated the ability of curcumin to both prevent cancer initiation and stimulate tumour regression. Thus, curcumin is regarded as a strong candidate for further research. However, clinical trials are impeded by its low bioavailability. Different strategies are currently being undertaken to improve the pharmacokinetics of curcumin. [4]

1.4.2 Berberine

Berberine is a quaternary ammonium salt that belongs to a group of plant secondary metabolites called alkaloids. It is commonly utilized as medicinal remedy in TCM. An increasing number of studies dedicated to the evaluation of berberine as a potential lead therapeutic compound have led to the discovery of its anti-tumour activity. Berberine has been shown, among other anti-tumour properties, to be effective against murine prostate cancer cells by promoting DNA damage in malignant cells. [5]

1.4.3 Genistein

Genistein is a polyphenolic molecule that belongs to a group of organic compounds called isoflavones, and can be found in soybeans. Genistein is currently being investigated in clinical trials for the evaluation of its therapeutic effect on prostatic neoplasm through the support of the National Cancer Institute. *In vitro* studies of cancer cells have been shown to undergo apoptosis after treatment with genistein. Further studies of genistein need to be conducted in order to map its underlying mechanisms of action in malignant diseases. [6]

1.5 Fungal and bacterial compounds

Several molecules of fungal origin have been reported in the literature to inhibit molecular pathways involved in tumour initiation and progression. Actinomycete strains have been shown to produce a number of molecules with potential effects on carcinogenesis. Examples of potential lead therapeutic compounds against cancer of fungal origin are CGP049090, PKF118-744 and ZTM000990. Also bacterial species have been shown to produce molecules with anti-tumour activities. An example of a bacterium that has been evaluated with regards to potential effects against different neoplasms is *Streptomyces conglobatus*, which in fact has yielded positive findings. [2]

1.6 Marine compounds

Continuous search for improved methods of cancer prevention and treatment by authorities worldwide, has led scientists to the discovery of new strategies. Research in the field of oncology has long been centred on the utilization of plants, herbs and terrestrial organisms as sources of potential chemotherapeutics. Lately, however, this focus has slightly shifted, or at least expanded, towards utilization of marine organisms as well. Several interesting molecules have been isolated from marine organisms, thus, it appears that our seas harbour enormous medicinal sources that remain unexploited.

Marine organisms have the ability to produce biologically active secondary metabolites due to the lack of innate immune systems. Secondary metabolites are utilized as protection against various diseases and harsh living conditions. Studies have demonstrated anti-tumour activity among several marine compounds. As a result, three marine compounds have in recent years been approved as chemotherapeutic agents by the FDA. These are trabectedin, cytarabine and eribulin mesylate. Biodiversity is much greater in the sea than on land, thus, marine organisms as a source of potential medicinal drugs will contribute immensely to the field of oncology for many years or even decades to come. [7]

2. Preclinical animal models of prostatic neoplasm

2.1 General considerations

The search for and development of novel therapeutic compounds, including anticancer drugs, can be a resource-intensive, time-consuming and costly process. In the field of oncology, such research usually starts with screening procedures of isolated and purified compounds in cell culture-based assays to detect and monitor anti-tumour activity. Properties characteristic of cancer cells (e.g. cell proliferation, migration, invasion) are utilized as targets in order to evaluate anti-tumour efficacy. Tumour specificity of novel chemotherapeutic agents can be assessed by monitoring apoptosis inducing effects in malignant cells compared to benign cells. Lately, cancer stem cell research has been dedicated increasing interest among scientists in the field of oncology. These cells are thought to be resistant towards conventional therapeutics and display high tumourigenic tendencies. Thus, future development of novel compounds against malignant diseases should also be focused on targeting cancer stem cells.

The goal of initial screening is to detect bioactive compounds. Initial screening and preclinical testing, together, serve to detect compounds with potential therapeutic applications, termed lead therapeutic compounds. Lead therapeutic compounds are utilized in modern drug development to develop novel therapeutic agents for use in patients. Thus, lead therapeutic compounds are necessary for the development of novel chemotherapeutics.

Different panels of chemical compounds are utilized in initial screening. Further description of screening goals and strategies will not be provided in this Master's thesis. For more information on this matter, a review article by Suggitt, M. and M.C. Bibby [8] is of more relevance. The goal of this Master's thesis is to provide an overview of available animal models for future investigation of selected compounds from initial screening tests. In accordance with the focus of the research group at the Gade Institute in collaboration with the MRCTCM, the emphasis of this Master's thesis is on animal models of prostatic neoplasm.

Professor Kalland's research group at the Gade Institute has developed a screening model for stepwise tumourigenesis of prostate cells based on physiological selection and adaptation by the use of benign, epithelial prostate cells. Such an approach is thought to yield a better representation of the clinical situation. Interestingly, cancer stem cells have been identified in this screening model. Thus, this model can be utilized to evaluate the efficacy of phytochemicals against the smaller sub-population of malignant cells represented by cancer stem cells.

2.2 Animal models

Rats are able to develop adenocarcinoma of the prostate spontaneously. The Dunning and the Lobund-Wistar rat model are examples. However, the use of rat models in prostatic neoplasm research is limited due to the low incidence of adenocarcinoma, prolonged latency and poor ability of tumours to metastasize. Dogs are similar to humans with respect to high incidence of prostatic neoplasms, late onset of clinical symptoms and high susceptibility for bone metastasis. Limiting their use in research is the fact that dogs display wide variability in tumour growth, the pathophysiological mechanisms behind canine prostatic neoplasms are poorly understood and the cost of sustaining dog experiments is high. Mice have a life expectancy of about 3 years, which enables scientists to study the development and progression of cancer over a relatively short period of time. Also most of the genes participating in cancer lend themselves to genetic manipulation. On the other hand, mice are both anatomically and structurally different from humans. While cancer in mice often develops in mesenchymal cells, cancer

in humans usually originates from epithelial cells. Differences in carcinogenesis have also been observed. Regardless of the disadvantages, mice models have provided invaluable information on both pathogenesis and mechanisms in adenocarcinoma of the prostate. Awareness of both advantages and limitations of mice models can provide more accurate extrapolation to pathogenesis in humans. [9]

2.3 Xenograft models

Transplantation of human neoplasms into immunodeficient mice has been of great importance in research on prostate adenocarcinoma. Xenograft models can be used to continuously assess disease progression and has long been considered to be the animal model that is most similar to the complex *in vivo* situation of human prostatic neoplasms. Some implants even metastasize, providing animal models that can be used in preclinical drug evaluations. Although xenograft models have proven to be immensely useful in prostatic neoplasm research, they are not without flaws, which include diminished immunologic response in host, low incidence of tumour establishment and physioanatomical differences between host and donor. Also the fact that xenografted neoplasms are advanced tumours has excluded the possibility to monitor cancer initiation and progression in healthy exocrine glands. Xenograft models do not focus on the interactions between host and xenografted neoplasm. In addition, other more clinically relevant models have been developed since their emergence. Thus lately, researchers have suggested that they should only be used as an intermediate step in preclinical evaluations, placed between cell cultures and immunocompetent mouse models. [9, 10]

2.4 Mouse prostate reconstitution (MPR) models

Foetal epithelial and/or stromal cells from the urogenital sinuses of mice are transplanted under the renal capsule of males with similar genetics, which produces an artificial organ. Oncogenes (e.g. ras and myc) are transferred to the transplanted cells through retroviral transduction. These cells have a high incidence of developing carcinomas. Metastasis can be increased by utilizing p53 knockout mice as donors of foetal urogenital sinus cells. The mouse prostate reconstitution model has been widely used for the evaluation of gene- and immunotherapy. On the negative side, these models require expertise in the field of molecular virology, the tumours are highly aggressive and bone metastasis is rarely achieved. Thus, evaluation of novel chemotherapeutics is highly restricted. [9, 10]

2.5 Transgenic models

Mutations in the mouse genome can provide excellent models for the study of adenocarcinoma of the prostate. As previously stated, no animal model is able to give a complete picture of the intricate tumour biology of prostatic neoplasms. Interpretation of data should for this reason rely on results from different animal models, thus, yielding a more detailed and accurate overview of cancer development and progression. Transgenic mice models are genetically engineered through the insertion of an exogenous gene into their genome by recombinant DNA technology. Molecular mechanisms of human carcinogenesis are conserved to some extent in mice, despite differences in species specific proteins. In models based on SV40 oncogenes, genes that code for tissue-specific viral oncoproteins are used to promote adenocarcinoma of the prostate. Two important mouse models are based on SV40 oncogenes, the TRAMP (transgenic adenocarcinoma of the mouse prostate) model and the LADY (LPB-Tag transgenic mice) model. The PSP-KIMAP model also displays promising features. [9, 10]

2.5.1 TRAMP models

These models exhibit a high degree of metastasis to lymph nodes and lungs (approx. 100 %). The inserted genes are called SV40 large and small t antigen. Large t antigen affects the functions of p53 and retinoblastoma tumour suppressor genes, thereby leading prostatic cells into uncontrolled cell proliferation. Small t antigen stimulates both growth and survival of tumour cells by inhibiting the function of protein phosphatase 2A. TRAMP models display several important characteristics: latency period is relatively short, metastasis progression resembles human cancer and castration does not inhibit growth. For these reasons the TRAMP model has been used to investigate the molecular pathways leading to adenocarcinoma of the prostate and to evaluate new medicines. [9]

2.5.2 LADY models

In the LADY model, only the large t antigen is inserted. The absence of small t antigen results in a less aggressive adenocarcinoma of the prostate compared to the TRAMP model. LADY models have been used to assess the effects of fat and antioxidants on prostatic neoplasm development. This model can also yield more aggressive tumours with a high degree of vascularisation which are called neuroendocrine tumours. These tumours give worse prognosis and have shown to be resistant to chemotherapy. LADY models with neuroendocrine tumours can thus be used to reveal molecular pathways in neuroendocrine carcinoma, angiogenesis in tumours and the development of new medicines. The use of highly aggressive LADY models is however limited due to the low incidence of neuroendocrine carcinoma of the prostate in humans (1-2 % of prostatic neoplasms). [9]

2.5.3 PSP-KIMAP models

Both large and small t antigens are inserted. These models display prostate-specific expression with prostatic neoplasm developing after a 2 month latency period and metastasis to lungs, lymph nodes and liver in about 80 % of the models. [9]

2.6 New transgenic models

The SV40 oncogene-based models just presented, display a range of important features: high tumour incidence, androgen independence and high metastasis incidence. Thus, these models have been of significant importance in researchers' quest to map the complexities of prostate carcinogenesis and to evaluate new drugs in preclinical studies. However, some drawbacks should be taken into consideration: neuroendocrine tumours might develop, carcinogenesis is induced by viral proteins and incidence of bone metastasis is low. Since the use of these models is focused on the investigation of late stage prostatic neoplasms, transgenic models of newer origin have been developed to study early stage prostatic neoplasms. This new generation of transgenic models, enabled due to advances in molecular technology, have alterations in a single or multiple genes. By gene deletion, mutation or insertion, a range of prostatic neoplasm models can be achieved. The most commonly employed targets for gene alterations are transcription factors, hormone receptors, growth factors and their receptors, and genes involved in cell cycle and apoptosis. For example, deletion of the PTEN gene in mice leads to development of prostatic intraepithelial neoplasm within a month or two. [9, 10]

2.7 Allograft models

Allograft models, also known as immunocompetent models, are developed by transplantation of tumour cells from one mouse into a genetically similar mouse. Thus, there is no need for athymic mice. Such a model allows the insertion of neoplasms at different stages in different locations.

Initiation of metastasis and further progression can be observed within a relatively short period of time, contributing to their great importance in the area of preclinical drug evaluation. Through the intracardiac injection of cell line derivatives from TRAMP mice, a new bone metastatic model of immunocompetent mice has been developed. Such a model provides an important platform for preclinical drug evaluations in the presence of an intact immune system. [9]

2.8 Mouse models in preclinical evaluations

The many molecular and cellular stages of adenocarcinoma of the prostate, from healthy exocrine gland to adenocarcinoma, are all features of an ideal, non-existing mouse model. Nevertheless, current transgenic models have provided reasonable indications as what to expect from new therapies with regards to anti-tumour effectiveness and toxicity. As the new generation of transgenic mouse models represent only early stage prostatic neoplasms, they are of no use in the evaluation of drug effectiveness in late stage prostatic neoplasms. Regardless of this fact, transgenic mouse models have been extensively used in preclinical drug evaluations. Due to their close resemblance to the clinical situation in humans, TRAMP models have most frequently been used for the evaluation of new treatment regimens. A wide range of compounds have been preclinically tested by the use of TRAMP models (e.g. polyphenolics, retinoic acid and anti-androgens). [9]

2.9 Stem cell research and cancer stem cells (CSCs)

CSCs share several properties, especially the dual capability for unlimited self-renewal and for giving rise to progeny cells through asymmetric division. Accumulating data suggest that CSCs are the indispensable and essential cells for tumour growth and are relatively resistant to existing chemotherapeutic drugs due to a variety of escape mechanisms. The conventional approach for anticancer drug discovery is to target cell proliferation rather than self-renewal and/or differentiation, and so, is often biased to select targets with homogeneous expression patterns and potent compounds that kill the bulk tumour cells. According to the CSC concept, conventional therapy does not treat the root of the problem, which is the main reason for re-growth of the tumour following seemingly successful remission. The CSC concept provides a rationale for several therapeutic strategies beyond traditional anti-proliferative agents by focusing on the putative tumourigenic cells, known as cancer stem cells (CSCs) in cell-based assays. Potential approaches to kill CSCs include blocking essential self-renewal signalling, inhibiting the survival mechanisms or inducing tumour cell differentiation, which can potentially be achieved by inhibiting developmental pathways. [11, 12]

2.10 Prostate cancer stem cell model developed at the Gade Institute

The EPT model of experimental stepwise prostate tumourigenesis developed in Bergen during the last 5 years, has led Professor Kalland's research group at the Gade Institute in Bergen to the establishment of a stepwise prostate cancer cell model based on physiological selection and adaptation. This model represents the first successful tumourigenic prostate model started from benign cells without the use of external oncogenes. The founding EP156T cells are human, benign epithelial cells with basal cell features. EP156T cells underwent EMT (epithelial-to-mesenchymal-transition) when they were grown at full confluence for 4 months to select for cells with loss of cell contact inhibition. The new mesenchymal type of progeny cells were called EPT1 cells and exhibited increased capabilities of migration and invasion. EPT1 cells were grown at full confluence for 3 to 4 weeks and foci appeared in the monolayer culture. Different from EP156T and EPT1 cells, cells from the foci form robust colonies in soft agar in 5 days. New EPT2 cells were cloned from soft agar colonies. In contrast to EPT1 cells, the majority of EPT2 cells can continue to divide after serum

depletion, exhibiting growth factor self-sufficiency. The selected EPT2 cells that grew well in serum free medium formed large subcutaneous tumours in mice. Cells obtained from the animal tumours were called EPT3. Following lentiviral introduction of a GFP-luciferase reporter construct, EPT3 cells were re-injected into mice prostate orthotopically. Extensive metastasis was found by *in vivo* bioluminescence imaging over 3 months. Cells collected from metastases were named EPT3-M1. DNA microsatellite analysis, karyotyping and genome-wide copy number analyses verified the genetic authenticity of all the different cells derived from this model and ruled out contamination with other laboratory cells. The identification and isolation of CSCs in the mice tumour and metastasis of this model makes it particularly interesting for testing compounds with activity against cancer stem cells. [13-15]

2.11 Predictive value of preclinical studies

So far, results from preclinical models have not been very predictive of results from clinical trials. Scientists and researchers have proposed several reasons for this inherent problem in the existing preclinical models. As mentioned earlier, no animal model gives a complete representation of prostate carcinogenesis. Thus, available preclinical models can be said to be inappropriate for prostatic neoplasm research. Another reason is that preclinical models might be used inappropriately due to discrepancies between the genotypic and phenotypic characteristics of a model and the therapeutic focus of the agent in question. A final reason might be due to major differences in the design of preclinical studies and human trials. The pharmacodynamic and pharmacokinetic variations in mice and humans should be accounted for in preclinical studies. Also drug administration and treatment of preclinical models should be the same in human trials. [16]

2.12 Metastasis

Metastasis requires a set of successive steps. The steps are divided as follows: (1) Tumour initiation: regulation of growth in a localized area is impaired and carcinogenesis is initiated. (2) Progression: further changes in regulatory mechanisms on both a molecular and cellular level promote invasiveness of tumour cells. (3) Intravasation: Acquired properties of tumour cells permit invasion of nearby tissue. (4) Transport: Invasion into blood vessels followed by subsequent transportation in the blood stream is dependent on the ability of the tumour cells to evade the immune system and suppress apoptosis. (5) Adhesion: transported tumour cells attach to blood vessels of other organs either non-specifically or through the use of receptors. (6) Extravasation: uptake of tumour cells from blood vessels and invasion of surrounding tissue due to further changes in tumour cell genome and physiology. (7) Metastasis: tumour cells survive and continue to grow into tumours in a different area from where carcinogenesis was initiated. Still, very little is known about what happens in metastasis on a molecular level. If only one step was to be targeted by a chemotherapeutic agent, it would undoubtedly have major implications on treatment and improve patient prognosis considerably. [17]

2.13 Useful models of metastasis

The search for an animal model with metastatic characteristics similar to those of humans, such as androgen independence and metastasis to specific organs, is of upmost concern in the study of metastasis. Obviously, no animal model can give an exact representation of cancer initiation and progression in humans. Before the development of genetically engineered mouse models, immunodeficient mouse models were the model of choice for the investigation of metastatic mechanisms in prostatic neoplasm. The most frequent site of metastasis in humans suffering from adenocarcinoma of the prostate is bone metastasis. Animal models that demonstrate bone metastasis

often have long latency periods and display somewhat different bone metastases from those observed in humans. [17]

Many articles covering xenograft models have been published and it is evident that such models can give valuable insights into the many stages of prostatic neoplasm. However, xenograft models have few, if any, good systems for the study of metastasis. Mouse prostate reconstitution (MPR) models where foetal epithelial and/or stromal cells from the urogenital sinuses of mice are implanted under the renal capsule of males can be used to assess cancer progression and detect host-tumour interactions. Highly specialized personnel and advanced equipment, though, is needed to perform studies in this type of model. Also no genetically engineered mouse model in current use has been able to produce bone metastasis to a desirable extent. Better metastatic models of human prostate cancer are necessary. Future metastatic models should lend themselves to facilitated use, display a high degree of bone metastasis and avoid the need for specialized knowledge in invasive implantation techniques. [10, 17]

3. Basic concepts in pharmacology

3.1 Pharmacokinetics

Pharmacokinetics is concerned with the effects the body has on the drug in question. Thus, immediately after a drug has entered the body, principles of pharmacokinetics are applicable. Pharmacokinetics is divided into four separate areas as the drug passes through the many functions and organs of the human body: absorption, distribution, metabolism and excretion. [18]

3.1.1 Absorption

The area of absorption is investigated to enable description of how much time it will take for the drug to exert its pharmacologic effect. Absorption is dependent on three general factors: route of administration, method of absorption and other factors influencing absorption. [18]

Administration can be further divided into enteral, parenteral and other non-enteral administration. Enteral administration means that the drug passes through the gastrointestinal tract and examples are oral, sublingual, buccal and rectal administration. Parenteral administration comprises the intravenous, intramuscular and subcutaneous route of administration. Other non-enteral administration forms constitute inhalation, topical, transdermal, intranasal, intrathecal, intraventricular and ophthalmic administration. [18]

Methods of absorption can be further divided into passive absorption, active absorption and pinocytosis. Passive absorption is the most prevalent method utilized by drugs in order to enter the systemic circulation. A concentration gradient across the luminal side of the enterocytes and their interior causes drugs to move (diffuse) from an area of high concentration (the lumen) to an area of low concentration (the cytoplasm of enterocytes). The drugs should be small and fat soluble in order to cross the plasma membrane solely by diffusion. Active absorption works oppositely from passive absorption. Input of energy is required and drugs are transported from an area of low concentration to an area of high concentration. In pinocytosis cells engulf drug particles to bring them across their plasma membranes. [18]

Other factors that can influence absorption are rate of dissolution, blood flow and contact time. The rate of dissolution has a great impact on absorption. If drugs dissolve quickly, their absorption times will be short and their pharmacologic effects will be observed short after administration. Blood flow

also affects absorption to a large extent. The intestine has high blood flow, thus drugs are more easily absorbed from the intestine compared to the stomach. Low blood flow in a cutaneous area where a drug is administered transdermally will reduce drug absorption, and as a consequence, pharmacologic effect. Contact time has to do with drug molecules being in contact with sites of absorption. If drug molecules find themselves away from sites of absorption for long periods of time, absorption will be reduced. Other drugs, food and beverages may also alter absorption. [18]

3.1.2 Bioavailability

Bioavailability is a term used to express the percentage of the administered drug that reaches the bloodstream. Bioavailability is strongly bound to and therefore affected by absorption. Along with the previously mentioned factors, bioavailability is also influenced by formulation, metabolism and surface area. Only intravenously administered drugs have 100 % bioavailability. Orally administered drugs never reach 100 % bioavailability due to the reasons mentioned above and because once absorbed by the intestine, drugs are transported to the liver by the hepatic portal vein (Latin: vena portae hepatis), where they are metabolized before being transported to the bloodstream. Surface area has to do with the area capable of absorbing drug molecules. The intestine has a large surface area, thus absorption is greater in the intestine compared to the stomach. Once in the bloodstream, drugs can be distributed throughout the body depending on drug characteristics and pharmacokinetic properties. Drugs can also bind to specific plasma proteins in the systemic circulation. Albumin is such a plasma protein. Bound drug is pharmacologically inactive (i.e. unable to exert therapeutic effect) while an unbound drug is pharmacologically active. [18]

3.1.3 Distribution

Drugs are first distributed to organs with the highest blood flow, meaning heart, liver, kidney and brain. Areas with lower blood flow then follow, meaning muscles, skin and fat. It is difficult to achieve high enough drug concentrations in bones due to very low blood flow in these parts of the body. Infections in the CNS might also be difficult to treat due to selective permeability of the blood-brain barrier. Volume of distribution is a commonly employed term used to describe to what extent the administered drug is distributed between the systemic circulation and the rest of the body. It is called volume of distribution because it is a calculation of the volume needed to reach the same concentration of the administered dose as the concentration found in a blood sample. In simpler terms, a large volume of distribution describes a drug that is highly distributed throughout the body while a small volume of distribution describes a drug that is poorly distributed throughout the body and stays largely within the systemic circulation. [18]

3.1.4 Metabolism

Metabolized drugs are often no longer pharmacologically active (at least less active) and more hydrophilic. Of course exceptions from this general rule exist. As an example, pro-drugs have to be metabolized to gain pharmacologic activity. Metabolism is primarily conducted by the liver, but can also be performed by the skin, lung, kidneys and gastrointestinal tract. The major group responsible for drug metabolism is a diverse group of proteins known as cytochrome P-450 enzymes. Interindividual differences in drug metabolism can be due to many factors, among them genetics, comorbidity and polypharmacy. [18]

3.1.5 Excretion

The body excretes drugs in many ways, some more important than others. Drugs can be excreted through urine, faeces, bile, exhaled air, sweat, tears, saliva and breast milk. Renal excretion comprises glomerular filtration, proximal tubular secretion and distal tubular reabsorption. Drugs are primarily excreted through urine. Half-life ($t_{1/2}$) is used to describe how long a drug will remain active before

being excreted. Half-lives vary greatly between drugs, ranging from only minutes to several days. A drug reaches steady state after about 5 half-lives. Thus, for a drug with a half-life of 1 hour, it will take 5 hours for the drug to be completely excreted from the body. Clearance is used to describe how much of the drug is removed from the systemic circulation per unit time. Total body clearance comprises hepatic clearance, renal clearance and pulmonary clearance among others. Glomerular filtration rate (GFR) is used to describe the amount of drug removed from the systemic circulation by the kidneys. Renal diseases can therefore cause severe toxicity in patients taking medications that are primarily excreted through urine. Creatinine clearance can be calculated by the use of the Cockcroft-Gault equation, which in turn gives an estimation of GFR. [18]

Tumours can have a profound effect on different organ systems and thereby alter their functions. This is particularly true for metastatic neoplasms. As a consequence, pharmacokinetics may also be affected to different extents.

3.2 Pharmacodynamics

Pharmacodynamics is concerned with the effects the drug in question has on the body. A graphic representation of drug response at different doses yields a graph similar to that of a normal distribution, called frequency distribution curve. Such a graph can be used to determine the median effective dose (ED_{50}), the dose that produces a therapeutic response in 50% of patients. The median lethal dose (LD_{50}), the dose that kills 50% of patients, is determined from preclinical studies alone. Median toxicity dose (TD_{50}), the dose that produces a toxic effect in 50% of patients, is determined from toxic effects recorded in animal studies and in clinical trials. Therapeutic index is determined by comparing ED_{50} to LD_{50}. A drug with a high therapeutic index is regarded much safer than a drug with a low therapeutic index. Blood samples are necessary to monitor drugs with low therapeutic indexes. Both the highest plasma concentration (peak level) and the lowest plasma concentration (trough level) are measured to prevent toxicity. [18]

Drugs used to treat the same diseases are compared on the basis of four different features: potency, efficacy, safety and cost. A potent drug displays a therapeutic effect at low doses. An efficacious drug yields a high maximal therapeutic response. A safe drug has a high therapeutic index and thus displays a low incidence of adverse and toxic effects. A costly drug can produce great income for the manufacturer, but can also be too expensive to take part in government funded health services. [18]

The mechanism of action for the majority of drugs is either to activate or inhibit a receptor, in a wide sense of the word. Drugs can be agonists, partial agonists or antagonists. An agonist produces the same response as the naturally occurring ligand. A partial agonist produces the same effect as the naturally occurring ligand, but is less efficacious. An antagonist competes with the naturally occurring ligand for binding site(s) and produces an opposite effect. [18]

Drug interactions can have major implications on chemotherapy. Drugs can interact with food, beverages and of course other drugs. Studies have shown a 50% risk of experiencing an adverse drug effect when taking 5 medications simultaneously. The risk for patients taking 8 or more medications is almost 100%. Drugs can either increase or decrease the efficacy of one or both of the drugs involved. Drug interactions are classified as additive, synergistic, antagonistic or incompatible. Additive means that drugs with similar mechanisms of action yield a therapeutic response that is the summation of individual therapeutic responses. Synergistic effects are therapeutic responses that exceed the summation of individual therapeutic responses. Antagonistic means that one drug reduces or completely erases the therapeutic effect of another drug. Incompatible drugs go through a chemical or physical reaction when mixed together. Incompatibility is most common for parenterally administered drugs. [18]

4. Preclinical pharmacology

4.1 Animal pharmacology

Although animal pharmacology is ranked second compared to animal toxicology, it can generate very useful information. As an example, previous work in the field of animal pharmacology has demonstrated the lethal dose in 10 % of mice (LD_{10} = C (concentration) × T (time)) to be the same as maximum tolerated dose in humans (MTD = C (concentration) × T (time)). This relationship has later been confirmed by several scientists. LD_{10} in mice thus provides an excellent dose escalation target in phase I clinical trials. [19]

4.2 Animal toxicology

In the United States of America, animal species normally employed in animal toxicology are mice or rats and beagle dogs. Primates are usually required for biological therapeutics (e.g. monoclonal antibodies). In Europe, toxicology studies in mice and rats are enough to advance to phase I clinical studies, given the fact that both species display similar dose-toxicity profiles. Animal models have been shown to yield reliable predictions of human toxicity. However, certain limitations are apparent. Toxicities in one species can be completely absent in another. Thus, although murine models display serious toxicities, it might be that dogs or primates do not do so due to differences in metabolism. Animal toxicology is crucial to the determination of dose for first human exposure. [19]

4.3 Modern drug development

Pharmacological objectives in modern drug development can be divided as follows:

1. Pharmacological profiling: any preclinical trial designed to assess drug affinity for a molecular target, its ability to stimulate, inhibit or otherwise affect a biochemical pathway, organs or another drug. [20]
2. Safety profiling: any preclinical trial designed to assess adverse effects and other unwanted effects of experimental drugs on biochemical pathways, organs or another drug. [20]

Specific recommendations are made for preclinical study design in order to enable applicability of data from animals to the pharmacology of humans. These recommendations are listed below (***Table 4.1***).

Table 4.1 Recommendations for preclinical study design

Design feature	Recommendation
Animal model	Conscious, no analgesics if possible, same as or close to phase I clinical trial conditions
Test subject	Rodent or other species normally used in toxicology, most appropriate species from a scientific point of view
Statistical consideration	Each animal should serve as its own control to reduce the amount of animals used
Number of test subjects	Enough to achieve statistically satisfying results
Control group	Perform placebo testing
Route of administration	Should be equal or close to phase I clinical trial route
Drug formulation	Should be equal or close to phase I clinical trial formulation
Dose range	Minimum three doses, including a maximum tolerated dose (MTD)
High dose	Investigate maximum tolerated dose, maximum dose limited by formulation, dose limit (1-2 g/kg)
Pharmacokinetics	Document systemic changes
Endpoints	Document blood pressure, heart rate, ECG, survival, death, etc.
Duration	Enough to document drug distribution, C_{max}, AUC
Study execution	Should be performed in accordance with Good Laboratory Practice Standards (GLPS)

Source: Kinter, L.B. and J.P. Valentin [20]

4.4 Biomarkers

Drugs can bind to and alter the functions of molecular targets such as proteins (e.g. receptors, enzymes, transporters), carbohydrates, lipids or nucleic acids and thus re-establish homeostasis in certain cells, tissues or organs. If the physiological response to the drug is over a distinct tolerable level, opposing physiological mechanisms are activated and the outcome may be an increase/decrease in cell size or number, apoptosis or carcinogenesis. [20]

Unwanted and toxic effects might be a direct consequence of the drug's mechanism of action or high concentrations of the drug in body fluids and tissue. However, signals involved in the progression from initial drug-target interaction to irreversible physiological mechanism can be detected and used to monitor unwanted effects, thus facilitating risk assessments. These signals are called biomarkers and are utilized in modern drug development to predict efficacy and safety of experimental drugs in humans before conducting phase I clinical trials. Biomarkers are associated with molecular mechanisms that can be physically measured when performing *in vitro* screening and *in vivo* studies. In other words, biomarkers are used to enable extrapolation of findings in preclinical trials to findings in clinical trials with greater certainty. Molecular markers in animals must be associated with endpoints measurable in humans in order to be termed biomarkers. [20, 21]

5. Magnetic resonance imaging

5.1 Doxorubicin in TRAMP models

In order to validate the use of MRI in efficacy studies, Degrassi et al. [22] employed the use of this non-invasive technique to assess tumour regression in TRAMP models treated with doxorubicin. Poorly differentiated and well differentiated adenocarcinomas were successfully separated through the use of MRI. 3 out of 6 mice with poorly differentiated adenocarcinomas relapsed within 40 days after completed treatment. The MR images of these mice showed heterogeneities due to necrotic and haemorrhagic tissue. The mice with well differentiated adenocarcinomas were followed for 4 weeks after completed treatment, by which time all of the mice had relapsed and tumour sizes were larger than pre-treatment sizes. The usefulness of MRI in preclinical trials is evident. Not only does it allow us to follow both tumour regression and relapse, but it can also be used to distinguish between poorly differentiated and well differentiated adenocarcinomas. [22]

6. I*n vivo* bioluminescence imaging (BLI)

6.1 Luciferase imaging

Major breakthroughs in the fields of molecular and cellular biology have enabled the development of several imaging techniques for use in real-time analysis. *In vivo* luciferase imaging is one well-known example. Natural light production is oftentimes brought about by a class of enzymes called luciferases. The best studied luciferase is the one derived from the North American firefly (Photinus pyralis). Luciferases generate light by catalyzing oxidation reactions. Luciferase from the North American firefly emits green light with a wavelength of 562 nm. Luciferase from the sea pansy (Renilla reniformis) generates blue light with a wavelength of 482 nm. [23]

6.1.1 Requirements

Before *in vivo* imaging can commence, cells under investigation need to be transfected with the bioluminescent reporter gene, thus, genetic engineering is required. Once engineered cells have been transferred to an animal model, their location can be detected through the provision of luciferase substrates. No cofactors are required for catalysis by Renilla luciferase. Catalysis by Photinus luciferase, on the other hand, requires oxygen, magnesium and energy input in the form of ATP. [23]

7. Small animal PET-CT and tumour assays

7.1 PET

PET (positron emission tomography) is enabled by the use of a radioisotope. Either a compound that is natural to the biology of the test animal or a drug under investigation is labelled with the radioisotope. The radioisotope emits a positron that annihilates with an electron yielding two γ-rays that travel in opposing directions. Both quantity and localization of the positron emitting substance is revealed. PET

sensitivity is generally considered to be very high. If two radioisotopes were to be used at the same time, the detector would not be able to distinguish between them. [21]

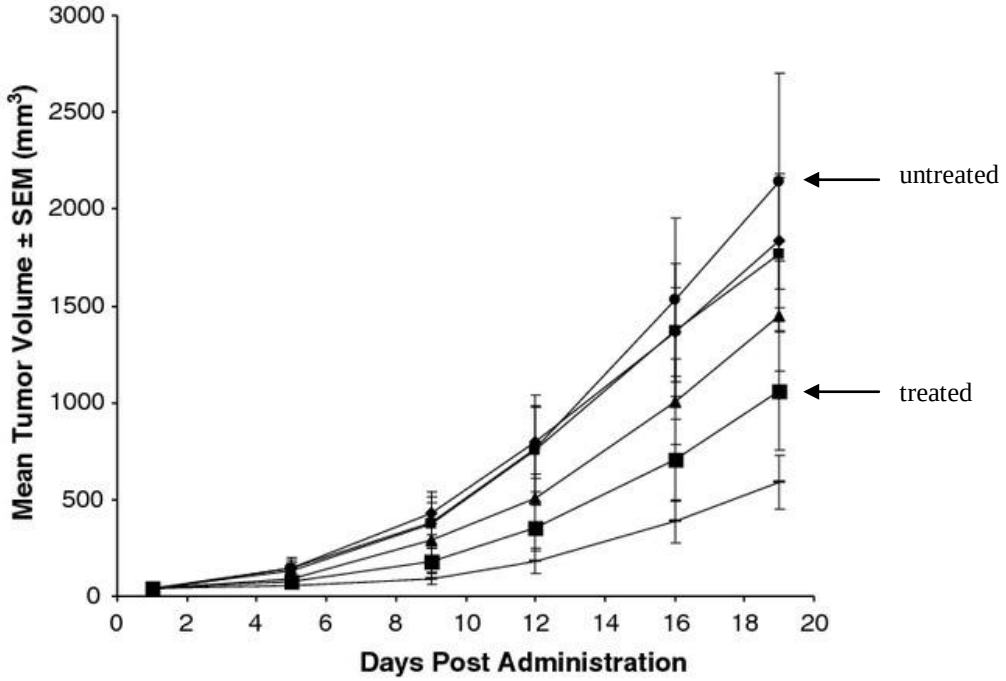
7.2 CT

In the case of CT (computed tomography), X-rays are projected towards an animal model by the use of an X-ray source. A detector on the opposite side of the X-ray source measures the extent of absorption. X-ray is projected from every angle to enable production of structural images. CT resolution is generally considered to be high. On the other hand, sensitivity is not very good and it is difficult to utilize contrast agents as labels. Thus, CT is used in combination with different molecular imaging techniques rather than by itself. In the clinic, PET-CT has become first-line of diagnosis among molecular imaging methods. [21]

7.3 Tumour assays

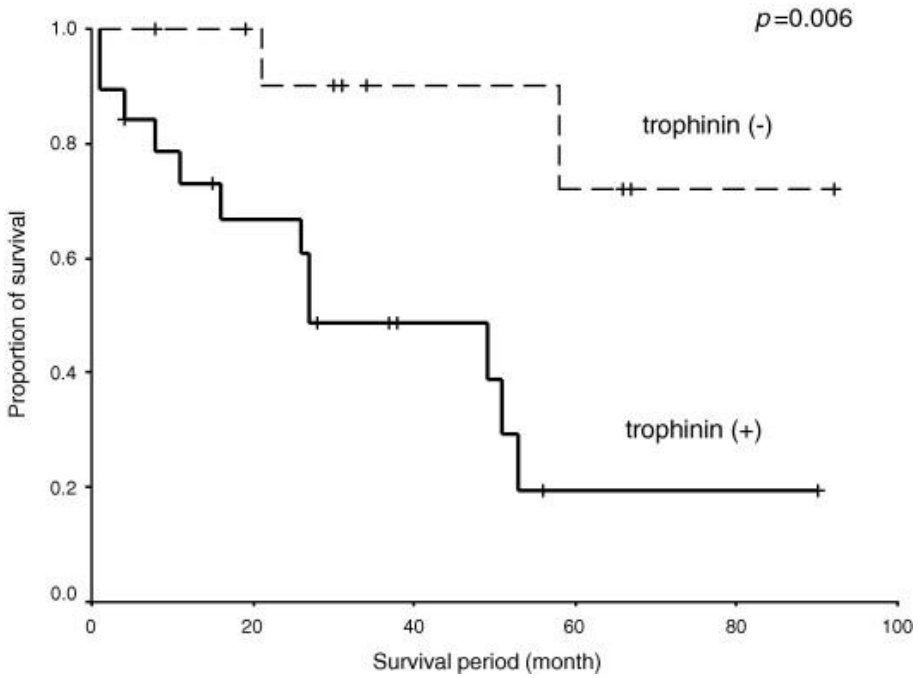
Many assays have been developed to evaluate drug efficacy. Tumour growth delay assay and survival time assay are commonly employed in modern drug development. Tumour delay is described as the time it takes for a treated tumour to reach a certain size minus the time it takes for an untreated tumour to reach the same size (***Figure 7.1***). Survival times can reach a maximum point, above which the adverse effects outweigh beneficial effects. Survival time is described as the time treated animal models survive minus the time untreated animal models survive (***Figure 7.2***). [24]

Figure 7.1 Tumour growth delay assay



Source: http://www.springerimages.com/Images/MedicineAndPublicHealth/1-10.1007_s00280-009- 1099-1-1

Figure 7.2 Survival time assay



Source: Chen, K.Y., et al [25]

8. Animals in research

8.1 Animal welfare

The well-being of laboratory animals has been greatly improved due to William Russell and Rex Burch. In 1959, they proposed the "3Rs", which apply to the use of animals in biomedical research. Their proposal were the following three principles: [26]

1. Studies should cause the minimum amount of pain and distress in animals, and should therefore be *refined* accordingly. [26]

2. In studies expected to cause pain and distress, the number of animals should be *reduced*. [26]

3. If studies permitted so, animals able of feeling pain and distress should be *replaced* by non-animal models. [26]

Russell and Burch's contributions to animal welfare are still essential to the requirements made today by regulatory authorities prior to approval of animal studies. In America, the local Institutional Animal Care and Use Committee (IACUC) evaluate animal study applications with two norms in mind: (1) infliction of untreated pain and distress in laboratory animals ***must*** be justified, and (2) infliction of untreated pain and distress in laboratory animals ***can*** be justified. "The Principles", published in 1985

by the United States Interagency Research Animal Committee, gives a set of guidelines in the use of animals in preclinical studies and other animal research. The major concepts presented in this publication, as well as in the two main laws in America currently regulating animal research, are the concepts that follow: [26]

- That which is painful to humans, should be assumed painful to animals. [26]
- Good scientific practice searches to minimize and even avoid pain and distress in animals. [26]
- Tranquilizers, anaesthesia, analgesia and even euthanasia can be withheld only when scientifically justifiable. [26]

8.2 Euthanasia in pain management

Killing, or euthanasia which is a more appropriate term, is regarded as the ultimate analgesic and is therefore often the first-line treatment of pain in research animals. Euthanasia does not require justification, but it is rather the avoidance of killing animals in pain and distress that necessitates justification. Pain due to cancer is a typical example. Pain management in the different forms of this disease requires increasingly more potent analgesics (e.g. fentanyl, morphine). Effective pain relief calls for intravenous drug administration, which would be rather difficult to implement in current mice cancer models. Analgesics can in addition have an effect on cancer progression. Euthanasia is thus the strategy of choice in cancer studies when addressing pain. [26]

8.3 Progresses and trends

The ultimate goal of public policies is to neither inflict pain nor distress in laboratory animals, something that seems rather idealistic and somewhat impossible. However, major progresses have been made to develop alternatives to animals and to reduce pain and distress in animals used in biomedical research. Whether the goal of zero pain and distress is realistic or not, consensus among regulatory authorities is that scientist at all times should strive for humane treatment of laboratory animals to ensure unnecessary infliction of pain. [26]

Based on annual reports for 2002-2009 from the IACUC to the U.S. Department of Agriculture, animals that are deliberately left untreated for pain represent just under 10% of all animals used in biomedical research and that the number of these animals declines for each year. However, the reports from the IACUC describe only Animal Welfare Act-covered species which are estimated to be only 1% of all research animals. Prostatic neoplasm research is performed mainly in mice, thus, it is impossible to know whether or not the annual reports from the IACUC give a true representation of pain management in mice since mice are not covered in the Animal Welfare Act. Another major drawback in the annual reports received by the U.S Department of Agriculture, is the fact that if previously conducted animal studies were to be performed by today's standards, many animals would be placed in the same pain management category, whereas previously they were kept in separate categories. Also the IACUC reports combine pain and distress, making it impossible to differentiate between animals with pain and those with distress but without pain. Regardless of the lack of data for mice and other rodents, the amount of pain in Animal Welfare Act-covered species must in reality be reducing due to the fact that the amount of animals left untreated for pain have diminished over the years and the threshold for placing animals in this category has been lowered. [26]

9. Modern Research Centre for Traditional Chinese Medicine (MRCTCM)

9.1 Objectives

The Modern Research Centre for Traditional Chinese Medicine is a research department at the Second Military Medical University in Shanghai. The objectives of this research department are to promote globalization of and establish a new research platform of TCM. Professors, scientists and students at the MRCTCM employ approaches and methods in structural biology, chemical biology, medicinal chemistry, analytical chemistry, pharmacology and toxicology.

9.2 Natural Products Library

342 plant families traditionally utilized in TCM have been systematically investigated through purification and identification. More than 6000 compounds have been characterized, wherefrom approximately 454 had not been reported in the literature prior to their isolation at the MRCTCM. Professors, scientists and students continuously publish results from their own work at this research department, and thus, contribute immensely to the globalization of TCM. The vast amount of work performed at the MRCTCM has enabled the construction of a Natural Products Library. About 8000 standard TCM extracts and over 6000 purified and characterized organic compounds constitute the Natural Products Library. This library is currently the largest library of natural products in the People's Republic of China.

9.3 Phytochemistry laboratory

The practical part of my Master's thesis was performed in the Phytochemistry laboratory at the MRCTCM by the help of Dr. Li Yong-Li. This man is a Ph.D. student at the Shanghai Jiao Tong University who performs his laboratory work at the MRCTCM. Upon my arrival to Shanghai, Dr. Li Yong-Li was gathering extracts from a plant called *Abies pindrow*. Therefore, the practical part consisted of isolation and characterization of compounds from this particular plant.

10. *Abies pindrow*

10.1 Background information

Abies pindrow (Family: Pinaceae) has a conical shape, levelled branches and needle-like leaves (***Illustration 10.1***). It grows in the Himalayas between the altitudes of 1600-4500 m above sea level. The use of this species as a medicinal plant is evident due to its mentioning in Ayurveda. *Abies pindrow* leaves have been utilized as traditional medicine in India to treat fever, inflammatory diseases, bronchitis and other ailments. [27, 28]

Illustration 10.1 Abies pindrow

Source: http://en.wikipedia.org/wiki/File:Abies_pindrow.jpg

10.2 Pharmacological actions

Studies have reported that extracts of *Abies pindrow* display anti-inflammatory, analgesic, anti-ulcerogenic, anti-stress and hypotensive activities in preclinical animal models. Previous isolation of compounds from *Abies pindrow* has led to the discovery that both the stem and the leaves contain flavonoids. [27, 28]

Methodological overview

11. Low-pressure column chromatography

11.1 Different techniques

The term column chromatography covers a wide range of chromatographic techniques, all central to the isolation of natural products. Although the methods within this group are numerous, they share a common feature; solvent (the mobile phase) of a certain composition is allowed to flow through the pores of densely packed sorbent (the stationary phase) with a given particle size. The separation of compounds is made possible through one or several types of interactions between the compounds and the phases. [29]

Compounds are selectively distributed to different extents between stationary and mobile phase. Differences in distribution coefficients are due to both chemical and physical characteristics of the phases and the compounds. Four important mechanisms of separation exist in column chromatography: adsorption, size-exclusion, partition and ion-exchange. [29]

Silica gel and alumina belong to the group of sorbents utilized in adsorption chromatography. If the interactions between a compound and the adsorbent are much greater than the interactions between the compound and the mobile phase, the rate of migration will be low. Evidently, if the affinity for the mobile phase is much greater, the rate of migration will be high. Some of the types of interactions in absorption chromatography are hydrogen bonding, van der Waals forces and dipole-dipole interactions. Separation is oftentimes due to a combination of these interactions. [29]

Size-exclusion chromatography, also called gel permeation chromatography, is as the name says a technique to exclude molecules based on size. The stationary phase consists of porous beads, which are easily penetrated by smaller molecules. Larger molecules, in contrast, are not able to penetrate the stationary phase particles. As a result, larger molecules elute faster than smaller molecules. [29]

A difference in distribution of compounds between two different liquids is the basis of partition chromatography. This type of chromatography employs both a liquid stationary phase, immobilized onto a solid support, and a liquid mobile phase. If a compound is largely distributed in the mobile phase compared to the stationary phase, obviously, that compound will elute first. [29]

For compounds that can carry a charge at a given pH value, the technique of ion-exchange chromatography is applicable. The sorbent contains charged groups that can interact with ionized groups on the compounds. Thus, separation is attributable to differences in charged groups. [29]

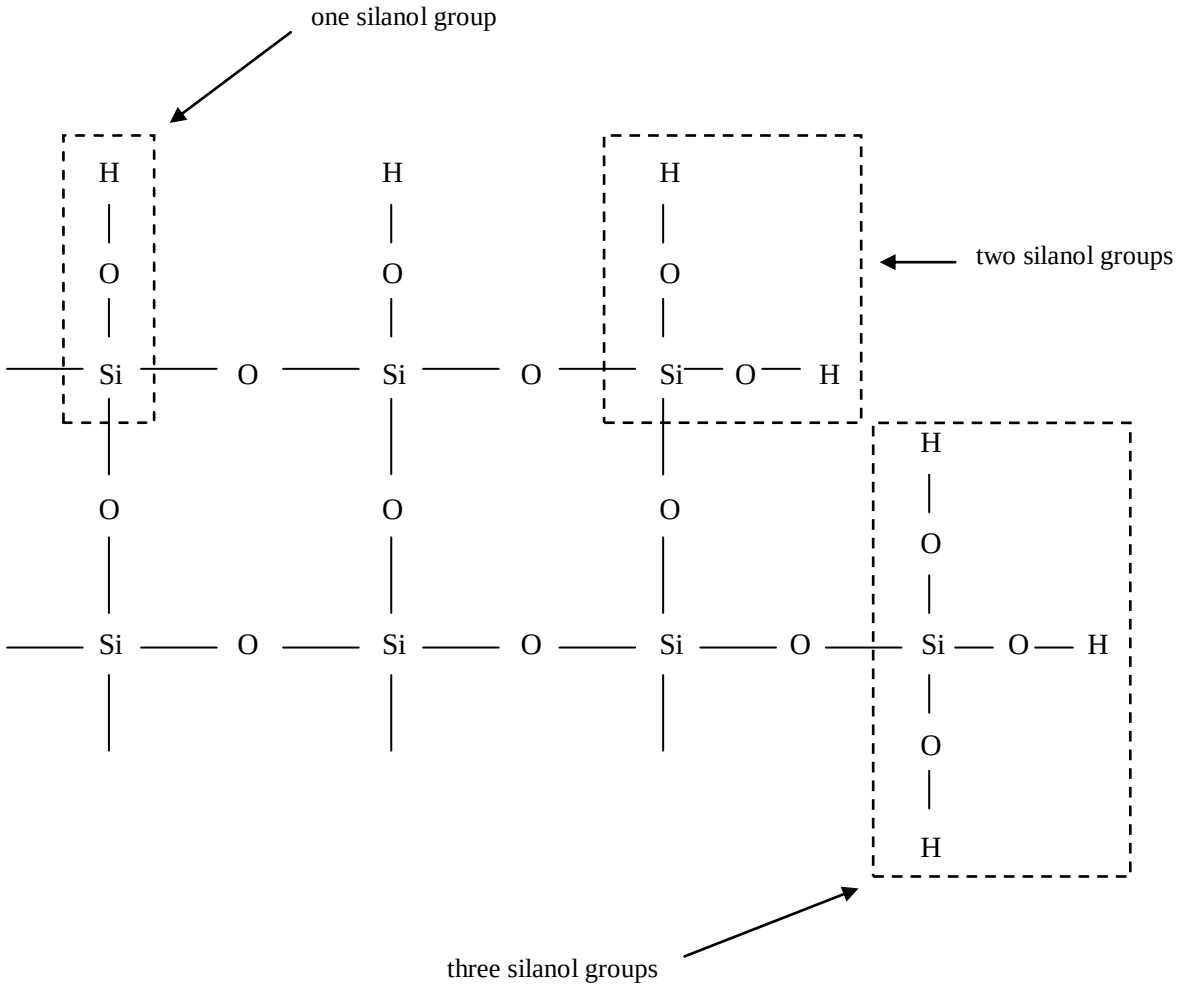
11.2 Silica gel chromatography

As mentioned above, silica gel is utilized in adsorption chromatography. Silica gel is in fact the most commonly employed adsorbent in low-pressure column chromatography. One of the greatest advantages of silica gel is that it is highly inert. The adsorbent in silica gel chromatography is polar, thus, the more polar compounds will be retained to a greater extent compared to the more non-polar compounds. [30]

11.2.1 Properties of silica gel

Silica gel is a highly porous polymer composed of silicon oxide units (SiO_2). In low-pressure column chromatography, silica gel particles in the size range of 40-200 µm are normally used. Silanol groups are exposed on the surface of silica gel particles (***Figure 11.1***). These silanol groups form the basis of adsorption to silica gel. Separated compounds can form many, few or no hydrogen bonds with the exposed silanol groups in the silica gel depending on characteristics of both the compounds and the mobile phase. [30]

Figure 11.1 Exposed silanol groups on silica gel



11.2.2 Mobile phases

Both non-polar solvents and polar solvents are employed in silica gel chromatography. If the mobile phase has many hydrogen bonding sites, the more capable it is of eluting polar compounds. Non-polar solvents such as hexane and dichloromethane are normally employed in silica gel chromatography. Conversely, regularly employed polar solvents are methanol, ethyl acetate and acetonitrile. Water and methanol can dissolve silica gel and consequently pollute the isolated products. [30]

11.3 Size-exclusion chromatography

A variety of polymers, such as polyacrylamide and different carbohydrates, can be utilized in size-exclusion chromatography.

11.3.1 Polyacrylamide

Polyacrylamide (Bio-Gel P) is used to separate macromolecules like carbohydrates and peptides. Polyacrylamide particles range in size from 45 to 180 µm. Bio-Gel P gels are almost always operated with water as the mobile phase. Different gels provide separation of different molecular weights. [30]

11.3.2 Carbohydrates

Carbohydrates serve as a highly inert group of polymers. In size-exclusion chromatography, polysaccharides are crosslinked and formed into beads. A commonly utilized polysaccharide is dextran. The size of the beads can range from 20 to 300 µm, all with varying porosities.

11.3.2.1 Sephadex

Sephadex is probably the most commonly employed polymer resin in size-exclusion chromatography. Its preparation consists in crosslinking dextran with epichlorohydrin. These gels are named after how much solvent they pick up upon swelling. As an example, Sephadex G-50 picks up 5.0 mL of solvent per gram of polymer. Sephadex is able to separate molecular weights ranging from 10 to 100,000 Da. The most commonly employed Sephadex gels in natural products isolation is Sephadex G-10, G-15 and LH-20.

Sephadex LH-20 is regularly used for the separation of non-polar and semi-polar compounds. This gel is used to separate natural products with molecular weights of 100 to 4,000 Da. When utilized solely on the basis of size-exclusion, only a single solvent is used.

12. Planar liquid chromatography

12.1 TLC

Among the techniques that belong to the group called planar liquid chromatography, thin liquid chromatography (TLC) is by far the most common one. This is greatly due to its inexpensiveness and its ease of use. TLC can be simply described as the separation of mixtures on thin layers of adsorbents coated on plastic, aluminium or glass. [29]

The mixture to be investigated is applied as a spot onto the adsorbent and placed in a tank with a solvent of appropriate quantity and composition. The solvent should wet the bottom of the plate but not the part where the spots are applied. The plate is left to develop in the tank, i.e. the solvent migrates through the sorbent by capillary action and separates the mixture along the way. [29]

12.2 Retardation factor (R_f)

The R_f value of a given compound with a given sorbent and solvent system is an important measure that is characteristic of each compound, and thus, serves to both detect and distinguish between similar and dissimilar compounds, respectively. The R_f value of a compound is calculated as follows: [29]

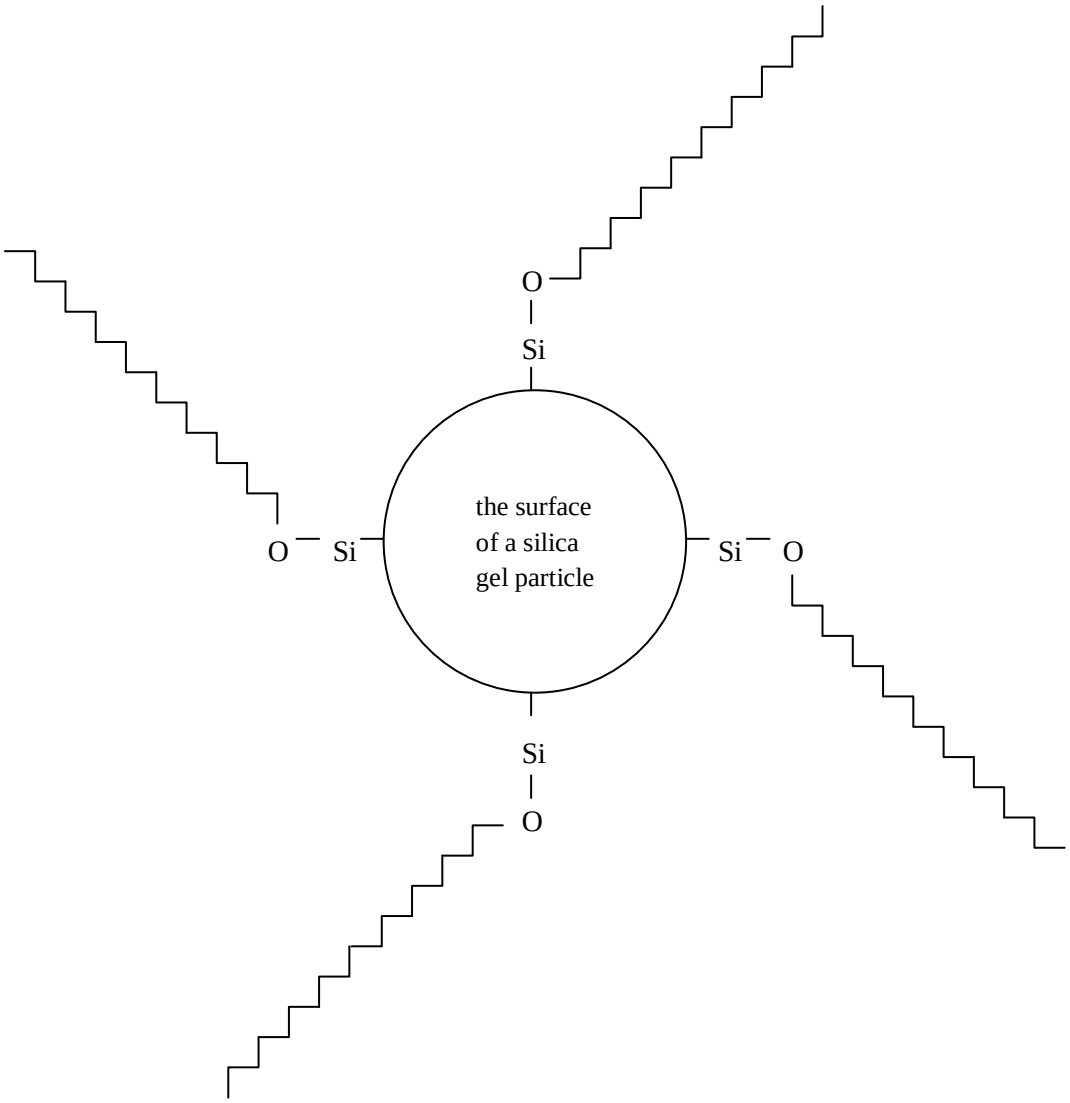
$$R_f = \frac{Migrated\ dis\tan ce\ of\ compound}{Migrated\ dis\tan ce\ of\ solvent}$$ [29]

As can easily be predicted from the above quotient, R_f values are always between 0 and 1. As previously mentioned, the R_f value of a compound will vary in different sorbent and solvent systems. In the case of silica and alumina sorbents, polar compounds will migrate to a lesser extent due to a greater affinity for the sorbent (stationary phase). Non-polar compounds, in contrast, will interact more with the solvent system (mobile phase) compared to the sorbent. Compounds are thus separated with regards to polarity, which in turn is dependent on the type and number of functional groups present on the compounds in the mixture. As a result, polar compounds will have small R_f values, whilst non-polar compounds will have large R_f values. [29]

12.3 Partition and reverse phase TLC

In most TLC experiments the mechanisms of separation are restricted to the mechanisms of adsorption and partition. The former has already been presented in the above section. Partition relies on differences in solubility of the compounds in the mixture with the stationary phase and the mobile phase. In other words, hydrophilic compounds will migrate less than hydrophobic compounds. In reverse phase TLC (i.e. non-polar stationary phase and polar mobile phase) the most commonly employed sorbent is octadecasilyl, which essentially is silica with attached C-18 aliphatic, unbranched units (***Figure 12.1***). In this mode, the extent of migration will be the other way around. Compounds that are hydrophilic will migrate to a greater extent compared to compounds that are hydrophobic. [29]

Figure 12.1 Octadecasilyl



12.4 Analytical TLC

During an isolation process it is routine to perform analytical TLC in order to identify and monitor compounds. Compounds contained in fractions from different elution techniques (e.g. silica gel chromatography, dextran gel chromatography) and from other chromatographic techniques (e.g. PTLC) are normally monitored by the use of TLC. Because different compounds may have matching R_f values, it is advisable to develop the same sample in two different solvent systems. This way, seemingly "pure" spots may be confirmed as consisting of several compounds and spots from different fractions may be confirmed to be the same compound. [29]

12.5 Solvent system

TLC is run either isocratically or by the use of a step-gradient. In the isocratic mode, the plate is developed in a solvent system of constant composition, whereas in the step-gradient mode, the plate is developed in a solvent system where the solvent polarity is gradually increased, thus, the power of elution is increased stepwise. [29]

12.6 Detection

Visualization of the isolated compounds is essential to the end result of the isolation process. Poor detection may lead to low yield of compounds and difficulties in obtaining compounds of required purity. Detection can be carried out in a destructive (e.g. spray reagent) or a non-destructive (e.g. UV-light, I_2) manner. A spray reagent consisting of 1 g vanillin powder, 90 mL of ethanol and 10 mL of concentrated sulphuric acid was routinely used during the isolation process. Destructive detection contaminates the compounds and renders recovery from the sorbent impossible. Non-destructive detection is therefore the method of choice if subsequent recovery is intended. [31]

13. PTLC

13.1 The role of PTLC

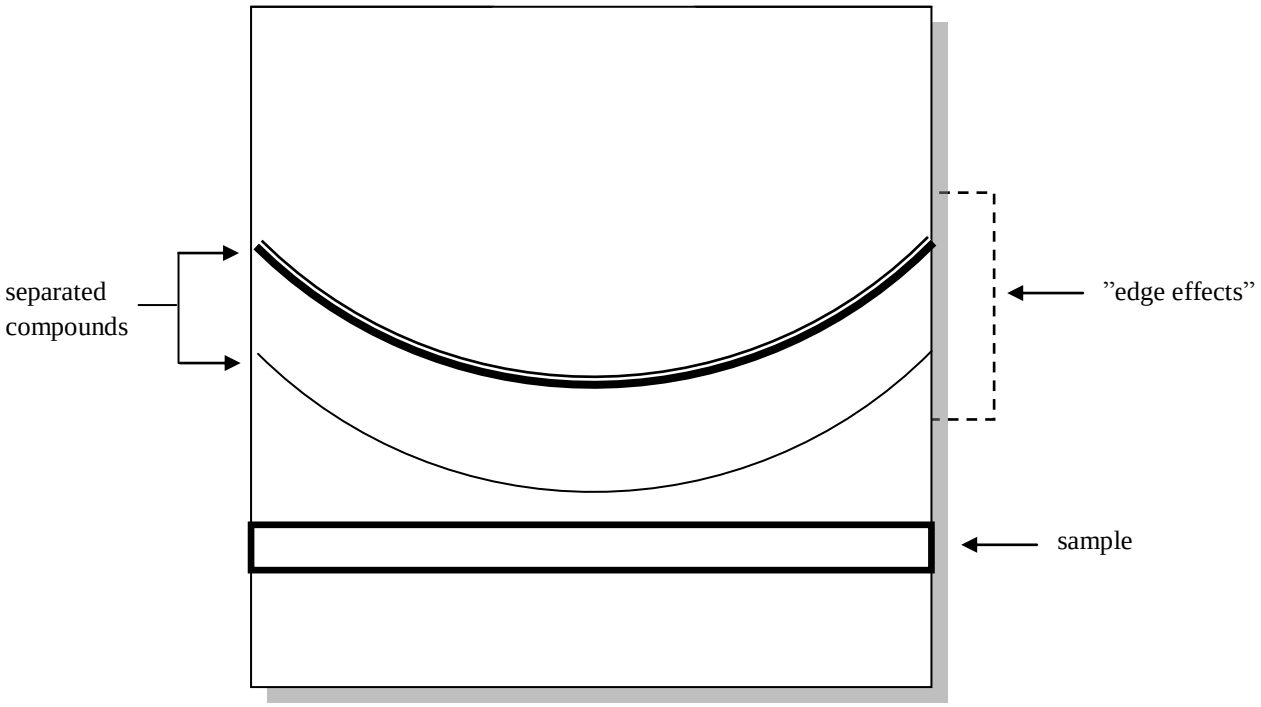
Preparative thin layer chromatography (PTLC) is a popular separation method among natural chemistry laboratories, for the same reasons as for TLC. Lately, though, an increase in the use of more advanced techniques such as high-performance liquid chromatography (HPLC) and counter current chromatography have slightly shadowed the use of PTLC. Even so, for reasons to be presented in the following sections, PTLC still holds an important role in the isolation of natural products. [29]

PTLC is oftentimes the last isolation step in an isolation process, and normally mixtures containing no more than 4 major components are developed. PTLC makes it is possible to isolate compounds in the 1 mg to 1 g range in a relatively short period of time, which is one of the main reasons why PTLC remains such an attractive separation method. Commercial plates are available in the sorbents silica, alumina, C-18 and cellulose, but it is also possible to make your own plates if cost is a concern or if the mixture in question requires enhanced separation. [29]

13.2 Procedure

The material to be separated on a PTLC plate should be dissolved in as little solvent as possible. The sample is applied approximately 1.5 cm from the bottom as a line about 5 mm in thickness by the use of a micro syringe or a capillary tube. Thinner capillary tubes give more concentrated lines, and thus, better separation. It is advisable to carefully draw a straight pencil line before applying the sample. The area about 1 cm from the plate edges should not be covered with sample because of "edge effects", i.e. rapid movement of solvent along the edges resulting in irregular separation bands (***Figure 13.1***). [29]

Figure 13.1 PTLC plate with "edge effects"



The tank used for developing plates is normally filled with 100 mL freshly made solvent. If the atmosphere in the tank is saturated with solvent, the separation process will be improved. Direct sunlight should be avoided during development due to the possibility of degradation. When the solvent front has either reached or is very close the top of the plate, the plate is taken out and left to air-dry in a fume cupboard. [29]

In the case of silica plates with UV fluorescent indicator incorporated into the sorbent, the compound of interest is lightly marked with a pencil and scraped off the backing plate onto a piece of paper with a spatula. If the compound of interest does not absorb UV-light, a piece from the side of the plate (approximately 2 cm) is cut off followed by spray detection. The contaminated compound is obviously not combined with the scrapings from the unsprayed plate. [29]

The sorbent particles are transferred to a conical flask, crushed to finer particles and added solvent. 30 minutes should be enough time to allow desorption, after which time the suspension is filtered. Desorption should be repeated at least two times. Recovery of the product can also be obtained by placing the sorbent particles in a sintered glass funnel on top of a glass Buchner flask to which vacuum is applied (***Illustration 13.1***). Desorption takes place by washing the particles with solvent, which is repeated until recovery is deemed complete. [29]

Illustration 13.1 Desorption by applying vacuum

Source: http://en.wikipedia.org/wiki/B%C3%BCchner_funnel

14. Mass Spectrometry (MS)

14.1 Function and instrumentation

This spectrometric technique is, as the name indicates, used to investigate the mass of compounds, either known or unknown. The sequence in a mass spectrometer is simple; the compound is first ionised, the resulting ions are then separated with regards to their mass/charge ratios and finally a spectrum presents the number of ions as a function of mass/charge (m/z). It is normal to couple a mass spectrometer to an isolation method (e.g. gas or liquid chromatography). Other spectrometric techniques such as IR and NMR are normally used in conjunction with MS to aid the identification process, especially for unknown compounds. [32]

The methods by which a compound is ionised and separated are in most cases independent of each other. Mass spectrometers can be divided into two major groups; low-resolution and high resolution instruments. The low-resolution instruments can separate ions with m/z of up to 3,000. The high-resolution instruments, on the other hand, can distinguish between ions with m/z of up to 100,000. As described earlier, entry of the sample into the ionisation chamber is often achieved by coupling the mass spectrometer to a chromatograph, but the sample can also be introduced by direct insertion. The methods of ionisation and separation are plentiful. [32]

14.2 Gas-phase ionisation methods

These methods of ionisation are among the most commonly employed methods. The only requirement for the use of these techniques is that the vapour pressure of the compound be above a certain limit. Mainly non-ionic organic compounds with molecular weights under 1,000 are ionized by gas-phase

ionisation methods. Examples of gas-phase ionisation methods are electron impact ionisation (EI) and chemical ionisation. [32]

14.3 Desorption ionisation methods

Desorption ionisation can be described as sample molecules being emitted directly into the vapour phase as ions. Compounds normally subjected to this type of ionisation are large, non-volatile or ionic. The main drawbacks are that the molecular ion is not always obvious and the spectra are oftentimes complicated by ions in the matrix. [32]

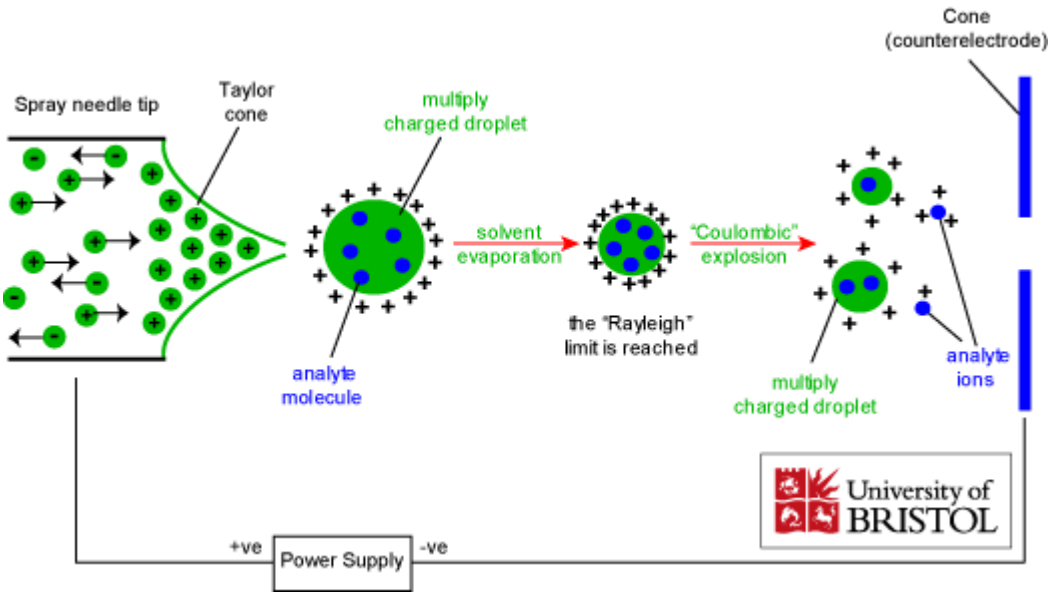
14.4 Evaporative ionisation methods

Evaporative ionisation methods have become very attractive since the direct coupling to a chromatograph was made possible. There exists two important methods; thermo spray ionisation and electro spray ionisation (ES). [32] Since the thermo spray method has been largely surpassed by the electro spray method, only the latter will be described.

14.4.1 Electro spray ionisation

Because the ionisation chamber in the electro spray mass spectrometer is operated at atmospheric pressure, this method is also called atmospheric pressure ionisation. The sample is introduced into the ionisation chamber through a capillary surrounded by a flow of nitrogen gas. The capillary tip is maintained at a high potential which in turn generates charged droplets. These droplets are directed towards the analyzer by the nitrogen gas. As the solvent evaporates, the electrostatic repulsion between the ions reaches a critical point which results in their release (“Coulombic explosion”) (***Figure 14.1***). Electro spray is mainly used on compounds able to carry multiple charges (e.g. proteins, peptides). [32]

Figure 14.1 Generation of droplets followed by columbic explosion in an electro spray ion source



Source: http://www.chm.bris.ac.uk/ms/theory/esi-ionisation.html

14.5 Mass analyzers

After ionisation, the ions need to be separated. This can be achieved in a number of ways, with each mass analyzer comprising its own set of features. Some of the most important mass analyzers are magnetic sector analyzers, quadrupole mass analyzers, ion trap analyzers, time-of-flight-analyzers and Fourier transform analyzers. Only the ion trap analyzer will be presented in greater detail.

14.5.1 Ion trap analyzer

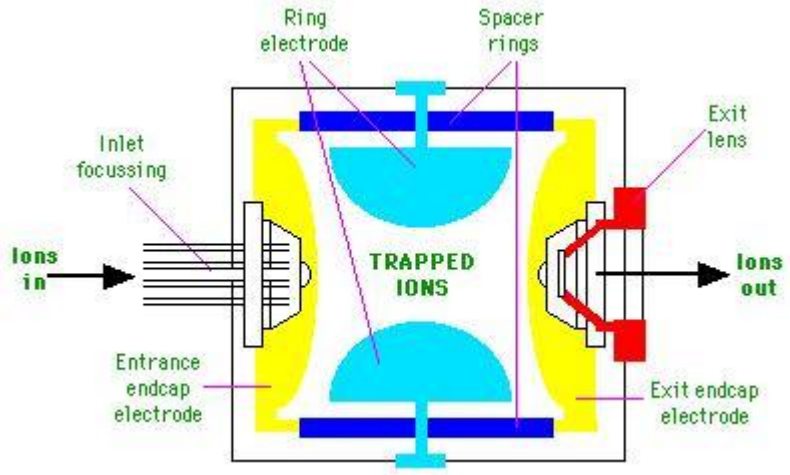
The ion trap analyzer has proven to be even more sensitive than the quadrupole mass analyzer. The name of this type of analyzer comes from the fact that ions can be "trapped" in the analyzer for a period of time before being ejected onto a detector. The ion trap analyzer is composed of three electrodes; one ring electrode and two end cap electrodes at both ends (***Figure 14.2***). The ring electrode is kept at a variable radio frequency, while the end caps can be maintained at ground potential, at a DC or at an AC voltage. There are three ways to separate ions with an ion trap analyzer: [32]

1. A starting radio frequency voltage is gradually increased.
   - Ions above a given m/z value will be trapped and ejected in order as the radio frequency voltage increases. [32]

2. A DC voltage is set across the end cap electrodes.
   - Ions both above and below a given m/z value will be trapped, which makes the ejection of ions highly selective. [32]

3. An auxiliary field is set between the end cap electrodes in addition to a DC voltage.
   - Selective ions gain kinetic energy which can be used to either further fragment ions or to reject unwanted ions. [32]

Figure 14.2 An ion trap mass analyzer



Source: http://people.stfx.ca/tsmithpa/Chem361/labs/ms.html

14.6 Index of hydrogen deficiency

Once the molecular formula of the compound has been obtained, it is possible to calculate what is called the "index of hydrogen deficiency". This term can be explained as a comparison of the discovered formula with the formula of the corresponding saturated compound, in order to calculate how many pairs of hydrogen atoms need to be removed from the corresponding saturated compound for the two formulas to be in agreement. Hydrogen deficiency arises from cyclization and multiple bond formation. [32]

For compounds containing only carbon and hydrogen atoms, the index of hydrogen deficiency is easily obtained by simple comparison of formulas. On the other hand, for more complex compounds containing not only carbon and hydrogen atoms, but also nitrogen, oxygen, sulphur, phosphate, silicon or halogen, a mathematical equation is more appropriate. For a compound with the generalized formula $\alpha_I\beta_{II}\gamma_{III}\delta_{IV,}$ the index of hydrogen deficiency can be calculated as follows: [32]

$$Index = (IV) - (I/2) + (III/2) + 1$$ [32]

α is any monovalent atom (e.g. H, D, halogen), β is any bivalent atom (e.g. O, S), γ is any trivalent atom (e.g. N, P) and δ is any tetravalent atom (e.g. C, Si). The number of atoms within each group is represented by the numerals I-IV. Although molecular formulas provide a great deal of information about a compound, they are not sufficient for structure elucidation. Several isomers are possible for

the same molecular formula, thus, different spectrometric techniques (e.g. NMR, MS) need to be carried out in order to get the definite structure of a compound under investigation. [32]

15. NMR Spectrometry

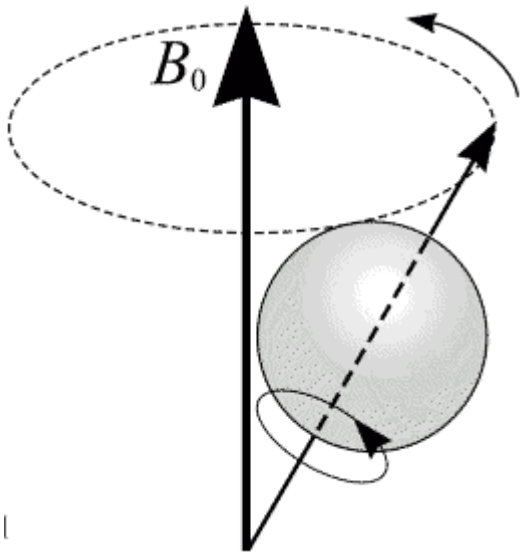
15.1 Function

NMR stands for nuclear magnetic resonance and is considered to be a type of absorption spectrometry (much like IR and UV). In its simplest form, NMR can be explained as a sample's characteristic absorption of radiofrequencies in the presence of an external magnetic field, which yields an NMR spectrum that can be used to determine the chemical environment of the sample. This powerful tool is usually used in conjunction with other spectrometric equipment in order to identify the exact structure of organic compounds. [32]

15.2 Magnetic properties of nuclei

Nuclei, as is well known, consist of both protons and neutrons, and thus, carry a net positive charge. When placed in an external magnetic field, these positive charges give some nuclei the magnetic ability to "spin", meaning that nuclei will spin on their own axes. Magnetic dipoles are generated along the axis of every nucleus as a result of this magnetic property (***Figure 15.1***). [32]

Figure 15.1 Magnetic dipole of a proton in an external magnetic field



Source: http://www.mikepuddephat.com/Page/1603/Principles-of-magnetic-resonance-imaging

An important property of nuclei regarding the ability to ”spin”, is their quantum spin number, I. Quantum spin numbers are given in values of 0, $^1/_2$, 1, $^3/_2$, 2, $^5/_2$, 3, and so on. Two different examples, but which are equally worth mentioning, are the ^{1}H and the ^{13}C nuclei. Both of these nuclei have quantum spin numbers of $^1/_2$, meaning that they have a uniform, spherical charge distribution and that the nuclei can assume two separate orientations when placed in a magnetic field. The number of orientations a given nuclei may have in a magnetic field, can be determined by the formula 2I + 1, where I is of course the quantum spin number of the nuclei in question. [32]

Many other nuclei have quantum spin numbers of $^1/_2$, e.g. ^{3}H, ^{15}N, ^{19}F and ^{31}P, but still the most widely used nuclei in NMR spectrometry are the nuclei ^{1}H and ^{13}C. [32] Both ^{1}H and ^{13}C NMR will be described next.

15.3 ^{1}H NMR

In a magnetic field, protons arrange themselves in two energy levels, with the lower energy level containing the highest amount of protons. Energy in the form of radiofrequency is applied to create a change in the population of these energy levels. Radiofrequency is given in units of megahertz (MHz). ^{1}H spectra are normally recorded by the use of 300 MHz instruments. When the protons resonate with the applied radiofrequency, they absorb energy, thus, the lower energy protons are raised to a higher energy state. [32]

15.3.1 Spectrometers

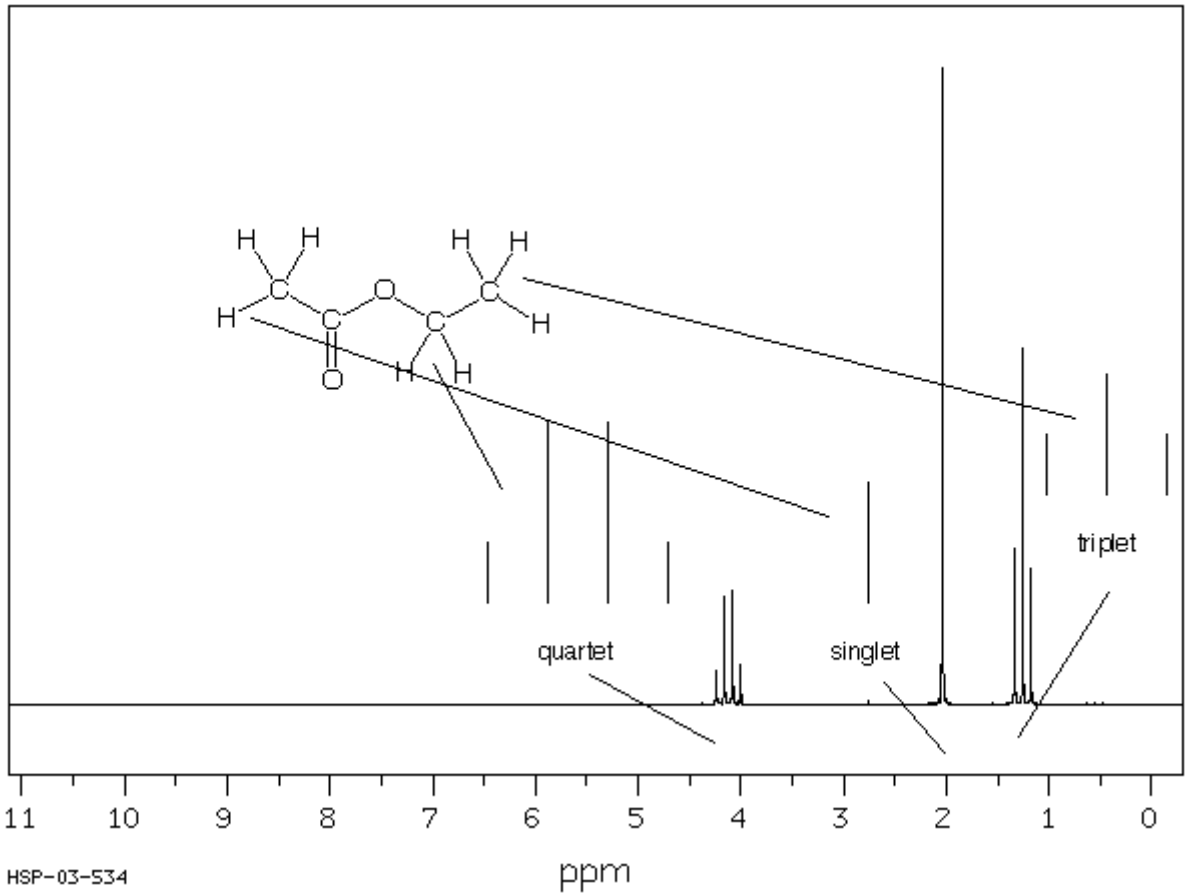
A spectrum can be recorded in two ways depending on the type of spectrometer; either by continuous-wave spectrometer or by pulsed Fourier transform spectrometer. The continuous-wave spectrometer irradiates the sample by sweeping through the frequency range necessary to yield resonance. The resulting spectrum thus presents the absorption of energy as a function of frequency. The pulsed spectrometer irradiates the sample with a frequency range sufficiently large enough to yield resonance in all the nuclei at the same time. A detector collects the absorbed energy which is radiated by the nuclei as they return to their ground state. The information is thus available as absorption of energy as a function of time, which is useless in means of determining the sample’s structure. A Fourier transformation (a mathematical process) is therefore needed to convert the information to an interpretable spectrum which displays absorption of energy as a function of frequency. [32]

The continuous-wave spectrometer has several disadvantages such as low sensitivity with both limited amount of sample and with nuclei of low natural abundance, each scan takes more than a few minutes and repeated scanning does either nothing or very little to improve the signal/noise ratio. For these reasons, the continuous-wave spectrometer has been greatly exceeded by pulsed Fourier transform spectrometer. Since protons return to their ground state in a matter of seconds or less than a second, pulses can be repeated rapidly. Also repeated pulses lead to signal accumulation, thus improving the signal/noise ratio. [32]

15.3.2 The proton spectrum

A proton spectrum is displayed as a series of peaks at different frequencies and with different intensities (***Figure 15.2***). The area under each peak is proportional to the number of protons it represents, thus, by means of integration; it is possible to identify exactly how many protons are present in the compound under investigation. This invaluable information can be used to decipher the molecular formula of the compound, to discover chemically equivalent protons and to assess the purity of the sample. [32]

Figure 15.2 Ethyl acetate ^{1}H NMR spectrum



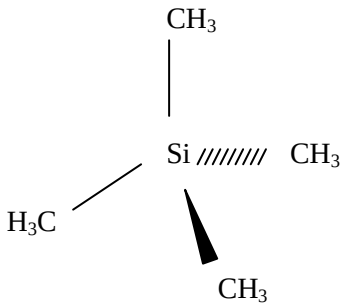
Source: http://www.chem2.bham.ac.uk/schools/ethyl-acetate-1h.htm

15.3.3 “Shielding” and “deshielding”

Every proton in a molecule is shielded by its electron cloud. The density of this electron cloud is different for protons in dissimilar chemical surroundings. These differences determine the position of each peak in a proton spectrum. The peaks are said to have different chemical shift positions. Chemical shift is described as the absorption position of a given NMR active nuclei relative to the absorption position of a reference nucleus. On the other hand, protons in similar chemical surroundings will have similar or identical chemical shifts. Thus, the peaks enable us to detect equivalent protons and to distinguish between non-equivalent protons. [32]

In proton NMR, the most widely used reference compound is tetramethylsilane (***Figure 15.3***). Tetramethylsilane (TMS) yields a single and sharp absorption peak, and the protons are one of the most shielded protons in organic compounds. The absorption peak of TMS is assigned zero and placed at the right end of the NMR spectrum, thus, frequency increases from right to left. Absorption peaks to the right of the spectrum are termed “shielded” while absorption peaks to the left are termed "deshielded". This is in accordance with the fact that the reference compound, which contains one of the most shielded group of protons present in organic compounds, is placed at the right end of the spectrum. [32]

Figure 15.3 Tetramethylsilane



15.3.4 ^{1}H NMR scale

The NMR scale can be set to Hz and parts per million (ppm), which is a dimensionless unit. For reasons explained below, the NMR scale is generally set to ppm. If a 300 MHz spectrometer and a 600 MHz spectrometer is used on the same sample, and the NMR scale is set to Hz, the absorption peak of a given proton in the sample will appear at a frequency in the 300 MHz spectrometer which is different from the frequency in the 600 MHz spectrometer. Alternatively, if the NMR scale is set to ppm, the absorption peak will appear on the same place. While Hz is dependent on the frequency of the operational spectrometer, ppm is not, thus, it is more practical to use this dimensionless unit for comparison purposes. [32]

15.4 ^{13}C NMR

As mentioned earlier, the ^{13}C nucleus has a quantum spin number of $^{1}/_{2}$, same as the ^{1}H nucleus. The overall sensitivity of ^{13}C compared to ^{1}H, though, is only 1/5700 (i.e. approximately 6000 times less sensitive). The low overall sensitivity of the ^{13}C nucleus is due to the combination of its low natural abundance compared to that of the ^{12}C nucleus (1.1 %) and the low sensitivity compared to that of the ^{1}H nucleus (1.6 %). [32]

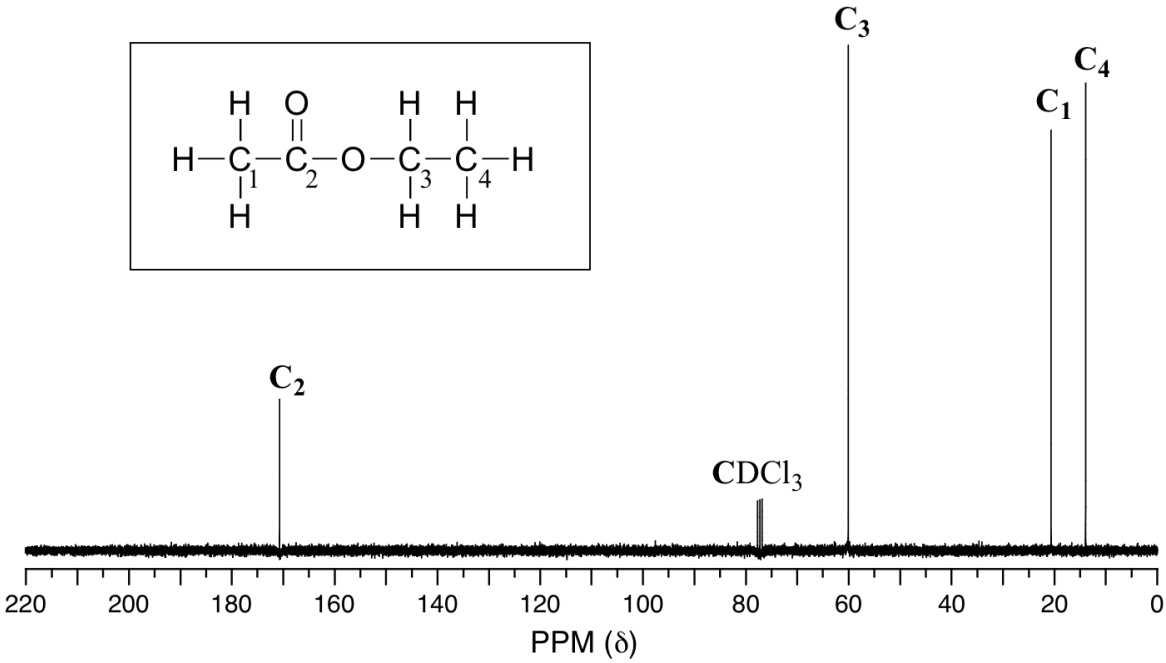
If the continuous-wave spectrometer is operated on ^{13}C nuclei, the investigation will be both time-consuming and require a large amount of sample. Therefore, the development of the pulsed Fourier transform spectrometer has led to a marked increase in the study of ^{13}C. In addition, analysis of ^{13}C and ^{1}H spectra on the same sample provides useful information that can be used to either confirm or disprove the presence of certain functional groups. [32]

15.4.1 Addressing coupling challenges

Whereas ^{13}C does not couple to ^{1}H in ^{1}H NMR spectra, coupling between ^{1}H and ^{13}C does occur in ^{13}C NMR spectra. This means, in practical terms, that ^{13}C NMR spectra show complex assemblies of absorption peaks, depending on the size of the compound under investigation, which are almost impossible to interpret. Coupling can be described as two NMR active nuclei, in different chemical surroundings, having a mutual effect on the "spin" of each other through intervening electrons. This mutual effect is made visible in NMR spectra through splitting of absorption peaks. For this reason, coupling complicates NMR spectra. Nevertheless, splitting can be avoided by irradiation of the ^{1}H nuclei which decouples ^{1}H nuclei from ^{13}C nuclei, thus, the absorption peaks appear as singlets with each peak corresponding to single or chemically equivalent ^{13}C nuclei. This type of spectrum is called

proton-decoupled ^{13}C spectrum, and is the most commonly employed spectrum for the study of ^{13}C (***Figure 15.4***). [32]

Figure 15.4 Proton-decoupled ^{13}C spectrum of ethyl acetate



Source: http://chemwiki.ucdavis.edu/Organic_Chemistry/Organic_Chemistry_With_a_Biological_Emphasis/Chapter__5%3A_Structure_Determination_II/Section_6%3A_13C-NMR_spectroscopy

15.4.2 ^{13}C NMR scale

Just like for ^{1}H, the NMR scale for ^{13}C is generally set to units of ppm. The range of the chemical shifts, however, is about 220 ppm from the ^{13}C nuclei in TMS to the left end of the spectrum. Absorption peaks rarely overlap due to the wide chemical shift range and the sharpness of each peak. [32]

15.4.3 Absorption peaks

While integration of absorption peaks in ^{1}H NMR spectra yields an area that is proportional to the number of protons it represents, the same cannot be said about the absorption peaks in ^{13}C NMR spectra. The peak intensities are affected by additional factors which render the area under each peak impossible to compare to one another. [32]

15.5 DEPT spectrum

This is a popular technique within ^{13}C NMR spectrometry which utilizes the differences in coupling constants between ^{1}H and ^{13}C in methine (-CH), methylene ($-CH_2$) and methyl ($-CH_3$) groups to generate an NMR spectrum that can be used to tell apart the different groups. In addition, this technique is more sensitive than the regular ^{13}C NMR method. Thus, DEPT (Distortion Enhancement by Polarization Transfer) has become routine in many laboratories when NMR experiments are required. [32]

Two separate spectrums can be produced through the use of DEPT depending on the angle of the pulsed radiofrequency, which is set to either 90° or 135°. The differences between the spectra is that in the 90° pulse sequence only ^{13}C nuclei in CH groups are detected, meanwhile in the 135° pulse sequence all of the ^{13}C nuclei are shown (except for quaternary carbons), but the CH_3 and CH carbons are phased up, while the CH_2 carbons are phased down. It is well worth mentioning that protons attached to other atoms (O, N, S, Si and P) are obviously not recorded, thus, there will be a difference in the number of protons in the DEPT spectrum and the ^{1}H spectrum if such groups are present in the compound under investigation. [32]

Materials

16. Solvents

16.1 Solvents in both isolation and structure determination

Many types of solvents were utilized in the isolation process. Most of which were organic solvents. Solvents were also mixed at different ratios in order to achieve a variety of solvent, eluent and developing systems with differing polarities. Only two types of solvents were employed in the structure determination, methanol-d_4 in NMR and regular methanol in MS (***Table 16.1***).

Table 16.1 Solvents from both the isolation and the identification of compounds

Solvent	**Supplier**
Methanol (MeOH)	Jiangsu Qiangsheng Chemical Co., Ltd
Trichloromethane ($CHCl_3$)	Sinopharm Chemical Reagent Co., Ltd
Dichloromethane (CH_2Cl_2)	Shanghai Desheng Chemical Co., Ltd
Ethyl acetate (EtOAc)	Shanghai Zaituo Trade Co., Ltd
Water (H_2O)	Shanghai Fudanyuan Purified Water Co., Ltd
Ethanol (EtOH)	Changshu Yangyuan Chemical Co., Ltd
Sulphuric acid (H_2SO_4)	Sinopharm Chemical Reagent Co., Ltd
Formic acid (HCOOH)	40 E. Main St., Newark, DE 19711, USA
Methanol-d_4 (CD_3OD)	Cambridge Isotope Laboratories Inc.

17. Materials

17.1 Materials from the isolation process

In order to purify a range of extracts and fractions, many chromatographic techniques need to be employed. The entire isolation process required different forms of low-pressure column chromatography and planar liquid chromatography. In addition to chromatographic materials, vanillin powder was necessary for the preparation of spray reagent and filter paper was utilized in gravity filtering (***Table 17.1***).

Table 17.1 Materials from the isolation of compounds

Material	Thickness (mm)	Particle size (µm)	Dimension (cm)	Supplier
Silica gel	-	100-200	-	Huiyou Silica Gel Development Co., Ltd
Sephadex LH-20	-	40-70	-	GE Healthcare Bio-Sciences AB
TLC plate	0.15-0.20	10-40	5×20	Yantai Jiangyou Silica Gel Development Co., Ltd
PTLC plate	0.4-0.5	10-40	20×20	Yantai Jiangyou Silica Gel Development Co., Ltd
Vanillin powder	-	-	-	Sinopharm Chemical Reagent Co., Ltd
Filter paper	-	-	11	Hangzhou Special Paper Co., Ltd

18. Equipment

18.1 Equipment in both isolation and structure determination

Specific equipment was required in order to purify extracts and fractions and to analyze the isolated compounds. Rotary evaporator and UV lamp were used throughout the isolation process. Purified compounds were made ready for NMR and MS analysis by the use of a micropipette, NMR tubes and microcentrifuge tubes (***Table 18.1***).

Table 18.1 Equipment from both the isolation and the identification of compounds

Equipment	Supplier
Rotary evaporator	Shanghai SENCO Technology Co., Ltd
Automatic fraction collector	Shanghai Qingpu-Huxi Instruments Factory: BSZ-100
UV lamp for TLC	WFH 203 Shanghai Jingke Industrial Co., Ltd
UV lamp for PTLC	WFH 203 Shanghai Jingke Industrial Co., Ltd
Micropipette, 20-200 µL	Axygen, Inc
NMR tubes, 5 mm o.d.	NORELL, Inc
Microcentrifuge tubes, 1.5 mL	Axygen, Inc
NMR spectrometer	Bruker Avance 600
Mass spectrometer	Agilent LC/MSD Trap XCT

Methods

19. Development of TLC plates

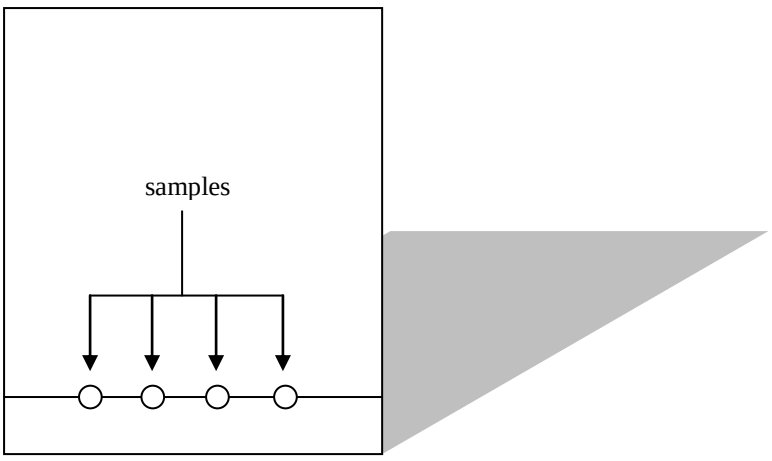
19.1 Use of TLC

TLC was performed continuously throughout the isolation process. Regardless of what type of sample was under investigation; that is raw material, chromatographic fractions, pooled fractions or purified compounds, TLC was performed before and after every step.

19.2 Procedure

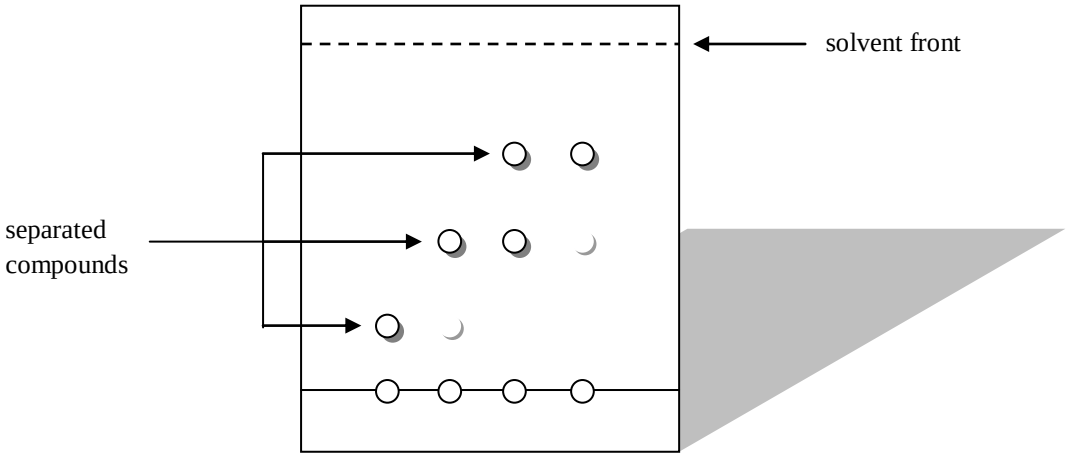
With a grey pencil and a ruler, TLC plates were marked with a fine line 6 mm above the bottom. A grey pencil was also used to draw small, fine circles on the line approximately 5 mm from one another. The outermost circles were drawn about 1 cm from the edges. The TLC plates were cut into appropriate sizes by the use of a scalpel. Thin capillary tubes were used to spot fractions on their respective circles. After a spot was made, the thin capillary tube was immersed in MeOH which was subsequently absorbed by a piece of toilet paper. This was done at least three times between each spot that was made to avoid contamination between fractions. A single TLC plate was used for as little as one or as many as twenty fractions at a time (***Figure 19.1***).

Figure 19.1 TLC plate before development



The plates were gently dried with a hairdryer immediately after the last spot was made. By the use of a large pair of tweezers, the plates were picked up and placed in a small tank saturated with a solvent of a certain composition. The tanks were filled with solvent about 2-3 mm in height. The plates were left to develop until the solvent front had almost reached the top, at which point the plates were taken out and gently dried with a hairdryer (***Figure 19.2***).

Figure 19.2 TLC plate after development



After development, the separated compounds were detected by the use of UV-light, molecular iodine or a spray reagent, or by a combination of these. The detection methods mainly performed were UV and spray detection. Routinely, the plates were first observed under UV-light set at 254 nm. The largest and strongest spots were gently circled with a grey pencil, a spray reagent was added, and finally the plates were heated on a cooking plate until either colouration was observed or heating was finished.

20. Isolation of compounds from Fraction 1

20.1 The isolation process

Fraction 1, Fraction 2 and Fraction 3 were dealt with in a sequential manner, meaning that compounds were isolated from Fraction 1 before commencing on Fraction 2 and so on. The isolation process comprised a variety of chromatographic techniques, each performed several times throughout the isolation process. Examples are low-pressure column chromatography and planar liquid chromatography. The first chromatographic technique performed was silica gel column chromatography. Isolation of compounds from Fraction 1 required all of the chromatographic techniques that would be performed, thus, when isolating compounds from Fraction 2 and Fraction 3, the same chromatographic techniques were employed. All the isolation and purification steps carried out on Fraction 1 are presented in ***Figure 25.1***.

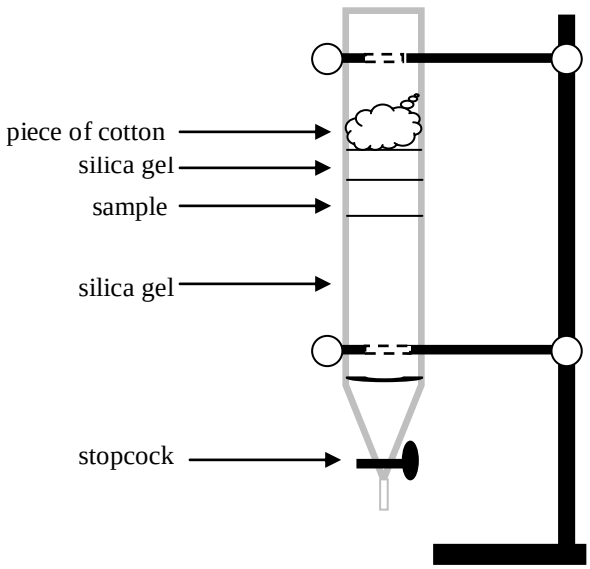
20.2 Prior to silica gel column chromatography

Fraction 1 was initially dry in an evaporating dish. As best as possible, a spatula was used to scrape off the dried fraction and transfer it to a clean, pre-weighed evaporating dish. Fraction 1 was calculated to weigh about 4 g. A mixture of dichloromethane (CH_2Cl_2) and methanol (MeOH) in a 10:1 ratio was added in enough quantities to dissolve Fraction 1 entirely. About 4.5 g silica gel was weighed and poured into the evaporating dish as well. Fraction 1 was left in a fume cupboard to dry off, thus, allowing dissolved components to adsorb onto the silica gel particles.

20.3 Preparing the silica gel column

To start with, the column was washed with MeOH. The silica gel column chromatography apparatus was then assembled. Approximately 120 g of silica gel, which is equal to 30 times the weight of Fraction 1, was weighed and transferred to the column. When all of the silica gel had been transferred, the column was dismounted and the tip of the column was gently hit against a soft, rubbery surface to compact the silica particles and release air bubbles within the adsorbent. The column was reattached to the rest of the apparatus and Fraction 1, which had adsorbed onto the silica gel particles in the evaporating dish, was transferred to the column by the use of a funnel and a spatula. Gentle taps on the side of the column made the sample more compact. Another layer of silica gel was added, just enough to cover the mixture of compounds. The column was tapped gently on the side one last time. A piece of cotton was placed on top of the last silica gel layer to protect the mixture of compounds (***Figure 20.1***).

Figure 20.1 Prepared silica gel column



20.4 Silica gel column chromatography

An eluent system consisting of a mixture of $CHCl_3$ and MeOH in a 30:1 ratio was first employed to separate the components in Fraction 1. Fractions of approximately 75 mL were collected throughout the silica gel column chromatography. Ten fractions were collected initially, wherefrom fractions 1 through 4 were pooled and fractions 8 through 10 were pooled. Thus, a total of five fractions were collected with this 30:1 eluent system.

$CHCl_3$ and MeOH were used throughout the silica gel column chromatography, but at different ratios. After the first eluent system, an eluent system of 25:1 was employed. Initially, 14 fractions were collected, wherefrom fractions 11 through 16 were pooled and fractions 17 through 24 were pooled. Thus, a total of two fractions were collected with this 25:1 eluent system.

A 20:1 eluent system followed. Seven fractions were collected initially and all of them were pooled. Thus, only one fraction was collected with this 20:1 eluent system. Interestingly, this fraction yielded a yellow colour on a TLC plate after spray detection followed by heating on a cooking plate. Thus, fraction 25-31 was set aside in order to further purify the mixture by polysaccharide size-exclusion chromatography.

Silica gel column chromatography was continued with a 15:1 eluent system. Six fractions were collected initially, wherefrom fractions 32 through 34 were pooled and fractions 35 through 37 were pooled. Thus, a total of two fractions were collected with this 15:1 eluent system.

A final eluent system of 10:1 was employed in the silica gel column chromatography. Initially, five fractions were collected, wherefrom fractions 38 through 41 were pooled. Thus, a total of two fractions were collected with this 10:1 eluent system. Fraction 38-41 gave several spots on a TLC plate both under UV light and after spray detection followed by heating. Therefore, polysaccharide size-exclusion chromatography was also performed on this fraction.

20.5 1st polysaccharide size-exclusion chromatography

Fraction 25-31 from the silica gel column chromatography was transferred to an Erlenmeyer bulb and the solvent was evaporated through the use of a rotary evaporator. Just enough MeOH was added to dissolve the dried fraction completely. Fraction 25-31 was then added to a prepared Sephadex LH-20 column with MeOH as the mobile phase.

20.5.1 Transfer of fraction 25-31 to the Sephadex LH-20 column

MeOH was eluted until it reached the bed of the Sephadex LH-20 particles. Fraction 25-31 was transferred to the column with a Pasteur pipette by slowly pressing out the sample in a circular motion along the inside of the column just over the bed of the Sephadex LH-20 particles. Such a transfer ensures an even distribution of components and, thus, a better separation. There was still sample left in the Erlenmeyer bulb, but a second Pasteur pipette with Fraction 25-31 was not added before the previous transfer had reached the bed of the Sephadex LH-20 particles. The rest of Fraction 25-31 and three Pasteur pipette washes of the Erlenmeyer bulb were transferred to the column in the same manner. When Fraction 25-31 had travelled sufficiently down the column, larger quantities of MeOH were added. MeOH was continually added to the column until enough fractions were collected.

20.5.2 Collecting fractions

The tip of the Sephadex LH-20 column was connected to an automatic fraction collector right before the compounds were about to elute. The automatic fraction collector was set on an interval of 800 seconds with fractions being collected in test tubes. Rate of elution was less than 1 drop per second. Initially, thirty-eight fractions were collected, wherefrom fractions 1 through 21 were pooled, fractions 22 through 27 were pooled and fractions 28 through 38 were pooled. Thus, a total of three fractions were collected in the 1st polysaccharide size-exclusion chromatography. Fraction 22-27 yielded two distinct spots both under UV-light and after spray detection followed by heating. The weight of fraction 22-27, however, was considered too low to be further purified by a simple chromatographic technique.

20.6 2nd polysaccharide size-exclusion chromatography

Fraction 38-41 from the silica gel column chromatography was dried, dissolved and transferred to a prepared Sephadex LH-20 column in the same way as done in the 1st polysaccharide size-exclusion chromatography. The tip of the Sephadex LH-20 column was connected to an automatic fraction collector right before the compounds were about to elute. The automatic fraction collector was set at an interval of 500 seconds with fractions being collected in test tubes. Rate of elution was approximately 1 drop per second. Initially, one hundred and five fractions were collected, wherefrom fractions 1 through 4 were pooled, fractions 5 through 11 were pooled, fractions 12 through 35 were pooled, fractions 36 through 39 were pooled, fractions 40 through 51 were pooled, fractions 52 through 81 were pooled, fractions 82 through 90 were pooled and fractions 91 through 105 were pooled. Thus, a total of eight fractions were collected in the 2nd polysaccharide size-exclusion chromatography.

20.6.1 Evaluation of collected fractions

Fraction 5-11 yielded an almost single purple spot under UV-light which was not visible after spray detection followed by heating. A PTLC was performed on fraction 5-11 to achieve purer results. Fraction 40-51 showed two separate yellow spots after spray detection followed by heating, thus, a PTLC was performed on this fraction as well. Fraction 91-105 yielded a single, clear yellow spot after spray detection followed by heating. The weight of this fraction was 69.1 mg. Fraction 91-105 was considered pure enough for structure determination and was therefore sent to another laboratory at the Department of Natural Medicinal Chemistry for NMR and MS analysis. Fraction 91-105 was given the name CAP-1. Every fraction that was considered pure enough for structure determination was given the name CAP followed by a number. CAP is an acronym for Carlos Abies pindrow. The numbers were given to seemingly pure fractions in a chronological order as they were being collected in the isolation process.

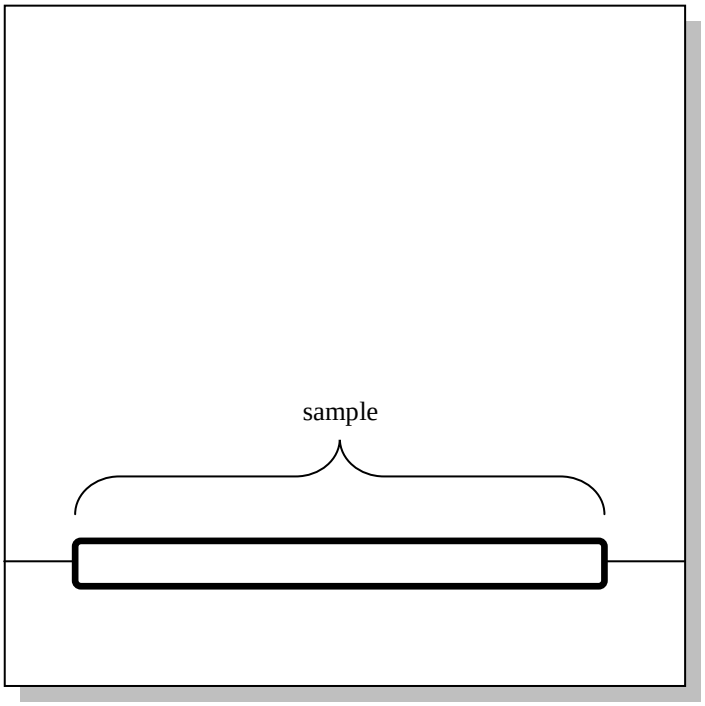
20.7 1st silica adsorbent PTLC

Fraction 5-11 from the 2nd polysaccharide size-exclusion chromatography was transferred to a pre-weighed Erlenmeyer bulb and the solvent was evaporated by the use of a rotary evaporator. The weight of the fraction was calculated to be 220.4 mg. Just enough MeOH was added to dissolve the dry fraction entirely. Fraction 5-11 was distributed on two PTLC plates and developed in the same twin trough chamber.

20.7.1 Development of PTLC plates

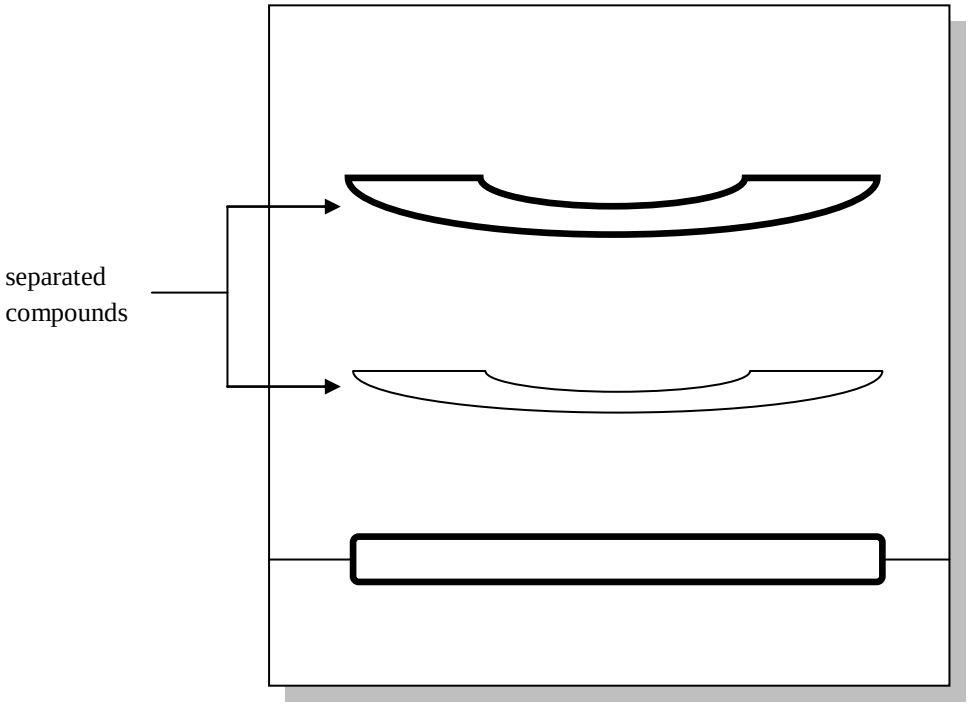
Fine lines about 1.5 cm above the bottom of the PTLC plates were marked with a grey pencil. Fraction 5-11 was evenly distributed along the grey pencil lines with a thick capillary tube. Approximately 1 cm on both sides of the PTLC plates were not covered with sample to avoid "edge effects" (***Figure 20.2***).

Figure 20.2 PTLC plate before development



A twin trough chamber was filled with 50 mL of a 20:1 ($CHCl_3$:MeOH) solvent system. Immediately before the PTLC plates were placed in the twin trough chamber, they were gently dried with a hairdryer to make sure that both MeOH and water particles were removed before development, since both can affect separation. Development was finished when the solvent front had reached the top of the plates (***Figure 20.3***).

Figure 20.3 PTLC plate after development



20.7.2 Desorption of compound from silica adsorbent

UV-light was used to visualize the compound of interest adsorbed onto the PTLC plates. Fine markings around the compound were made with a grey pencil. Silica adsorbent was gently scraped off the glass backing plates and onto a paper of A4 format with a spatula. Silica adsorbent particles from each PTLC plate were poured into separate 50 mL beakers and crushed into finer particles. Desorption was enabled by the use of a 3:1 (CH_2Cl_2:MeOH) solvent system at a volume about double of that occupied by the silica adsorbent particles. Two simple assemblies, each consisting of a 100 mL Erlenmeyer flask, a funnel and a folded filter paper, were used for gravity filtering of each beaker. The extracts in the beakers were transferred onto the filter papers with a Pasteur pipette. Desorption was performed three times in total. The final extracts were combined and purity assessed by TLC. The compound was purple under UV-light, but no colour was visible after spray detection followed by heating. Weight of the isolated compound was calculated to be 149.9 mg. This compound was given the name CAP-4.

20.8 2^{nd} silica adsorbent PTLC

Fraction 40-51 from the 2^{nd} polysaccharide size-exclusion chromatography was dried, dissolved and developed in the same way as done in the 1^{st} silica adsorbent PTLC. Two PTLC plates were evenly distributed with fraction 40-51 and placed in a twin trough chamber filled with 50 mL of a 6:1 ($CHCl_3$:MeOH) developing solvent. When development was finished, two separate bands could be seen on each plate under UV-light. Desorption of these compounds was performed in the same way as done in the 1^{st} silica adsorbent PTLC.

20.8.1 Assessment of compounds

The highest band (i.e. the one with the highest R_f value) on the PTLC plates was weighed after desorption. Calculated weight was found to be 11.7 mg. This compound was given the name CAP-2. The lower band (i.e. the one with the lowest R_f value) on the PTLC plates seemed less pure, thus, a polysaccharide size-exclusion chromatography was performed on this extract.

20.9 3rd polysaccharide size-exclusion chromatography

A polysaccharide size-exclusion chromatography was performed on the extract with lowest R_f value from the 2nd silica adsorbent PTLC. The extract was dried, dissolved and transferred to a small, self-prepared Sephadex LH-20 column in the same way as that done in previous polysaccharide size-exclusion chromatography. Fractions were collected in 50 mL beakers. Fractions were collected right before the compounds were about to elute. Each fraction was approximately a volume of 50 mL.

20.9.1 Preparing the Sephadex LH-20 column

Sephadex LH-20 particles had already been added to the column, but it was all dried out. Elution was hindered by turning the stopcock at a 90° angle to the column. A chromatography reservoir was attached to the column and the column was filled with MeOH until approximately ¼ of the chromatography reservoir contained MeOH. A ground stopper was attached to the chromatography reservoir, the column was dismounted from the rest of the apparatus and everything was turned upside down repeatedly until all the Sephadex LH-20 particles were wet. This made the Sephadex LH-20 particles pack neatly in the column. The column was reattached to the rest of the apparatus and subsequent elution made the Sephadex LH-20 particles settle to a compact gel layer.

20.9.2 Collected fractions

Ten fractions were collected initially, wherefrom fractions 1 and 2 were pooled, fractions 3 through 7 were pooled and fractions 8 through 10 were pooled. Thus, a total of three fractions were collected in the 3rd polysaccharide size-exclusion chromatography. Fraction 3-7 gave a single, purple spot under UV-light. The weight of this fraction was calculated to be 75.7 mg. Fraction 3-7 was given the name CAP-3.

21. Isolation of compounds from Fraction 2

21.1 Purification of Fraction 2

When the isolation process on Fraction 1 was finished, purification was continued on Fraction 2. Only two chromatographic steps were employed on Fraction 2. Thus, isolation of compounds from Fraction 2 took considerably less time compared to isolation of compounds from Fraction 1. Both polysaccharide size-exclusion chromatography and silica adsorbent PTLC was performed. The isolation process carried out on Fraction 2 is presented in ***Figure 26.1***.

21.2 Polysaccharide size-exclusion chromatography

A polysaccharide size-exclusion chromatography was performed on Fraction 2 from the initial extraction and isolation carried out by Dr. Li Yong-Li. Fraction 2 was dried, dissolved and transferred to a prepared Sephadex LH-20 column in the same way as done in previous size-exclusion chromatography. The tip of the column was connected to an automatic fraction collector right before the compounds were about to elute. The automatic fraction collector was set at an interval of 600 seconds with fractions being collected in test tubes. Rate of elution was approximately 1 drop per second. Initially, fifty-one fractions were collected, wherefrom fractions 1 through 6 were pooled, fractions 7 through 15 were pooled, fractions 18 through 20 were pooled and fractions 21 through 50 were pooled. Thus, a total of seven fractions were collected in this polysaccharide size-exclusion chromatography. Silica adsorbent PTLC was performed on fraction 7-15. Fraction 51 was collected in a 250 mL Erlenmeyer flask to make sure that all of the compounds had eluted. Fraction 51 reached a final volume of 150 mL.

21.3 Silica adsorbent PTLC

Fraction 7-15 from the polysaccharide size-exclusion chromatography was dried, dissolved and developed in the same way as done in previous silica adsorbent PTLCs. Before development, fraction 7-15 was calculated to weigh 130.1 mg, thus, two PTLC plates were employed. Both PTLC plates were evenly distributed with fraction 7-15 and placed in a twin trough chamber filled with 50 mL of a 3:1 ($CHCl_3$:MeOH) developing system and 5 mL HCOOH, which served to improve separation. When development was finished, desorption was performed in the same way as done in previous silica adsorbent PTLCs by the use of a 2:1 ($CHCl_3$:MeOH) solvent system. The compound was purple under UV-light and yellow after spray detection followed by heating. Calculated weight of the compound was found to be 85.3 mg. This compound was given the name CAP-5.

22. Isolation of compounds from Fraction 3

22.1 Final isolation steps

Fraction 3 was the last major fraction to be purified. Both column chromatography and planar chromatography were employed. Fraction 3 required significantly more isolation steps compared to Fraction 2. The first chromatographic technique to be performed on Fraction 3 was polysaccharide size-exclusion chromatography. All of the isolation and purification steps carried out on Fraction 3 are presented in ***Figure 27.1***.

22.2 1st polysaccharide size-exclusion chromatography

Polysaccharide size-exclusion chromatography was employed on Fraction 3 from the initial extraction and isolation carried out by Dr. Li Yong-Li. Fraction 3 was dried, dissolved and transferred to a prepared Sephadex LH-20 column in the same way as done in previous size-exclusion chromatography. The tip of the column was connected to an automatic fraction collector right before the compounds were about to elute. The automatic fraction collector was set at an interval of 600 seconds with fractions being collected in test tubes. Rate of elution was approximately 1 drop per second. Forty-one fractions were collected initially, wherefrom fractions 1 through 4 were pooled,

fractions 5 through 19 were pooled, fractions 20 through 31 were pooled and fractions 32 through 41 were pooled. Thus, a total of four fractions were collected in this 1st polysaccharide size-exclusion chromatography. Silica gel column chromatography was performed on fraction 5-19 while silica adsorbent PTLC was performed on fraction 20-31.

22.3 Silica gel column chromatography

A silica gel column chromatography was performed on fraction 5-19 from the 1st polysaccharide size-exclusion chromatography. Fraction 5-19 was calculated to weigh 377.2 mg. Both the sample and the silica gel column were prepared in the same way as done in previous silica gel column chromatography, with the exceptions that half the column was filled with silica gel instead of approximately 30 times the weight of the sample and two teaspoons of silica gel were poured into the evaporating dish. Fractions of about 75 mL were collected throughout the silica gel column chromatography.

22.3.1 Collecting fractions with a 10:1 eluent system

A 10:1 (CH_2Cl_2:MeOH) eluent system was first employed to separate the compounds in fraction 5-19. Initially, ten fractions were collected, wherefrom fractions 1 through 3 were pooled, fractions 4 through 7 were pooled and fractions 8 through 10 were pooled. Thus, a total of three fractions were collected with this 10:1 eluent system.

22.3.2 Collecting fractions with a 5:1 eluent system

A second and final eluent to be employed was a 5:1 (CH_2Cl_2:MeOH) eluent system. Six fractions were collected initially, wherefrom fractions 11 and 12 were pooled and fractions 13 through 16 were pooled. Thus, a total of two fractions were collected with this 5:1 eluent system.

22.3.3 Further purification of fractions

Fraction 4-7 was calculated to weigh 142.2 mg, thus, there was enough material to perform a silica adsorbent PTLC on this fraction. Fraction 8-10 was first pooled with fraction 11-12 and subsequently combined with the extract from the lowest band in the 2nd silica adsorbent PTLC performed on fraction 4-7. The resulting fraction was named fraction Low R_f + 8-12 and was further purified in a polysaccharide size-exclusion chromatography.

22.4 1st silica adsorbent PTLC

Fraction 20-31 from the 1st polysaccharide size-exclusion chromatography was dried, dissolved and developed in the same way as done in previous silica adsorbent PTLCs. Two PTLC plates were evenly distributed with fraction 20-31 and placed in a twin trough chamber filled with 50 mL of a 4:1 ($CHCl_3$:MeOH) developing system. Desorption was performed in the same way as done in previous silica adsorbent PTLCs by the use of a 2:1 ($CHCl_3$:MeOH) solvent system. The compound was purple under UV-light and slight yellow after spray detection followed by heating. Weight of the compound was calculated to be 65.6 mg. This compound was given the name CAP-6.

22.5 2nd silica adsorbent PTLC

Fraction 4-7 from the silica gel column chromatography was dried, dissolved and developed in the same way as performed in previous silica adsorbent PTLCs. Two PTLC plates were evenly distributed with fraction 4-7 and placed in a twin trough chamber filled with 50 mL of a 3:1 ($CHCl_3$:MeOH) developing system. Two separate bands could be seen on the PTLC plates under UV-light after development. Desorption of these compounds was performed in the same way as done in previous silica adsorbent PTLCs by the use of a 2:1 (CH_2Cl_2:MeOH) solvent system.

22.5.1 Evaluation of compounds

The highest band (i.e. the one with the highest R_f value) on the PTLC plates was weighed after desorption. The compound was purple under UV-light and strong yellow after spray detection followed by heating. Weight of the compound was calculated to be 23.0 mg. This compound was given the name CAP-7. On the basis of TLC results, the lower band (i.e. the one with the lowest R_f value) on the PTLC plates was combined with fractions 8-10 and 11-12 from the silica gel column chromatography after desorption. A polysaccharide size-exclusion was performed on this fraction.

22.6 2nd polysaccharide size-exclusion chromatography

As explained earlier, a polysaccharide size-exclusion chromatography was performed on fraction Low R_f + 8-12, which is a combined fraction from both the 2nd silica adsorbent PTLC and the silica gel column chromatography. Fraction Low R_f + 8-12 was dried, dissolved and transferred to a prepared Sephadex LH-20 column in the same way as done in previous polysaccharide size-exclusion chromatography. The tip of the column was connected to an automatic fraction collector right before the compounds were about to elute. The automatic fraction collector was set at an interval of 500 seconds with fractions being collected in test tubes. Rate of elution was approximately 1 drop per second. Initially, nineteen fractions were collected, wherefrom fractions 1 through 3 were pooled, fractions 4 and 5 were pooled, fractions 6 through 10 were pooled, fractions 11 through 15 were pooled and fractions 16 through 19 were pooled. Thus, a total of five fractions were collected in this 2nd size-exclusion chromatography.

22.6.1 Assessment of fractions

Fraction 4-5 seemed to contain only one compound, which was purple under UV-light. Spray detection followed by heating yielded a yellow colour. Weight of the compound was calculated to be 5.0 mg. This compound was given the name CAP-8. Final isolation step was silica adsorbent PTLC performed on fraction 11-15.

22.7 3rd silica adsorbent PTLC

Fraction 11-15 from the 2nd polysaccharide size-exclusion chromatography was dried, dissolved and developed in the same way as done in previous silica adsorbent PTLCs. Calculated weight of fraction 11-15 was found to be only 29.3 mg. Such a small amount of sample is generally considered too low to be further purified by silica adsorbent PTLC. Nonetheless, an attempt to isolate one final compound in sufficient quantities for structure determination was done by the use of a single PTLC plate. The PTLC plate was evenly distributed with fraction 11-15 and placed in a twin trough chamber filled with 50 mL of a 20:3.4:2.7 (EtOAc:MeOH:H_2O) developing solvent. After development, desorption was performed in the same way as done in previous silica adsorbent PTLCs by the use of a 2:1 ($CHCl_3$:MeOH) solvent system.

22.7.1 Last isolated compound

The last isolated compound was purple under UV-light and strong yellow after spray detection followed by heating. Weight was calculated to be 15.6 mg. This compound was given the name CAP-9.

23. NMR and MS analysis

23.1 Preparing isolated compounds for NMR

Isolated compounds deemed pure enough for structure determination were analyzed both by NMR and MS. In the case of NMR, a solvent without protons is employed to enable correct interpretation of absorption peaks on the resulting spectra. Compounds were almost completely dissolved with 0.6 mL of CD_3OD. A micropipette was used to transfer the solutions to NMR tubes. Each NMR tube was named after the isolated compound and Dr. Li Yong-Li was in charge of transporting the samples to another laboratory at the Department of Natural Medicinal Chemistry for NMR analysis.

23.2 Preparing isolated compounds for MS

As explained in the section above, not all the amounts of the isolated compounds were dissolved by CD_3OD, thus, residual amounts were used in MS. Since deuterated solvents are not needed for MS analysis, dilute solutions of the isolated compounds were made by the addition of MeOH. Solutions were considered to be dilute when they displayed fairly weak colouration. By the use of a micropipette, 0.5 mL of these dilute solutions were transferred to separate microcentrifuge tubes. Each microcentrifuge tube was named after the isolated compound and Dr. Li Yong-Li was in charge of transporting the samples to another laboratory at the Department of Natural Medicinal Chemistry for MS analysis.

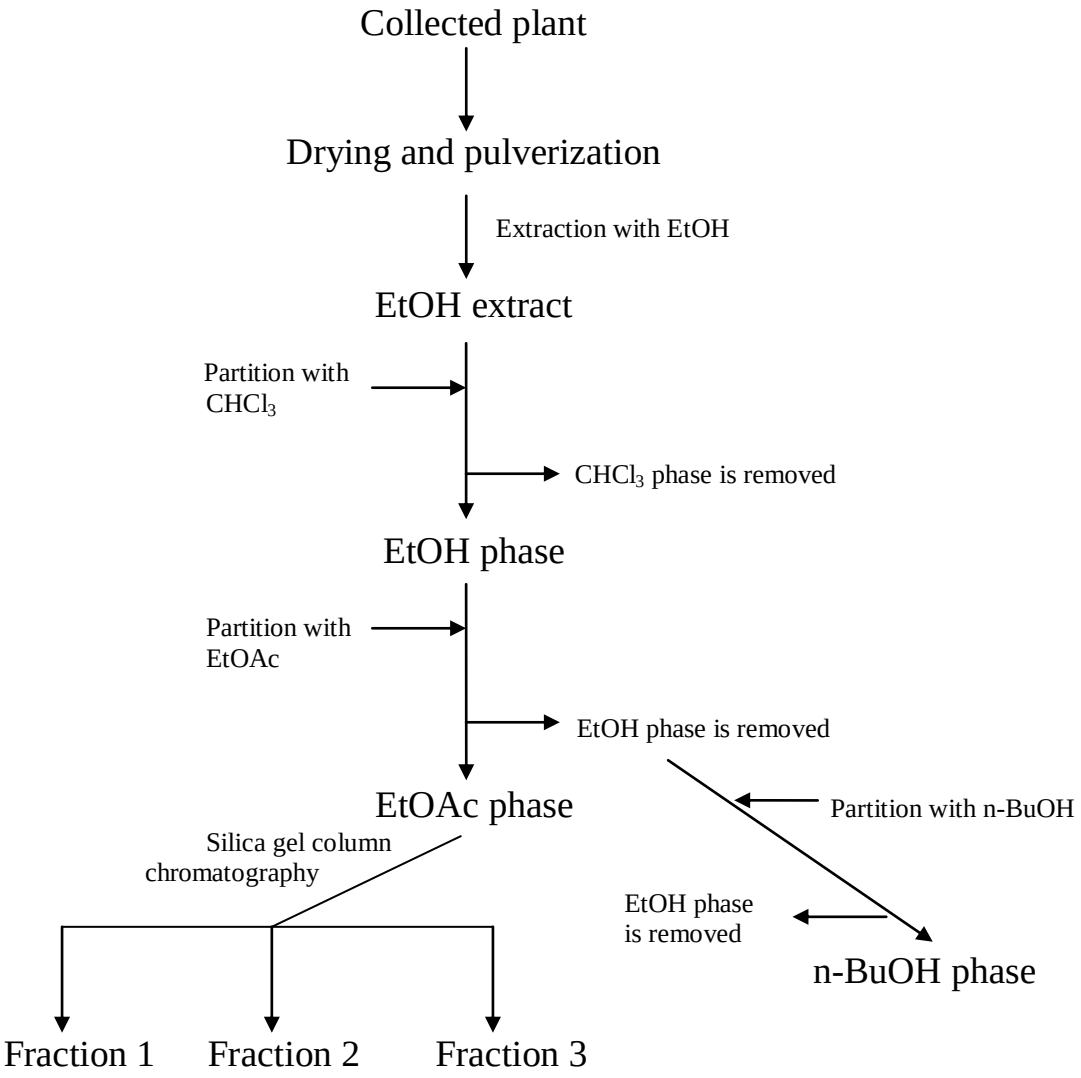
Results and discussion

24. Prior work

24.1 Initial steps

Prior to my involvement, Dr. Li Yong-Li had dried and pulverized both branches and leaves from *Abies pindrow*. Pulverization was followed by a series of extraction steps and a final column chromatography (***Figure 24.1***).

Figure 24.1 Initial extraction and isolation carried out by Dr. Li Yong-Li prior to my involvement in the purification process



24.2 Initial extraction and partition

After the plant had been dried and pulverized, Dr. Li Yong-Li proceeded with extraction by the use of different solvents. The pulverized branches and leaves were first extracted with EtOH. The solid mass left after initial extraction was removed. Compounds extracted with EtOH were subsequently partitioned with $CHCl_3$. Purification was intended to continue on the EtOH phase, thus, the $CHCl_3$ phase was removed. Another partition with EtOAc was performed on the EtOH phase. This time the EtOH phase was removed, but purification was continued on both solvent phases. While the EtOH phase went through a final partition with n-BuOH, a silica gel column chromatography was performed on the EtOAc phase.

24.3 Initial silica gel column chromatography

As described above, Dr. Li Yong-Li performed a silica gel column chromatography on the EtOAc phase from the initial partition steps. This was the last isolation step performed prior to my involvement in the isolation process. The silica gel column chromatography generated three separate fractions, which I have chosen to name Fraction 1, Fraction 2 and Fraction 3.

25. Purification of Fraction 1

The entire range of simple chromatographic techniques was employed in the isolation of compounds from Fraction 1. Silica gel column chromatography was the first isolation step carried out on Fraction 1. All of the isolation and purification steps are presented in ***Figure 25.1***.

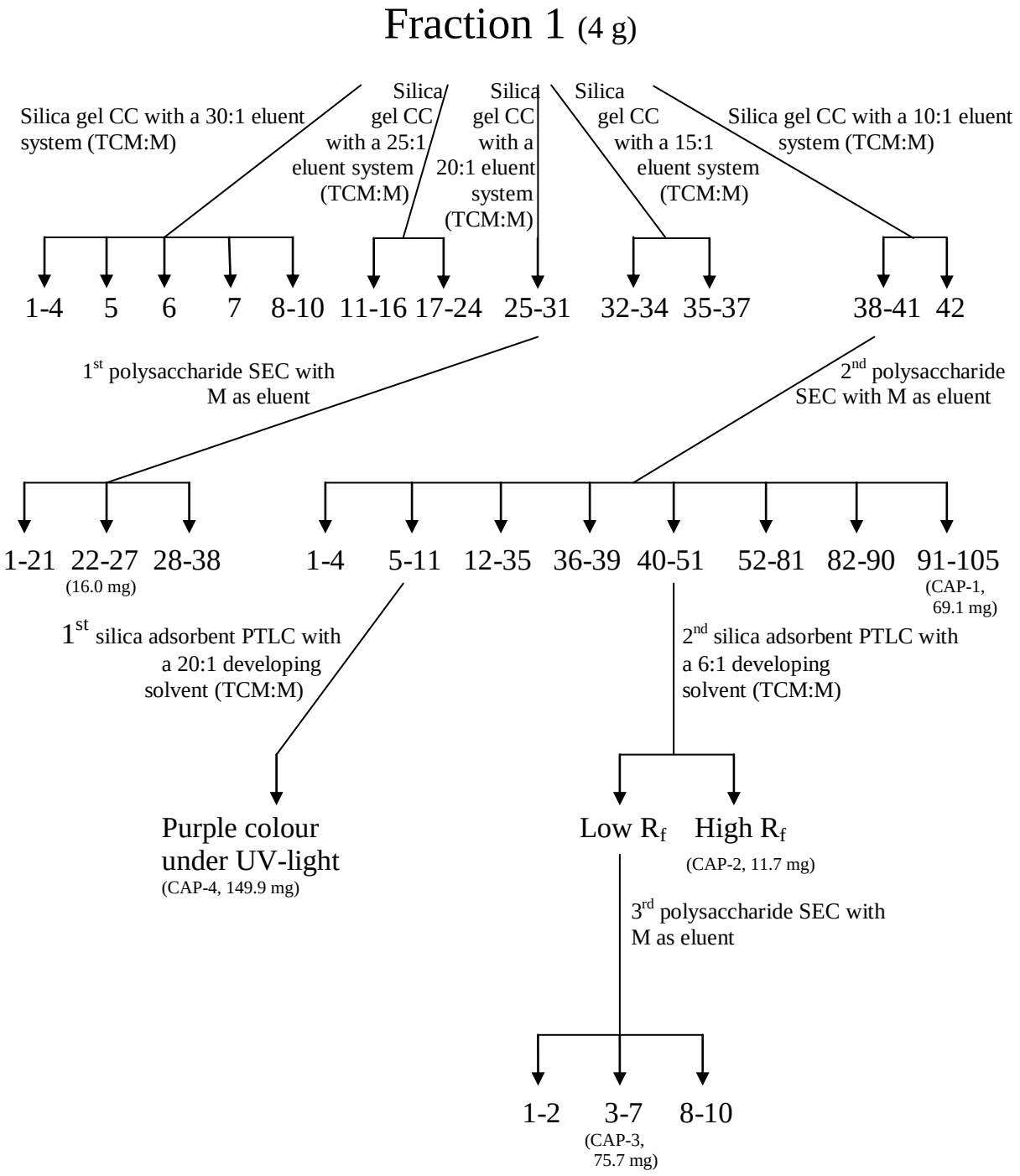
26. Purification of Fraction 2

Only two chromatographic steps were employed on Fraction 2. A polysaccharide size-exclusion chromatography was carried out first followed by a silica adsorbent PTLC. The isolation process carried out on Fraction 2 is presented in ***Figure 26.1***.

27. Purification of Fraction 3

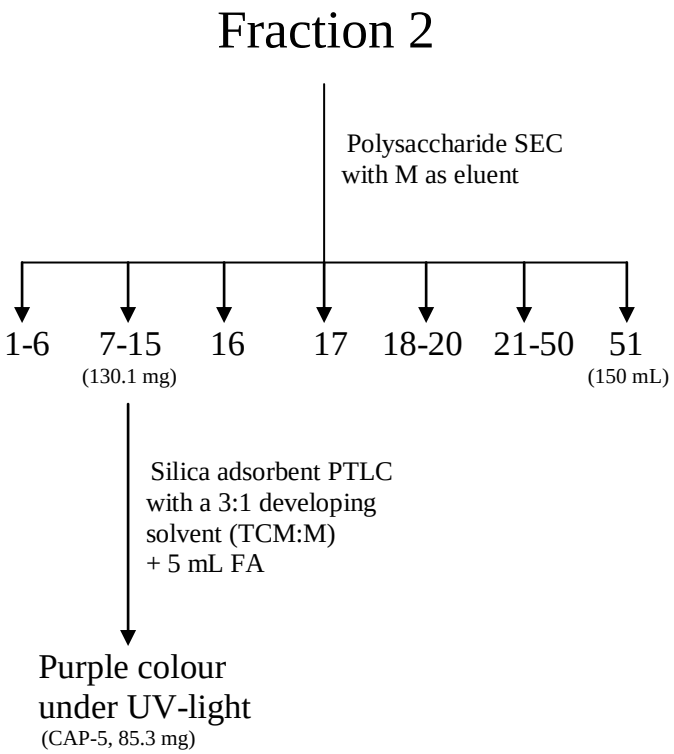
Isolation of compounds from Fraction 3 required the entire range of simple chromatographic techniques as well. A polysaccharide size-exclusion chromatography was the first chromatographic technique to be employed on Fraction 3. All of the isolation and purification steps are presented in ***Figure 27.1***.

Figure 25.1 Isolation steps carried out on Fraction 1 including weights of fairly pure fractions



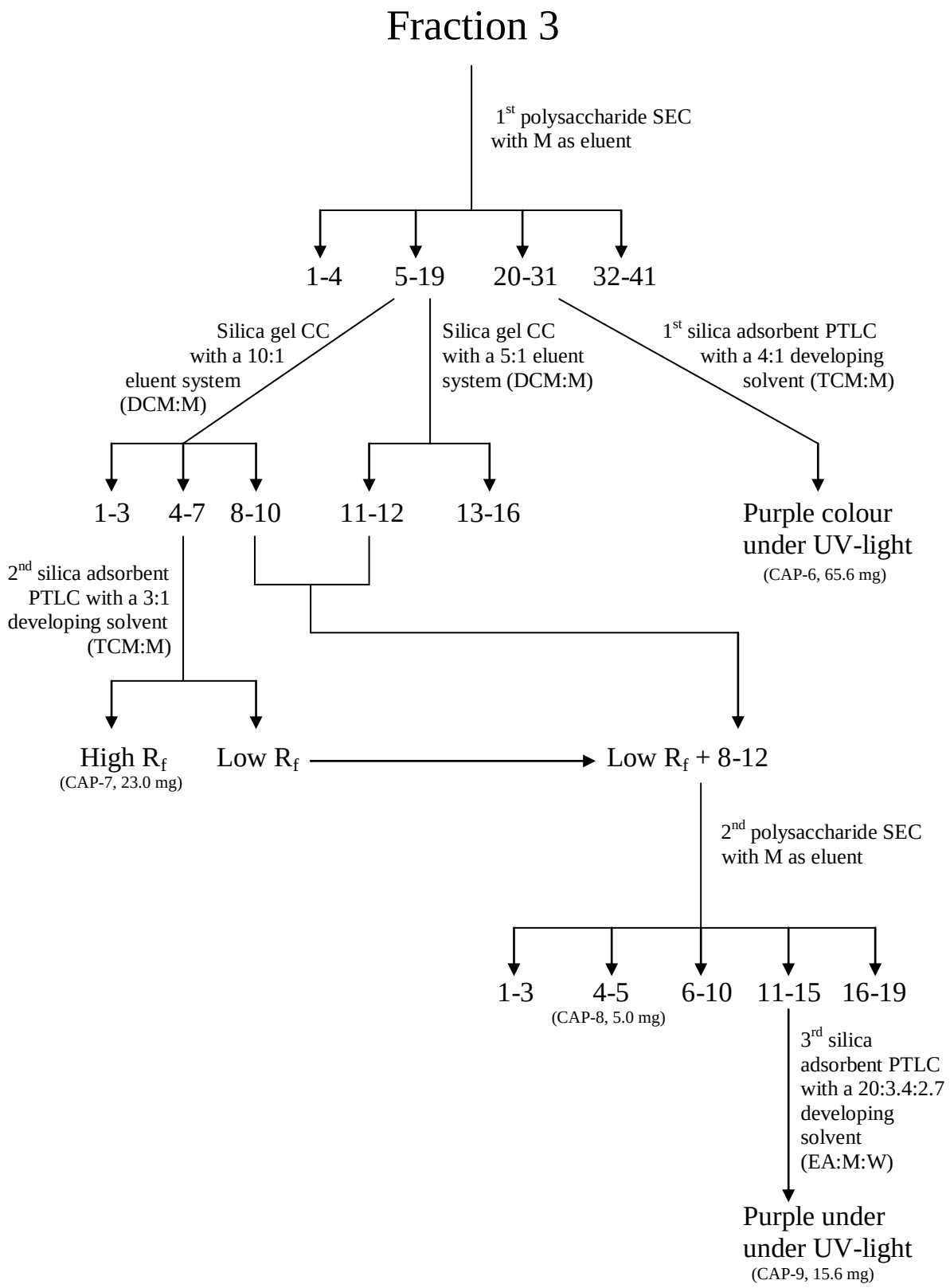
CC = column chromatography, TCM = trichloromethane, M = methanol, SEC = size-exclusion chromatography, PTLC = preparative thin layer chromatography, R_f = retardation factor.

Figure 26.1 Isolation steps carried out on Fraction 2 including weights of fairly pure fractions



FA = formic acid.

Figure 27.1 Isolation steps carried out on Fraction 3 including weights of fairly pure fractions



DCM = dichloromethane, EA = ethyl acetate, W = water.

28. Evaluation of fractions

28.1 Fractions that were not further purified

Collected fractions from every isolation step were evaluated with regards to purity and polarity. Some fractions were also evaluated with regards to weight. Majority of fractions were not further purified due to a number of reasons: too many compounds were present, compounds weighed too little to be further purified by a simple chromatographic technique, compounds had already been isolated, no compounds were present and/or polarity of compounds was too low or too high.

28.2 Further purified fractions

Fractions that were further purified displayed one or several desirable traits: compounds were of appropriate polarity, compounds weighed enough to be further purified by a simple chromatographic technique, no more than five compounds were present and/or compounds had not been isolated earlier in the isolation process. Purification of fractions continued until they were considered pure enough (i.e. only one compound was present) for structure elucidation.

29. Compounds prepared for structure determination

29.1 Pure fractions

Fractions considered pure enough for structure determination were, as previously explained, given the name CAP followed by a number. Before preparing compounds for NMR and MS analysis, they were assessed in two different developing solvents to avoid seemingly "pure" spots resulting from compounds with matching R_f values. In addition to R_f values, calculated weights and the colour of every compound, both under UV-light and after spray detection, were recorded as well prior to NMR and MS analysis (***Table 29.1***).

Table 29.1 Characteristics of isolated compounds

Compound	Colour			Developing solvent	R_f	Weight (mg)
	Under UV-light (254 nm)	After spray detection	No detection method			
CAP-1	Purple	Clear yellow	-	5:1 ($CHCl_3$:MeOH)	0.68	69.1
CAP-2	-	-	Strong yellow	30:1 ($CHCl_3$:MeOH)	0.62	11.7
CAP-3	Purple	Slight yellow	-	5:1 ($CHCl_3$:MeOH)	0.51	75.7
CAP-4	Purple	No colour	-	20:1 ($CHCl_3$:MeOH)	0.54	149.9
CAP-5	Purple	Yellow	-	3:1 ($CHCl_3$:MeOH) + 5 drops HCOOH	0.59	85.3
CAP-6	Purple	Slight yellow	-	3:1 ($CHCl_3$:MeOH	0.63	65.6
				20:3.4:2.7 (EA:M:W)	0.75	
CAP-7	Purple	Strong yellow	-	3:1 ($CHCl_3$:MeOH	0.63	23.0
				20:3.4:2.7 (EA:M:W)	0.75	
CAP-8	Purple	Yellow	-	20:3.4:2.7 (EA:M:W)	0.54	5.0
CAP-9	Purple	Strong yellow	-	20:3.4:2.7 (EA:M:W)	0.56	15.6

29.2 CAP-1

CAP-1 was made visible through the use of UV-light and spray detection followed by heating. A single, clear yellow spot appeared on a TLC plate developed in a 5:1 ($CHCl_3$:MeOH) developing system. R_f was calculated to be 0.68 and weight was calculated to be 69.1 mg.

29.3 CAP-2

CAP-2 was the only compound that was visible without the use of any detection method. A strong yellow spot appeared on a TLC plate developed in a 30:1 ($CHCl_3$:MeOH) developing system. R_f was calculated to be 0.62 and weight was calculated to be 11.7 mg.

29.4 CAP-3

CAP-3 was visible both under UV-light and after spray detection followed by heating. A slight yellow spot appeared on a TLC plate developed in a 5:1 ($CHCl_3$:MeOH) developing system. R_f was calculated to be 0.51 and weight was calculated to be 75.7 mg.

29.5 CAP-4

CAP-4 was made visible only under UV-light, where it was purple. No colour was visible after spray detection followed by heating. The compound was developed in a 20:1 ($CHCl_3$:MeOH) developing system. In order to calculate R_f, the purple spot was gently circled with a grey pencil. R_f was calculated to be 0.54 and weight was calculated to be 149.9 mg.

29.6 CAP-5

CAP-5 was made visible by the use of UV-light and spray detection followed by heating. A yellow spot appeared on a TLC plate developed in a 3:1 ($CHCl_3$:MeOH) developing system added 5 drops of formic acid. R_f was calculated to be 0.59 and weight was calculated to be 85.3 mg.

29.7 CAP-6

CAP-6 was visible both under UV-light and after spray detection followed by heating. A slight yellow spot appeared both on a TLC plate developed in a 3:1 ($CHCl_3$:MeOH) developing system and on a TLC plate developed in a 20:3.4:2.7 (EtOAc:MeOH:H_2O) developing system. R_f was calculated to be 0.63 in the former and 0.75 in the latter developing system. Weight was calculated to be 65.6 mg.

29.8 CAP-7

CAP-7 was made visible by the use of UV-light and spray detection followed by heating. This compound exhibited the exact same R_f values as CAP-6 in the same developing systems. Thus, CAP-7 and CAP-6 were considered to be the same compound. However, CAP-7 was strong yellow after spray detection followed by heating and weight was calculated to be 23.0 mg. CAP-7 seemed purer than CAP-6, thus, only CAP-7 was sent to another laboratory at the Department of Natural Medicinal Chemistry for NMR and MS analysis.

29.9 CAP-8

CAP-8 was visible both under UV-light and after spray detection followed by heating. A yellow spot appeared on a TLC plate developed in a 20:3.4:2.7 (EtOAc:MeOH:H_2O) developing system. R_f was calculated to be 0.54 and weight was calculated to be 5.0 mg.

29.10 CAP-9

CAP-9 was visible both under UV-light and after spray detection followed by heating. A strong yellow spot appeared on a TLC plate developed in a 20:3.4:2.7 (EtOAc:MeOH:H_2O) developing system. R_f and weight were calculated to be 0.56 and 15.6 mg, respectively.

30. Structure determination

30.1 Employed techniques

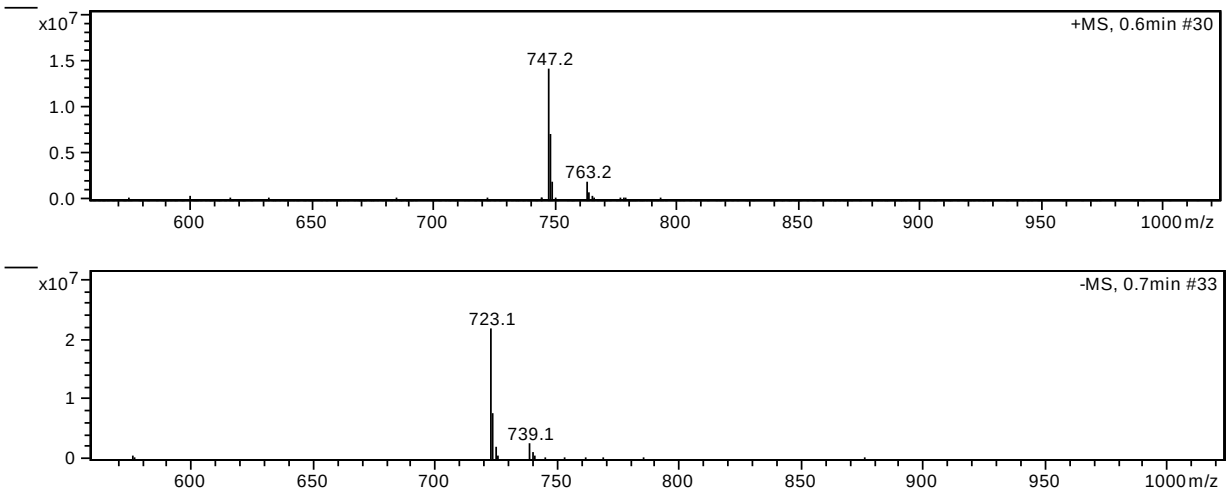
Both MS and NMR were employed in the structure determination of the different isolated compounds. MS was performed by electro spray ionisation and ion trap analysis. Compounds were analysed in both positive ion mode and negative ion mode, i.e. the molecular ions appeared as $[M+Na]^+$ and $[M-H]^-$ on the MS spectra, respectively. Thus, molecular weights were easily obtained by MS. As for NMR, compounds were analysed both by ^1H and ^{13}C NMR. DEPT-135 spectra were also obtained.

Chemical shifts from NMR spectra were compared to published chemical shifts from previously isolated organic compounds. DEPT-135 spectra were used to detect dissimilar carbons and to confirm proposed structures.

30.2 Structure elucidation of CAP-1

MS analysis of CAP-1 yielded quasi-molecular ions at m/z: 747 $[M+Na]^+$ and 723 $[M-H]^-$ in the positive ion mode and negative ion mode, respectively (***Figure 30.1***). Thus, the molecular weight of CAP-1 was calculated to be 724 Da.

Figure 30.1 MS spectra from CAP-1



30.2.1 Interpretation of ^{1}H NMR spectra

Two ^{1}H NMR spectra from CAP-1 can be found between the last pages of this Master's thesis (***Supplementary material 1.1*** and ***Supplementary material 1.2***). One spectrum displays all the peaks yielded by the protons in CAP-1, while the other spectrum is a close-up of peaks yielded by the most deshielded protons in CAP-1. The former spectrum ranges from 8.0-0.5 ppm, while the latter ranges from 7.9-4.0 ppm. These NMR spectra were interpreted prior to comparison of chemical shifts with published chemical shifts from a previously isolated organic compound (***Table 30.1***).

Table 30.1 Interpretation of ^{1}H NMR spectra from CAP-1

ppm	Multiplicity and coupling constant (Hz)	No. of protons	Assignment
7.79-7.76	Doublet, 9	2	Aromatics
7.65-7.60	Doublet, 15	2	Aromatics
7.54-7.52	Doublet, 6	2	Aromatics
7.47-7.44	Doublet, 9	2	Aromatics
7.04-7.01	Doublet, 9	2	Aromatics or alkenes
6.83-6.80	Doublet, 9	2	Aromatics, alkenes or α-disubstituted aliphatics
6.80-6.77	Doublet, 9	2	Aromatics, alkenes or α-disubstituted aliphatics
6.43-6.38	Doublet, 15	1	Aromatic, alkene or α-disubstituted aliphatic
6.34	Singlet	1	Aromatic, alkene or α-disubstituted aliphatic
6.32-6.27	Doublet, 15	1	Aromatic, alkene or α-disubstituted aliphatic
6.18	Singlet	1	Aromatic, alkene or α-disubstituted aliphatic
5.76	Singlet	?	Alkene(s) or α-disubstituted aliphatic(s)
5.58	Singlet	1	Alkene or α-disubstituted aliphatic
Approx. 5.00	Large, broad peak	?	Alkene(s) or α-disubstituted aliphatic(s)
4.21-4.17	Double doublet, 3, 9	1	α-monosubstituted or α-disubstituted aliphatic
Approx. 3.30	Large, thin peak	?	α-monosubstituted or α-disubstituted aliphatic(s)
0.87-0.85	Doublet, 6	3	Methyl

As can be deduced from ***Table 30.1***, CAP-1 consists of at least twenty-one chemically dissimilar protons. If assumed that the question marks represent one proton each, a total of twenty-four chemically dissimilar protons are present in CAP-1. Protons bound to oxygen are not included, thus, the total number of protons is probably higher than twenty-four. Assignment of functional groups gives a strong reason to believe that CAP-1 consists of several aromatic rings, conjugated alkenes and an aliphatic portion. It seems that only one methyl group is present in CAP-1.

30.2.2 Interpretation of ^{13}C NMR spectra

Two ^{13}C NMR spectra from CAP-1 are placed between the final pages of this Master's thesis (***Supplementary material 1.3*** and ***Supplementary material 1.4***). One spectrum displays the peaks yielded by all the carbons in CAP-1, while the other spectrum is a close-up of the carbons further downfield (to the left). The former spectrum ranges from 185-10 ppm, while the latter ranges from 170-113 ppm. These NMR spectra were interpreted prior to comparison of chemical shifts with published chemical shifts from a previously isolated organic compound (***Table 30.2***).

Table 30.2 Interpretation of ^{13}C NMR spectra from CAP-1

ppm	Assignment
179.1	Carbonyl
168.5	Carbonyl or alkene (conjugated or bound to a polar substituent)
168.2	Carbonyl or alkene (conjugated or bound to a polar substituent)
165.9	Carbonyl or alkene (conjugated or bound to a polar substituent)
161.7	Carbonyl or alkene (conjugated or bound to a polar substituent)
161.3	Carbonyl or alkene (conjugated or bound to a polar substituent)
159.3	Carbonyl or alkene (conjugated or bound to a polar substituent)
158.5	Carbonyl or alkene (conjugated or bound to a polar substituent)
147.4	Aromatic or alkene (both conjugated and unconjugated)
147.0	Aromatic or alkene (both conjugated and unconjugated)
134.7	Aromatic or alkene (both conjugated and unconjugated)
133.9	Aromatic or alkene (both conjugated and unconjugated)
132.0	Aromatic or alkene (both conjugated and unconjugated)
131.3	Aromatic or alkene (both conjugated and unconjugated)
127.1	Aromatic or alkene (both conjugated and unconjugated)
122.5	Aromatic or alkene (both conjugated and unconjugated)
116.8	Aromatic or alkene (both conjugated and unconjugated)
116.6	Aromatic or alkene (both conjugated and unconjugated)
114.9	Aromatic or alkene (both conjugated and unconjugated)
114.6	Aromatic or alkene (both conjugated and unconjugated)
105.9	Alkene or aromatic
100.0	Alkene or aromatic
99.2	Alkene or aromatic
94.9	Alkene or aromatic
74.6	C-O-C or CH-OH
73.1	C-O-C or CH-OH
69.7	C-O-C or CH-OH
68.5	C-O-C or CH-OH
17.7	Methyl

Twenty-nine chemically different carbons are present in CAP-1. ***Table 30.2*** strongly suggests that there is at least one carbonyl group present in CAP-1, but there may be several as well. It appears that CAP-1 consists of aromatic rings, conjugated alkenes, ether groups and/or alcohol groups. This is consistent with interpretations from the ^{1}H NMR spectra. Only one methyl groups is confirmed to be present in CAP-1.

30.2.3 Interpretation of DEPT-135 spectrum

A single DEPT-135 spectrum from CAP-1 can be found between the final pages of this Master's thesis (***Supplementary material 1.5***). Only carbons with chemical shifts from 155-15 ppm were recorded. Interpretation of this DEPT-135 spectrum was done prior to comparison of chemical shifts with published chemical shifts of a previously isolated organic compound (***Table 30.3***).

Table 30.3 Interpretation of DEPT-135 spectrum from CAP-1

ppm	Signal	Assignment
147.4	Positive	CH (aromatic or alkene)
147.0	Positive	CH (aromatic or alkene)
134.7	-	Quaternary carbon
133.9	Positive	CH (aromatic or alkene)
132.0	Positive	CH (aromatic or alkene)
131.3	Positive	CH (aromatic or alkene)
127.1	-	Quaternary carbon
122.5	-	Quaternary carbon
116.8	Positive	CH (aromatic or alkene)
116.6	Positive	CH (aromatic or alkene)
114.9	Positive	CH (aromatic or alkene)
114.6	Positive	CH (aromatic or alkene)
105.9	-	Quaternary carbon
100.0	Positive	CH (aromatic or alkene, alkane only if bound to a polar substituent)
99.2	Positive	CH (aromatic or alkene, alkane only if bound to a polar substituent)
94.9	Positive	CH (aromatic or alkene, alkane only if bound to a polar substituent)
74.6	Positive	CH (CH-O-C or CH-OH)
73.1	Positive	CH (CH-O-C or CH-OH)
69.7	Positive	CH (CH-O-C or CH-OH)
68.5	Positive	CH (CH-O-C or CH-OH)
17.7	Positive	CH_3

It is obvious from ***Table 30.3*** that there are no methylene groups in CAP-1, even though the carbons furthest downfield were not recorded on the DEPT-135 spectrum. This is true because carbons that are furthest downfield are either carbonyls or conjugated alkenes as discovered in the interpretation of the ^{13}C NMR spectra. A total of four quaternary carbons are present in CAP-1. Majority of carbons are either aromatics or alkenes. Only a small portion of carbons are aliphatics.

30.2.4 Comparison of CAP-1 with a published compound

Due to the many possibilities of structures that can be proposed from the interpretations of the ^{1}H NMR spectra, the ^{13}C NMR spectra and the DEPT-135 spectrum, chemical shifts from CAP-1 have been compared to published chemical shifts from a previously isolated organic compound (***Table 30.4*** and ***Table 30.5***).

Table 30.4 ^{1}H NMR chemical shifts from CAP-1 compared with published chemical shifts from kaempferol-3-O-(2'', 4''-di-E-p-coumaroyl)-α-L-rhamnopyranoside

Proton	CAP-1 (δ), multiplicity and coupling constant (Hz)	Published (δ), multiplicity and coupling constant (Hz)
H-6	6.18 (s)	6.23 (s)
H-8	6.34 (s)	6.40 (s)
H-2′	7.79 (d, 9)	7.82 (d, 8.5)
H-3′	7.04 (d, 9)	7.05 (d, 8.5)
H-5′	7.01 (d, 9)	7.05 (d, 8.5)
H-6′	7.76 (d, 9)	7.82 (d, 8.5)
H-1″	5.76 (s)	5.78 (s)
H-2″	5.58 (s)	5.56 (s)
H-3″	4.17 (dd, 3, 9)	4.18 (dd, 2.5, 10.0)
H-4″	Approx. 5.00 (br s)	4.99 (t, 10.0)
H-5″	Approx. 3.30 (N/A)	3.30 (N/A)
H-6″	0.87 (d, 6)	0.87 (d, 6.0)
H-2‴	7.47 (d, 9)	7.51 (d, 8.5)
H-3‴	6.80 (d, 9)	6.82 (d, 8.5)
H-5‴	6.77 (d, 9)	6.82 (d, 8.5)
H-6‴	7.44 (d, 9)	7.51 (d, 8.5)
H-7‴	7.65 (d, 15)	7.70 (d, 16.0)
H-8‴	6.43 (d, 15)	6.44 (d, 16.0)
H-2⁗	7.54 (d, 6)	7.55 (d, 8.5)
H-3⁗	6.83 (d, 9)	6.85 (d, 8.5)
H-5⁗	6.80 (d, 9)	6.85 (d, 8.5)
H-6⁗	7.52 (d, 6)	7.55 (d, 8.5)
H-7⁗	7.60 (d, 15)	7.59 (d, 16.0)
H-8⁗	6.32 (d, 15)	6.32 (d, 16.0)

Source: Wang, G. J., et al [33]

s = singlet, d = doublet, dd = double doublet, t = triplet, N/A = not applicable

As can be seen from ***Table 30.4***, chemical shifts from protons in CAP-1 are fairly similar to chemical shifts from protons in the published compound. Multiplicities and coupling constants are practically the same. H-2⁗ and H-6⁗ display the largest differences in coupling constants (6 Hz in CAP-1 and 8.5 Hz in the published compound). Not only are the chemical shifts similar, but also the number of protons are the same. To further prove their similarity, chemical shifts from carbons were compared as well (***Table 30.5***).

Table 30.5 ^{13}C NMR chemical shifts from CAP-1 compared with published chemical shifts from kaempferol-3-O-(2'', 4''-di-E-p-coumaroyl)-α-L-rhamnopyranoside

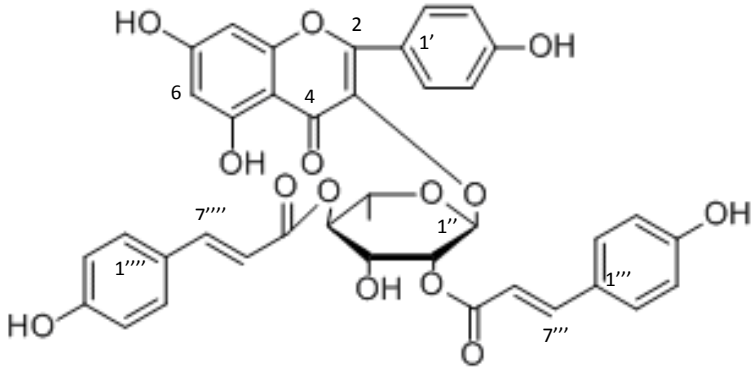
Carbon no.	CAP-1 (δ)	Published (δ)
2	159.3	259.4
3	134.7	134.7
4	179.1	179.2
5	161.7	163.3
6	100.0	100.0
7	165.9	166.0
8	94.9	94.8
9	158.5	158.6
10	105.9	105.9
1'	122.5	122.5
2'	133.9	132.0
3'	116.6	116.7
4'	161.7	161.8
5'	116.6	116.7
6'	132.0	132.0
1''	99.2	99.2
2''	73.1	73.1
3''	68.5	68.5
4''	74.6	74.6
5''	69.7	69.8
6''	17.7	17.7
1'''	127.1	127.2
2'''	131.3	131.4
3'''	116.8	116.8
4'''	161.3	161.4
5'''	116.8	116.8
6'''	131.3	131.4
7'''	147.4	147.4
8'''	114.6	114.7
9'''	168.2	168.2
1''''	127.1	127.2
2''''	131.3	131.4
3''''	116.8	116.8
4''''	161.3	161.4
5''''	116.8	116.8
6''''	131.3	131.4
7''''	147.0	146.9
8''''	114.9	115.0
9''''	168.5	168.5

Source: Wang, G.J., et al [33]

A total of thirty-nine carbons are present in the published compound, but only twenty-nine of them are chemically different, which is in agreement with the interpretation of the ^{13}C NMR spectra. Chemical shifts are so alike that they are practically the same. C-5 and C-2′ display the biggest differences in chemical shifts (δ 161.7 and δ 133.9 in CAP-1 and δ 163.3 and δ 132.0 in the published compound, respectively).

The molecular formula of the published compound is $C_{39}H_{32}O_{14}$, thus , the index of hydrogen deficiency is twenty-four. It consists of four aromatic rings, three conjugated alkenes, one sugar moiety, three carbonyl groups, four quaternary carbons and one methyl group. In addition, no methylene groups are present and its molecular weight is 724 Da. All of this is consistent with the interpretations of the MS spectra, the ^{1}H NMR spectra, the ^{13}C NMR spectra and the DEPT-135 spectrum. Thus, CAP-1 was characterized as kaempferol-3-O-(2″, 4″-di-E-p-coumaroyl)-α-L-rhamnopyranoside (***Figure 30.2***).

Figure 30.2 Molecular structure of kaempferol-3-O-(2'', 4''-di-E-p-coumaroyl)-α-L-rhamnopyranoside



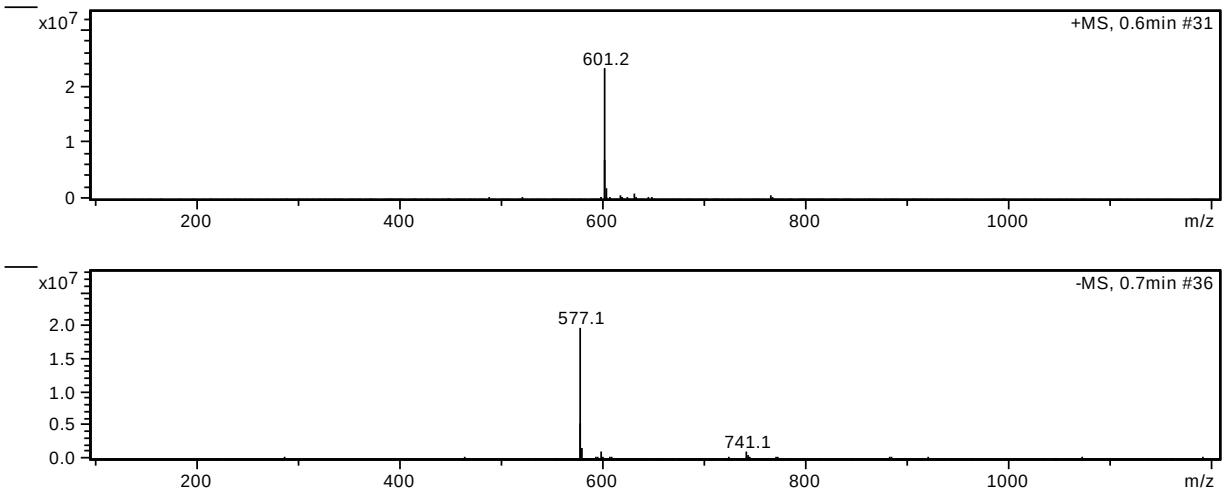
30.3 Structure elucidation of CAP-2

Results from NMR and MS analysis of CAP-2 were inconclusive due to impurities in the sample. Further purification by a simple chromatographic technique would also be difficult to perform due to the small weight of the compound (11.7 mg). Thus, CAP-2 was not characterized.

30.4 Structure elucidation of CAP-3

MS analysis of CAP-3 yielded quasi-molecular ions at m/z: 601 $[M+Na]^+$ and 577 $[M-H]^-$ in the positive ion mode and negative ion mode, respectively (***Figure 30.3***). Thus, the molecular weight of CAP-3 was calculated to be 578 Da.

Figure 30.3 MS spectra from CAP-3



30.4.1 Comparison of CAP-3 with a published compound

Due to the similarity between ^{1}H NMR spectra, ^{13}C NMR spectra and DEPT-235 spectra of CAP-1 and CAP-3, chemical shifts from CAP-3 were compared with published chemical shifts from a previously isolated organic compound (***Table 30.6*** and ***Table 30.7***).

Table 30.6 ^{1}H NMR chemical shifts from CAP-3 compared with published chemical shifts from kaempferol-3-O-α-L-(4″-E-p-coumaroyl)-rhamnoside

Proton	CAP-3 (δ), multiplicity and coupling constant (Hz)	Published (δ), multiplicity and coupling constant (Hz)
H-6	6.17 (s)	6.18 (d, 1.9)
H-8	6.33-6.31 (d, 6)	6.34 (d, 1.9)
H-2′	7.74-7.71 (d, 9)	7.77 (d, 8.8)
H-3′	Approx. 7.00 (m)	6.95 (d, 8.8)
H-5′	Approx. 7.00 (m)	6.95 (d, 8.8)
H-6′	7.74-7.71 (d, 9)	7.77 (d, 8.8)
H-1″	5.62 (s)	5.49 (br s)
H-2″	4.25 (s)	4.23 (br s)
H-3″	Approx. 3.90 (dd, N/A)	3.94 (dd, 3.4, 9.4)
H-4″	Approx. 5.00 (br s)	4.95 (t, 9.4)
H-5″	Approx. 3.30 (N/A)	3.30 (m)
H-6″	0.80-0.78 (d, 6)	0.80 (d, 6.0)
H-2‴	6.33-6.31 (d, 6)	6.31 (d, 16.0)
H-3‴	7.67 (m)	7.60 (d, 16.0)
H-5‴	7.52-approx. 7.48 (d, 12)	7.53 (d, 8.6)
H-6‴	6.82-6.79 (d, 9)	6.82 (d, 8.6)
H-8‴	6.82-6.79 (d, 9)	6.82 (d, 8.6)
H-9‴	7.52-approx. 7.48 (d,12)	7.53 (d, 8.6)

Source: Yang, N.Y., et al [34]

m = multiplet, br s = broad singlet

Clearly, CAP-3 is not as pure as thought prior to MS and NMR analysis. This is evident by studying the many multiplets and unclear peaks displayed on the ^{1}H NMR spectrum. Thus, many approximations had to be made when CAP-3 was compared to a published compound. However, only three peaks on the ^{1}H NMR spectrum were not included in ***Table 30.6*** (δ 7.50, δ 6.74 and δ 6.72), which gives a strong reason to believe that CAP-3 and the published compound are in fact the same compound. In spite of impurities, chemical shifts from CAP-3 and the published compound are very close to one another. Correct multiplicities and coupling constants are the most difficult to obtain from an impure ^{1}H NMR spectrum. Majority of multiplicities are in agreement with the published compound, but majority of coupling constants are not. A total of fourteen chemically dissimilar protons are present both in the published compound and in CAP-3. Although the number of chemically dissimilar protons are the same, impurities can both display "false" peaks and hide "true" peaks on the spectrum. Thus, this number is rather uncertain. Chemical shifts from carbons in CAP-3 were also compared to chemical shifts from carbons in the published compound (***Table 30.7***).

Table 30.7 ^{13}C NMR chemical shifts from CAP-3 compared with published chemical shifts from kaempferol-3-O-α-L-(4''-E-p-coumaroyl)-rhamnoside

Carbon no.	CAP-3 (δ)	Published (δ)
C-2	161.2	159.3
C-3	133.9	135.5
C-4	179.3	179.5
C-5	163.1	163.3
C-6	99.9	100.0
C-7	165.7	166.4
C-8	94.8	95.0
C-9	158.4	158.7
C-10	105.9	105.7
C-1′	122.6	122.0
C-2′	131.3	131.8
C-3′	116.8	116.8
C-4′	161.6	161.7
C-5′	116.5	116.8
C-6′	131.9	131.8
C-1″	102.1	102.1
C-2″	71.7	72.0
C-3″	70.1	70.3
C-4″	74.7	75.0
C-5″	69.6	70.1
C-6″	17.6	17.8
C-1‴	168.7	168.8
C-2‴	115.1	115.4
C-3‴	146.7	146.8
C-4‴	127.1	127.1
C-5‴	131.9	132.1
C-6‴	116.8	116.9
C-7‴	161.5	161.0
C-8‴	116.5	116.9
C-9‴	132.0	132.1

Source: Yang, N.Y., et al [34]

A total of twenty-six chemically dissimilar carbons are present in the published compound, while twenty-eight chemically dissimilar compounds are found in CAP-3. As explained earlier, this discrepancy might be due to impurities in the sample, but it might also be due to deviations in spectrometer measurements. Chemical shifts from the carbons in CAP-3 and chemical shifts from the carbons in the published compound are relatively similar. C-2 and C-3 display the largest differences in chemical shifts (δ 161.2 and δ 133.9 in CAP-3 and δ 159.3 and δ 135.5 in the published compound, respectively). Chemical shifts from the carbons in CAP-3 are far more similar to the published compound than the chemical shifts from the protons in CAP-3. This strengthens the suggestion that CAP-3 and the published compound are the same.

30.4.2 Interpretation of DEPT-135 spectrum

A single DEPT-135 spectrum from CAP-3 can be found among the final pages of this Master's thesis (***Supplementary material 1.8***). The entire chemical shift range was recorded. Interpretation of this DEPT-135 spectrum from CAP-3 was used to confirm similarity (***Table 30.8***).

Table 30.8 Interpretation of DEPT-135 spectrum from CAP-3

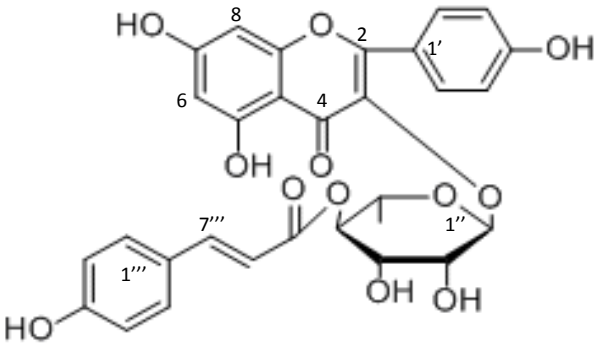
ppm	Signal	Assignment
179.3	-	Quaternary carbon?
168.7	-	Quaternary carbon?
165.7	-	Quaternary carbon?
163.1	-	Quaternary carbon?
161.6	-	Quaternary carbon?
161.5	-	Quaternary carbon?
161.2	-	Quaternary carbon?
158.4	-	Quaternary carbon?
146.7	Positive	CH (aromatic or alkene)
133.9	Positive	CH (aromatic or alkene)
132.0	Positive	CH (aromatic or alkene)
131.9	Positive	CH (aromatic or alkene)
131.3	Positive	CH (aromatic or alkene)
127.1	-	Quaternary carbon?
122.6	-	Quaternary carbon?
116.8	Positive	CH (aromatic or alkene)
116.5	Positive	CH (aromatic or alkene)
115.8	Positive	CH (aromatic or alkene)
115.1	Positive	CH (aromatic or alkene)
105.9	-	Quaternary carbon?
102.1	Positive	CH (aromatic or alkene, alkane only if bound to a polar substituent)
99.9	Positive	CH (aromatic or alkene, alkane only if bound to a polar substituent)
94.8	Positive	CH (aromatic or alkene, alkane only if bound to a polar substituent)
74.7	Positive	CH (CH-O-C or CH-OH)
71.7	Positive	CH (CH-O-C or CH-OH)
70.1	Positive	CH (CH-O-C or CH-OH)
69.6	Positive	CH (CH-O-C or CH-OH)
17.6	Positive	CH_3

According to ***Table 30.8***, as many as eleven quaternary carbons might be present in CAP-3. This is obviously incorrect due to the fact that carbons furthest downfield are carbonyls, aromatics or alkenes. Eleven quaternary carbons would require a substantial number of aromatic rings, cyclic aliphatics or alkynes. If eleven quaternary carbons were due to aromatics, a whole lot more peaks would be present on the ^{13}C NMR spectrum; if eleven quaternary carbons were due to cyclic aliphatics, chemical shifts would concentrate upfield on the ^{13}C NMR spectrum; if eleven quaternary carbons were due to alkynes, peaks would concentrate downfield on the ^{13}C NMR spectrum; and if eleven quaternary

carbons were due to a combination of these structures, all the effects mentioned would appear on the ^{13}C NMR spectrum. Thus, it unreasonable to believe that there are eleven quaternary carbons present in CAP-3. Since all of the NMR spectra from CAP-3 resemble the NMR spectra from CAP-1 to a great extent, it is more reasonable to assume that CAP-3 consists of only three quaternary carbons. Majority of carbons are therefore either aromatics or alkenes. Only a small portion of carbons are aliphatics. No methylene groups are present.

The molecular formula of the published compound is $C_{30}H_{26}O_{12}$, thus, the index of hydrogen deficiency is eighteen. It consists of three aromatic rings, two conjugated alkenes, one sugar moiety, two carbonyl groups, three quaternary carbons and one methyl group. In addition, no methylene groups are present and its molecular weight is 578 Da. Although impurities in CAP-3 impeded comparison of chemical shifts and interpretation of DEPT-235 spectrum, there exists strong proof that CAP-3 and the published compound are the same. Thus, CAP-3 was characterized as kaempferol-3-O-α-L-(4″-E-p-coumaroyl)-rhamnoside (***Figure 30.4***).

Figure 30.4 Molecular structure of kampferol-3-O-α-L-(4''-E-p-coumaroyl)-rhamnoside



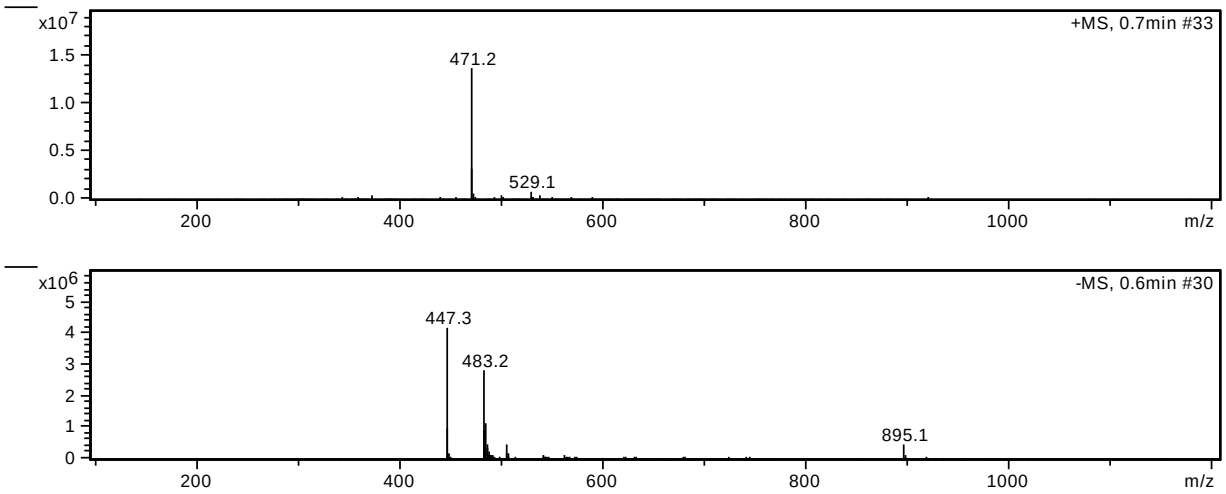
30.5 Structure elucidation of CAP-4

Results from NMR and MS analysis of CAP-4 were inconclusive due to impurities in the sample. CAP-4 consisted of enough material for it to be further purified by a simple chromatographic technique (149.9 mg). Unfortunately, when the MS and NMR results came back, there was not enough time left to continue purification of CAP-4. Thus, CAP-4 was not characterized.

30.6 Structure elucidation of CAP-5

MS analysis of CAP-5 yielded quasi-molecular ions at m/z: 471 $[M+Na]^+$ and 919 $[2M+Na]^+$ in the positive ion mode and quasi-molecular ions at m/z: 447 $[M-H]^-$ and 895 $[2M-H]^-$ in the negative ion mode (***Figure 30.5***). Thus, the molecular weight of CAP-5 was calculated to be 448 Da.

Figure 30.5 MS spectra from CAP-5



30.6.1 Comparison of CAP-5 with a published compound

^1H NMR spectra, ^{13}C NMR spectra and DEPT-135 spectra from CAP-3 and CAP-5 are similar to one another, except for the fact that fewer protons and carbons are displayed on the ^1H NMR spectrum and on the ^{13}C NMR spectrum from CAP-5, respectively. This strongly suggests that CAP-5 is a smaller molecule than CAP-3, but that they share many similar structures. Chemical shifts from CAP-5 were compared to chemical shifts from a previously isolated organic compound (***Table 30.9*** and ***Table 30.10***).

Table 30.9 ^{1}H NMR chemical shifts from CAP-5 compared with published chemical shifts from quercitrin

Proton	CAP-5 (δ), multiplicity and coupling constant (Hz)	Published (δ), multiplicity and coupling constant (Hz)
H-6	6.12 (s)	6.15 (d, 1.6)
H-8	6.28 (s)	6.32 (d, 2.0)
H-2′	7.30 (s)	7.29 (d, 2.1)
H-5′	6.89-6.86 (d, 9)	6.87 (d, 8.4)
H-6′	7.25-7.22 (d, 9)	7.26 (dd, 2.1, 8.0)
H-1″	5.31 (s)	5.31 (br s)
H-2″	4.22 (s)	3.96 (br s)
H-3″	3.78-3.75 (m)	3.33 (m)
H-4″	Approx 3.30 (m)	3.09 (m)
H-5″	Approx. 3.30 (m)	3.09 (m)
H-6″	0.91-0.89 (d, 6)	0.79 (d, 5.6)

Source: Yang, N.Y., et al [34]

A total of ten chemically dissimilar protons are present in the published compound, which is exactly the same number of chemically dissimilar protons in CAP-5. Chemical shifts on both compounds are fairly similar. H-2″, H-3″, H-4″ and H-5″ display the least similar chemical shifts (δ 4.22, δ 3.78-3.75, δ 3.30 and δ 3.30 in CAP-5 and δ 3.96, δ 3.33, δ 3.09 and δ 3.09 in the published compound, respectively). Large differences in these chemical shifts might be due to deviations in spectrometer measurements and impurities in the sample. It is also worth mentioning that chemical shifts from H-2″, H-3″, H-4″ and H-5″ in CAP-3 come much closer to CAP-5 than the published compound. This strengthens similarity between CAP-5 and quercitrin because CAP-3 has been identified to be an organic compound that is very similar to quercitrin. Majority of multiplicities are in accordance with the published compound. Coupling constants are very small in places where multiplicities are not in accordance, thus, split peaks might be left out from the full scale ^{1}H NMR spectrum. Perhaps a close-up would enable detection of splitting patterns with small coupling constants. Peaks with larger coupling constants are assigned both multiplicities and coupling constants that are in agreement with the published compound. To further prove their similarity, chemical shifts from carbons were also compared (***Table 30.10***).

Table 30.10 ^{13}C NMR chemical shifts from CAP-5 compared with published chemical shifts from quercitrin

Carbon no.	CAP-5 (δ)	Published (δ)
C-2	159.1	159.2
C-3	136.1	136.1
C-4	179.4	179.4
C-5	162.9	162.9
C-6	99.8	99.7
C-7	165.8	165.7
C-8	94.7	94.7
C-9	158.3	158.4
C-10	105.7	105.8
C-1′	122.9	122.9
C-2′	116.9	116.9
C-3′	146.1	146.3
C-4′	149.5	149.6
C-5′	116.3	116.3
C-6′	122.9	122.7
C-1″	103.3	103.4
C-2″	71.9	71.9
C-3″	71.9	72.0
C-4″	73.2	73.2
C-5″	71.8	71.8
C-6″	17.6	17.7

Source: Yang, N.Y., et al [34]

A total of twenty-one chemically dissimilar carbons are present in the published compound, while nineteen chemically dissimilar carbons are present in CAP-5. This discrepancy might be due to similar chemical shifts between two pairs of carbons that make them appear on the same place on the ^{13}C NMR spectrum. Thus, two chemically dissimilar carbons are left out. This explanation is confirmed by studying the DEPT-135 spectrum from CAP-5 which displays four peaks from δ 73.2-71.8, whereas the ^{13}C NMR spectrum only displays three peaks in the same area. Chemical shifts from the carbons in CAP-5 and chemical shifts from the carbons in the published compound are so similar that they are practically equal. C-3′ and C-6′ display the largest differences in chemical shifts (δ 146.1 and δ 122.9 in CAP-5 and δ 146.3 and δ 122.7 in quercitrin, respectively). However, differences in chemical shifts are so small that CAP-5 and quercitrin can be considered to be the same compound.

30.6.2 Interpretation of DEPT-135 spectrum

A single DEPT-135 spectrum from CAP-5 can be found between the final pages of this Master's thesis (***Supplementary material 1.11***). Only carbons with chemical shifts from 137-15 ppm were recorded. Interpretation of this DEPT-135 spectrum from CAP-5 was used to confirm similarity (***Table 30.11***).

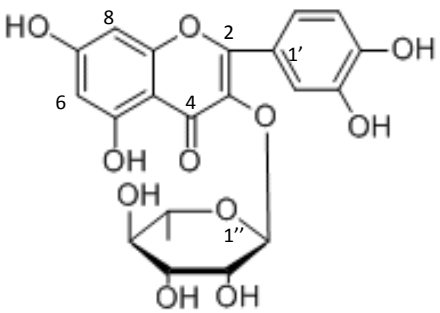
Table 30.11 Interpretation of DEPT-135 spectrum from CAP-5

ppm	Signal	Assignment
136.1	-	Quaternary carbon
122.9	Positive	CH (aromatic or alkene)
116.9	Positive	CH (aromatic or alkene)
116.3	Positive	CH (aromatic or alkene)
105.7	-	Quaternary carbon
103.3	Positive	CH (aromatic or alkene, alkane only if bound to a polar substituent)
99.8	Positive	CH (aromatic or alkene, alkane only if bound to a polar substituent)
94.7	Positive	CH (aromatic or alkene, alkane only if bound to a polar substituent)
73.2	Positive	CH (CH-O-C or CH-OH)
71.9	Positive	CH (CH-O-C or CH-OH)
71.8	Positive	CH (CH-O-C or CH-OH)
17.6	Positive	CH_3

It is clear from ***Table 30.11*** that there are only two quaternary carbons present in CAP-5, although the carbons furthest downfield were not recorded. Reasons for this have previously been explained in the interpretation of DEPT-135 spectrum from CAP-3. CAP-5 consists mostly of aromatics and/or alkenes. Only a small portion of carbons are aliphatics, which most likely belong to a sugar moiety as in CAP-1 and CAP-3. Also there are no methylene groups present.

The molecular formula of quercitrin is $C_{21}H_{20}O_{11}$, thus, the index of hydrogen deficiency is twelve. It consists of two aromatic rings, one conjugated alkene, one sugar moiety, one carbonyl group, two quaternary carbons and one methyl group. In addition, no methylene groups are present and its molecular weight is 448 Da. All of this is consistent with comparison of NMR spectra and interpretation of the DEPT-135 spectrum. Thus, CAP-5 was characterized as quercitrin (***Figure 30.6***).

Figure 30.6 Molecular structure of quercitrin



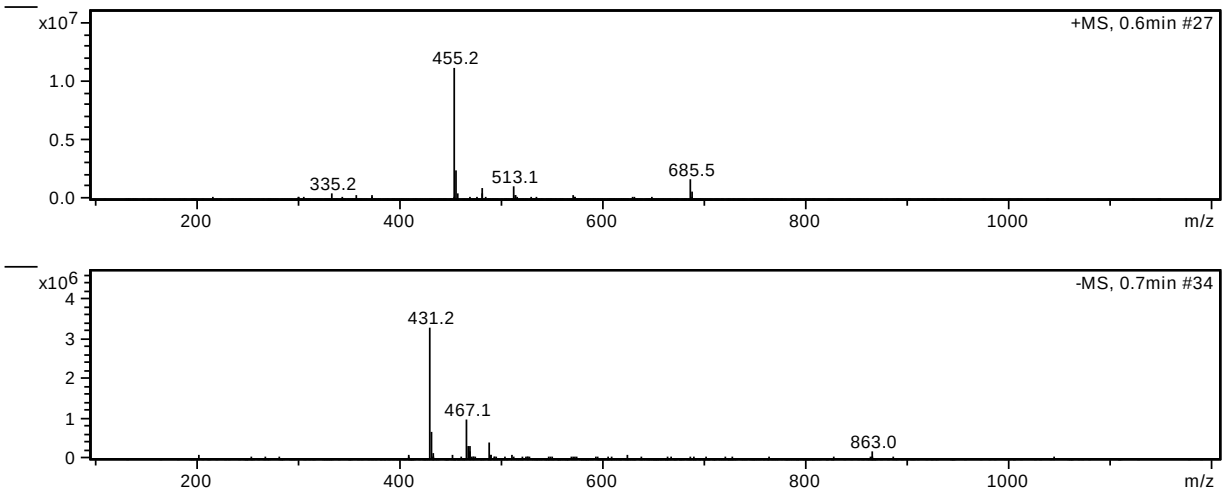
30.7 Structure elucidation of CAP-6

CAP-6, as previously explained, was not sent to another laboratory at the Department of Natural Medicinal Chemistry for MS and NMR analysis because analytical TLC confirmed that CAP-6 and CAP-7 was the same compound. Thus, only one of them was analyzed. CAP-7 seemed purer and was therefore the obvious choice.

30.8 Structure elucidation of CAP-7

MS analysis of CAP-7 yielded quasi-molecular ions at m/z: 455 $[M+Na]^+$ and 431 $[M-H]^-$ in the positive ion mode and negative ion mode, respectively (***Figure 30.7***). Thus, the molecular weight of CAP-7 was calculated to be 432 Da.

Figure 30.7 MS spectra from CAP-7



30.8.1 Comparison of CAP-7 with a published compound

Resemblance with previously characterized compounds, strongly suggests that CAP-7 might be similar in structure as well. Due to difficulties in obtaining a single structure for CAP-7 by interpretation of NMR spectra alone, chemical shifts from CAP-7 were compared with chemical shifts from a previously isolated organic compound (***Table 30.12***).

Table 30.12 ^{1}H NMR chemical shifts from CAP-7 compared with published chemical shifts from afzelin

Proton	CAP-1 (δ), multiplicity and coupling constant (Hz)	Published (δ), multiplicity and coupling constant (Hz)
H-6	6.19-6.18 (d, 3.0)	6.15 (d, 2.0)
H-8	6.37-6.36 (d, 3.0)	6.31 (d, 2.0)
H-2′	7.77-7.74 (d, 9.0)	7.73 (dd, 2.8, 8.8)
H-3′	6.94-6.92 (d, 6)	6.89 (dd, 2.8, 8.4)
H-5′	6.94-6.92 (d, 6)	6.89 (dd, 2.8, 8.4)
H-6′	7.77-7.74 (d, 9.0)	7.73 (dd, 2.8, 8.8)
H-1″	5.37 (s)	5.35 (s)
H-2″	4.23 (br s)	4.21 (br s)
H-3″	3.73 (m)	3.71 (m)
H-4″	Approx. 3.30 (m)	3.33 (m)
H-5″	Approx. 3.30 (m)	3.33 (m)
H-6″	0.93-0.91 (d, 6.0)	0.91 (d, 6.0)

Source: Yang, N.Y., et al [34]

Chemical shifts from protons in CAP-7 are fairly similar to chemical shifts from protons in the published organic compound. H-8 displays the least similar chemical shift (δ 6.37-6.36 in CAP-7 and δ 6.31 in the published compound). Multiplicities and coupling constants are practically the same as well. Major differences between CAP-7 and the published compound are found in protons H-2′, H-3′, H-5′ and H-6′. These protons should display double doublets, but they yield only doublets in the ^{1}H NMR spectrum. Perhaps a close-up of the range 8.0-6.5 ppm would have made it possible to detect double doublets in the ^{1}H NMR spectrum instead of only doublets. A total of nine chemically dissimilar protons are present both in CAP-7 and in the published compound. To further prove their similarity, chemical shifts from carbons were also compared (***Table 30.13***).

Table 30.13 ^{13}C NMR chemical shifts from CAP-7 compared with published chemical shifts from afzelin

Carbon no.	CAP-7 (δ)	Published (δ)
2	159.3	158.7
3	136.2	133.9
4	179.6	179.1
5	163.1	162.2
6	99.9	99.8
7	165.8	165.3
8	94.8	94.5
9	158.5	158.3
10	Approx. 105	104.2
1′	122.6	122.5
2′	131.9	132.2
3′	116.5	116.2
4′	161.6	159.4
5′	116.5	116.0
6′	131.9	132.2
1″	103.4	102.5
2″	72.0	71.6
3″	72.1	72.3
4″	73.2	73.2
5″	71.9	71.4
6″	17.6	17.8

Source: Bilia, A.R., et al [35]

A total of nineteen chemically dissimilar carbons were found in CAP-7, while twenty dissimilar carbons are present in the published compound. A reason for this discrepancy might that the chemical shifts of two carbons are so close that they appear as one single peak. Perhaps a close-up of these carbons would display two peaks instead of only one. Chemical shifts between the two compounds are so similar that they are practically the same. C-3 and C-4′ display the most dissimilar chemical shifts (δ 136.2 and δ 161.6 in CAP-7 and δ 133.9 and δ 159.4 in the published compound, respectively). Both comparison of ^{1}H NMR spectra and ^{13}C NMR spectra strongly suggest that CAP-7 is in fact afzelin.

30.8.2 Interpretation of DEPT-135 spectrum

A single DEPT-135 spectrum from CAP-7 can be found between the final pages of this Master's thesis (***Supplementary material 1.14***). The entire chemical shift range was recorded. Interpretation of this DEPT-135 spectrum from CAP-7 was used to confirm similarity (***Table 30.14***).

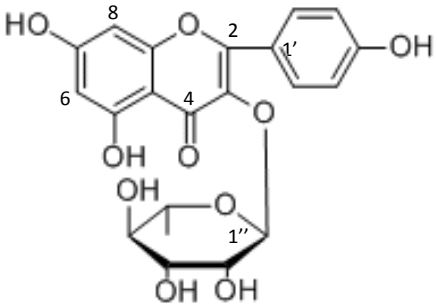
Table 30.14 Interpretation of DEPT-135 spectrum from CAP-7

ppm	Signal	Assignment
179.6	-	Quaternary carbon?
165.8	-	Quaternary carbon?
163.1	-	Quaternary carbon?
161.6	-	Quaternary carbon?
159.3	-	Quaternary carbon?
158.5	-	Quaternary carbon?
136.2	-	Quaternary carbon?
131.9	Positive	CH (aromatic or alkene)
122.6	-	Quaternary carbon?
116.5	Positive	CH (aromatic or alkene)
Approx. 105	-	Quaternary carbon?
103.4	Positive	CH (aromatic or alkene, alkane only if bound to a polar substituent)
99.9	Positive	CH (aromatic or alkene, alkane only if bound to a polar substituent)
94.8	Positive	CH (aromatic or alkene, alkane only if bound to a polar substituent)
73.2	Positive	CH (CH-O-C or CH-OH)
72.1	Positive	CH (CH-O-C or CH-OH)
72.0	Positive	CH (CH-O-C or CH-OH)
71.9	Positive	CH (CH-O-C or CH-OH)
17.6	Positive	CH_3

Table 30.14 suggests that there are as many as nine quaternary carbons present in CAP-7. This number is obviously too high to be correct, due to the fact that majority of carbons are further downfield, which indicates that they are carbonyls, alkenes or aromatics. The same reasons explained for CAP-3 with regards to the number of quaternary carbons present also apply to CAP-7. Due to similarities with previously identified compounds and the number of chemically dissimilar carbons present in CAP-7, it is more likely that CAP-7 consists of only two quaternary carbons. Peaks furthest upfield in the DEPT-135 spectrum strongly suggests that there exists a similar sugar moiety in CAP-7 as those found in CAP-1, CAP-3 and CAP-5. No methylene groups are present.

Afzelin has a molecular formula of $C_{21}H_{20}O_{10}$, thus, the index of hydrogen deficiency is twelve. It consists of two aromatic rings, one conjugated alkene, one sugar moiety, one carbonyl group, two quaternary carbons and one methyl group. In addition, no methylene groups are present and its molecular weight is 432 Da. All of this is consistent with comparisons of chemical shifts from CAP-7 with chemical shifts from afzelin and interpretation of the DEPT-135 spectrum. Thus, CAP-7 was characterized as afzelin (***Figure 30.8***).

Figure 30.8 Molecular structure of afzelin



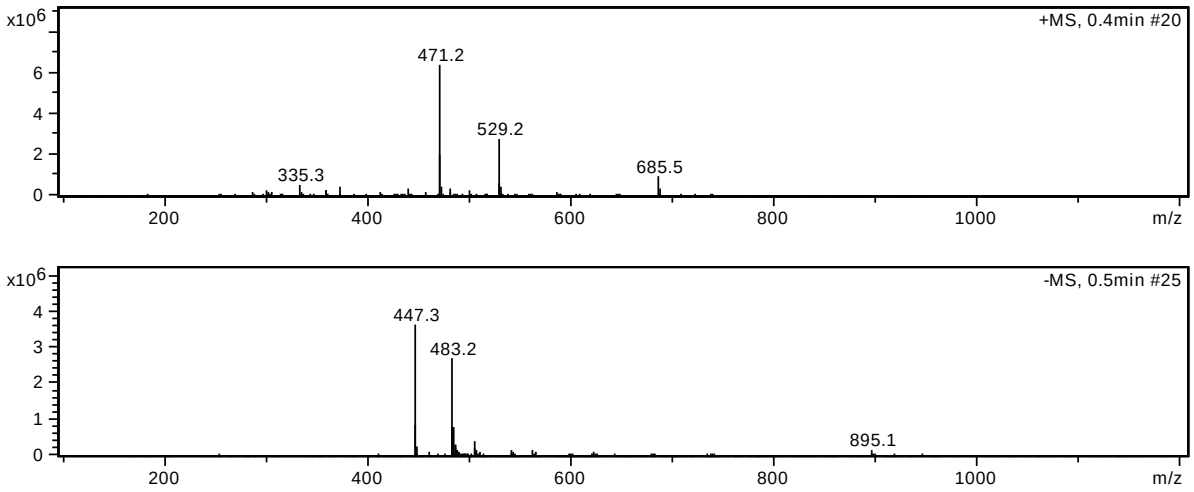
30.9 Structure elucidation of CAP-8

Results from NMR and MS analysis of CAP-8 were inconclusive due to impurities in the sample. Further purification by a simple chromatographic technique would be difficult to perform due to the low weight of the compound (5.0 mg). Thus, CAP-8 was not characterized.

30.10 Structure elucidation of CAP-9

MS analysis of CAP-9 yielded quasi-molecular ions at m/z: 471 $[M+Na]^+$ in the positive ion mode and quasi-molecular ions at m/z: 447 $[M-H]^-$ and 895 $[2M-H]^-$ in the negative ion mode (***Figure 30.9***). Thus, the molecular weight of CAP-9 was calculated to be 448 Da. This is the same molecular weight of CAP-5, which was identified as quercitrin.

Figure 30.9 MS spectra from CAP-9



30.10.1 Comparison of CAP-9 with a published compound

Since CAP-5 and CAP-9 yielded the same peaks on the MS spectra, it was natural to compare their NMR spectra as well. Not only were their MS spectra the same, but also their NMR spectra displayed a high degree of similarity. After such a comparison was made, the initial thought was that CAP-5 and CAP-9 was in fact the same compound. Thus, as for CAP-5, chemical shifts from CAP-9 were compared to chemical shifts from quercitrin (***Table 30.15***).

Table 30.15 ^{1}H NMR chemical shifts from CAP-9 compared with published chemical shifts from quercitrin

Proton	**CAP-9 (δ), multiplicity and coupling constant (Hz)**	**Published (δ), multiplicity and coupling constant (Hz)**
H-6	6.20-6.19 (d, 3)	6.15 (d, 1.6)
H-8	6.37 (d, N/A)	6.32 (d, 2.0)
H-2′	7.35-7.34 (d, 3)	7.29 (d, 2.1)
H-5′	6.93-6.90 (d, 9)	6.87 (d, 8.4)
H-6′	7.32-7.29 (d, 9)	7.26 (dd, 2.1, 8.0)
H-1″	5.35 (s)	5.31 (br s)
H-2″	4.22 (s)	3.96 (br s)
H-3″	3.78-3.74 (m)	3.33 (m)
H-4″	3.34 (m)	3.09 (m)
H-5″	3.34 (m)	3.09 (m)
H-6″	0.94-0.92 (d, 6)	0.79 (d, 5.6)

Source: Yang, N.Y., et al [34]

A total of ten chemically dissimilar protons are present in the published compound, which is exactly the same number of chemically dissimilar protons in CAP-9. Chemical shifts on both compounds come somewhat close to one another. H-2″, H-3″, H-4″ and H-5″ display the least similar chemical shifts (δ 4.22, δ 3.78-3.74, δ 3.34 and δ 3.34 in CAP-9 and δ 3.96, δ 3.33, δ 3.09 and δ 3.09 in the published compound, respectively). These large differences in chemical shifts might be attributed to deviations in spectrometer measurements and impurities in the sample. It is also worth mentioning that chemical shifts from H-2″, H-3″, H-4″ and H-5″ in CAP-5 come much closer to CAP-9 than the published compound, which strengthens similarity between CAP-9 and quercitrin since CAP-5 has already been identified as quercitrin. Almost all multiplicities are in accordance with the published compound. Even small coupling constants are detected in the ^{1}H NMR spectrum, many of which were not detected in the ^{1}H NMR spectrum from CAP-5. Peaks with larger coupling constants are assigned both multiplicities and coupling constants that are in agreement with the published compound. To further prove similarity between CAP-9 and quercitrin, chemical shifts from carbons were also compared (***Table 30.16***).

Table 30.16 ^{13}C NMR chemical shifts from CAP-9 compared with published chemical shifts from quercitrin

Carbon no.	CAP-9 (δ)	Published (δ)
C-2	159.3	159.2
C-3	136.2	136.1
C-4	179.6	179.4
C-5	163.1	162.9
C-6	99.8	99.7
C-7	165.8	165.7
C-8	94.7	94.7
C-9	158.5	158.4
C-10	105.9	105.8
C-1′	123.0	122.9
C-2′	116.9	116.9
C-3′	146.3	146.3
C-4′	149.7	149.6
C-5′	116.4	116.3
C-6′	122.9	122.7
C-1″	103.5	103.4
C-2″	72.0	71.9
C-3″	72.1	72.0
C-4″	73.2	73.2
C-5″	71.9	71.8
C-6″	17.6	17.7

Source: Yang, N.Y., et al [34]

A total of twenty-one chemically dissimilar carbons are present in the published compound, which is exactly the same number of chemically dissimilar carbons present in CAP-9. Chemical shifts from the carbons in CAP-9 and chemical shifts from the carbons in the published compound are so similar that they are practically the same. The largest difference in chemical shift between two similar carbons is δ 0.2, which is regarded as a minuscule, insignificant difference. Thus, ***Table 30.16*** strongly suggests that CAP-9 and CAP-5 is the same compound, meaning that they both can be identified as quercitrin.

30.10.2 Interpretation of DEPT-135 spectrum

A single DEPT-135 spectrum from CAP-9 can be found as the last page of this Master's thesis (***Supplementary material 1.17***). Only carbons with chemical shifts from 140-15 ppm were recorded. Interpretation of this DEPT-135 spectrum from CAP-9 was used to confirm similarity (***Table 30.17***).

Table 30.17 Interpretation of DEPT-135 spectrum from CAP-9

ppm	Signal	Assignment
136.2	-	Quaternary carbon
123.0	-	Quaternary carbon?
122.9	Positive	CH (aromatic or alkene)
116.9	Positive	CH (aromatic or alkene)
116.4	Positive	CH (aromatic or alkene)
105.9	-	Quaternary carbon
103.5	Positive	CH (aromatic or alkene, alkane only if bound to a polar substituent)
99.8	Positive	CH (aromatic or alkene, alkane only if bound to a polar substituent)
94.7	Positive	CH (aromatic or alkene, alkane only if bound to a polar substituent)
73.2	Positive	CH (CH-O-C or CH-OH)
72.1	Positive	CH (CH-O-C or CH-OH)
72.0	Positive	CH (CH-O-C or CH-OH)
71.9	Positive	CH (CH-O-C or CH-OH)
17.6	Positive	CH_3

There exists a possibility that CAP-9 consists of three quaternary carbons. However, there also exists a possibility that two carbons have come so close on the DEPT-135 spectrum that they have been detected as one single carbon. In addition, three quaternary carbons would require detection of more carbons on the ^{13}C NMR spectrum. Thus, CAP-9 is more likely to consist of only two quaternary carbons. CAP-9 consists mostly of aromatics and/or alkenes. Only a small portion of carbons are aliphatics, strongly suggesting the presence of a sugar moiety. Also there are no methylene groups present.

Quercitrin, as previously mentioned, consists of two aromatic rings, one conjugated alkene, one sugar moiety, one carbonyl group, two quaternary carbons and one methyl group. This is all consistent with comparison of NMR spectra and interpretation of the DEPT-135 spectrum. CAP-9 and quercitrin also share the same molecular weight. Thus, CAP-9 was also characterized as quercitrin (***Figure 30.6***).

31. End results

31.1 Summary of findings

A total of nine compounds were isolated in the isolation process. Two of these were found to be the same compound on the basis of final TLC results, namely CAP-6 and CAP-7. Thus, only eight compounds were analyzed by MS and NMR. Comparison of MS and NMR spectra led to the discovery that CAP-5 and CAP-9 was the same compound as well. In addition, three out of the eight compounds sent for structure analysis were not pure enough to allow for structure characterization. As a result, out of the nine initially isolated and purified compounds, only four structurally dissimilar compounds were finally characterized (***Table 31.1***).

Table 31.1 Isolated and identified compounds

Name	Identified as	Weight (mg)
CAP-1	Kaempferol-3-O-(2″, 4″-di-E-p-coumaroyl)-α-L-rhamnopyranoside	69.1
CAP-2	N/A	11.7
CAP-3	Kaempferol-3-O-α-L-(4″-E-p-coumaroyl)-rhamnoside	75.7
CAP-4	N/A	149.9
CAP-5	Quercitrin	85.3
CAP-6	Afzelin	65.6
CAP-7	Afzelin	23.0
CAP-8	N/A	5.0
CAP-9	Quercitrin	15.6

Conclusion

Compounds originating from *Abies pindrow* branches and leaves were isolated and characterized. *Abies pindrow* is utilized in Ayurveda for the treatment of fever, inflammatory diseases, bronchitis and other ailments. Studies of *Abies pindrow* extracts have in fact been shown to exert therapeutic activity in preclinical animal models. As a consequence; isolation, characterization, screening and preclinical testing of compounds from this plant species could potentially lead to the discovery of novel lead therapeutic compounds. [28]

A series of extractions and an initial silica gel column chromatography was performed by Dr. Li Yong-Li prior to my involvement in the purification process (***Figure 24.1***). His work yielded three separate fractions, namely Fraction 1, Fraction 2 and Fraction 3. These fractions were purified in a sequential manner through the use of simple chromatographic techniques. A total of four compounds were isolated from Fraction 1 (***Figure 25.1***), one compound was isolated from Fraction 2 (***Figure 26.1***) and four compounds were isolated from Fraction 3 (***Figure 27.1***).

Interpretation of MS spectra and NMR spectra led to the discovery that two out of five samples that were pure enough for structure determination, namely CAP-5 and CAP-9, were the same compound. Thus, a final count of four structurally dissimilar compounds were isolated and identified in this Master's thesis. More precisely: CAP-1 was identified as kaempferol-3-O-(2″, 4″-di-E-p-coumaroyl)-α-L-rhamnopyranoside (***Figure 30.2***); CAP-3 was identified as kaempferol-3-O-α-L-(4″-E-p-coumaroyl)-rhamnoside (***Figure 30.4***); CAP-5 and CAP-9 were identified as quercitrin (***Figure 30.6***); and CAP-7 was identified as afzelin (***Figure 30.8***).

All identified compounds belong to a class of plant secondary metabolites called flavonoids. Humans are unable to synthesize flavonoids. Previous studies have shown that these compounds display antioxidant, anti-inflammatory and other cardiovascular protective properties in humans, even in small amounts. Thus, flavonoids are being extensively studied with regards to their potential therapeutic effects on human diseases, including cancer. The flavonoids isolated in this Master's thesis are more specifically called flavonoid glycosides. [36, 37]

Unfortunately, % yield of identified compounds in the branches and leaves of *Abies pindrow* is impossible to calculate due to the fact that no dry weight of material was provided. Only four structurally dissimilar compounds were identified although a total of nine compounds were initially isolated. All of the characterized compounds have previously been reported in the literature. The fact that three of the isolated compounds were not pure enough for structure elucidation could perhaps have been avoided by the use of more advanced chromatographic techniques such as HPLC, gas chromatography (GC) or counter current chromatography. Simple chromatographic techniques such as those described in this Master's thesis are most commonly employed at the MRCTCM.

Characterized compounds from this Master's thesis will be incorporated to the Natural Products Library at the MRCTCM. The MRCTCM has developed an MDL database to provide a platform for professors, scientists and students to update and add new information about their compounds and to allow for easy access of all the research material performed at the MRCTCM.

Larger amounts of compounds would be required to perform both *in vitro* screening and preclinical testing of the characterized compounds in this Master's thesis. Thus, future work with these flavonoid glycosides depends on the isolation of larger quantities of material. If sufficient quantities are

obtained, future work could involve the study of these compounds on a molecular and on a cellular level. Hopefully, antitumor activity from initial screening tests would support further investigation in animal models.

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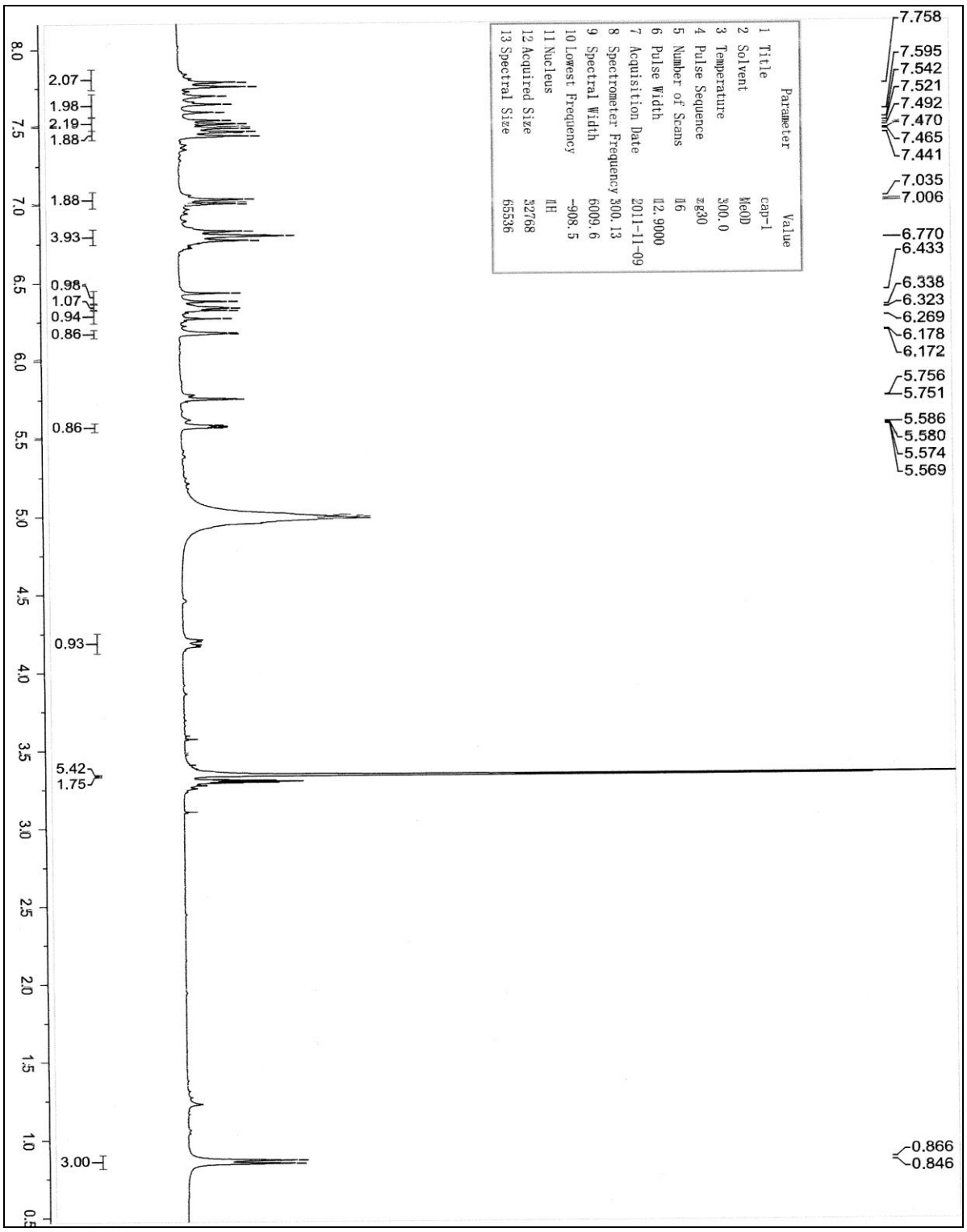
Supplementary material 1.1



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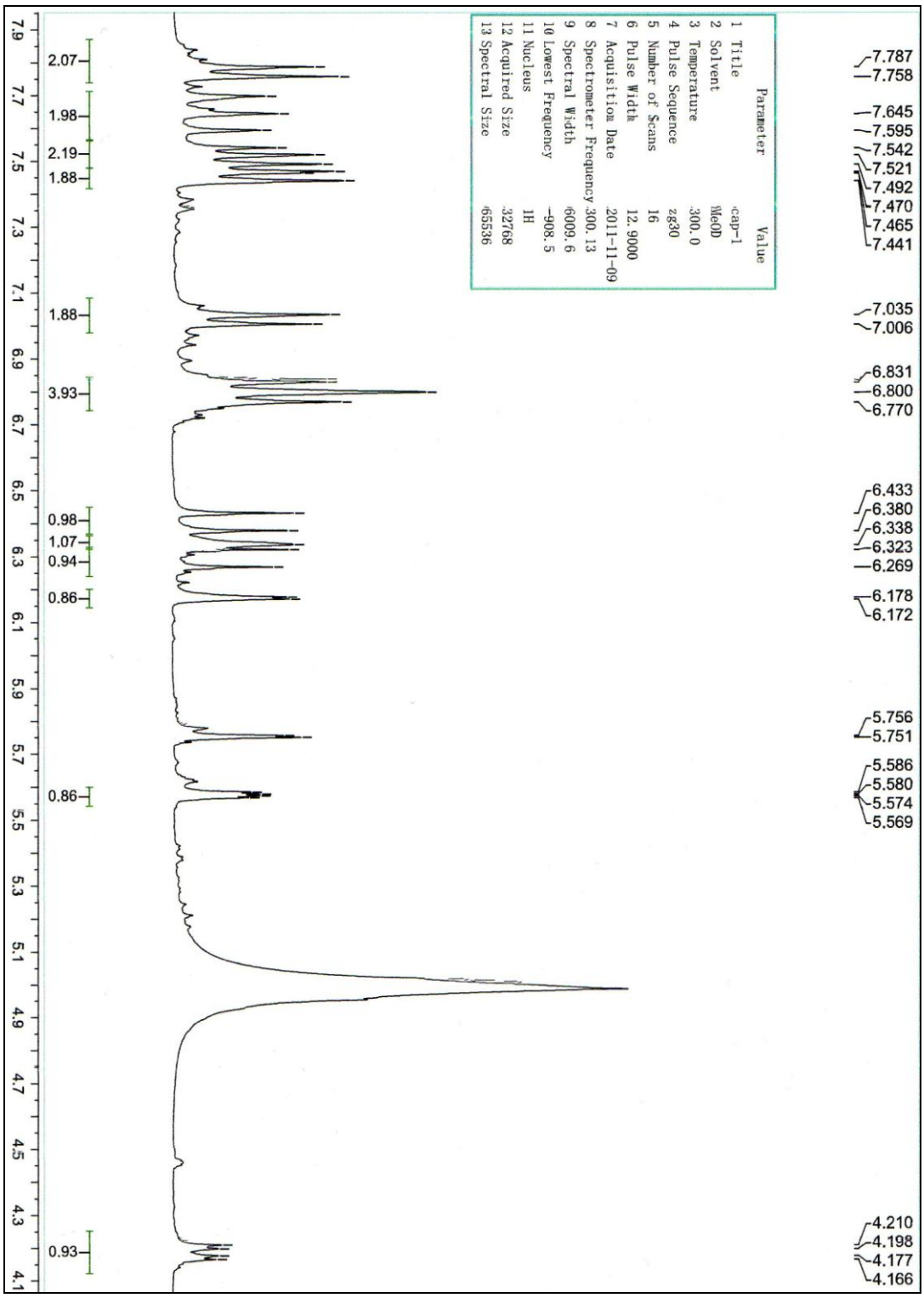

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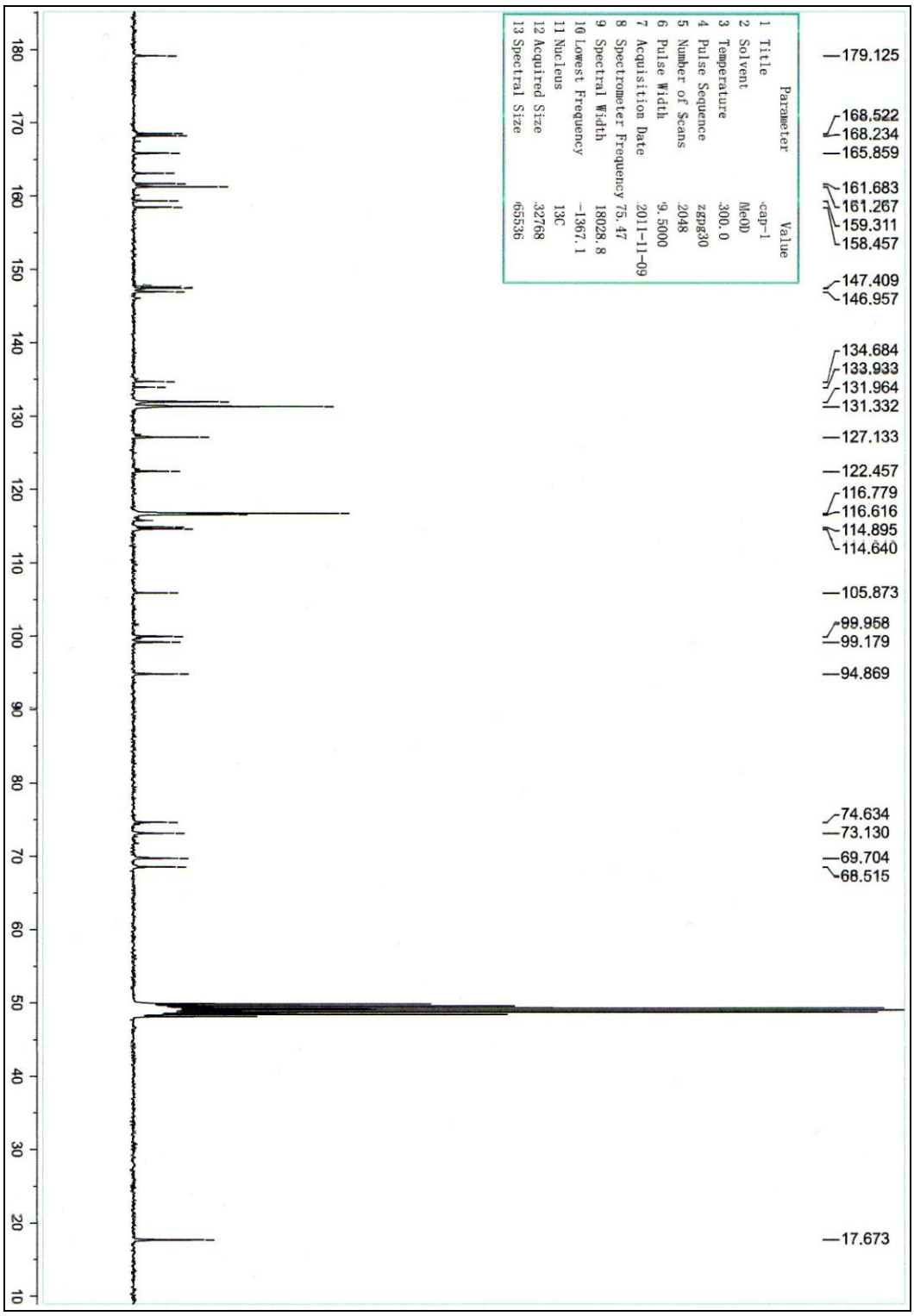

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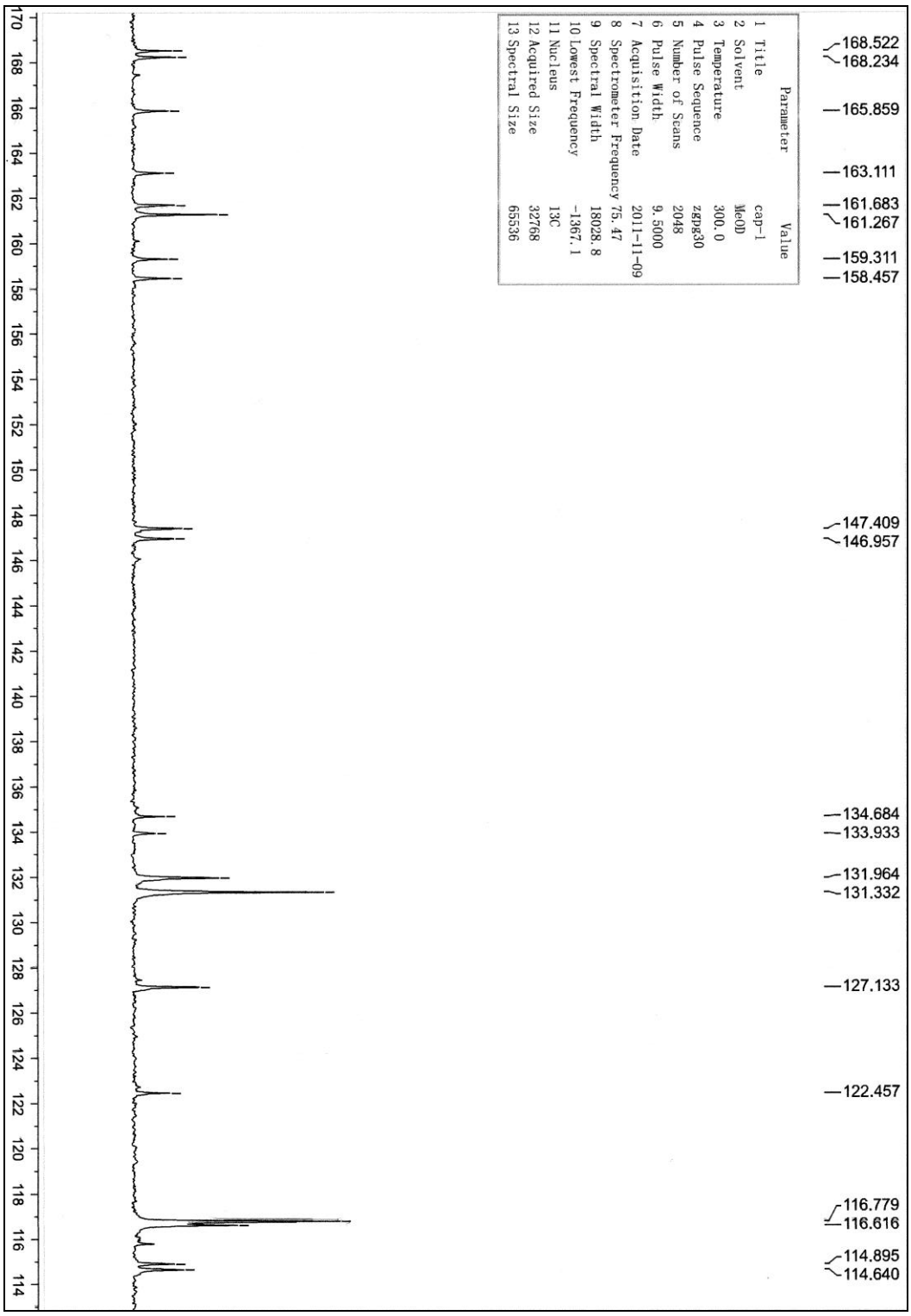

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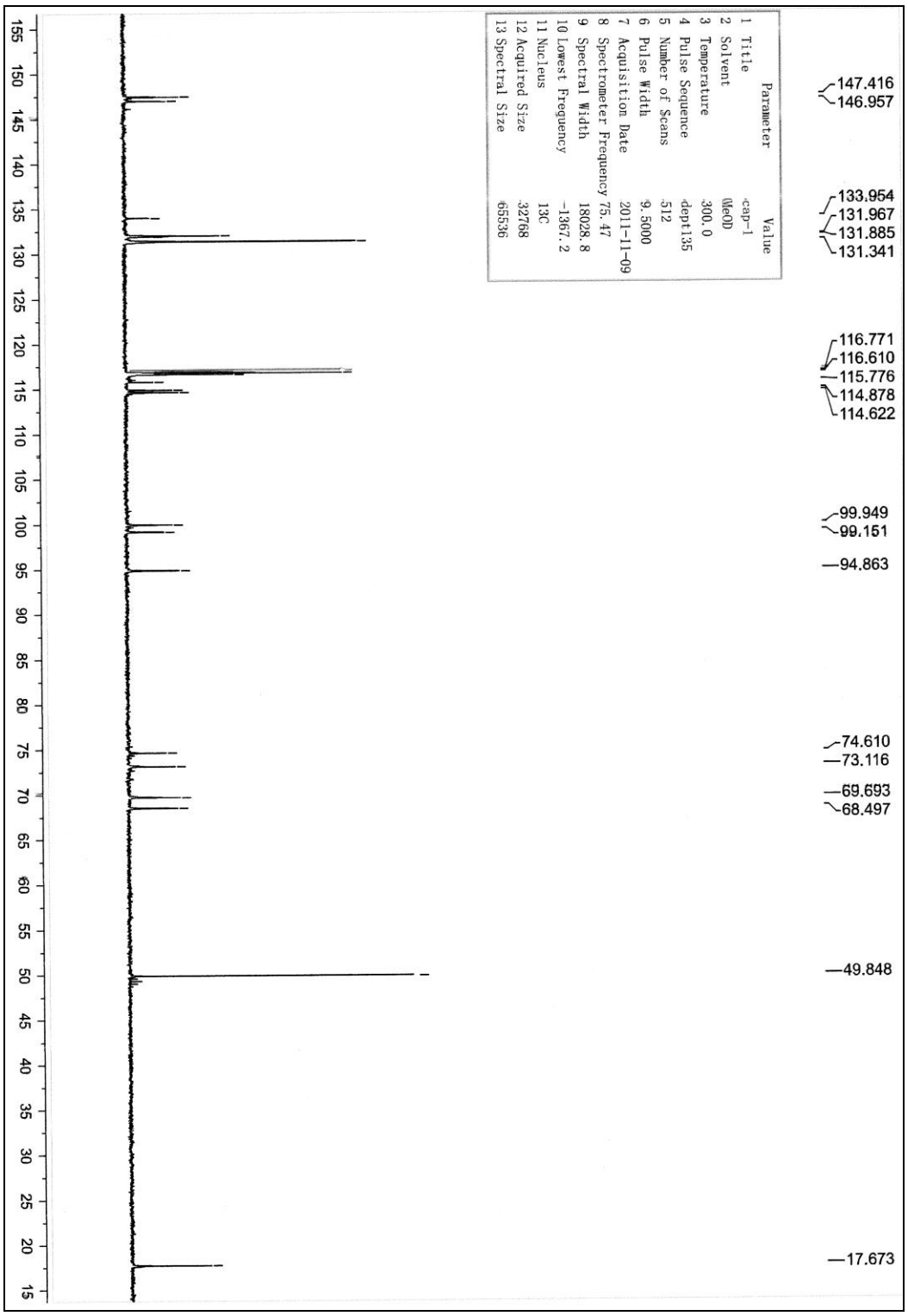

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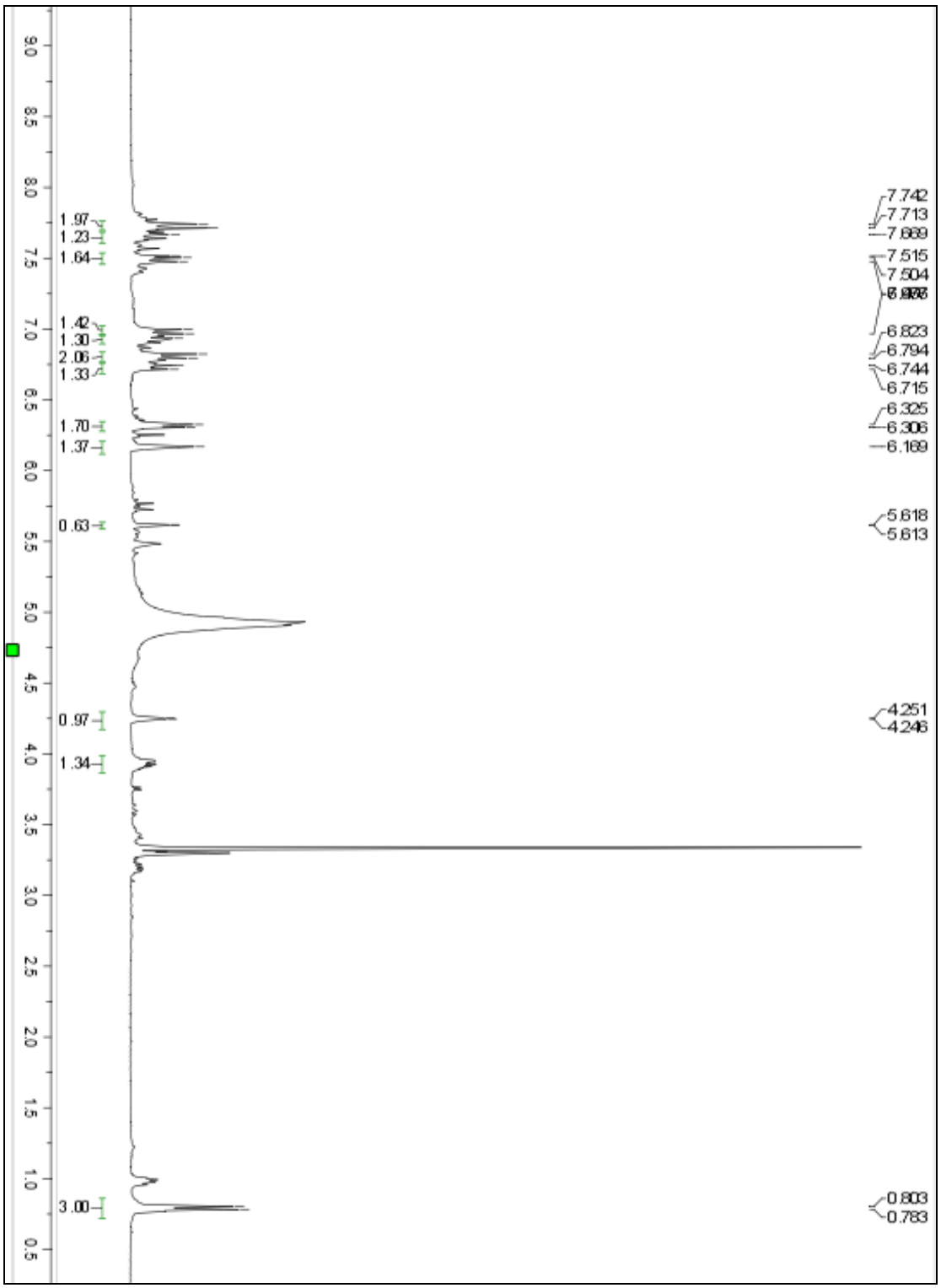

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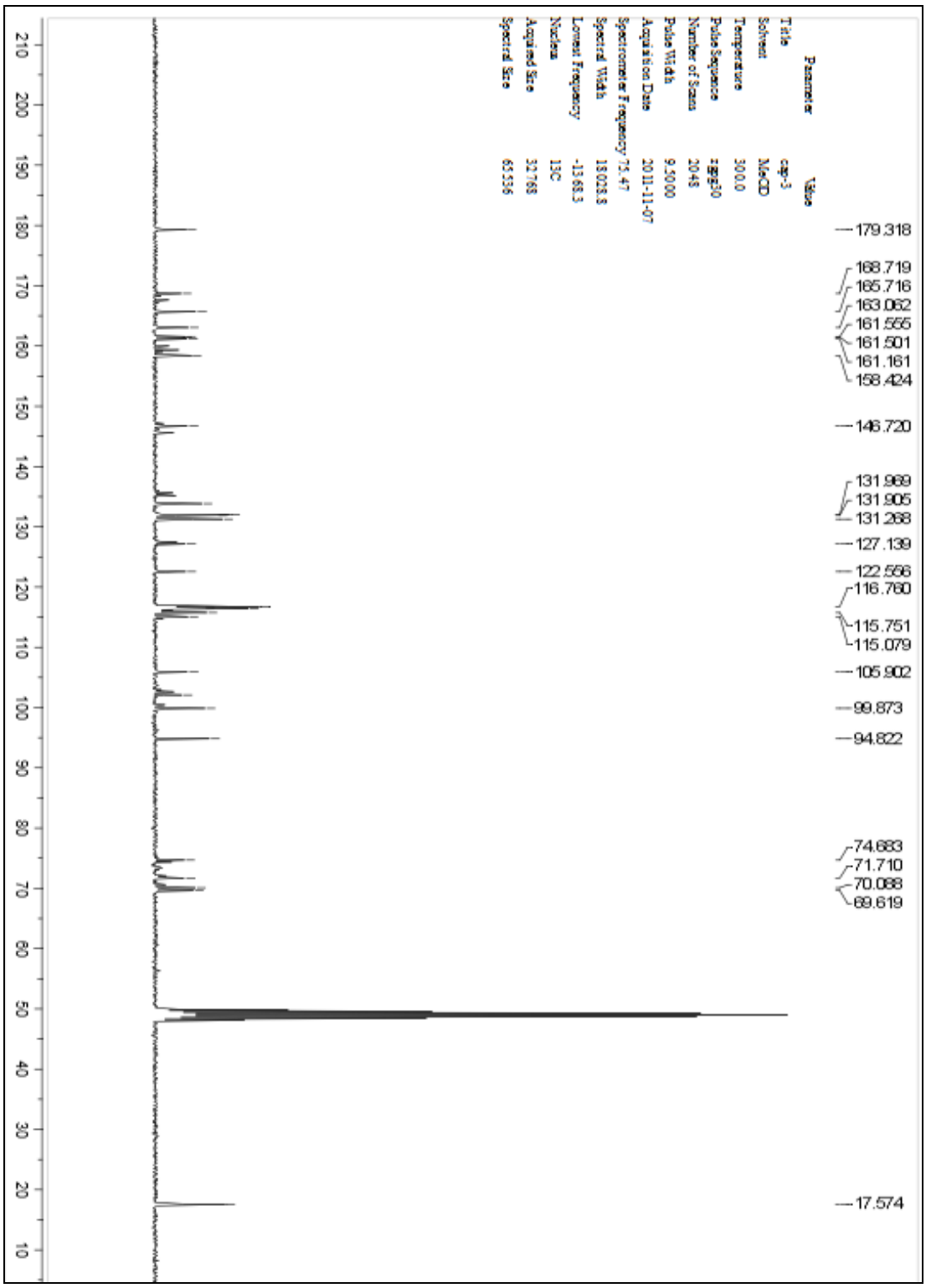

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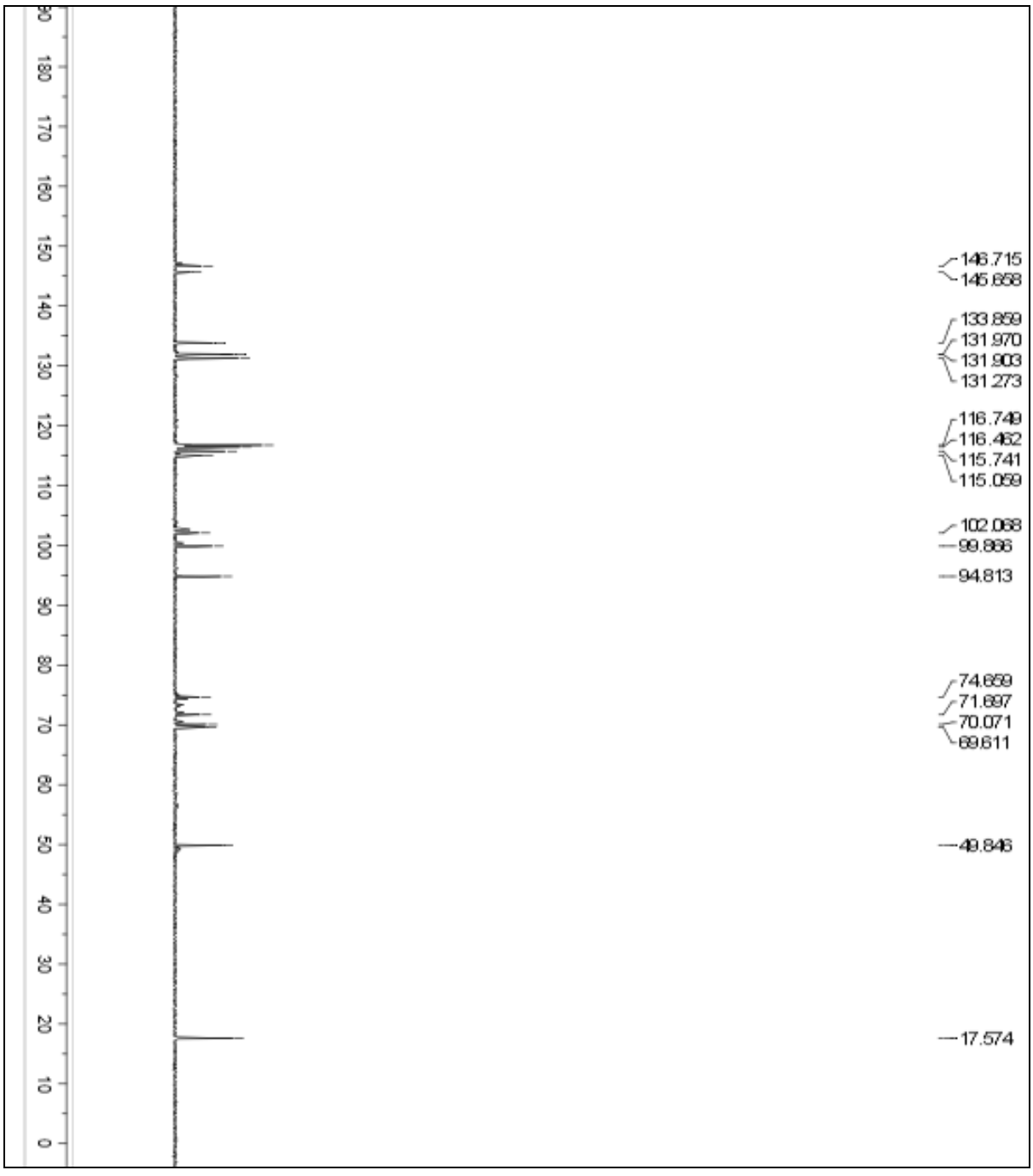

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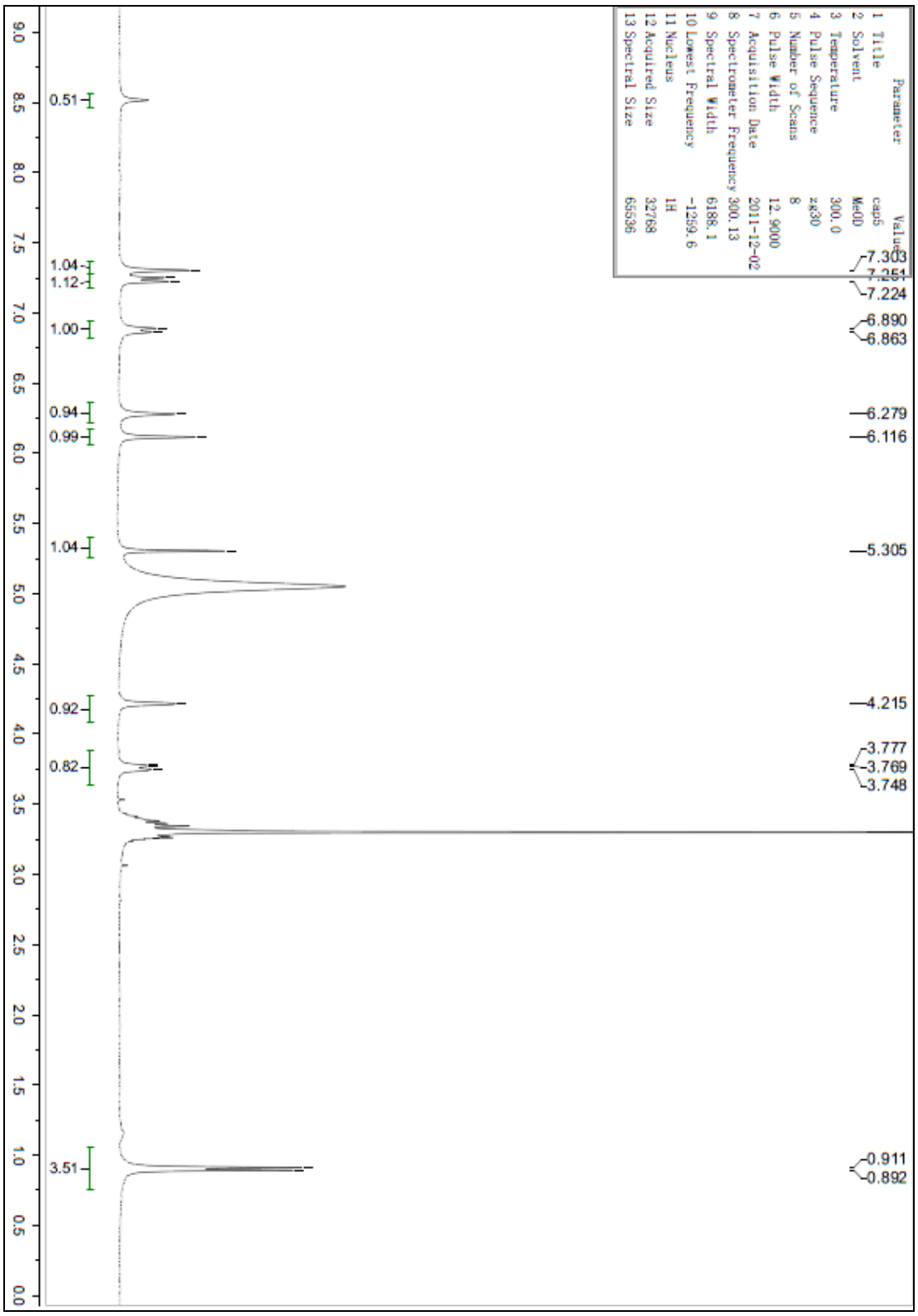

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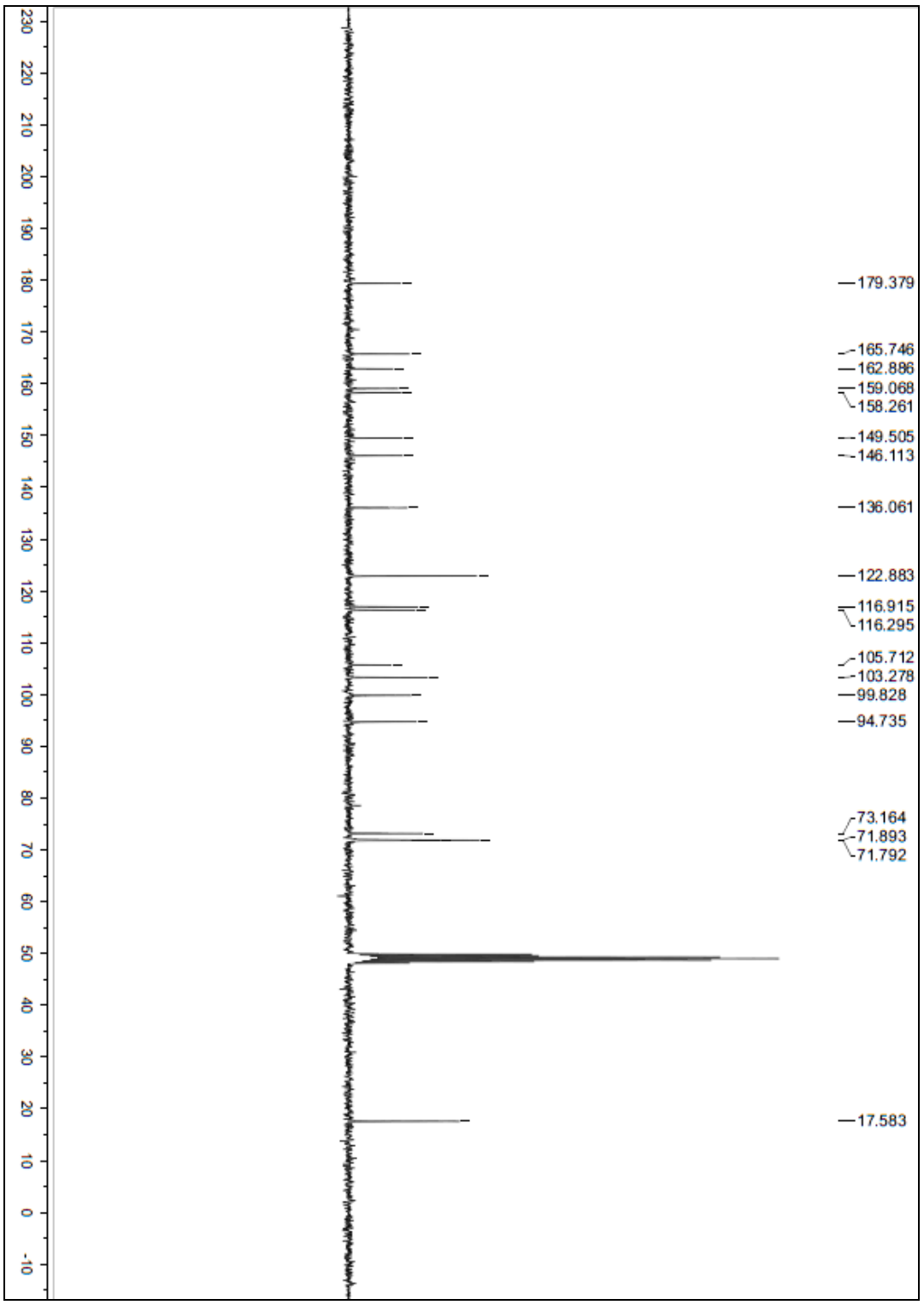

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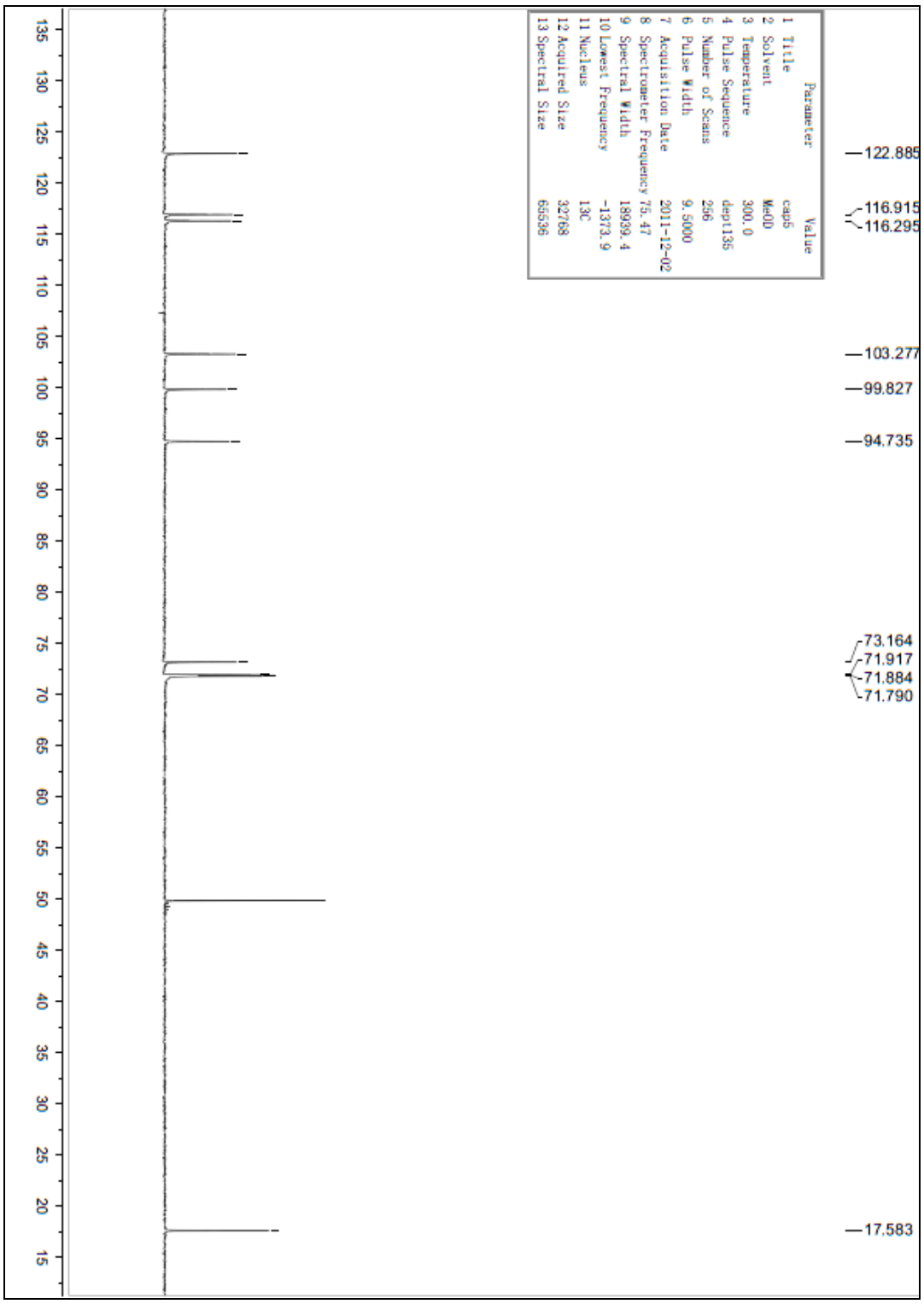

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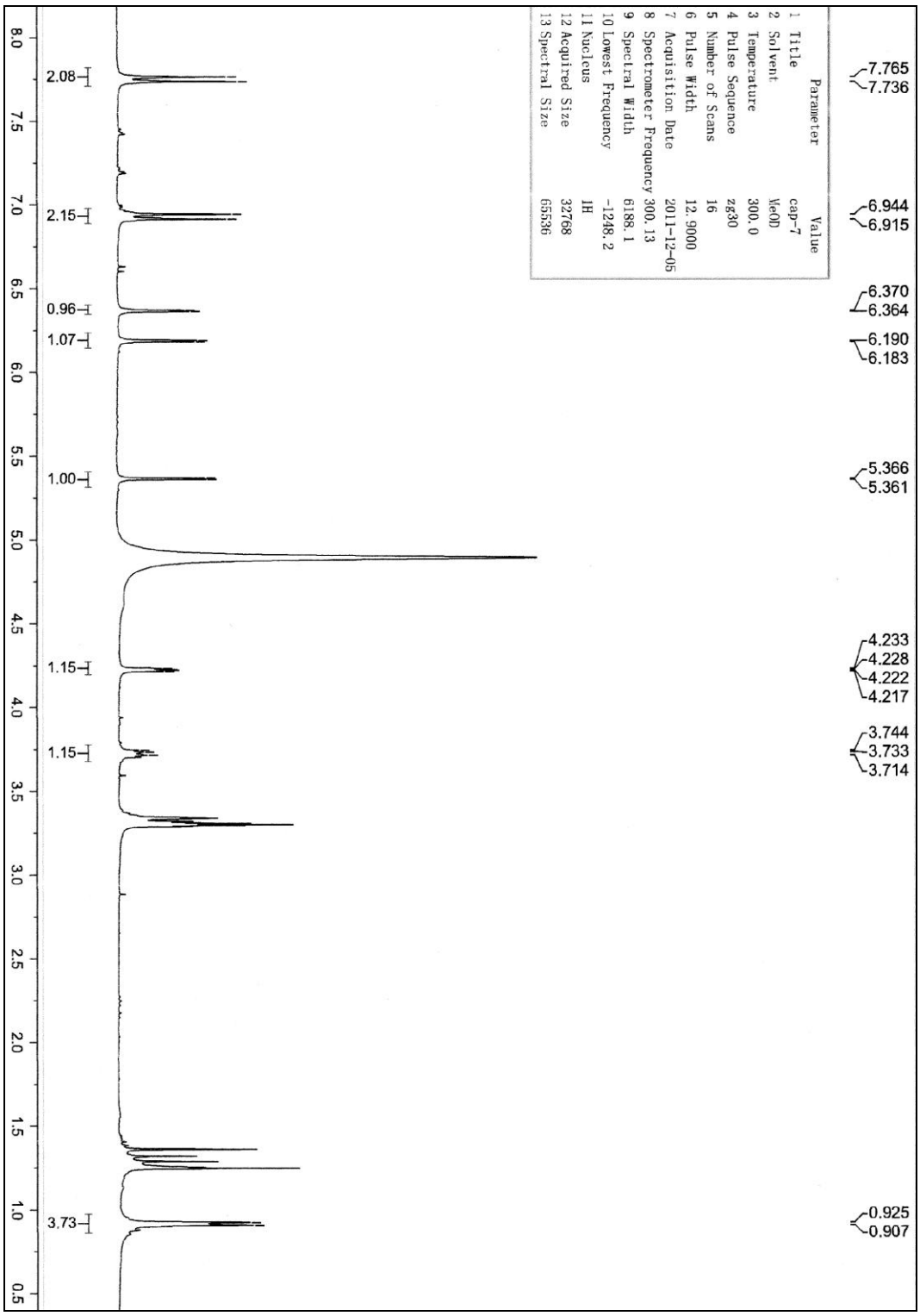

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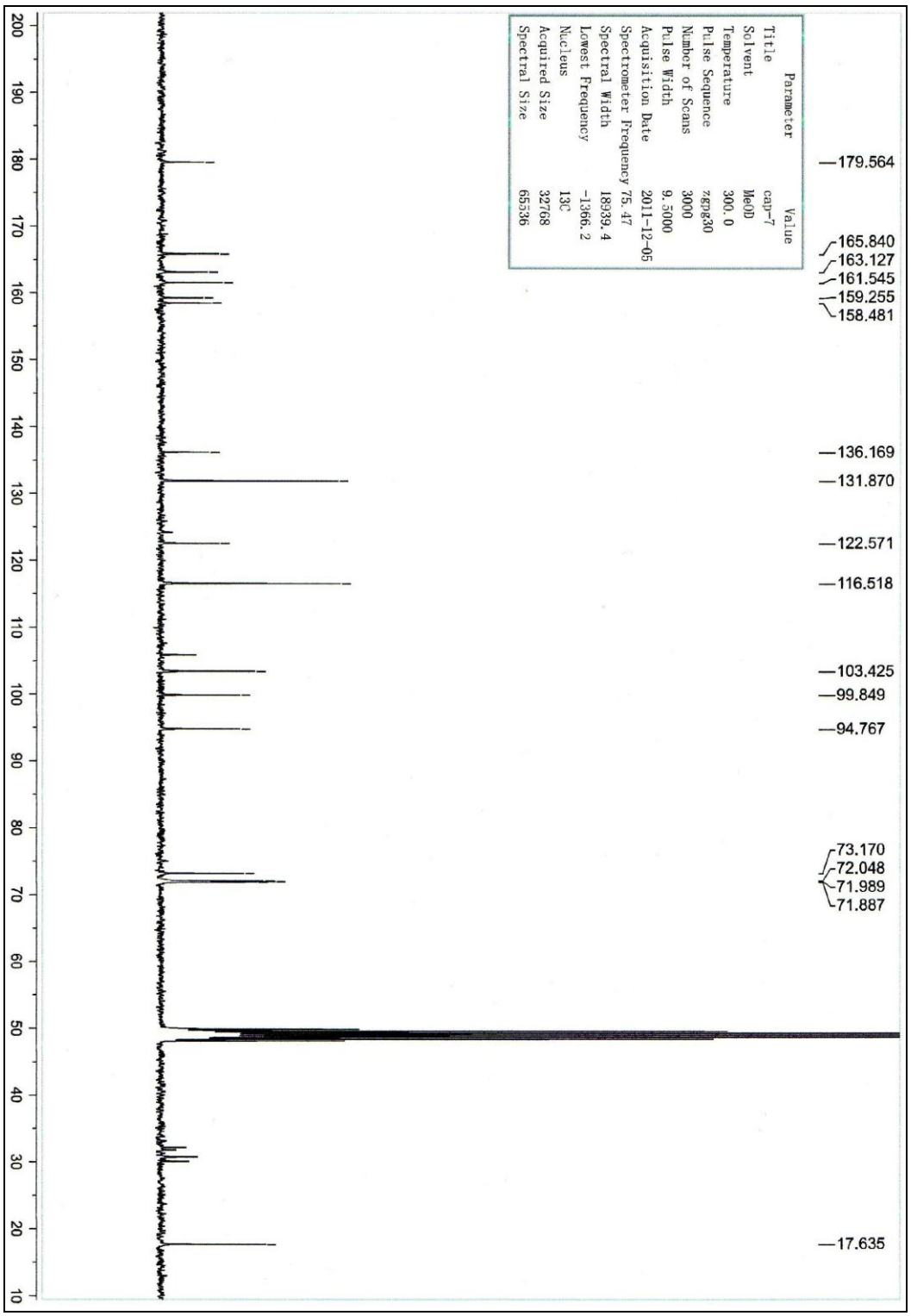

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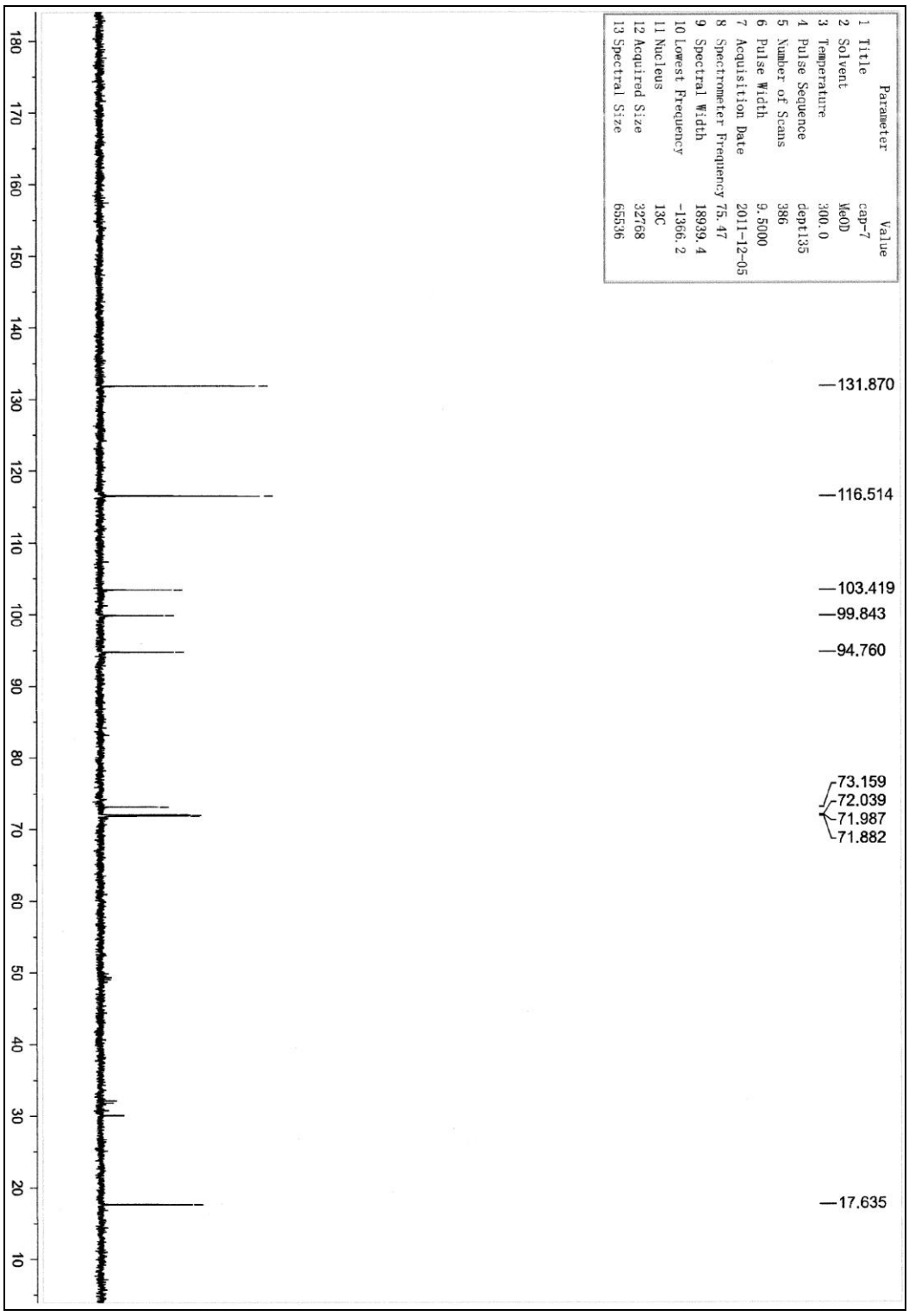

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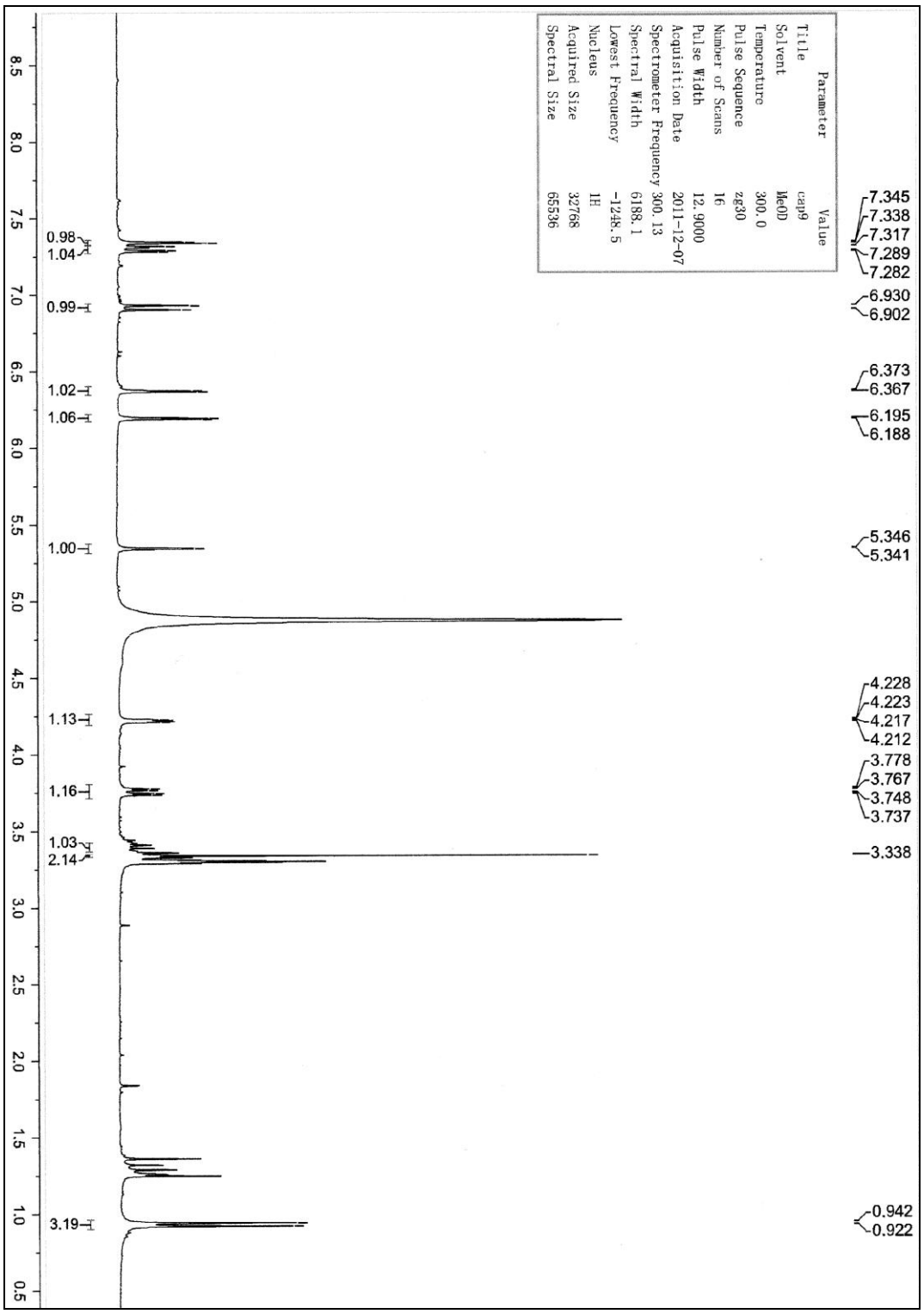

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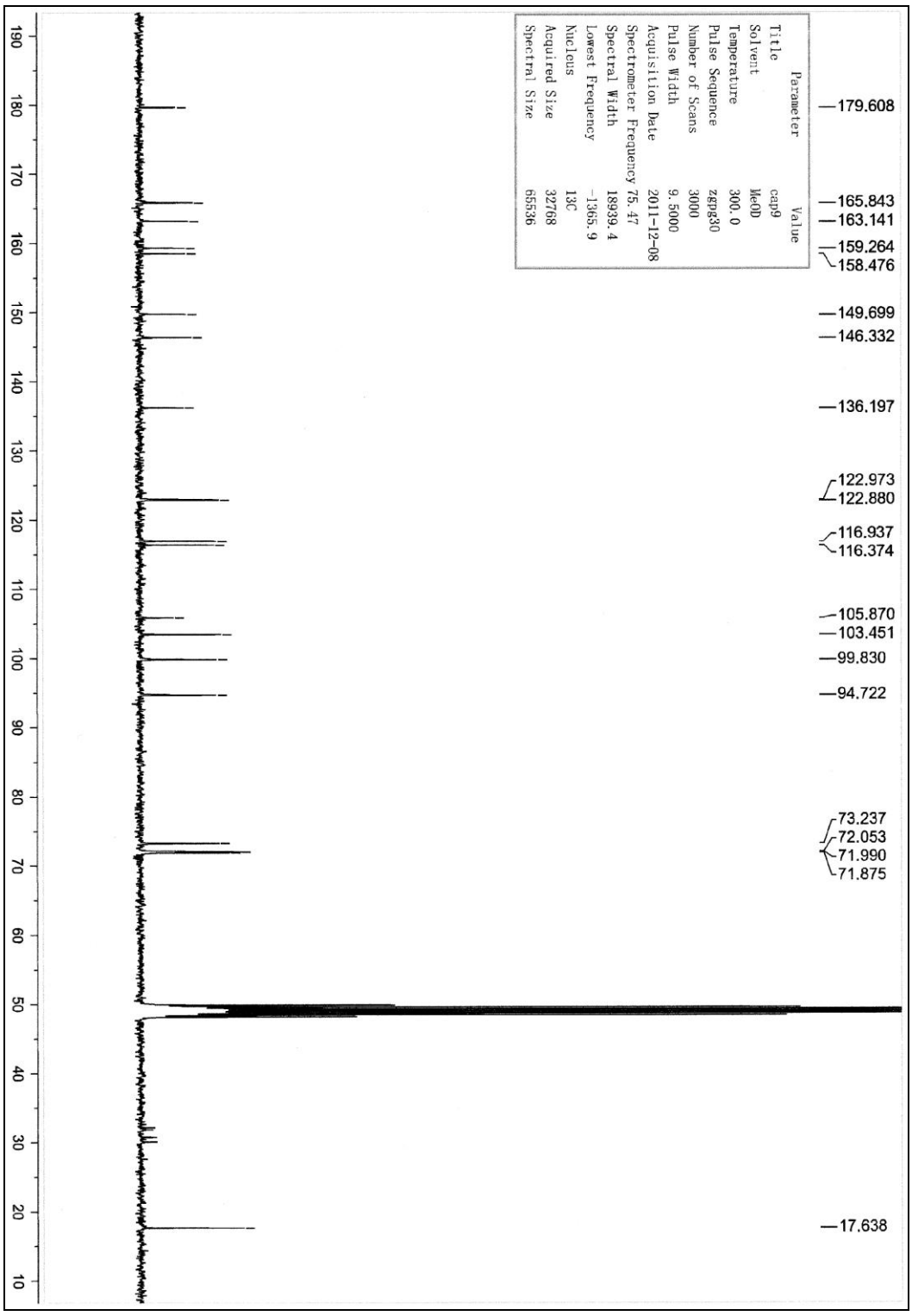

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