# The extraction of high value chemicals from heather (*Calluna vulgaris*) and bracken (*Pteridium aquilinum*)

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# Abstract

The aim of the project is to extract and identify high value chemicals with potential applications as pharmaceutical drugs or precursors from heather (*Calluna vulgaris*) and bracken (*Pteridium aquilinum*). Supercritical carbon dioxide extracts of heather were analysed for their triterpenoid content. Several triterpenoids were identified in heather supercritical carbon dioxide extracts and many of them were already reported to have potential valuable biological activities. Seasonal variation of total triterpenoid content in heather was also evaluated. The result indicated that summer flowering heather contains the highest concentration of total triterpenoid (15400 μg/g dry plant) and spring heather picked in March exhibited the second highest total triterpenoid content (11200 μg/g dry plant). Optimal harvest and extraction time of heather is also identified based on its triterpenoid seasonal variation trend.

In this study, supercritical carbon dioxide extraction of bracken was achieved for the first time. Naturally occurring pterosin B and pterosin F were identified in bracken supercritical $CO_2$ extract and the seasonal variation in pterosin content and other major compounds in bracken were also evaluated. Bracken crozier was found to contain the highest content of pterosins, and the pterosin content reduced dramatically when the frond grew towards its maturity. Geographical difference was also found to cause significant variation in the level of pterosins. It was found that Welsh bracken contains much less pterosin B and pterosin F compared to the Yorkshire sample. This distinct difference may be due to different pH value of the soil in Kilburn (Yorkshire) and Wales. Pterosins were recently proved to have strong anti-diabetic and anti-obesity activities. Although pterosin B and F were not identified to have certain anti-diabetic or anti-obesity activities in previous research, these pterosins, especially pterosin F, have high possibility to be used as precursors to other more effective pterosins because of its active chlorinated side chain.

# List of Contents

# List of tables

# List of illustrations

# Acknowledgments

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# Declaration

Some of the results presented in this thesis were obtained by, or in collaboration with other workers, who are fully acknowledged in the text. All the other results are the work of the author.

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# *Heather and bracken as potential resource of high value chemicals*

# Chapter 1

## 1.1 Project background

It is acknowledged that moorland habitats dominated by heather (*Calluna vulgaris)* and related undershrubs are of high conservation value within the European Union and especially the United Kingdom.[1] British moorland represents 20% of the European resource of this high conservation value community.

British upland moorlands are usually designated as National Parks for the sake of protecting vegetation and fauna. The North York Moors National Park contains the largest patch of continuous heather moorland (50,100 ha) in England, representing over 10% of the country's resource.[1] Within the National Park 88% (44,088 ha) of moorland habitat is designated as a Site of Special Scientific Interest (SSSI), Special Protection Area (SPA) and Special Area for Conservation (SAC). Dry heath vegetation covers 26,500 ha and forms the main land cover on the western, southern and central moors. Heather or ling is the dominant species over this entire area with patches of bell heather. Heather is also abundant on the wet heath (8,700 ha), which occurs on moister soils. The most noticeable value of heather is as bird habitats for breeding and feeding. To suit the bird's different phases of growth, moorland needs to be cut to various heights. Burning, alongside grazing, is still the predominant method of managing moorland vegetation.[2] If more value can be added to this undeveloped asset, then the farmers in upland areas or the national parks authority could harvest it rather than burn it. The extraction of chemicals from such plants by environmental benign techniques would hopefully give a new life to this generally considered waste resource.

As well as heather, bracken fern (*Pteridium aquilinum*) is a widely distributing species which dominates the vegetation of large areas in many UK upland regions. The amount of land dominated by bracken range from 2880 $km^2$, which is

approximately 1.3% of the land area of Great Britain, to 6361 $km^2$, 2.8% of the land area.[3] The total area of bracken is estimated at 12,300 ha (24.6%) in North York Moors National Park. Bracken is a major weed problem in Britain, particularly in the uplands of the west and north. The major ecological effect of an expansion of bracken is the loss of large areas of semi-natural vegetation. The loss of heather moorland through bracken invasion is viewed as disastrous especially for the upland fauna which is already affected by fragmentation of moorland habitat. Moreover, the continuing spread of bracken can be seen as a threat to farming in the uplands as well. The increasing area under bracken could lead to overgrazing elsewhere, which could help the spread of bracken and also accelerate moorland degradation to grass.

Bracken fern is a perennial fern, it's also one of the higher plants known to cause cancer naturally in animals.[4] In the past it was an important rural resource and was used for animal bedding, thatch, potash, soap and compost. Since the rural and industrial applications declined, it no longer regarded as an asset.[5] Meanwhile, a number of well-recognized toxic effects on livestock have been known since the end of the 19th century. In cattle, it can cause a syndrome named cattle bracken poisoning. It can induce urinary bladder cancer, and can also initiate thiamine deficiency in pigs and horses, haemorrhagic syndrome in cattle, bright blindness in sheep and upper gastrointestinal tracts tumour in all ruminants.[4]

Currently the use of the herbicide Asulam (methyl *N*-[(4-aminophenyl)sulfonyl] carbamate) is most widely used for bracken control.[3] It has a low toxicity for animals and affects few other plants; however it still has some disadvantages such as high cost and spray drift which can affect populations of other ferns. Bracken can also be controlled by mechanical means such as cutting or rolling. This method can be well-targeted at bracken fronds to prevent spread, however it requires to be carried out regularly, e.g. twice a year for 12 years, to gives good control but not elimination.[3] If

any valuable compounds can be extracted from bracken with a potential to be applied as pharmaceutical drugs or precursors, more value would be added to this plant which is currently viewed as a problem.

The major toxin and carcinogen has been shown to be ptaquiloside, a nor-sesquiterpene glycoside which can be present in bracken at high concentration.[6] Its hydrolysate pterosins are a large group of biologically active sesquiterpenes. Although none of the pterosins were found to be carcinogenic, some of them were reported to be cytotoxic to HeLa cells.[7] Other properties such as antimicrobial[8] and smooth muscle relaxant activity[9] of some pterosins were also identified in the last decade. Because of these properties and the link between pterosins and bracken toxin, synthetic methods of different pterosins were widely researched. Since pterosins can be easily converted from the unstable precursor ptaquiloside by mild acid or base treatment, from a green chemistry perspective it is better to develop a conversion route from ptaquiloside to certain pterosins rather than complex synthesis using large quantities of chemicals and catalysts. The high content of ptaquiloside may afford a large scale of preparation of the pterosins without producing much chemical waste.

Figure 1.1 *Calluna vulgaris*

Figure 1.2 *Pteridium aquilinum*

## 1.2 Project Aims

The core aim and focus of this project was to extract and characterise natural chemicals with high value from heather (*Calluna vulgaris*) and bracken (*Pteridium aquilinum*) using green chemical technologies that can be used directly as bioactive molecules or as precursors for new pharmaceuticals. These technologies used include supercritical fluid extraction in combination with other benign solvents. The extracts were analysed using a number of analytical techniques such as GC, GC-MS, HPLC, LC-MS and Supercritical Fluid Chromatography.

Summary of aims:

- Extraction of high value chemicals with biological activities such as triterpenoids from heather using supercritical carbon dioxide extraction and conventional green solvent extraction.
- Extraction of pterosins with potential pharmaceutical applications from bracken and analysis of conversion routes to specific molecules.
- Investigate environmental benign co-solvents to enhance the selectivity of $CO_2$ to polar components.
- Isolate, purify and identify triterpenoids and other high value natural chemicals from crude extracts, and also identify applications for these molecules.
- Examine the seasonal variation in heather triterpenoids content and bracken pterosins content.
- Improve conventional methods of analysis by using a greener solvent.

## 1.3 Green chemistry

Green Chemistry is the design, development, and implementation of chemical products and processes to decrease or eliminate the use and production of substances harmful to human health and the environment. Green Chemistry is a basic fundamental way to implement sustainable development and the application of the Twelve Principles of Green Chemistry has proved that it is possible to arrive at a compromise between society and economy, resources and environment, equity and efficiency, mankind and nature by designing in sustainability at the molecular level.[10] Table 1.1 shows the 12 Principles of Green Chemistry. Application of the 12 fundamental principles of Green Chemistry can achieve higher efficiency and less environmental pressure during chemical synthesis.

Table 1.1： The 12 fundamental principle of Green Chemistry[11]

| | *12 Fundamental principles* |
|---|---|
| *1* | It is better to prevent waste than to treat or clean up waste after it is formed. |
| *2* | Synthetic methods should be designed to maximize the incorporation of all materials used in the process into the final product. |
| *3* | Wherever practicable, synthetic methodologies should be designed to use and generate substances that possess little or no toxicity to human health and the environment. |
| *4* | Chemical products should be designed to preserve efficacy of function while reducing toxicity. |
| *5* | The use of auxiliary substances should be made unnecessary wherever possible and, innocuous when used. |
| *6* | Energy requirements should be recognized for their environmental and economic impacts and should be minimized. Synthetic methods should be conducted at ambient temperature and pressure. |
| *7* | A raw material of feedstock should be renewable rather than depleting wherever technically and economically practicable. |
| *8* | Unnecessary derivatisation should be avoided whenever possible. |
| *9* | Catalytic reagents are superior to stoichiometric reagents. |
| *10* | Chemical products should be designed so that at the end of their function they do not persist in the environment and break down into innocuous degradation products. |
| *11* | Analytical methodologies need to be further developed to allow for real-time, in-process monitoring and control prior to the formation of hazardous substances. |
| *12* | Substances and the form of a substance used in a chemical process should be chosen so as to minimize the potential for chemical accidents. |

According to the 7$^{th}$ principle, the use of renewable raw materials is of great importance to green chemistry practice and there is growing urgency to develop bio-based products produced from renewable resource. Bio-derived material is made from renewable agricultural and forestry feed stocks including wood, wood waste and residues, grasses, crop and crop by-product. Using bio-derived material as a renewable resource is not only a solution to growing environmental threat but also a solution to the uncertainty of petroleum supply.[12]

The extraction of natural wax and lipid from plants normally requires large amount of solvent,[13] but with the application of supercritical carbon dioxide extraction, the solvent usage and extraction time can be dramatically reduced. In addition, supercritical $CO_2$ extraction also shows preferable selectivity in some cases.[2] Moreover, the recent development of analytical techniques such as supercritical fluid chromatography has brought quicker analysis and better selectivity into practice. In a word, for a sustainable future of chemistry, all the processes must reach equilibrium for the highest efficiency, safety and economy.

## 1.4 Chemicals from heather and bracken

Heather and bracken are excellent feedstocks for natural extracts with a wide range of biological activities. The existence of several groups of natural chemicals with anticancer, antioxidant and antimicrobial activities has been identified in heather. Bracken contains a major toxin, ptaquiloside, which is well known for its carcinogenicity. However antitumor activity and cytotoxicity to some cancer cells has also been reported.

### 1.4.1 Natural chemicals from *Calluna Vulgaris*

The confirmation of the biological activities of triterpenoids has gained more and more attention. Triterpenoids are ubiquitous throughout the plant kingdom in the form of free acids or aglycones of saponins and exhibit various biological activities.[14] Already, more than 80 triterpenoids have been identified from plants, and the number of papers describing their bioactive effects has increased sharply during the last decade.[15] Specifically some of them have already been used as anticancer and anti-inflammatory agents in Asian countries.[16]

At the same time, heather is also rich in phenolic acids, which are well known for their antioxidant ability by donating hydrogen or electrons. Meanwhile, their stable radical intermediates can prevent the oxidation of many food ingredients, especially fatty acids and oils.[17] Lambropoulos and co-workers[18] proved that red wine phenolic extracts at 100mg/L inhibited the oxidation of corn oil stripped of tocopherols to a greater extent than butylated hydroxyanisole at 200 mg/L. Previous research has also indicated that phenolic compounds have antimutagenic, anticarcinogenic, and antiglycemic beneficial properties. To utilize these properties, phenolic compounds can be added to health promoting food to prevent potential chronic diseases.[19]

1.4.1.1 Ursolic acid

Ursolic acid (3-hydroxy-urs-12-en-28-oic acid) is a natural pentacyclic triterpenoid carboxylic acid and is the major component of some traditional medicine herbs. It is well known for its biological effects, for instance antioxidation, anti-inflammation, and anticancer activities. In recent years it has attracted considerable attention because of its pharmacological effects combined with a relatively low toxicity.[16]

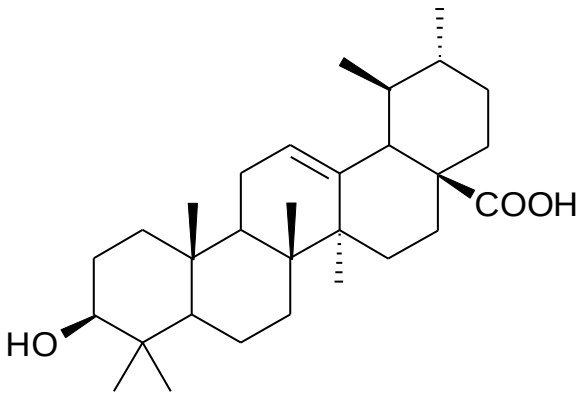



Figure1.3: Chemical structure of ursolic acid

Ursolic acid exists naturally in many plants species including fruits and herbs (Table 1.2). Conventional applications of plants containing ursolic acid in folk medicine are

plentiful. For instance, the leaf and shoot tips of the Labrador tea plant (*Ledum groenlandicum Retzius*), an *Ericaceae* species widely spread throughout North America, are used in traditional American medicine to fight against some inflammatory diseases such as asthma, rheumatism, and also diseases of the liver and kidney. Lately, several instances of medicative efficacy of plants containing ursolic acid have been shown and this may contribute to the further use of plants that contain this chemical in both folk medicine and clinical research.[15]

More and more reports indicate that some natural plant compounds have conspicuous inflammatory and cancer preventive effects.[20,21] Ursolic acid is regarded as one such compound since it possesses many biological activities, such as anti-oxidation, anti-inflammatory, anticancer, and hepato-protection, as well as the ability to induce apoptosis. In the early 1990s, the antioxidative activities of ursolic acid against free radical-induced damage were studied.[22, 23] In these experiments, ursolic acid showed remarkable anti-oxidative activities by scavenging free radicals to four standard chemical (ascorbic acid, carbon tetrachloride, ADP/iron, and adriamycin) induced lipid peroxidation in isolated rat liver and heart microsome *in vitro*.

Table 1.2: Representative example of distribution of ursolic acid in folk medicines and its pharmacological activities[15]

| Plant name | Botanical name | Biological activity |
|---|---|---|
| **Apple** | Malus pumila | Anti-proliferation<br>Anti-cancer |
| **Basil** | Ocimum basilicum | Antiviral |
| **Blueberry** | Vaccinium spp. | Anti-cancer |
| **Cranberry** | Vaccinium macrocarpon | Anti-cancer |
| **Ground ivy** | Glechoma hederacea L. | Anti-cancer |
| **Guava** | Psidium guajava | Unknown |
| **Heather flower** | Calluna vulgaris | Anti-inflammatory |
| **Japanese cherry** | Prunus serrulata var. spontanea | Unknown |
| **Labrador tea** | Ledum groenlandicum Retzius | Antioxidant<br>Anti-inflammatory<br>Anti-cancer |
| **Loquat** | Eribotrya japonica Lindl. | Anti-mutagenic |
| **Olive** | Olea europaea | Antioxidant<br>Anti-atherosclerotic<br>Anti-hypertensive |
| **Oregano** | Origanum vulgare | Anti-leukemic<br>Antioxidant |
| **Persimmon** | Diospyros leucomelas | Antioxidant<br>Anti-inflammatory |
| **Plantain** | Plantago major L. | Antioxidant<br>Anti-inflammatory |
| **Rosemary** | Rosmarinus officinalis L. | Anti-inflammatory<br>Anti-cancer |
| **Sage** | Salvia officinalis L. | Anti-inflammatory |
| **Thyme** | Thymus | Anti-inflammatory |

### 1.4.1.2 Oleanolic acid

Oleanolic acid (3b-hydroxyolean-12-en-28-oic acid) exists in ginseng root at a high concentration. Along with ursolic acid, oleanolic acid has been found in more than 120 plants in the form of free acid or aglycones of the saponin group of triterpenoids.[20] Plants that contain oleanolic acid have been used as a drug for longevity in East Asian countries since ancient times.[15]

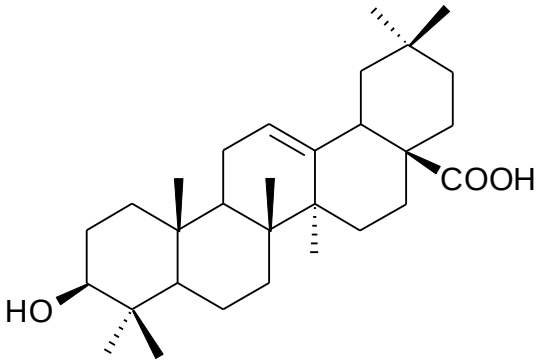



Figure1.4: Chemical structure of oleanolic acid

Oleanolic acid has been used for treatment of non-lymphatic leukemia[24] and has been shown to have the same biological activities as ursolic acid, including antioxidative, [22,23] anti-inflammation,[25-27] anticancer,[28-30] and anti-hepatotoxic[31-33] activities. Among these biological effects, the protection it provides to the liver against acute chemical induced liver damage and chronic liver fibrosis and cirrhosis is the most widely known. Oleanolic acid has been used individually or combined with other hepato-protective components as an oral drug.[14] The effective function of this triterpenoid for combating liver diseases could be due to its anti-oxidant and anti-inflammatory effects, and their actions on drug-metabolising enzymes. Oleanolic acid is an efficient revulsant of metallothionein, a small cysteine-rich protein acting like glutathione in the body's defence against toxin invasion.[14]

As well as ursolic acid, oleanolic acid has various anticarcinogenic effects including inhibiting tumour genesis and development, inducing cancer cell differentiation and apoptosis.[14] Oleanolic acid and its derivatives are also effective in inhibiting angiogenesis, tumour cells infiltration and metastasis, and more and more are used as a new class of chemotherapeutics.[34] The mechanisms of the anticancer effects by oleanolic acid need further investigation.

#### 1.4.1.3 Lupeol

Lupeol (lup-20(29)-en-3b-ol), is a naturally occurring triterpene found in a range of edible fruits such as olive, mango, strawberry, red grape, fig, and some medicinal herbs, and is also widely used as a folk medicine in the Far East, North America, Latin America, and the Caribbean islands.[35-37] Lupeol and some analogues have many proven biological activities such as antioxidative, anti-inflammatory and anticancer.[38] Since they are widely distributed in abundant plants, lupeol and its analogues are easier to obtain than other pharmaceuticals that are being used at the moment, therefore make them a good potential herbal medicine to prevent cancer, coronary and hepatic diseases. Moreover, it's also revealed that lupeol showed low cytotoxicity on healthy cells and can act as synergist in combined therapies, which makes it worth investigating for single use or as an auxiliary drug to complement anti-inflammatory, antineoplatic and anti-hypertensive therapies that are already used.[38]

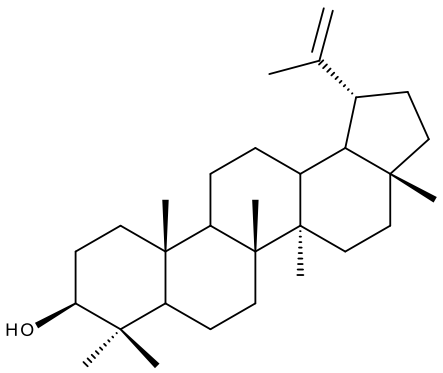



Figure 1.5: Chemical structure of lupeol

### 1.4.1.4 Phenolic compounds

Phenolics are one of the largest and most widespread groups of plant secondary compounds with multiple bioactivities such as antioxidation, antibiotic, anti-ulcer, and anti-inflammatory. Polyphenols also have many important industrial applications such as in the production of paints, paper and cosmetics, as tanning agents and in the food industry as additives. Previous researchers indicated that *Calluna vulgaris* tissues have a high total phenolic content, moreover, quantitative and qualitative determination of phenolic composition and seasonal variation of phenolic content are well understood.[39]

Table 1.3: Seasonal variation in phenolic compounds of *Calluna vulgaris* shoots and roots[39]

| | Jan | Feb | Mar | Apr | May | Jun | Jul | Aug | Sep | Oct | Nov | Dec |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Caffeic acid | - | 1 | 2 | 2 | 1 | - | - | - | - | - | - | - |
| Orcinol | - | - | 2 | 1 | - | - | - | - | - | - | - | - |
| Orcinol-β-D-glucoside | - | -- | - | - | 2 | 2 | 3 | 1 | 2 | 2 | 3 | 2 |
| Arbutin | 1 | - | - | - | 1 | - | 1 | - | - | 1 | 1 | 2 |
| Chlorogenic acid | 2 | 3 | 1 | 2 | 4 | 3 | 3 | 3 | 3 | 3 | 2 | 4 |
| Isochlorogenic acid | - | - | 1 | 2 | - | - | - | - | - | - | - | - |
| Callunin | - | - | - | 2 | 4 | 3 | - | 4 | 2 | 2 | - | 2 |
| Kaempherol | - | - | 1 | 1 | 1 | 1 | 1 | - | 1 | - | 1 | 1 |
| Quercetin | 1 | 1 | 1 | 1 | 2 | 3 | 3 | 1 | 4 | 1 | 4 | 2 |
| Kaemphherol 3-O-glucoside | - | - | - | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | - |
| Kaemphherol-3-O-galacoside | - | - | - | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | - |

| | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Kaemphherol 3-O-arabinoside | - | - | - | 1 | 1 | 1 | 1 | 1 | - | - | 1 | - |
| Quercetin 3-O-glucoside | 4 | 1 | 1 | 2 | 3 | 4 | 3 | 4 | 4 | 3 | 3 | 3 |
| Quercetin 3-O-galacoside | 4 | 1 | 1 | 2 | 3 | 4 | 3 | 4 | 4 | 3 | 3 | 3 |
| Quercetin 3-O-arabinoside | 1 | 1 | 1 | 1 | 2 | 2 | 1 | 1 | 2 | 2 | 2 | 1 |
| (+)-Catechin | 1 | 1 | - | 1 | 2 | 3 | 3 | 3 | 2 | 3 | 4 | 3 |
| (-)-Epicatechin | 1 | 1 | - | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 2 | 1 |
| Procyanidin D1 | 1 | 1 | 2 | 2 | 2 | 3 | 2 | - | 1 | 3 | 2 | 2 |
| Procyanidin B1 | - | 1 | 1 | 1 | 1 | 1 | 1 | - | - | 1 | 1 | 1 |
| Procyanidin B2 | - | - | - | - | 1 | - | 1 | 1 | 1 | 1 | 1 | 1 |
| Procyanidin B3 | - | - | - | 1 | 1 | 1 | 1 | - | 1 | - | - | - |
| ProcyanidinB4 | - | - | - | - | - | 1 | 1 | - | - | - | - | - |
| Procyanidin B5 | - | - | - | - | - | 1 | 1 | - | 1 | - | - | - |
| Procyanidin C1 | - | 1 | 1 | - | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 |
| Total phenolic content in shoots (%dry wt) | 16.0 | 15.6 | 15.3 | 16.2 | 23.4 | 21.7 | 26.5 | 19.4 | 28.4 | 23.0 | 21.9 | 19.8 |

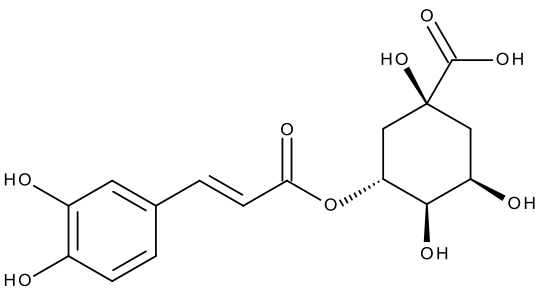



Figure 1.6: Chemical structure of chlorogenic acid

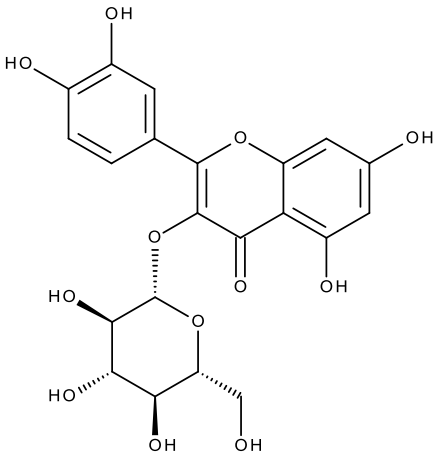



Figure 1.7: Chemical structure of quercetin 3-O-glucoside

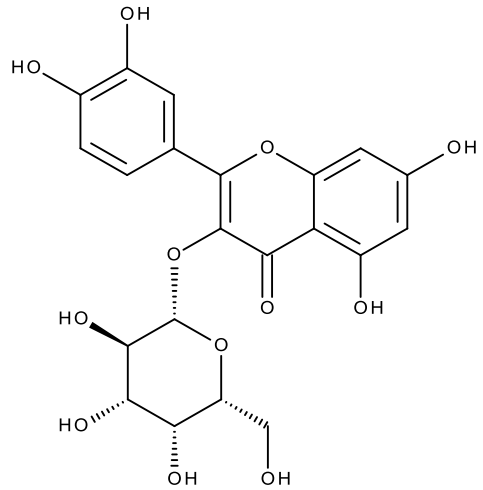



Figure 1.8: Chemical structure of quercetin 3-O-galactoside

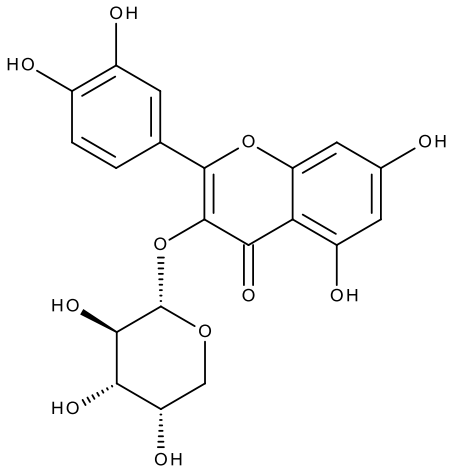



Figure 1.9: Chemical structure of quercetin 3-O-arabinoside

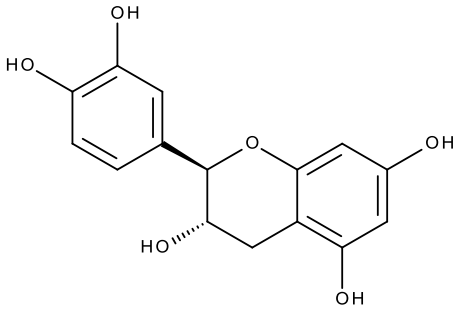



Figure 1.10: Chemical structure of (+)-catechin

It is well known that phenolic compounds are powerful antioxidants, it has attracted increasing interest in the food industry because they can slow oxidative degradation of lipids and thereby improve the quality and nutritional value of food.[40] In addition, all aerobic life suffers oxidative pressure from reactive oxygen species (ROS), excess ROS can lead to a series of pathological changes through lipid peroxidation and protein and nucleic acid damage, and these changes may eventually induce chronic diseases including cancer, cardiovascular disease, aging and neurodegenerative disorders.[41] Thus dietary phenolic antioxidants have been paid increasingly attention as preventive ingredient.[42]

As well as antioxidation, the antimicrobial activity of phenolic compounds is also significant. Corrales and co-workers tested the antimicrobial ability of phenolic acids from grape seed extract on *Listeria monocytogenes, Salmonella enterica serovar typhimurium, Staphylococcus aureus, Escherichia coli, Enterococcus faecium, Enterococcus faecalis,* and *Brochothrix thermosphacta,* and the result showed a inhibition of all the Gram-positive bacteria tested.[43] This result corroborates many previous reports and mechanisms of Gram-positive inhibition are proposed in Corrales's article.[43]

### 1.4.2 Natural chemicals from *Pteridium aquilinum*

#### 1.4.2.1 Ptaquiloside

Ptaquiloside ($C_{20}H_{30}O_8$) is a colourless amorphous compound which can be easily extracted by water from every part of bracken fern. Since it exhibits strong toxicity and water solubility, much research has been undertaken to analyse the soil contamination caused by rain water flushed ptaquiloside. In 1983, the isolation of ptaquiloside was first achieved by Yamada and co-workers, two years later, they modified the isolation method and achieved a higher yield and a simpler procedure.[6]

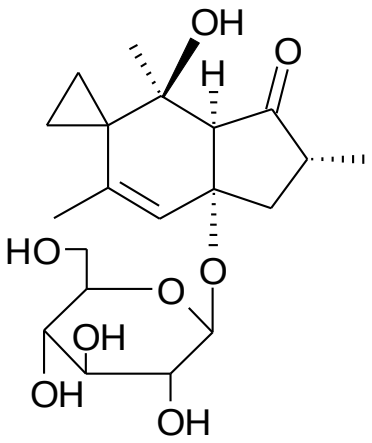



Figure 1.11: Chemical structure of ptaquiloside

Ptaquiloside is readily soluble in water and easily decomposes in aqueous solution depending on the pH.[6] In acidic conditions, ptaquiloside gradually converts to pterosin B through acidic hydrolysis. Meanwhile, in weak alkaline conditions, ptaquiloside will hydrolyse to a very unstable conjugated dienone named bracken dienone, which will itself rapidly convert to pterosin B in weak acidic conditions as shown in figure 1.12.

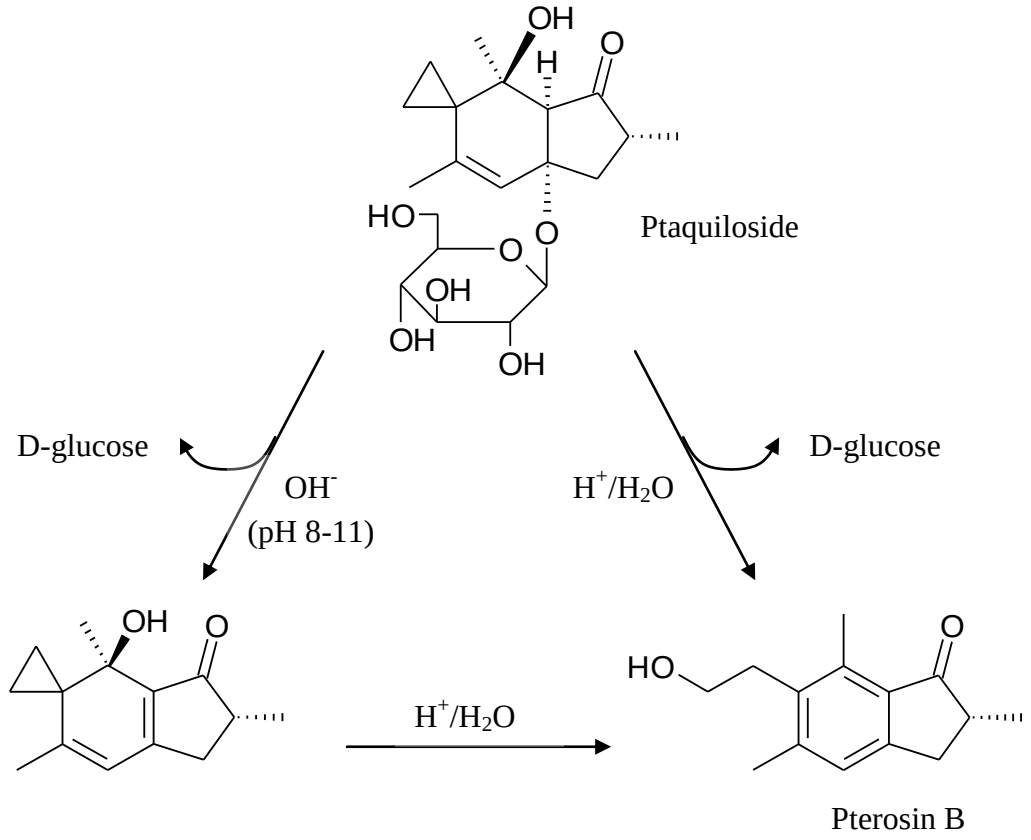


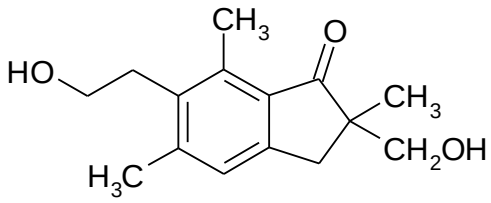

Figure 1.12: Ptaquiloside reactions

The toxicity and carcinogenicity of bracken fern were first reported in 1960 and Ptaquiloside was confirmed as the main carcinogen in *Pteridium aquilinum* in 1984: the peritoneal injection done by Yamato group to rats induced mammary cancer by 100% and ileal cancer by 91%.[6] Hirono's group also showed that administration of ptaquiloside via an oral route induced thrombocytopenia, myeloid aplasia and

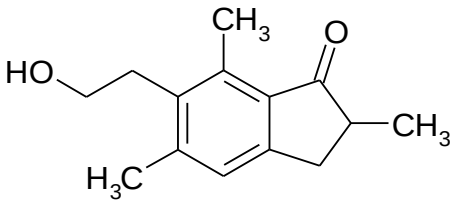
neutropenia in a calf typical of the acute haemorrhagic syndrome.[44] Possible mechanism of action of ptaquiloside and related cancer model are also well discussed in previous reports.[44]

1.4.2.2 Pterosins

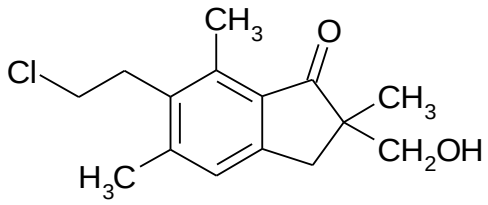
The pterosins are a large group of sesquiterpenes and norsesquiterpenes first isolated from *Pteridium aquilinum* in Japan. These compounds have been found to occur widely in various fern species and they are also present in certain fungi of the class basidiomycetes.[45] More than one pterosin will be present after hydrolysis of the unstable precursor ptaquiloside. Castillo and co-workers isolated pterosin A, B, K, Z at the same time after treatment of ptaquiloside with base then acid, the HPLC and NMR data are all well recorded.[46]



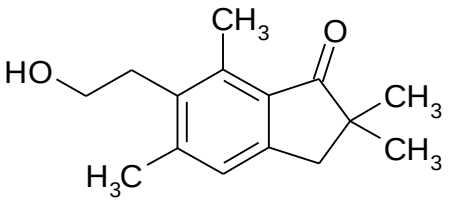

Pterosin A




Pterosin B




Pterosin K




Pterosin Z

Figure 1.13: Chemical structures of some bracken pterosins

During the last decade many reports based on biological activities of pterosins have been published. When Natoris group isolated ptaquiloside and related pterosins and tested their carcigenicity respectively, none of the pterosin was found to be carcinogenic although some of them were cytotoxic to HeLa cells.[7] The report of Kobayashi and co-workers showed that several pterosins produce cytotoxic effects on the ciliate, *Paramecium caudatum*, and also induced abnormal development of the urchin embryos.[47] Pterosin O was isolated from *Pteris inequalis*, and was showed to be active against *Bacillus subtilis*.[48] Pterosin C, which has been isolated from a plateau plant *Pteris wallichiana* which grows in the Himalaya and was reported to be active against *Staphylococcus aureus*.[49] Pterosin Z has been shown to be a smooth muscle relaxant,[9] and has also been used in Guyana for treatment of venereal diseases.[49]

Recently in 2010, pterosins are discovered to have anti-diabetic and anti-obesity activities.[50] Totally 84 different pterosins or pterosin related compounds were synthesized or extracted from plants and have been tested in vitro and in vivo for their anti-diabetic and anti-obesity activities. Fifteen of them expressed very strong biological activities against type 1 and type 2 diabetes and obesity and among these 15 pterosins, four of them are naturally occurring in bracken fern and other plant materials. These pterosin compounds were indicated to significantly lowered blood glucose levels in STZ-induced diabetic mice, and also dramatically enhanced the insulin sensitivity and glucose consumption in vitro. It was suggested that the pterosin compounds may activate AMPK (AMP-activated protein kinase), which in turn regulates insulin control of carbohydrate and fatty acid metabolism, therefore these compounds can be considered as a potential anti-diabetic and anti-obesity agents. Further results also indicated that these pterosin compounds can significantly reduce serum lipids and exhibited anti-obesity effects in high fat-diet fed mice.

Most pterosin compounds are synthesized through a series of complex processes. For example, McMorris and co-workers reported that pterosin F can be produced by mixing pterosin B and triphenylphosphine in carbon tetrachloride and refluxing for 3 hours.[51] The starting material pterosin B was obtained from pterosin O by demethylation with 48% hydrobromic acid in acetic acid. Moreover, pterosin O was afforded through catalytic hydrogenation with 5% Pd on carbon in ethyl acetate from an intermediate indandione, and this key intermediate was formed through a Friedel-Crafts bisacylation of the methyl ether of 2-(2,6-dimethylpheny1)ethanol with methylmalonyl chloride. The synthesis processes are very complicated and will generate large amounts of chemical waste and solid waste. Therefore, if naturally occurring valuable pterosins can be extracted from bracken by using environmental benign technologies, it will not only add value to this troublesome natural plant, but also make some contribution to the green chemistry application in this research field.

## 1.5 Extraction and analysis methods

### 1.5.1 Supercritical Carbon Dioxide Extraction

Supercritical fluids extraction (SFE) is increasingly replacing the organic solvents that are used in sample extraction and preparation prior to the analysis of compounds in natural product matrices and supercritical fluid extraction plants are operating at throughputs of up to 40,000 tons/yr in the food industry.[52] The extractions of caffeine from coffee and bitter acids from hops are well known processes performed on an industrial scale. Coffee and tea are decaffeinated via supercritical fluid extraction and most global brewers use extracts that are prepared from hops using supercritical fluids. At the same time, many other applications of supercritical fluids have been investigated. Some of these include the refining of triglycerides and fatty acids, the production of flavours, spices and essential oil extracts from a variety of natural

products, the production of antioxidants, the production of low fat and low cholesterol foods and the selective separation of nicotine from tobacco.[52] Moreover, SFE processes are also being commercialized in the polymer, pharmaceutical, specialty lubricants and fine chemicals industries.[53] To conclude, SFE are advantageously applied to increase product performance to levels that cannot be achieved by traditional processing technologies, and such applications for SCF offer the potential for both technical and economic benefit.

Table 1.4: Comparison of physical and transport properties of gases, liquids, and SCF[54]

| Property | Density(kg/m$^3$) | Viscosity(cP) | Diffusivity(mm$^2$/s) |
|---|---|---|---|
| Gas | 1 | 0.01 | 1-10 |
| SCF | 100-800 | 0.05-0.1 | 0.01-0.1 |
| Liquid | 1000 | 0.5-1.0 | 0.001 |

A supercritical fluid is characterized by physical and thermal properties that are between those of the pure liquid and gas. The fluid density is a strong function of the temperature and pressure. The diffusivity of supercritical fluid is much higher than that for a liquid and SCF can readily penetrates porous and fibrous solids.

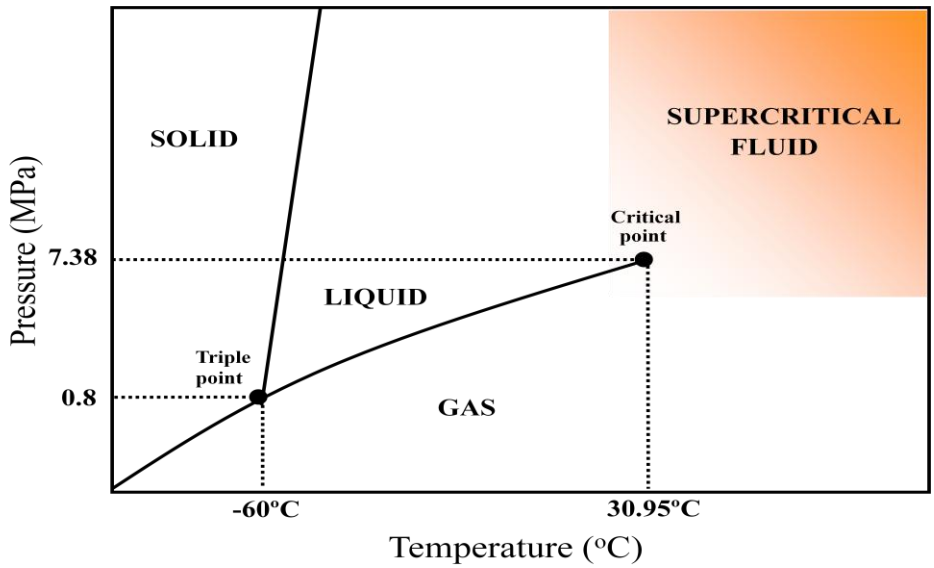



Figure 1.14: Phase diagram for supercritical $CO_2$

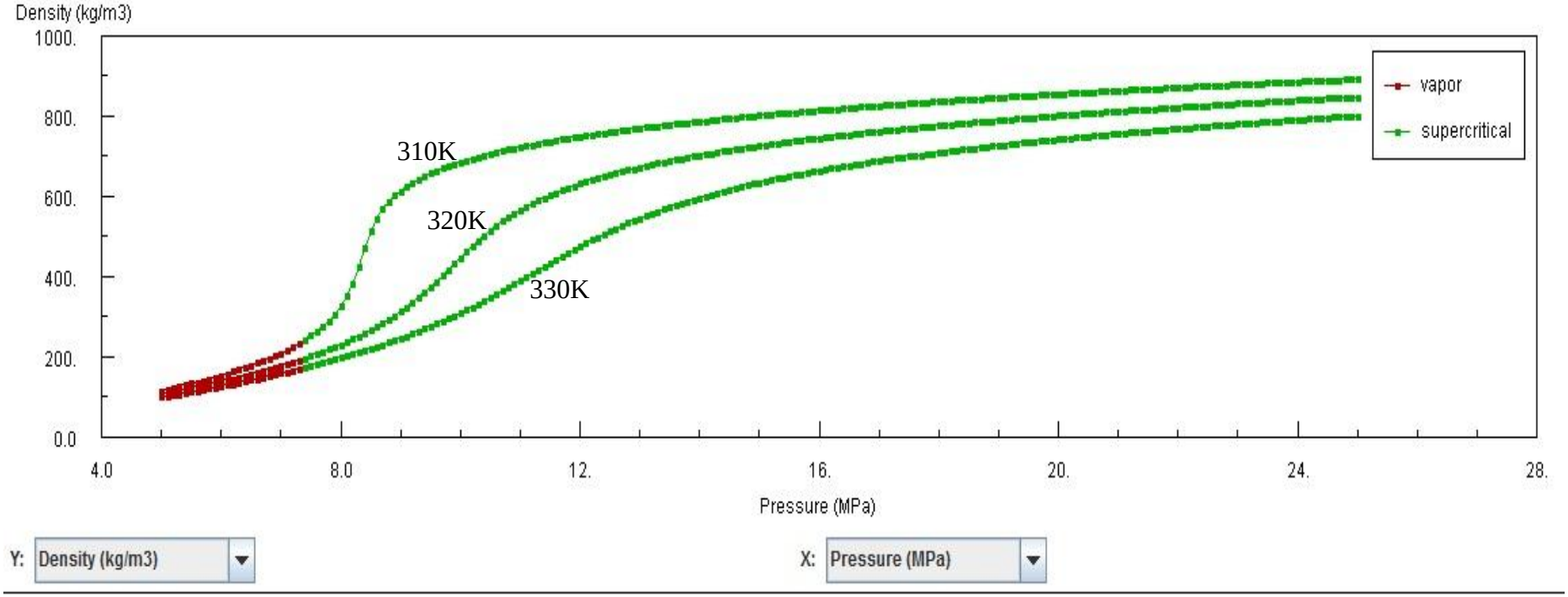



Figure 1.15: Variation of density with pressure for carbon dioxide[55]

Carbon dioxide is a perfect selective extraction solvent since it is non-toxic, environmentally safe and cheap. Carbon dioxide has the advantage over liquid solvents that it's selectivity or solvent power is adjustable and can be set to values ranging from gas to liquid-like by changing pressure and by adding co-solvents, among other variables.[56]

Supercritical $CO_2$ is capable of extracting a wide range of diverse compounds from a variety of sample matrices. Many non-polar to moderately polar compounds from triglycerides to steroids can be extracted with pure carbon dioxide, whereas more polar compounds such as phenolics, glycosidic compounds and alkaloids can be extracted with carbon dioxide modified with entrainer solvents. Adding polar co-solvents to the supercritical $CO_2$ is the most common way to greatly increase the solubility of polar components. Frequently used co-solvents include methanol, ethanol, acetonitrile, acetone, water, diethyl ether and dichloromethane. Methanol is the most commonly used because it is an effective polar modifier and is up to 20% miscible with $CO_2$. However, ethanol may be a better choice in SFE of nutraceuticals and food ingredients because of its lower toxicity.[57] Some research has indicated that ethanol and methanol improve the solubility of $scCO_2$ to triterpenoids and sterols by up to two orders of magnitude at a ethanol concentration of several mole percent.[52]

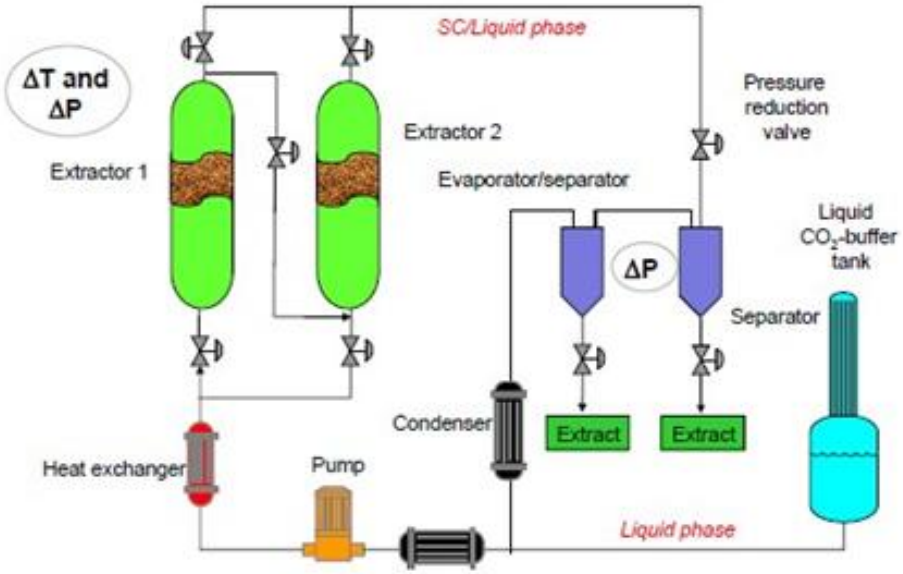



Figure 1.16: A detailed diagram of supercritical $CO_2$ extraction apparatus[72]

Table 1.5: Co-solvents used to modify carbon dioxide for SFE[52]

| Modifier | *Tc* (°C) | *Pc* (atm) | Molecular mass | Dielectric constant at 20°C | Polarity index |
|---|---|---|---|---|---|
| Methanol | 239.4 | 79.9 | 32.04 | 32.70 | 5.1 |
| Ethanol | 243.0 | 63.0 | 46.07 | 24.3 | 4.3 |
| Propan-1-ol | 263.5 | 51.0 | 60.10 | 20.33 | 4.0 |
| Propan-2-ol | 235.1 | 47.0 | 60.10 | 19.3 | 3.9 |
| Hexan-1-ol | 336.8 | 40.0 | 102.18 | 13.3 | 3.5 |
| 2-Methoxyethanol | 302 | 52.2 | 76.10 | 16.93 | 5.5 |
| THF | 267.0 | 51.2 | 72.11 | 7.58 | 4.0 |
| 1,4-Dioxane | 314 | 51.4 | 88.11 | 2.25 | 4.8 |
| Acetonitrile | 275 | 47.7 | 41.05 | 37.5 | 4.8 |
| DCM | 237 | 60.0 | 84.93 | 8.93 | 3.1 |
| Chloroform | 263.2 | 54.2 | 119.38 | 4.81 | 4.1 |
| Propylene carbonate | 352.0 | --- | 102.09 | 69.0 | 6.1 |
| N,N-Dimethylacetamide | 384 | --- | 87.12 | 37.78 | 6.5 |
| DMSO | 465.0 | --- | 78.13 | 46.68 | 7.2 |
| Formic acid | 307 | --- | 46.02 | 58.5 | --- |
| Water | 374.1 | 217.6 | 18.01 | 80.1 | 10.2 |
| Carbon disulphide | 279 | 78.0 | 76.13 | 2.64 | --- |

Compared to supercritical carbon dioxide, liquid $CO_2$ has its own special advantages. Liquid $CO_2$ extractions are desirable for high value thermolabile product such as plant essential oils that require mild extraction conditions. It was reported by Moyler that cleaner essential oil extract can be obtained by liquid $CO_2$ which resembled much closer the natural aroma of the original vegetable matrix compared to extraction with supercritical $CO_2$.[4]

Table 1.6 shows the selected solubility of some organic compounds in liquid $CO_2$. Some features of liquid $CO_2$ can be observed from this data. Generally, liquid $CO_2$ is a good solvent for aliphatic hydrocarbons and most small aromatic hydrocarbons. It behaves mostly like a hydrocarbon solvent, but with some points of difference (for instance methanol miscibility). Halohydrocarbon, aldehydes, esters, ketones and low carbon alcohols are readily soluble in liquid $CO_2$. Phenols, anilines, hydroquinone and other polyhydroxy aromatics are mainly insoluble. Polar compounds like amide, ureas and urethanes also have very low solubility. Ordinarily, liquid $CO_2$ can only dissolve compounds with a molecular weight under 500 of any structural type.[5]

Table 1.6: Selected solubility of organic compounds in liquid $CO_2$[5]

| Compound | Solubility, wt% |
|---|---|
| **n-heptane** | Miscible |
| **n-dodecane** | Miscible |
| **n-hexadecane** | 8 |
| **n-tetracosane** | 1-2 |
| **β-carotene** | 0.01-0.05 |
| **p-xylene** | 4-25 |
| **Pentamethylbenzene** | 17 |
| **Biphenyl** | 2 |
| **Anthracene** | ＜0.02 |
| **Benzotrichloride** | 2 |
| **Methanol** | Miscible |
| **tert-butyl alcohol** | Miscible |
| **7-tridecanol** | 11 |
| **p-benzoquinone** | 7 |
| **Benzophenone** | 4 |
| **Cholestanone** | 1.5 |
| **Methyl benzoate** | Miscible |
| **Diethyl phthalate** | 10 |
| **n-butyl hexadecanoate** | 3 |
| **N,N-dimethylaniline** | Miscible |
| **Aniline** | 3 |
| **Diphenylamine** | 1 |
| **Phenol** | 3 |
| **p-isopropylphenol** | 6 |
| **Hydroquinone** | ＜0.01 |
| **4-hydroxybiphenyl** | 0.05 |
| **a-tocopherol** | 1 |
| **Acetic acid** | Miscible |
| **Phenylacetic acid** | ＜0.1 |
| **Lauric acid** | 1 |
| **2,4-dinitrotoluene** | 24 |
| **2,4-dinitrochlorobenzene** | 11 |
| **Dychlorohexyl-18-crown-6** | 1 |
| **glucose** | 0 |

To conclude, extraction with liquid or supercritical carbon dioxide is an outstanding environmentally benign extraction system with the following advantages:

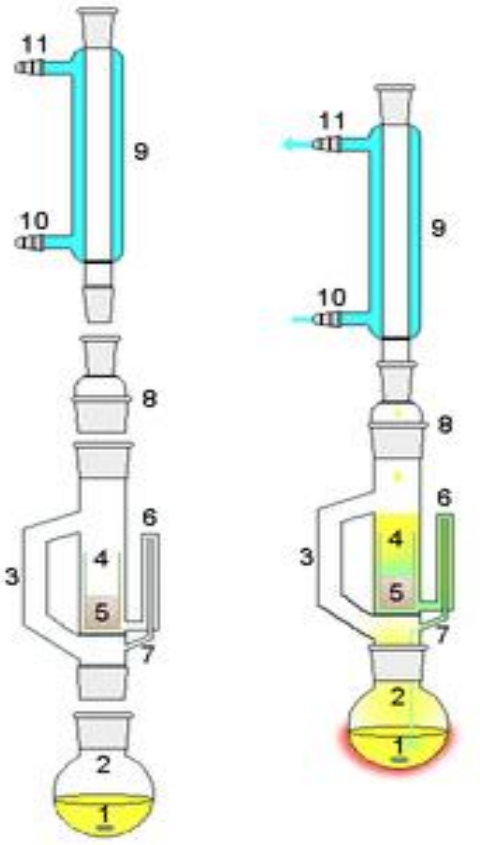
- $CO_2$ is non-toxic, odourless and leaves no solvent residues making it ideal for food or pharmaceutical applications.
- $CO_2$ is tuneable across a wide range of temperatures and pressures and can selectively extract groups of molecules. In addition, with the help of co-solvents, the solubility to polar compounds in $CO_2$ can be extended significantly. The use of a green co-solvent such as bio-ethanol will make $scCO_2$ extraction of more profound significance.
- $CO_2$ provides an inert and neutral environment for the extraction of many thermolabile compounds at low temperatures and provides cleaner plant extracts because the degradation of certain compounds by lengthy exposure to high temperatures, low pH or oxygen is avoided
- Chlorophylls are insoluble in $scCO_2$.[58] This is important in many areas of natural product extractions and pharmaceutical processes.
- SFE extracts are also more concentrated.[58]

### 1.5.2 Soxhlet extraction

Soxhlet extraction is a very efficient method to extract compounds from solid materials by using a hot, pure solvent. Soxhlet extraction is a general and well established technique that outperforms other conventional extraction techniques except for, in a limited field of applications, the extraction of thermolabile compounds.[59]

In a typical Soxhlet extraction a tube of ground plant material (for increasing solid-liquid contact area) is placed in a thimble-holder, and filled with condensed fresh solvent from a round-bottom flask. When the liquid reaches the overflow level, a

siphon aspirates the extract liquid and drains it back into the round-bottom flask. In the solvent flask, solute is separated from the solvent using distillation. By repeating this, solid material can be extracted continuously by pure solvent and the extract will be enriched in the round-bottom flask. The operation is repeated until complete extraction is achieved.




**1:** Stirrer bar or anti-bumping granules
**2:** Still pot (round bottom flask) -still pot should not be overfilled and the volume of solvent in the still pot should be 3 to 4 times the volume of the soxhlet chamber.
**3:** Distillation path
**4:** Soxhlet Thimble
**5:** Extraction solid (residue solid)
**6:** Syphon arm inlet
**7:** Syphon arm outlet
**8:** Expansion adapter
**9:** Condenser
**10:** Cooling water in
**11:** Cooling water out

Figure 1.17: A schematic diagram of soxhlet extraction device

Choosing a suitable solvent for soxhlet extraction is essential. Different solvents will yield different extracts and extract compositions.[60] The most widely-used solvent to extract edible oils from plant sources is hexane. It is an excellent oil solvent due to its oil solubility and ease of recovery. However, n-hexane, the main component of commercial hexane, is listed as No. 1 on the list of 189 hazardous air pollutants by the US Environmental Protection Agency.[61] The use of alternative solvents such as

bio-ethanol, hydrocarbons, and even water, has been investigated due to environmental, health, and safety considerations. It has been described that supercritical carbon dioxide has extraction ability similar to that of hexane since it has been proved to have low polarity which sits between that of hexane and toluene.[62] Since hexane and toluene have been demonstrated to be excellent solvents for extracting lipid, supercritical $CO_2$ also has high potential to be good solvent for extraction of triterpenoids and plant lipids. Entrainers are sometimes added into supercritical fluid order to increase the polarity of the liquid phase. A mixture of solvents such as isopropanol and hexane has been reported to increase the yield and improve the kinetics of extraction.[57, 63]

Table 1.7: The advantages and disadvantages of conventional Soxhlet extraction[57]

| **Advantages** | | **Disadvantages** | |
|---|---|---|---|
| 1 | Transfer equilibrium is obtained by recycling solvent repeatedly | 1 | The extraction time is long |
| 2 | Maintains a relatively high extraction temperature | 2 | A large amount of solvent is used which requires an evaporation procedure |
| 3 | No filtration requirement after leaching | 3 | Agitation cannot be provided in the Soxhlet device to accelerate extraction rate |
| 4 | The Soxhlet method is simple and cheap | 4 | Thermolaible components can't be extracted by soxhlet |

### 1.5.3 Supercritical fluid chromatography

Supercritical fluid chromatography (SFC) is a relative new chromatographic technique which has been commercially used since only the end of the last century. The principles of operation of SFC are similar to those of HPLC, though SFC requires a supercritical fluid (typically carbon dioxide) with an organic modifier as mobile phase and operates in normal phase rather than reverse phase. Because of the unique

benefit of supercritical fluid, SFC has some significant advantages over other separation techniques such as gas chromatography (GC) and high performance liquid chromatography (HPLC). SFC is able to give a good separation and determination of group of compounds that are not conveniently handled by either GC or LC. It can analyse and purify compounds with high boiling point and low volatility that cannot be analysed by GC and can achieve higher analysing speed and better resolution compared with HPLC. For instance, GC is not able to analyse any non-volatile or thermally unstable compounds. Similarly, LC cannot be used for compounds with some functional groups that cannot be detected by either spectroscopic or using electrochemical detectors. However compared with these conventional techniques, SFC is relatively recent and there is a large amount of research currently underway both in SFC method development and in hardware development.

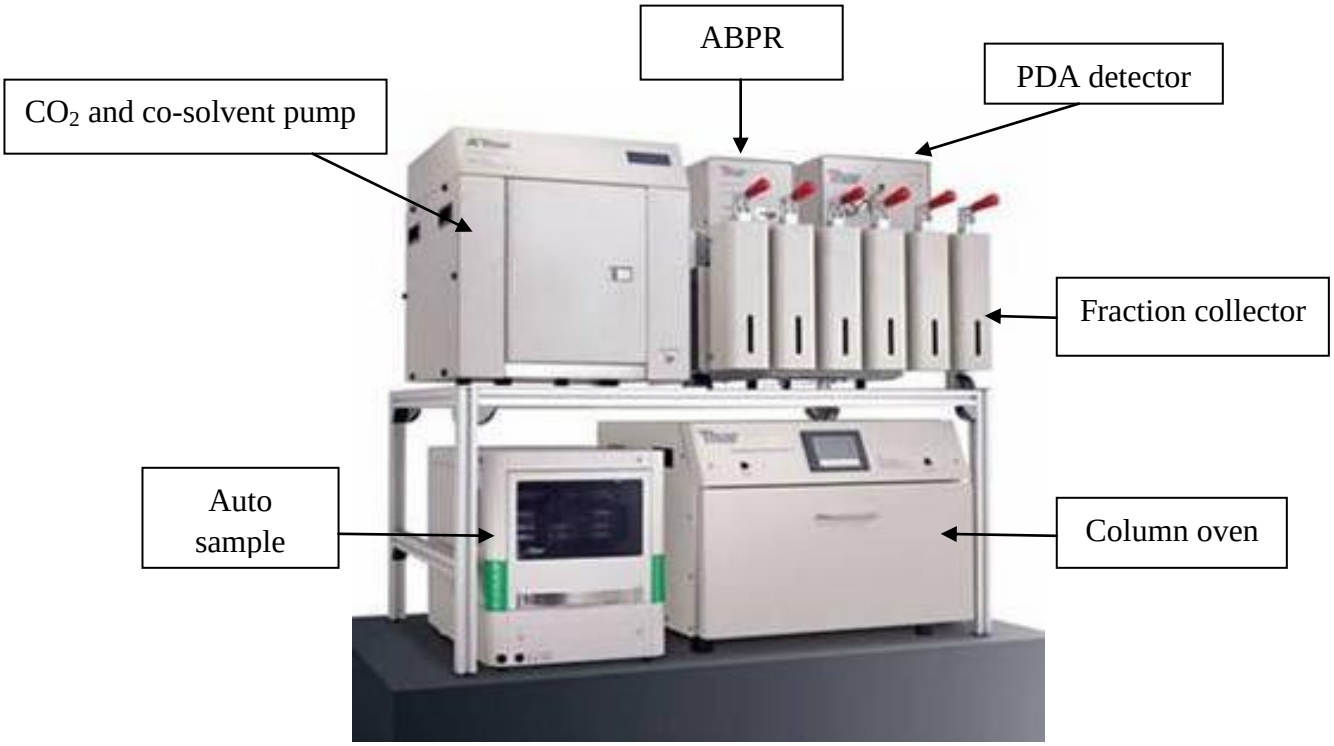



Figure 1.18: Semi-prep scale supercritical fluid chromatography in Green Chemistry group, University of York

There are many obvious advantages of using SFC. As the diffusion coefficient of supercritical fluid is much greater than a liquid, SFC can achieve better resolution and faster analysis speed at same condition than using HPLC with a liquid mobile phase. Viscosity is also a major reason that SFC is superior to HPLC. Since the viscosity of supercritical fluid is similar to that of gas, the pressure drop across the column will be much lower than a HPLC column in operation. The low viscosity and low pressure drop also allow higher flow rate in SFC. Generally, the optimal flow rate of SFC is 3-5 times higher than a HPLC. Thus, higher throughput can be achieved and many more samples would be analysed within a certain period of time. Due to the unique properties of supercritical fluid, re-equilibration could be much faster than HPLC as well so cycle time for each sample will be greatly reduced. Generally speaking, SFC is able to accelerate every step in sample analysis and massive analysing time will be saved by using SFC.

SFC columns are normally packed under 1000 bar, so compared with HPLC columns they are more robust and trustworthy to give out reliable data. It is also proven that SFC is better for separating isomers, enantiomers and structurally related compounds. Besides, SFC is particularly useful for the analysis of polar compounds that have less selectivity on reverse phase LC.[64]

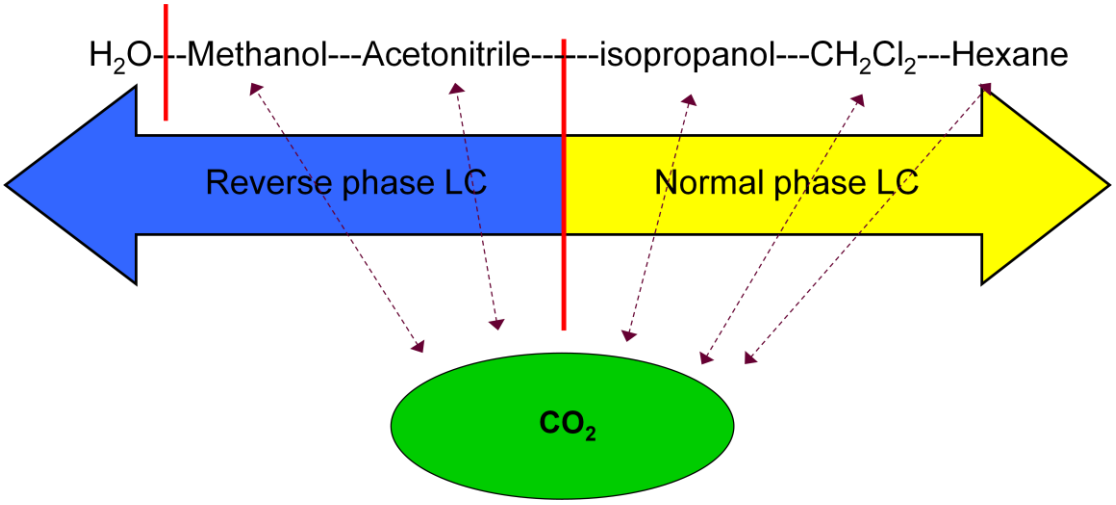



Figure 1.19: Polarity comparison between modified $CO_2$ with common LC solvents

Normally, different solvents are used for HPLC due to their various polarities. Common solvents used are hexane, DCM and isopropanol for normal phase and acetonitrile, methanol and water for reverse phase. With the help of organic modifier, supercritical $CO_2$ can reach a broad range of polarity from hexane up to methanol. The use of SFC reduces solvent volumes and therefore cost, the only thing that modified $CO_2$ cannot compete with is water. Overall, supercritical fluid chromatography has shown superior performance in analysing compounds that are very difficult to measure and separate by conventional GC and LC, and can also make the whole analysing process easier and quicker. SFC is an ideal complement to GC and HPLC, but new applications and development are needed to consolidate this technique.

# *Supercritical carbon dioxide extractions of heather*

# Chapter 2

## 2.1 Supercritical carbon dioxide extractions of heather

Triterpenoids have been reported to be present in heather at a quite high concentration; however they are not essential for plant development.[2] Prior studies indicated that the triterpenoids within *Calluna vulgaris* were found to be $\Delta^{12}$-unsaturated triterpenoids with the double bond positioned on the 12 carbon.[2] These unsaturated triterpenoids included α-amyrin, β-amyrin, oleanolic acid and ursolic acid.

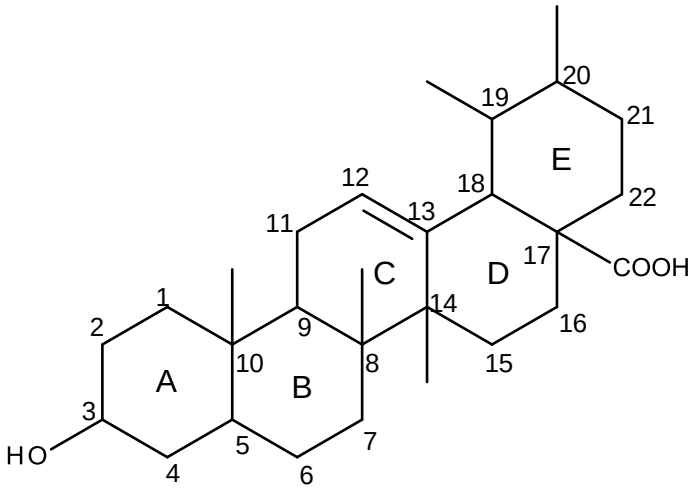



Figure 2.1: Numbering of carbon skeleton of triterpenoids

Pancost and co-workers quantified the content of triterpenoids in *Calluna vulgaris* and another heather species *Erica tetralix*.[65] Their results indicated that *Calluna vulgaris* contain much more triterpenoids in stems and leaves than *Erica tetralix*, but less in roots. Table 2.1 shows the abundances of triterpenoids in these two heather species.

Table 2.1 Abundances (in μg/g dry plant) of triterpenoids in leaves, stems and roots of *Calluna vulgaris* and *Erica tetralix*[65]

| | *Erica tetralix* | | | *Calluna vulgaris* | | |
|---|---|---|---|---|---|---|
| | Fine roots | Coarse roots | Stems | Leaves | Roots | Stems and leaves |
| α-amyrin | 3100 | 1800 | 620 | 35000 | 370 | 3900 |
| β-amyrin | 910 | 710 | 1800 | 13000 | 550 | 5700 |
| Lupeol | 780 | 600 | 120 | 19000 | 0 | 0 |
| Oleanolic acid | 1800 | 1000 | 600 | 11000 | 55 | 14000 |
| Taraxerol | 0 | 0 | 0 | 0 | 610 | 0 |
| Taraxer-4-one | 0 | 0 | 0 | 0 | 490 | 0 |
| Ursolic acid | 12000 | 2900 | 2400 | 52000 | 130 | 34000 |
| Unsaturated ursolic acid | 1700 | 1300 | 600 | 26000 | 20 | 7500 |

Among these triterpenoids, ursolic acid is present in the greatest concentration in *Calluna vulgaris* with α-amyrin, β-amyrin and oleanolic acid at lower concentrations. Previous results indicated that ursolic acid can make up 2.5% of the dried plant of heather in flowering season.[31] Ursolic acid and oleanolic acid have been shown to have anticancer properties against leukaemia cells, gastric tumours and breast cancer cells *in vitro*.[66] Other biological activities of triterpenoids such as anti-inflammation, anti-ulcer, antimicrobial and liver protection against chemical damage has also been demonstrated.[31]

Since triterpenoids are found to be more concentrated in stems and leaves in *Calluna vulgaris*, aerial parts of heather were harvested in October 2007, January 2008, March 2008 and August 2008 to be representative of heather from different seasons. All the

heather samples are harvested from Hole of Horcum, North York Moors National Park (Grid reference SE 84558 93633). All the plant samples were dried and milled to a particle size of ≤2 mm and then stored in the fridge at 2 $^{o}$C prior to extraction.

Heather supercritical carbon dioxide extracts are reported to have high proportion of triterpenoids.[2] Depending on the extraction conditions, supercritical $CO_2$ extractions can yield higher total triterpenoids than corresponding hexane Soxhlet extractions. Therefore, in this study, heather samples were extracted by supercritical $CO_2$ with and without the presence of ethanol entrainer. Extraction was carried out under supercritical conditions at a pressure of 35 MPa, a temperature of 50 $^{o}$C and a $CO_2$ flow rate of 40 g/min, for 4 hours. When extraction was carried out with the addition of ethanol as entrainer the ethanol flow rate was set to 4 ml/min (10% of $CO_2$ flow). Several triterpenoids have been discovered in these heather extracts, and some other triterpenoids such as taraxerol which have not been previously found in *Calluna vulgaris* stems and leaves are also found.

## 2.2 General yield of supercritical carbon dioxide extractions

The yield of heather supercritical $CO_2$ and supercritical $CO_2$ with ethanol entrainer extractions are recorded. The seasonal variation in extract yield in both dry plant and fresh plant can be seen from figure 2.2 and 2.3. It is clear that summer flowering sample harvested in August 2008 exhibited the highest crude yield in both dry plant and fresh plant and January winter heather, by contrast, showed the lowest yield in dry plant and fresh plant. The yields from heather supercritical $CO_2$ extraction with ethanol entrainer are generally higher than that of supercritical $CO_2$ only extraction by approximately 0.8%-1% in dry plant and 0.7%-1% in fresh plant. However, the seasonal variation trend in crude yield of these supercritical extractions are exactly the same as the lowest point found in winter sample and the peak reached in summer.

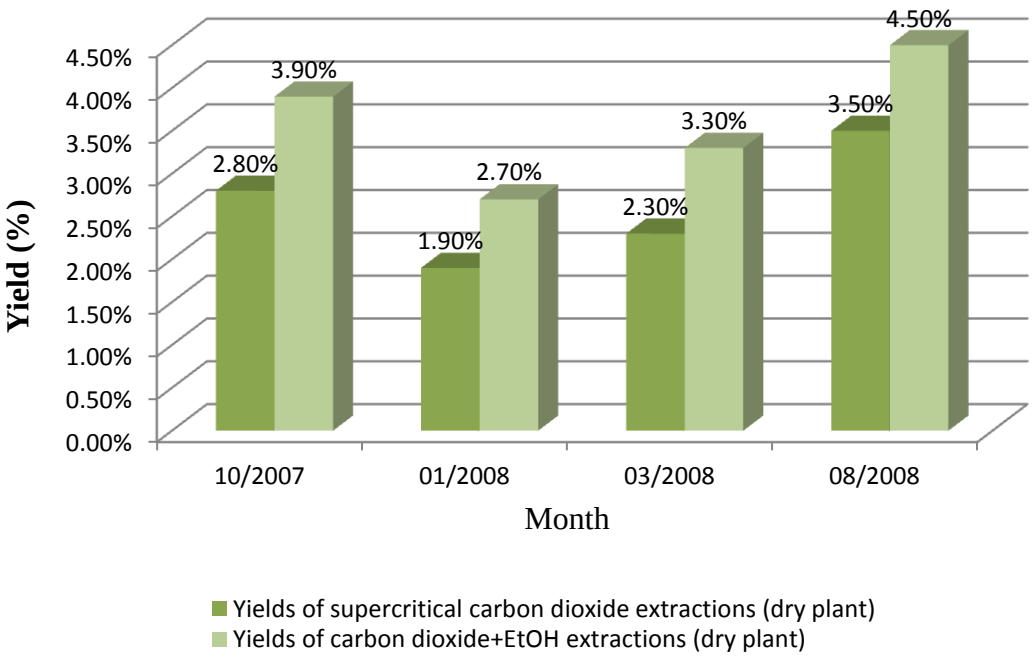



Figure 2.2: Extraction yield from dried heather using supercritical $CO_2$ and supercritical $CO_2$ with ethanol entrainer

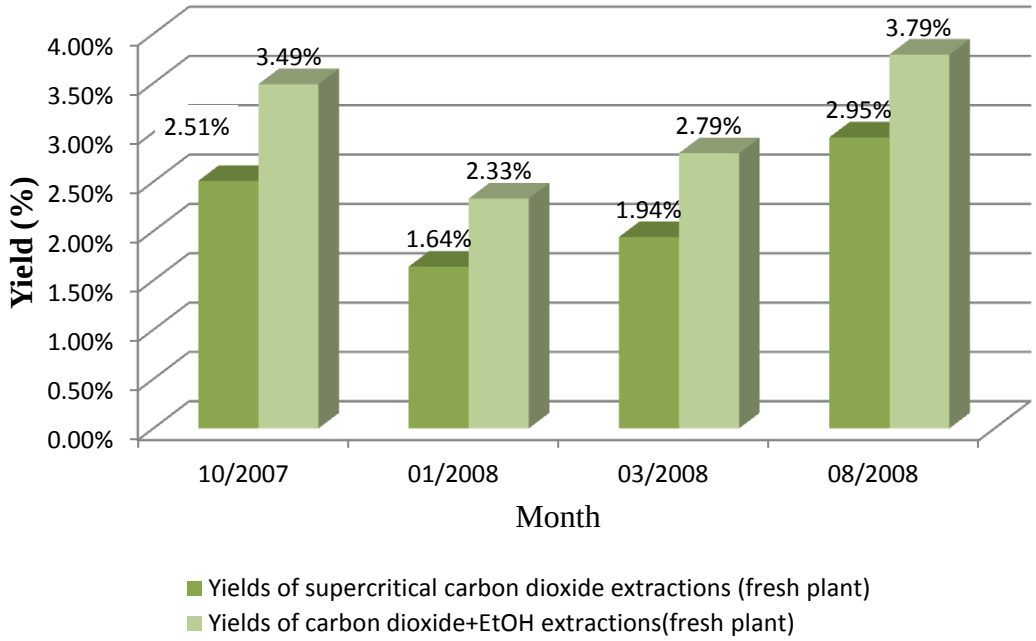



Figure 2.3: Extraction yield from fresh heather using supercritical $CO_2$ and supercritical $CO_2$ with ethanol entrainer

## 2.3 Column fractionation of heather supercritical $CO_2$ and supercritical $CO_2$ with ethanol entrainer extracts and identification of triterpenoids

Column chromatography was used to fractionate summer heather supercritical $CO_2$ and supercritical $CO_2$ with entrainer extracts in conjunction with thin layer chromatography to help purify and isolate fractions rich in triterpenoids. Several triterpenoids were discovered in flowering heather supercritical dioxide extracts.

### 2.3.1 Fractionation of heather supercritical $CO_2$ extract

The method used for column chromatography and thin layer chromatography are stated in chapter 7.3.3 and 7.3.4. 56 initial fractions were collected from the column and they are combined based on their spot positions revealed by TLC (figure 2.4). Eventually, 14 combined fractions were obtained, and the weight of each fraction was recorded after evaporating the solvent using a rotary evaporator. GC-MS samples were then made up in dichloromethane for each fraction at a concentration of 20 mg/ml.

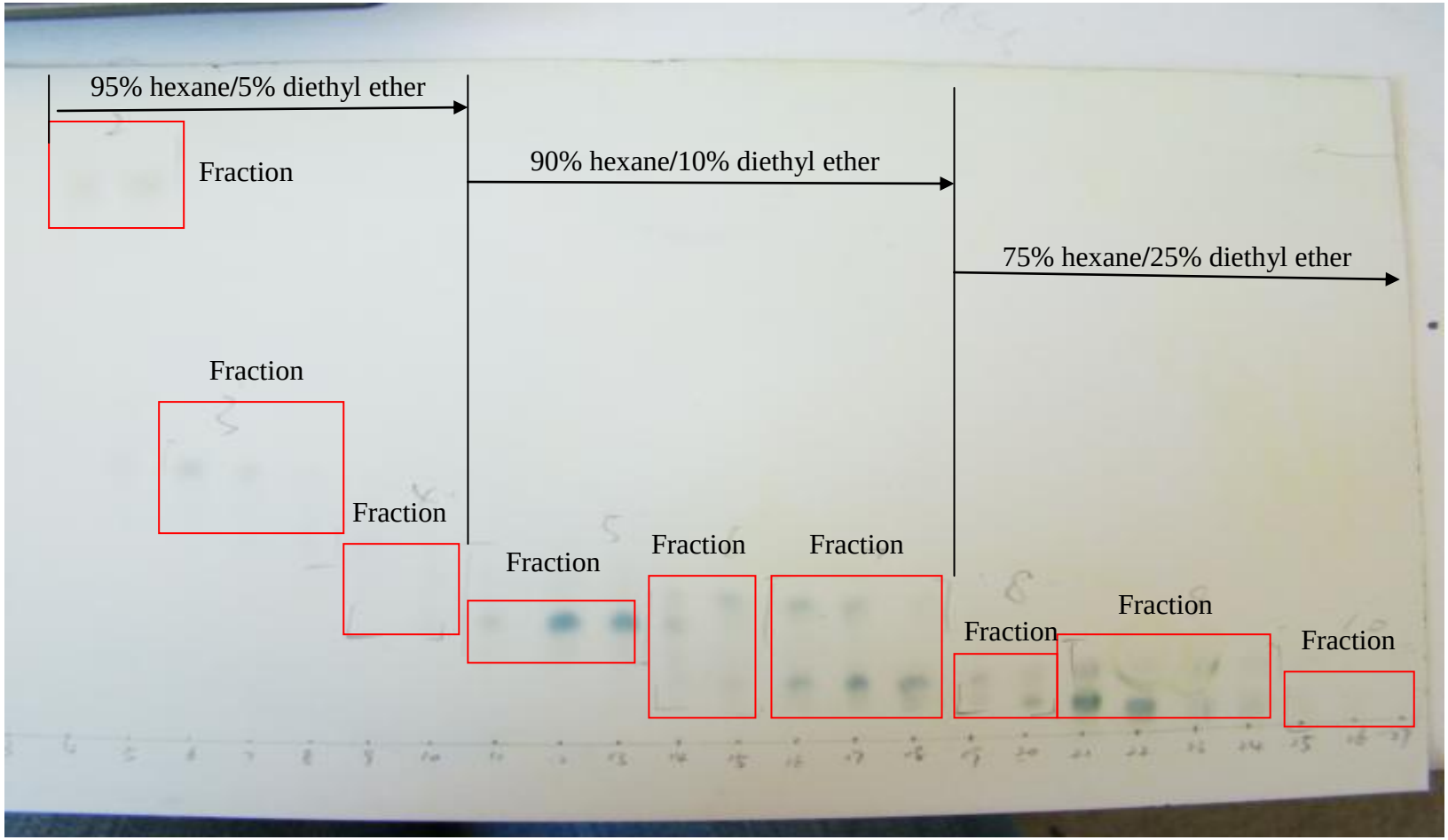



Figure 2.4: TLC plate with separated spots revealed from the column of supercritical $CO_2$ extract

2.3.1.1 Molecular ion adjustment in GC-MS analysis

It is noticed that throughout the GC-MS analysis of this work, all compounds with a molecular mass over 400 showed their molecular ion as $[M+1]^{+\bullet}$ rather than $M^{+\bullet}$. Although this phenomenon has been observed in the analysis process, the reason for it is not understood clearly. It is likely that the high mass calibration of the Perkin Elmer Clarus 560S mass spectrometer used within this study was malfunctioning therefore the molecular ion for compounds which have over 400 molecular mass were not measured very precisely. To prove this phenomenon, several standards which represent different groups of compounds with higher than 400 molecular mass were analysed and their mass spectrum were compared with a standard spectrum obtained from NIST library. Squalene (MW 410), α-amyrin (MW 426) and stigmasterol (MW 412) all displayed molecular ion as $[M+1]^{+\bullet}$ rather than $M^{+\bullet}$. Figure 2.5, 2.6 and 2.7 show the mass spectra obtained from Perkin Elmer mass spectrometer compared with Nist library standards.

From the spectrum of injected standards, it is clear that due to a unique instrumental conditions, all parent ions were displayed as $[M+1]^{+\bullet}$, not $M^{+\bullet}$. Therefore in later GC-MS analysis and compound identification process, all the $[M+1]^{+\bullet}$ parent ions will be regarded as normal $M^{+\bullet}$ parent ion.

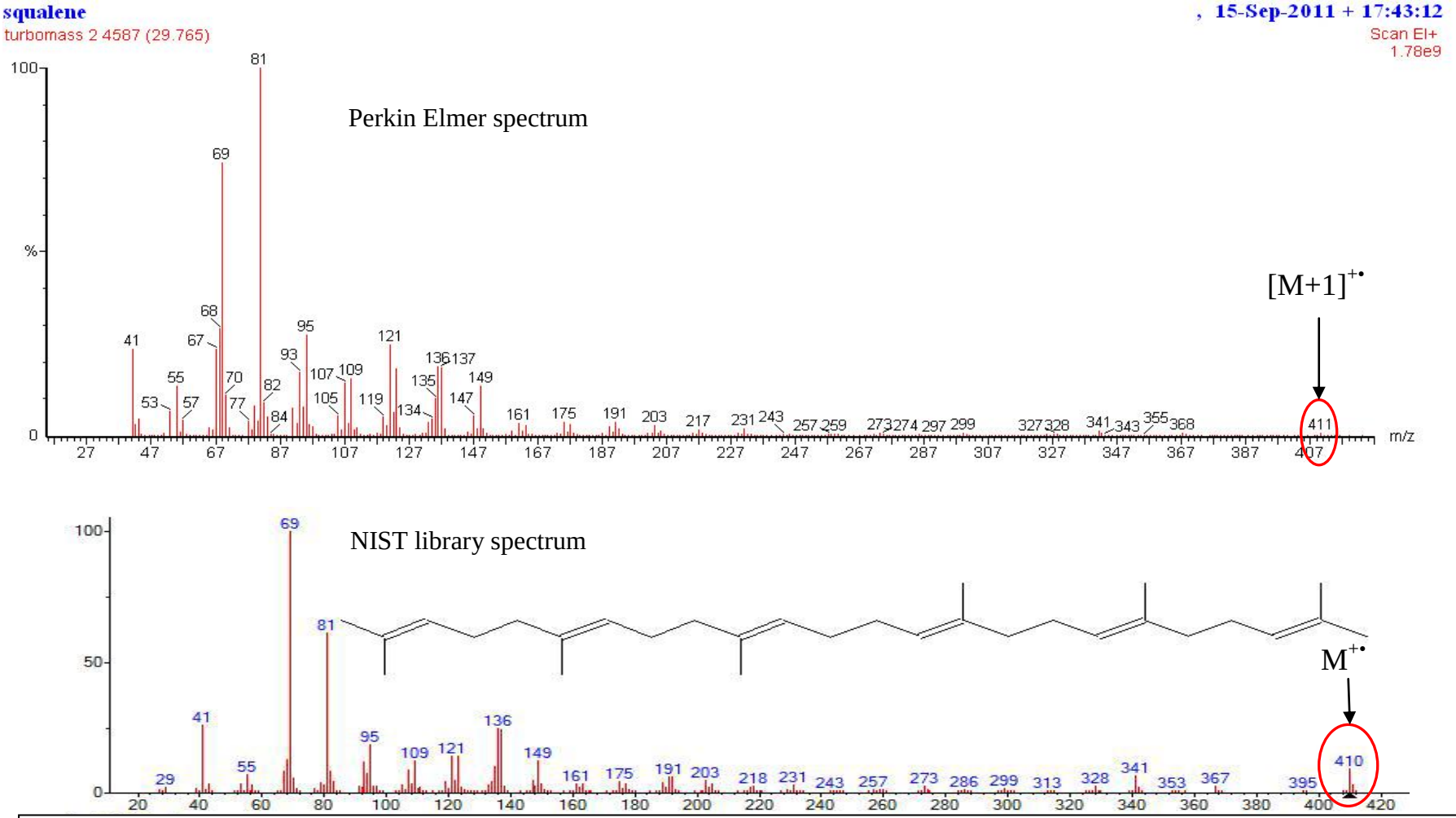



Figure 2.5: Mass spectrum of squalene obtained from Perkin Elmer Clarus 560S mass spectrometer compared with NIST library standard

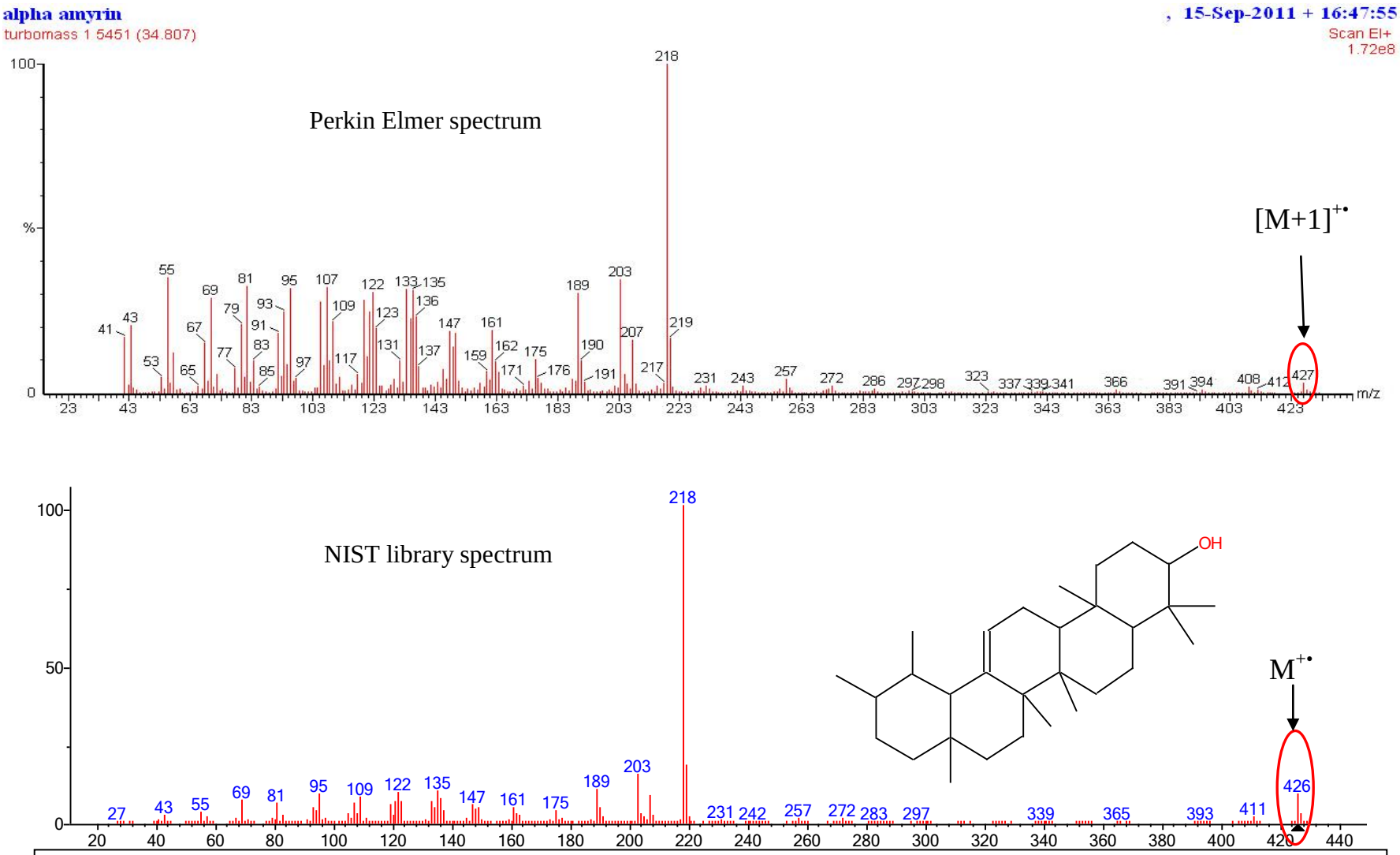



Figure 2.6: Mass spectrum of α-amyrin obtained from Perkin Elmer Clarus 560S mass spectrometer compared with NIST library

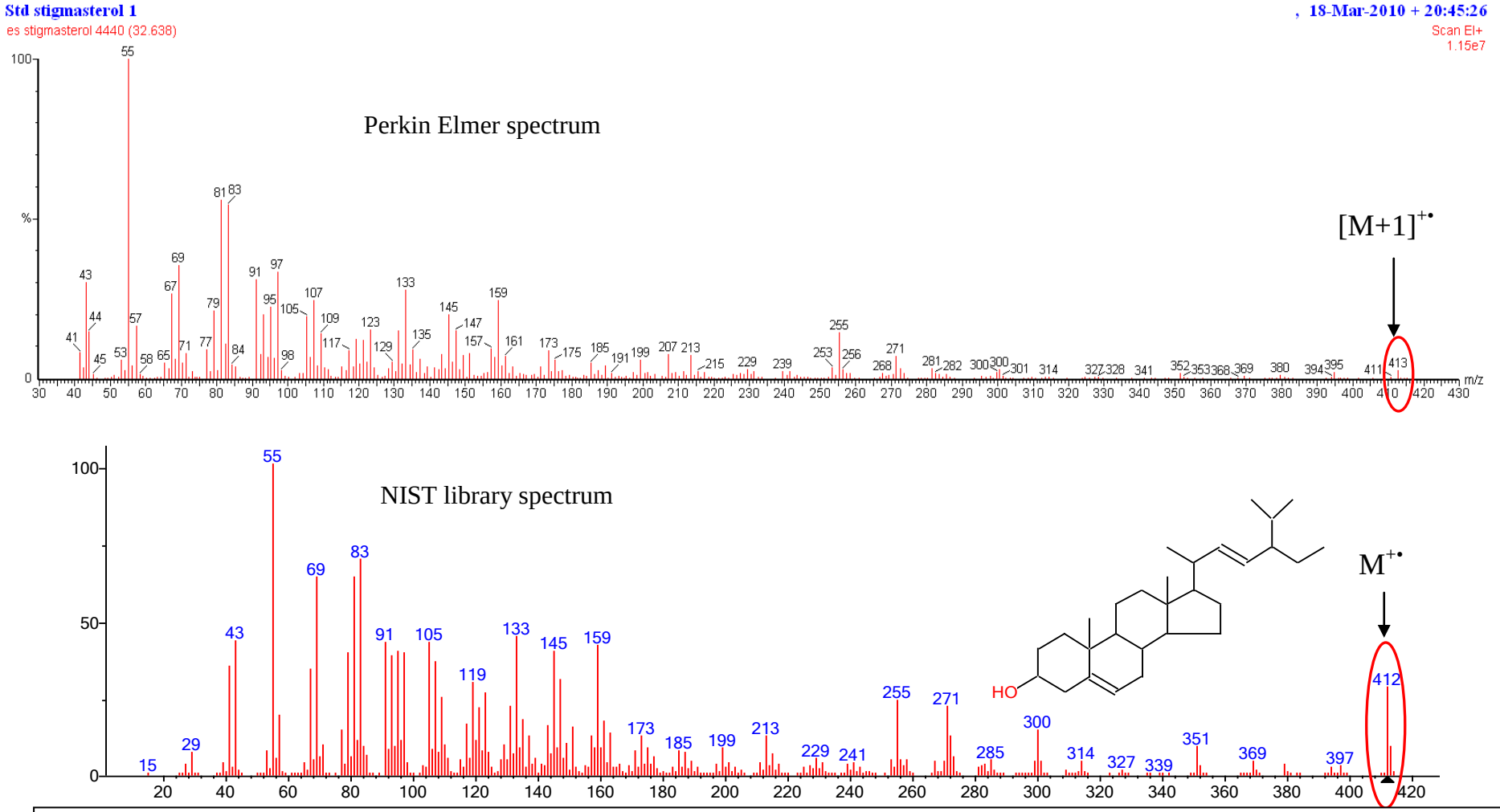



Figure 2.7: Mass spectrum of stigmasterol obtained from Perkin Elmer Clarus 560S mass spectrometer compared with NIST library standard

2.3.1.2 Identification of alkanes in heather supercritical $CO_2$ extract hexane fraction

The hexane eluent was collected from the column and combined as one fraction (25.2 mg) and analysed by GC (figure 2.8).

Most of the peaks in this fraction are identified as primary alkanes as they all have identical spectra to hydrocarbon standards. These n-alkanes within *Calluna vulgaris* exhibited chain lengths in the range of C21 to C35. The major peaks appeared at 31.17 min and 32.72 min were identified as hentriacontane and tritriacontane after comparison of their retention times with C12-C60 hydrocarbon standards. In this fraction, it is also clear that odd numbered alkanes generally show greater peak areas than even number alkanes. This finding is consistent with previous results and it is suggested that the production of these compounds in plants is through a elongation-decarboxylation mechanism of corresponding even carbon numbered fatty acids to yield odd carbon chain length alkanes.[67]

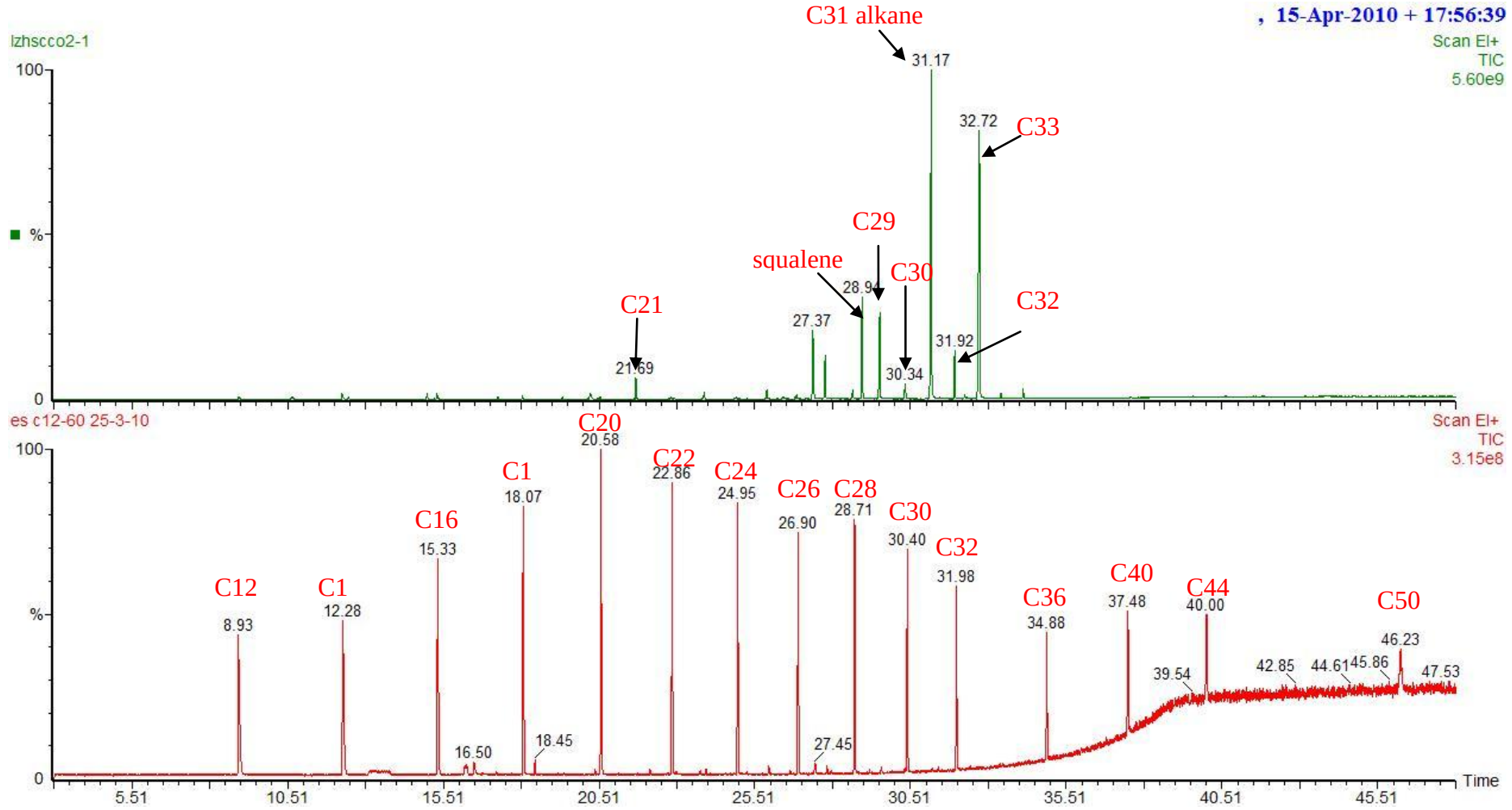



Figure 2.8: Gas chromatogram of fraction 1 compared with C12-50 hydrocarbon standards

Squalene was also identified in this fraction at 28.94 min. Squalene occurs naturally in plants as a precursor in the biosynthesis of sterols.[68] When campesterol and sitosterol are synthesized in the endoplasmic reticulum via the mevalonate pathway, production of the precursor squalene would take place at the same time.[69] Figure 2.9 shows the mass spectrum of the peak at 28.94 min and the standard spectrum of squalene obtained from the NIST library.

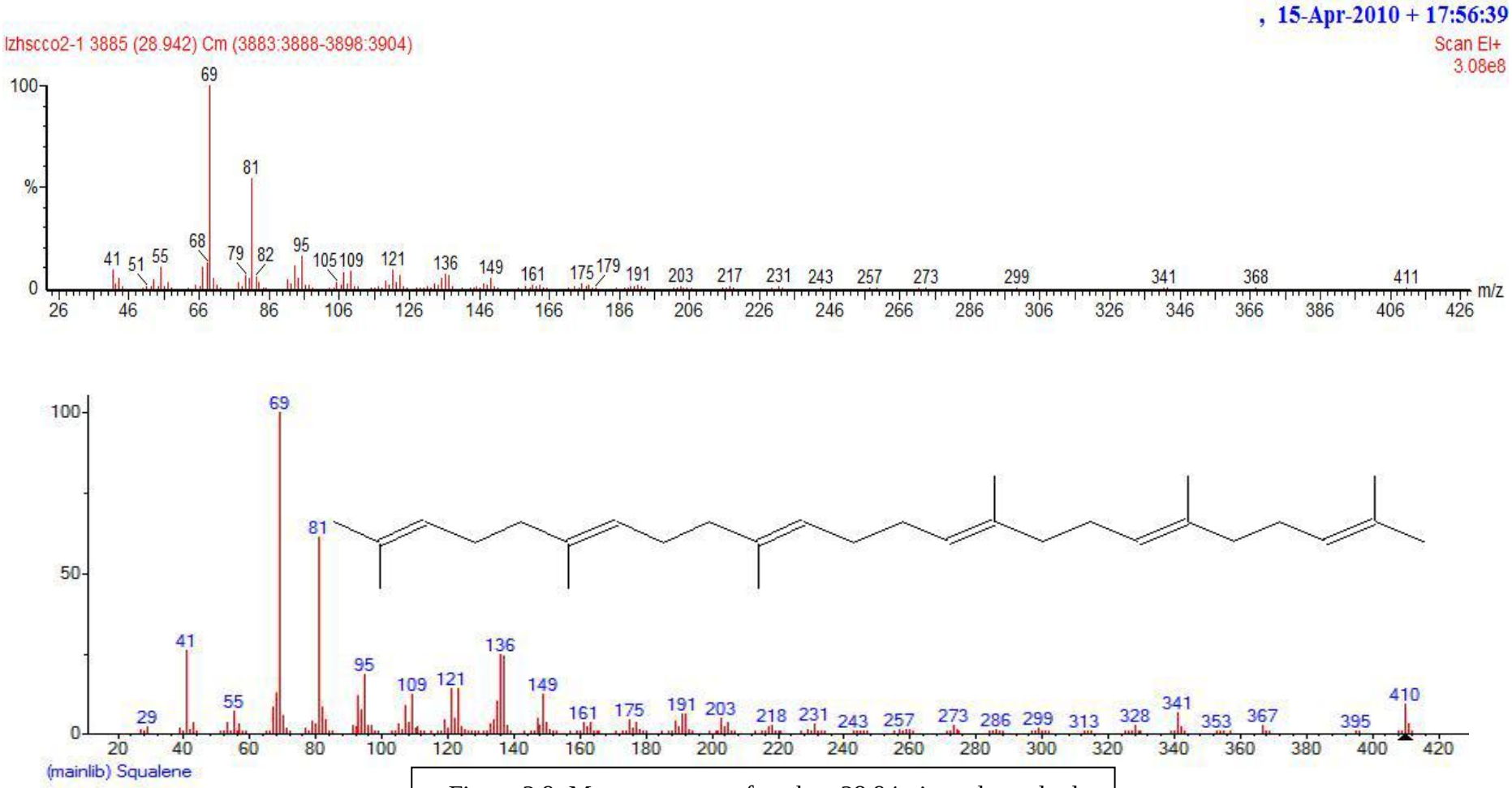



Figure 2.9: Mass spectrum of peak at 28.94min and standard spectrum of squalene obtained from NIST library

### 2.3.1.3 Quantification of triterpenoid

The quantification of each identified triterpenoid compound was carried out with the application of oleanolic acid as a standard and assuming that the response factor for structurally similar triterpenoids would be similar. Standard solutions of oleanolic acid at different concentrations were made up and analysed by GC. The absolute areas were recorded and a standard curve was determined. Figure 2.10 shows the standard curve of oleanolic acid and the regressive equation. A correlative coefficient of 0.9967 was obtained from this curve.

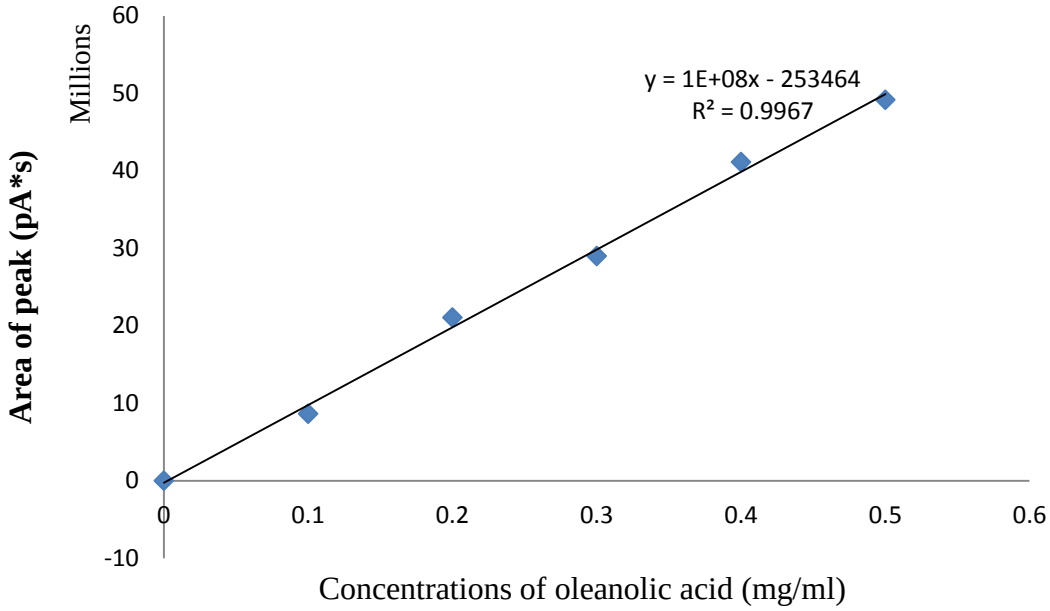



Figure 2.10: The standard curve of oleanolic acid for heather triterpenoid compound quantification

### 2.3.1.4 Identification of triterpenoids in heather supercritical $CO_2$ extract fractions

Triterpenoid compounds are mainly found in fractions 2 to fraction 5 which eluted from the silica column by hexane/diethyl ether mixture in different ratios. The weight and triterpenoid components found in each fraction are listed in table 2.2

Table 2.2 Triterpenoid compounds found in heather supercritical $CO_2$ extract fractions

| | Weight | Triterpenoids identified | Concentration of each triterpenoid (dry plant) |
|---|---|---|---|
| Fraction 2 | 83.2mg | Taraxerone<br>Friedelin<br>13,27-cycloursane<br>D: C-Friedoolean-8-en-3-one | 326 μg/g<br>234 μg/g<br>91 μg/g<br>77 μg/g |
| Fraction 3 | 12.3mg | Taraxerol<br>Friedelin<br>Friedelinol<br>13,27-cycloursane<br>9-methyl-19-Norlanosta-5,24-dien-3-ol<br>α-amyrin<br>β-amyrin | 81 μg/g<br>155 μg/g<br>362 μg/g<br>108 μg/g<br>80 μg/g<br>131 μg/g<br>46 μg/g |
| Fraction 4 | 45.7mg | α-amyrin<br>β-amyrin | 494 μg/g<br>221 μg/g |
| Fraction 5 | 29.3mg | α-amyrin<br>β-amyrin | 68 μg/g<br>25 μg/g |

Figures 2.11 and 2.12 show the gas chromatogram of fractions 2-5 with triterpenoid group highlighted. Quantification of each identified triterpenoid compound was also accomplished by using oleanolic acid as a standard. Figure 2.13 showed the expanded gas chromatogram of fraction 2 with each identified triterpenoid compound highlighted.

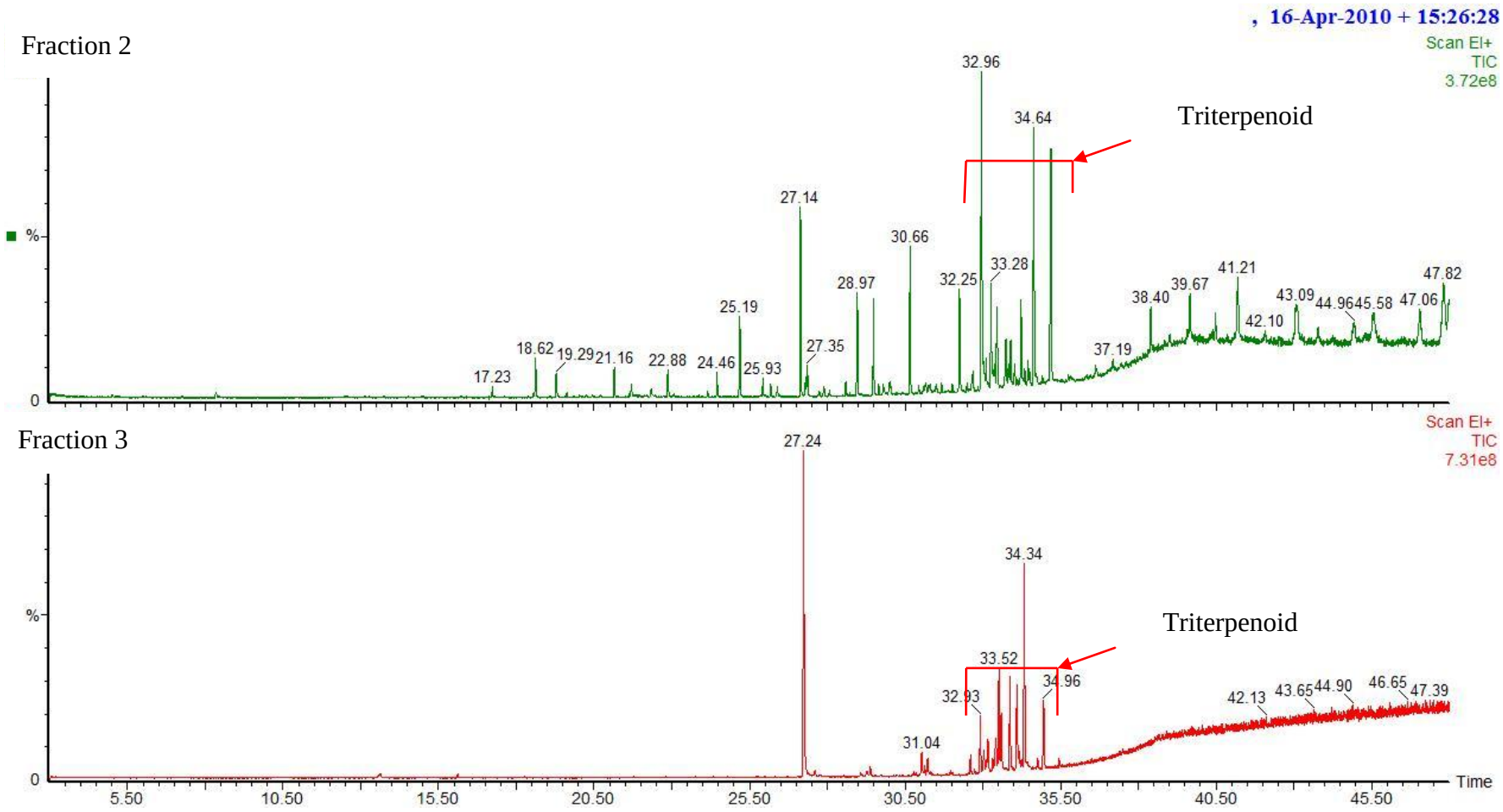



Figure 2.11: Gas chromatogram of fraction 2 and fraction 3 with triterpenoid group highlighted

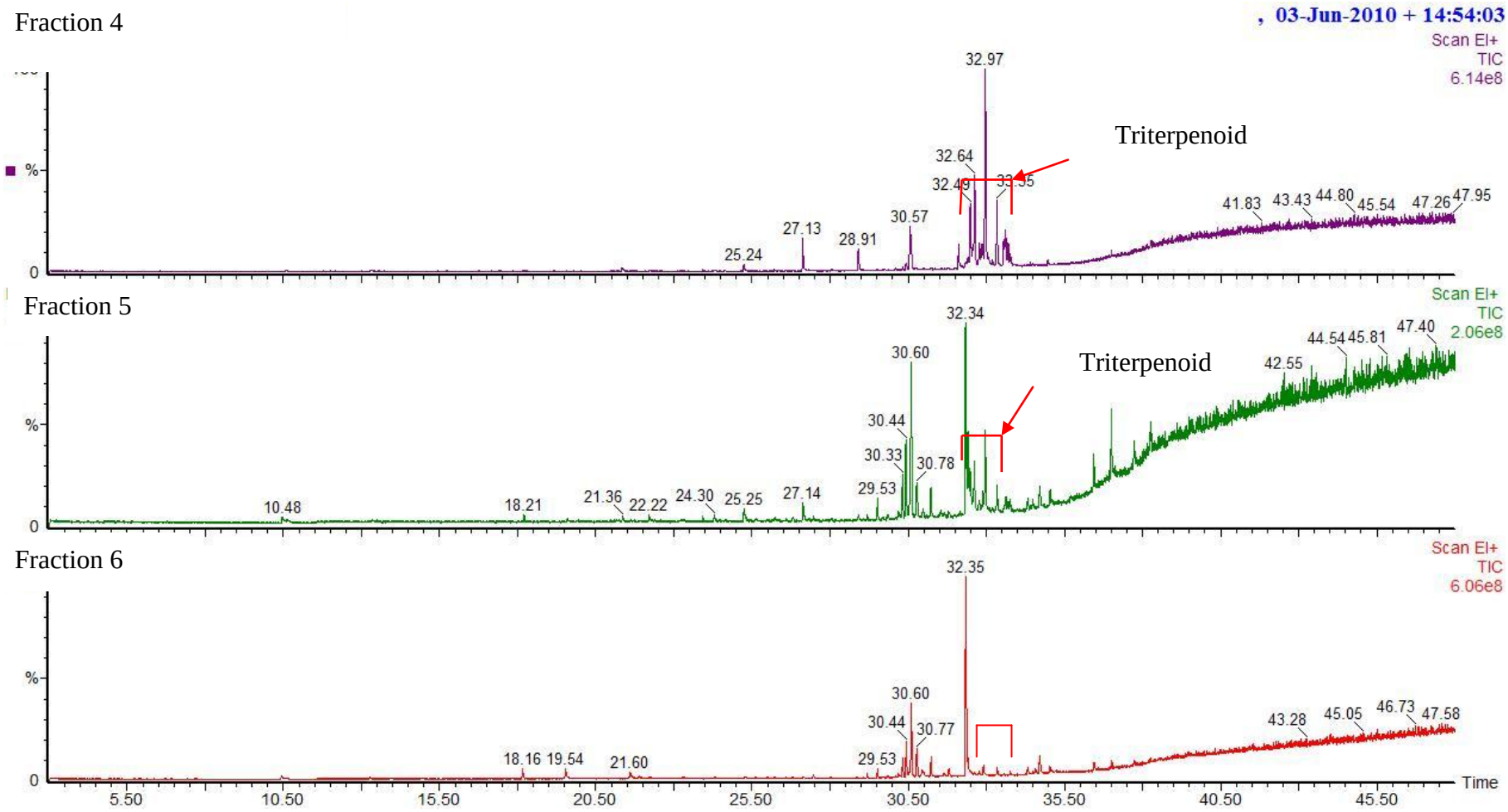



Figure 2.12: Gas chromatogram of fractions 4, 5, and 6 with triterpenoid group highlighted

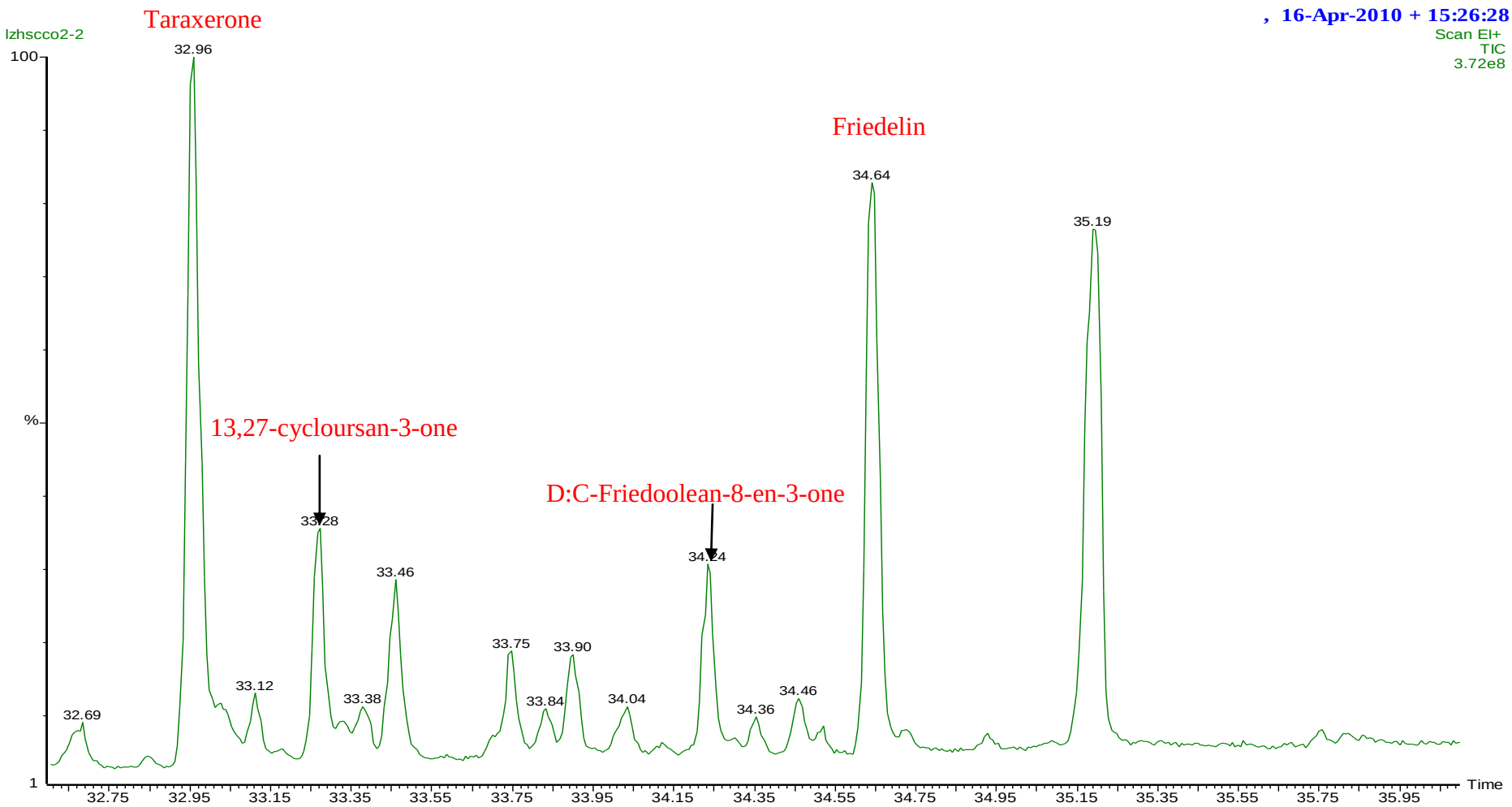



Figure 2.13: Expanded gas chromatogram of fraction 2 with each identified triterpenoid compound highlighted

Taraxerone ($C_{30}H_{48}O$, also called D-Friedoolean-14-en-3-one, MW 424) was found in fraction 2 with a retention time of 32.96 min. Taraxerone exhibits a fragmentation pattern of m/z 424, 409, 300, 285 204 and 133. Figure 2.15 shows the mass spectrum comparison of the peak at 32.96 min with taraxerone standard obtained from NIST library. Taraxerone was present at 326 μg/g in August heather dry plant.

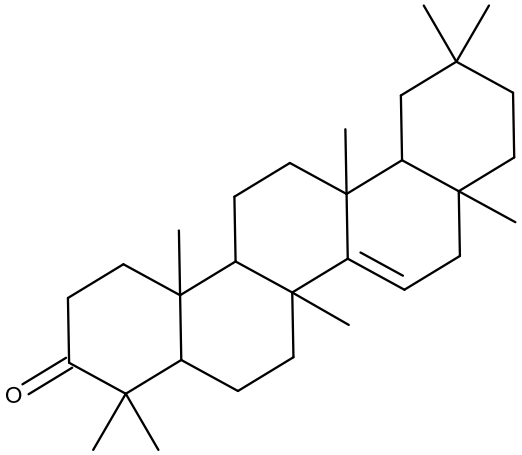
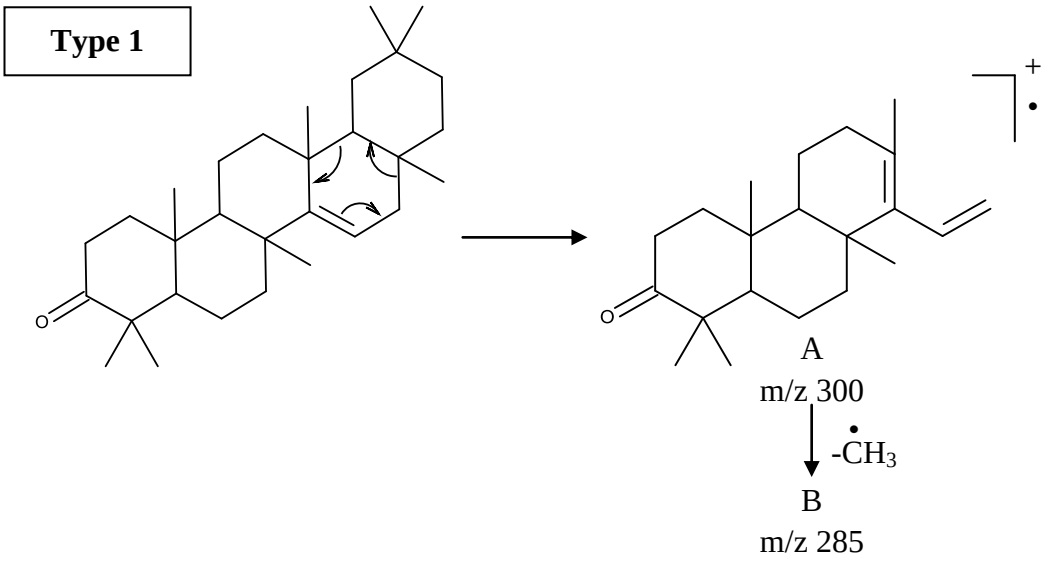


Figure 2.14 Chemical structure of taraxerone

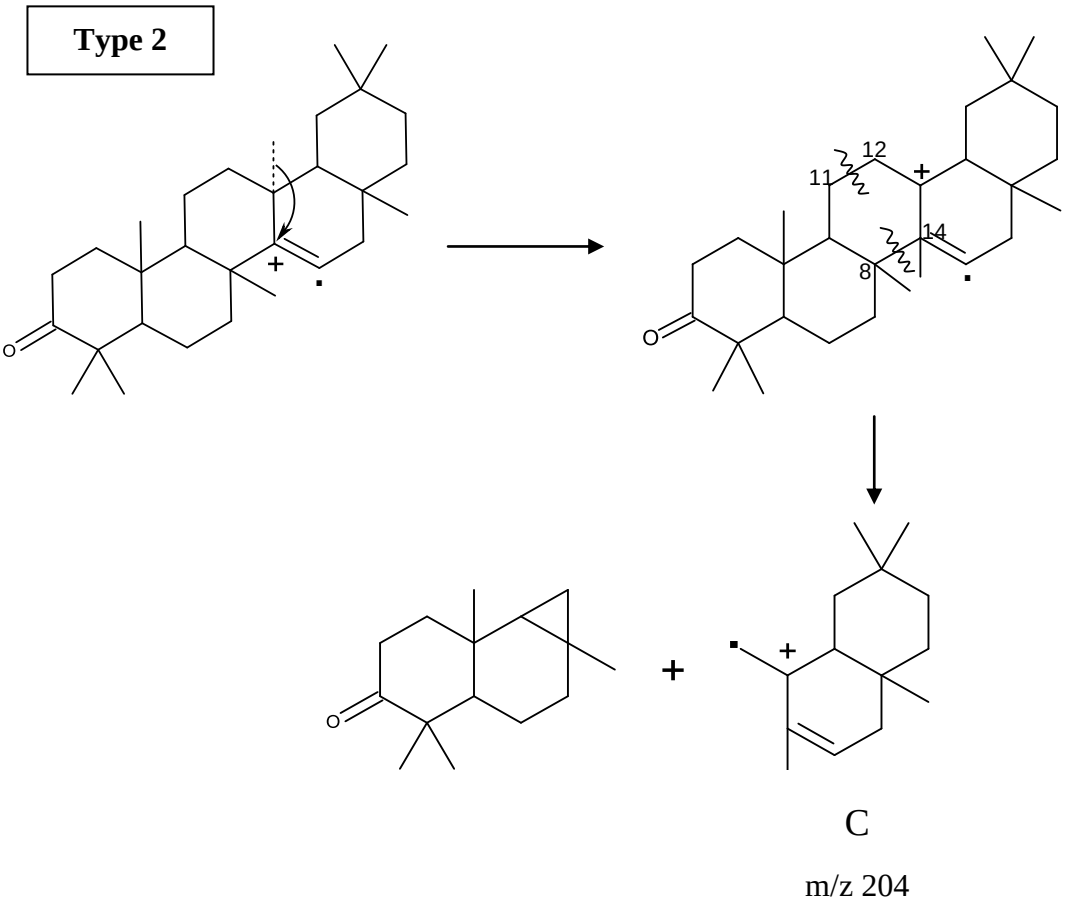
An important cleavage process in this compound is the retro Diels-Alder collapse of ring D (Type 1) as shown below yielding diene fragment A gives the characteristic ion at m/z 300; further loss of a methyl group yields ion B (m/z 285).




Scheme 2.1: Proposed mechanism for fragmentation of taraxerone (type 1)

A second important cleavage in this compound occurs in ring C involving rupture of the 8, 14 and 11, 12 bonds (Type 2). Previous researchers found it very difficult to explain this fragmentation process, however they postulated a mechanism in which the molecular ion of taraxerone generates a new carbonium ion, which then leads to the ion C by appropriate cleavage.[70] Ion C (m/z 204) is the most abundant in the spectrum of taraxerone.

The postulated mechanism of type 2 fragmentation is not entirely convincing, but as mentioned before, this fragmentation is difficult to explain. The mechanism of the type 2 fragmentation showed in scheme 2.2 is only a tentative mechanism based on previous researcher's postulation.




Scheme 2.2: Proposed mechanism for fragmentation of taraxerone (type 2)

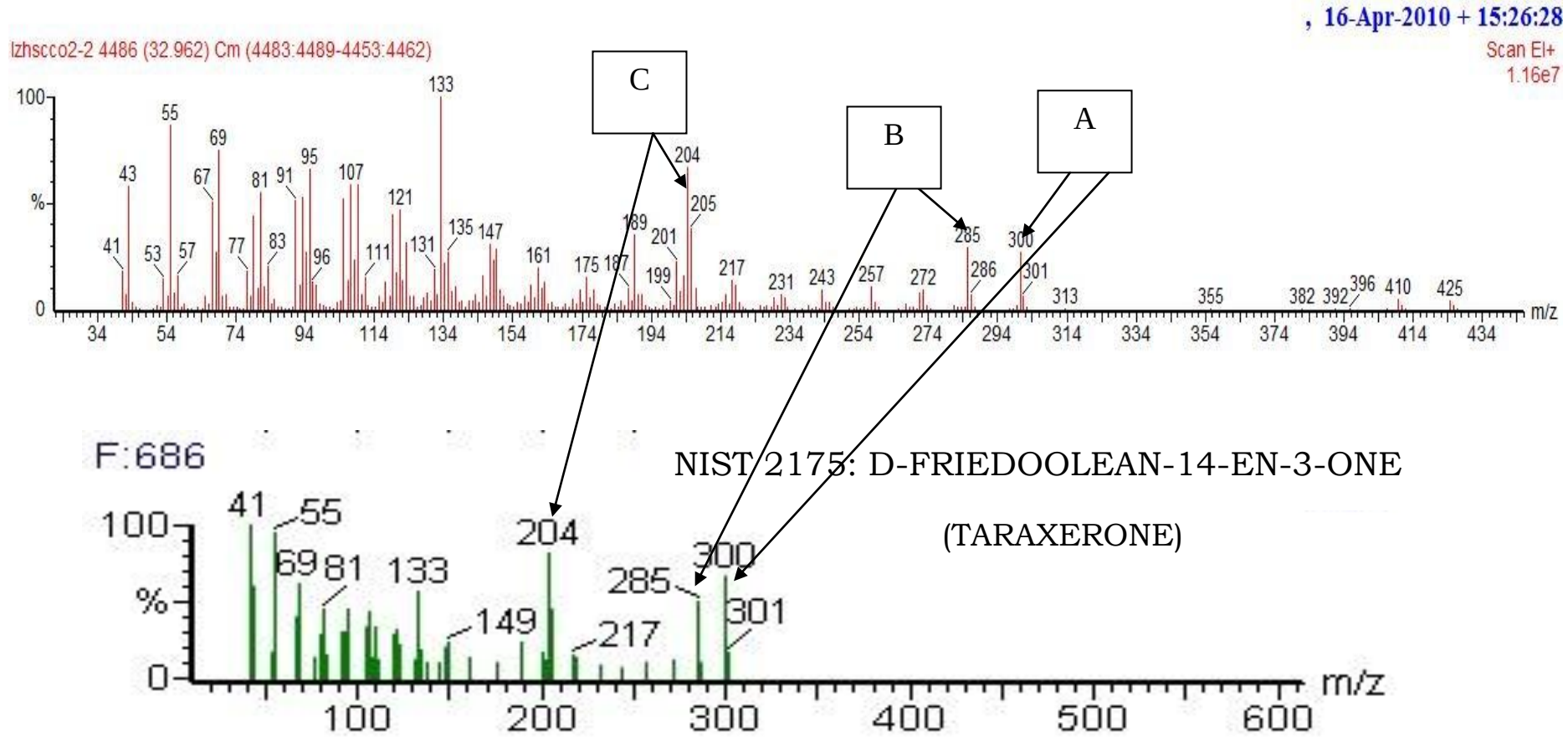



Figure 2.15: Mass spectrum of the peak at 32.96min in fraction 2 compared with standard taraxerone spectrum

The peak with a retention time of 32.93 min in fraction 3 was identified as taraxerol ($C_{30}H_{50}O$, MW 426) by directly comparing its mass spectrum with standard taraxerol spectrum. Taraxerol gives characteristic fragmentation of m/z 426, 411, 302, 287 and 204. Figure 2.17 shows the comparison between the spectrum of peak at 32.93 min and the standard spectrum of taraxerol obtained from NIST library. Taraxerol showed a concentration of 81 μg/g in heather dry plant.

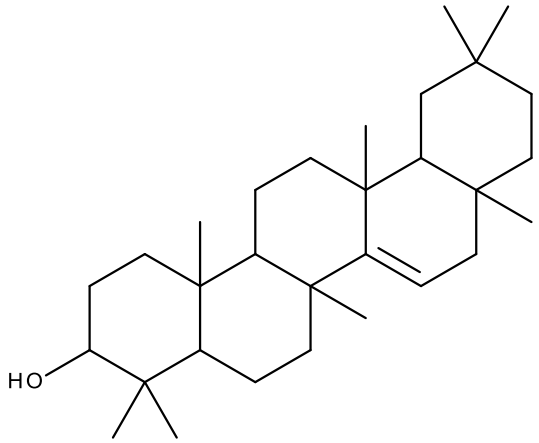
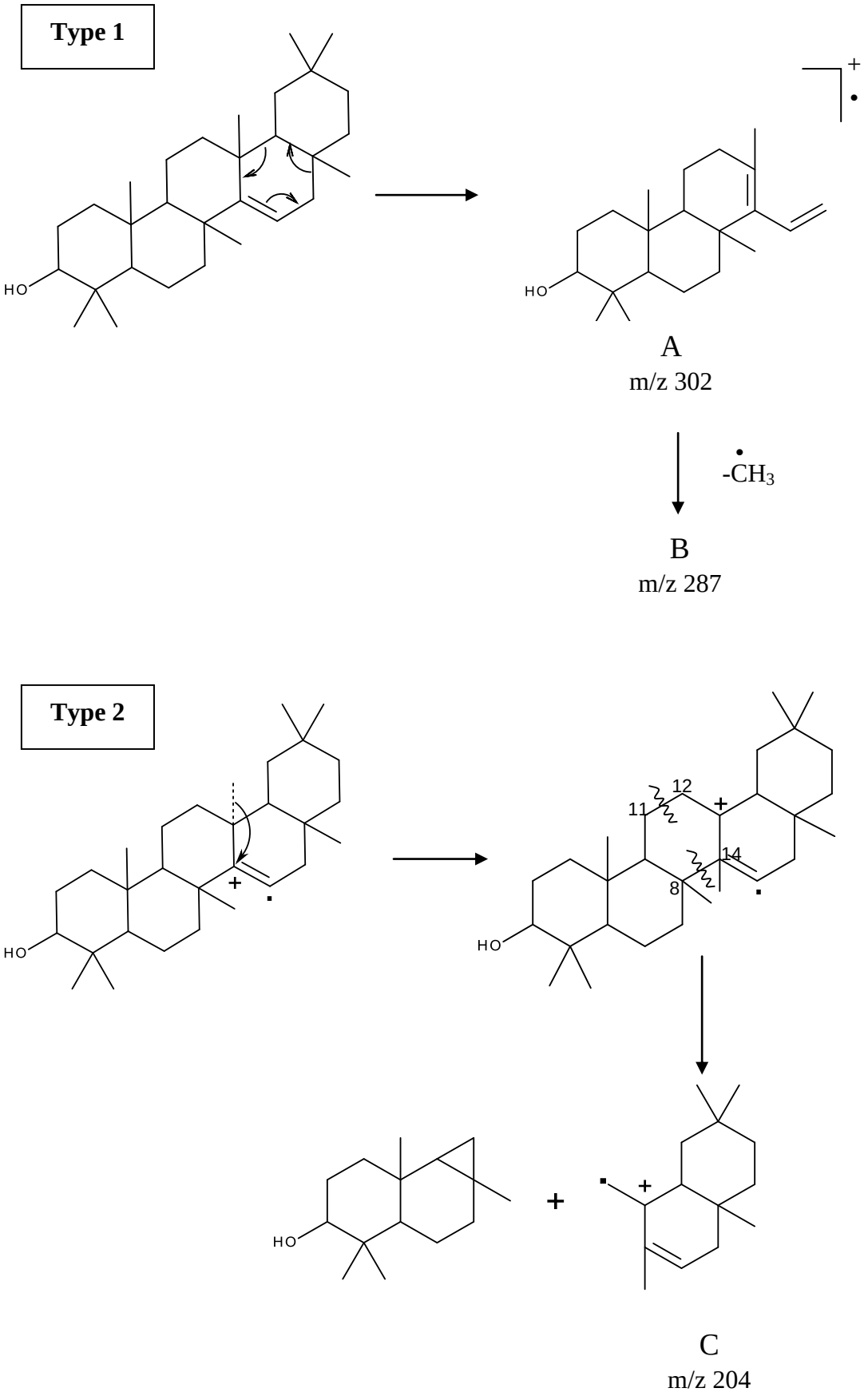



Figure 2.16 Chemical structure of taraxerol

The same retro Diels-Alder collapse of ring D (Type 1) occurs during taraxerol fragmentation process and affords the characteristic ion A at m/z 302. A further loss of a methyl group yields ion B (m/z 287). Similarly, rupture of the 8, 14 and 11, 12 bonds occurs in ring C and yield the characteristic ion C (m/z 204).[70]




Scheme 2.3: Proposed mechanism for fragmentation of taraxerol

According to Pancost and co-workers,[65] taraxerone and taraxerol were only detected in the roots of *Calluna vulgaris*. Their results indicated that taraxerone and taraxerol were not detected in aerial part includes stems and leaves. However, in this study, taraxerone and taraxerol were both found in heather aerial parts, the supercritical carbon dioxide extract showed a high concentration of taraxerone and was a major component in this fraction.

Taraxerol and taraxerone are both reported to have biological activities such as anti-cancer and anti-inflammatory. Taraxerone showed in vitro anti-leishmanial activity against promastigotes of *Leishmania donovani* and anti-tumour activity on K562 leukemic cell line.[71] Taraxerol was reported to have significant activity against dermatophytes *T. rubrum* and *T. mentagrophytes* with MIC values between 6.2 and 25 μg/ml, additionally, taraxerol also expresses moderate activity against *Aspergillus niger*.[72]

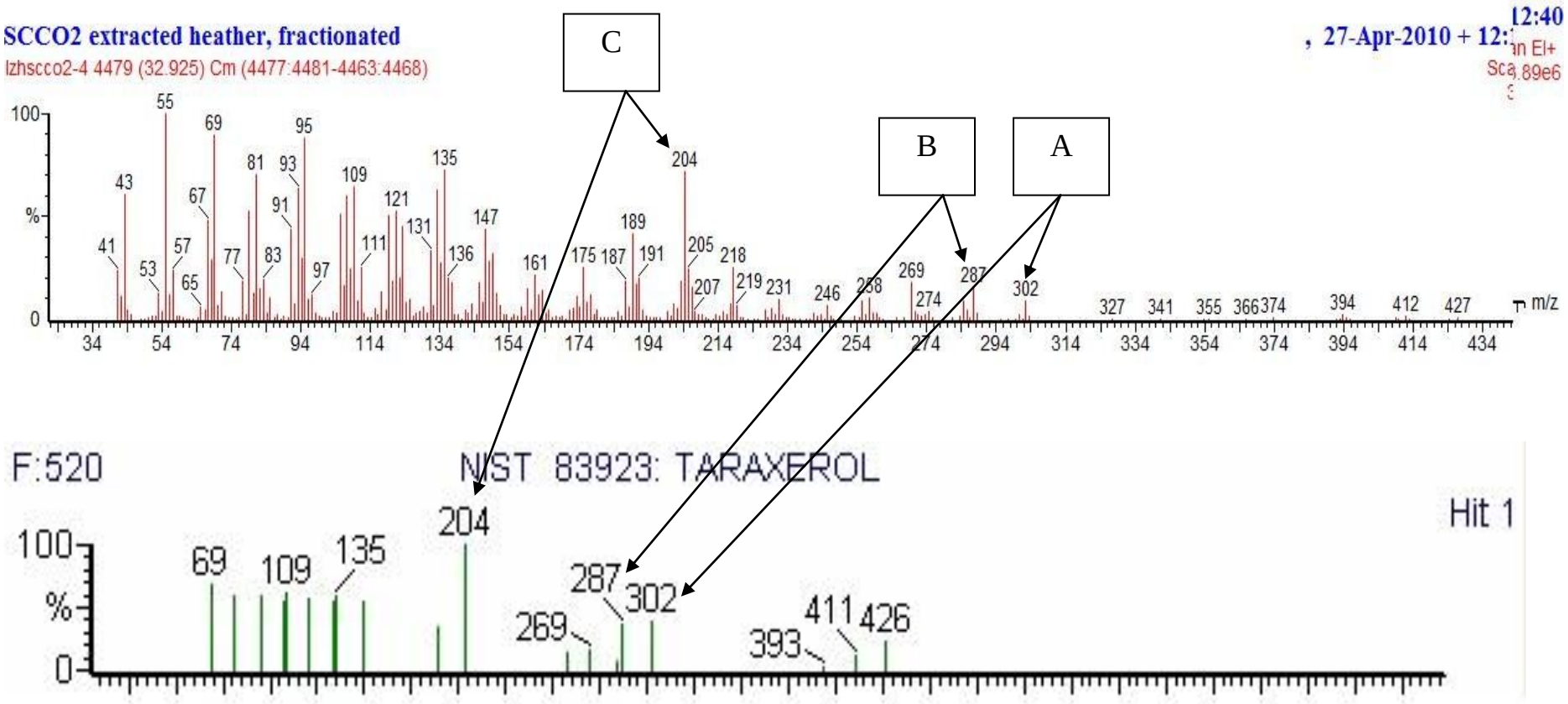
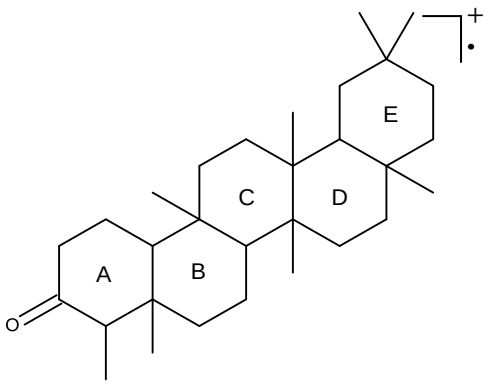


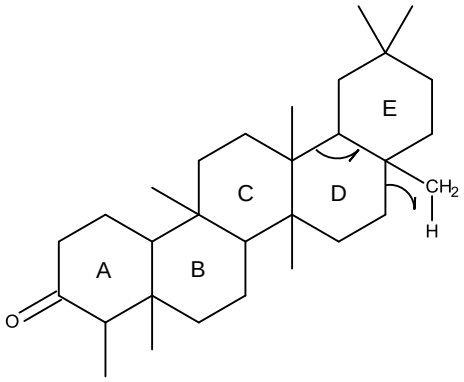

Figure 2.17: Mass spectrum of peak at 32.93min in fraction 3 compared with standard taraxerol spectrum

The peaks at 34.64 min in fraction 2 and 34.11 min in fraction 3 which expressed quite high absolute areas were identified as friedelin ($C_{30}H_{50}O$, MW 426). Friedelin exhibited a fragmentation pattern of m/z 411, 273, 123 and 109. The mass fragmentation patterns of friedelin were presented in Scheme 2.3. The molecular ion peak of this compound was observed at m/z 426. The loss of a methyl group was indicated by the presence of a peak at m/z 411. Other significant ions at m/z 341, 273, 205 and 123 were attributed to the fragmentation of A, B, C and D rings respectively. Figure 2.18 shows the mass spectrum of the peak at 34.63 min in fraction 2 compared with standard spectrum of friedelin. The concentration of friedelin in was calculated as 234 μg/g in fraction 2 and 155 μg/g in fraction 3. Therefore, its total concentration in heather dry plant is 389 μg/g.

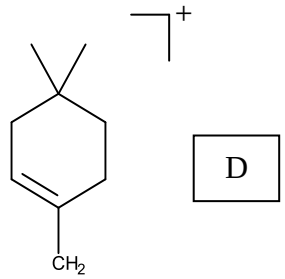
Friedelin and several derivatives of it have been tested on their biological activities. Friedelin has been tested for its cytotoxicity on human leukemia cell HL-60, human ovarian cancer cell SK-OV-3, human lung adenocarcinoma cell A549 and human colon cancer HT-29 cell lines.[73] Friedelin showed significant cytotoxic activity against all cell lines with $GI_{50}$ values in the range of 11.1 to 13.5 μM. Friedelin has also been evaluated for its anti-inflammatory effects in non-cytotoxic concentrations (1-100 nM).[73] The result shows that friedelin expressed moderate inhibitory activity of TNF-α secretion in the presence of LPS in a murine macrophage cell line at 100 nM. Other researchers reported that the anti-inflammatory activity of friedelin can be tripled by introducing hydroxyl group into its 3β position.[74] Therefore, friedelin not only has valuable biological activities on its own, but also has a good potential to be applied as pharmaceutical precursor.



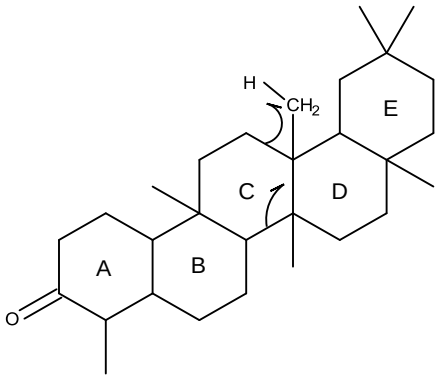

$\overset{\bullet}{-CH_3}$ → m/z 411



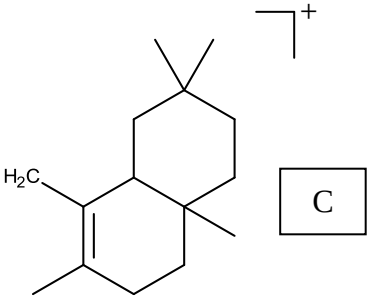

D-ring cleavage →



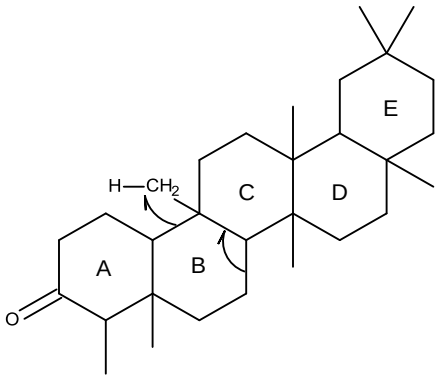

m/z 123



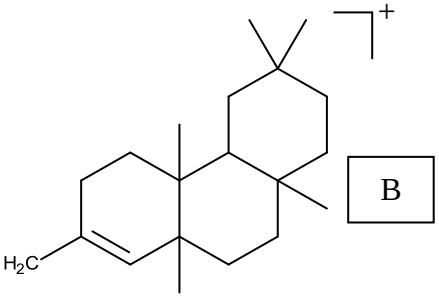

C-ring cleavage →



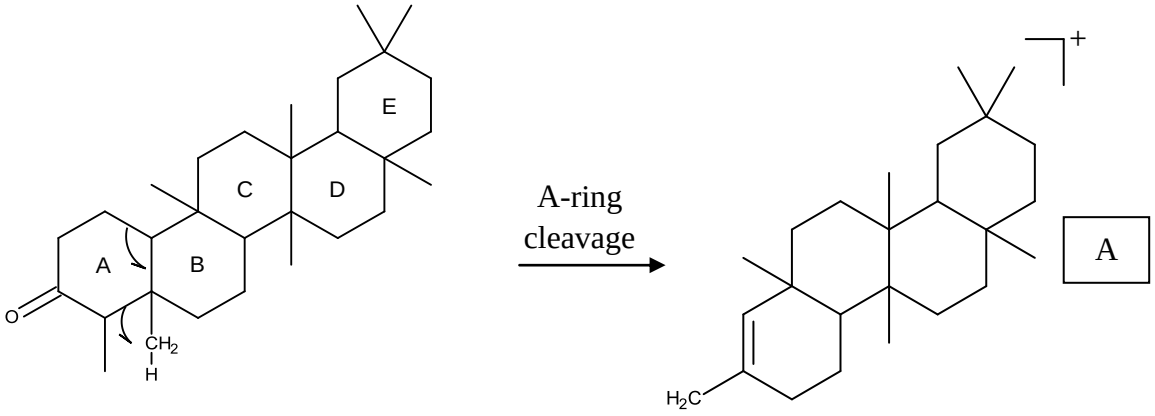

m/z 205




B-ring cleavage →




m/z 273




m/z 341

Scheme 2.4: Proposed mechanism for fragmentation of friedelin

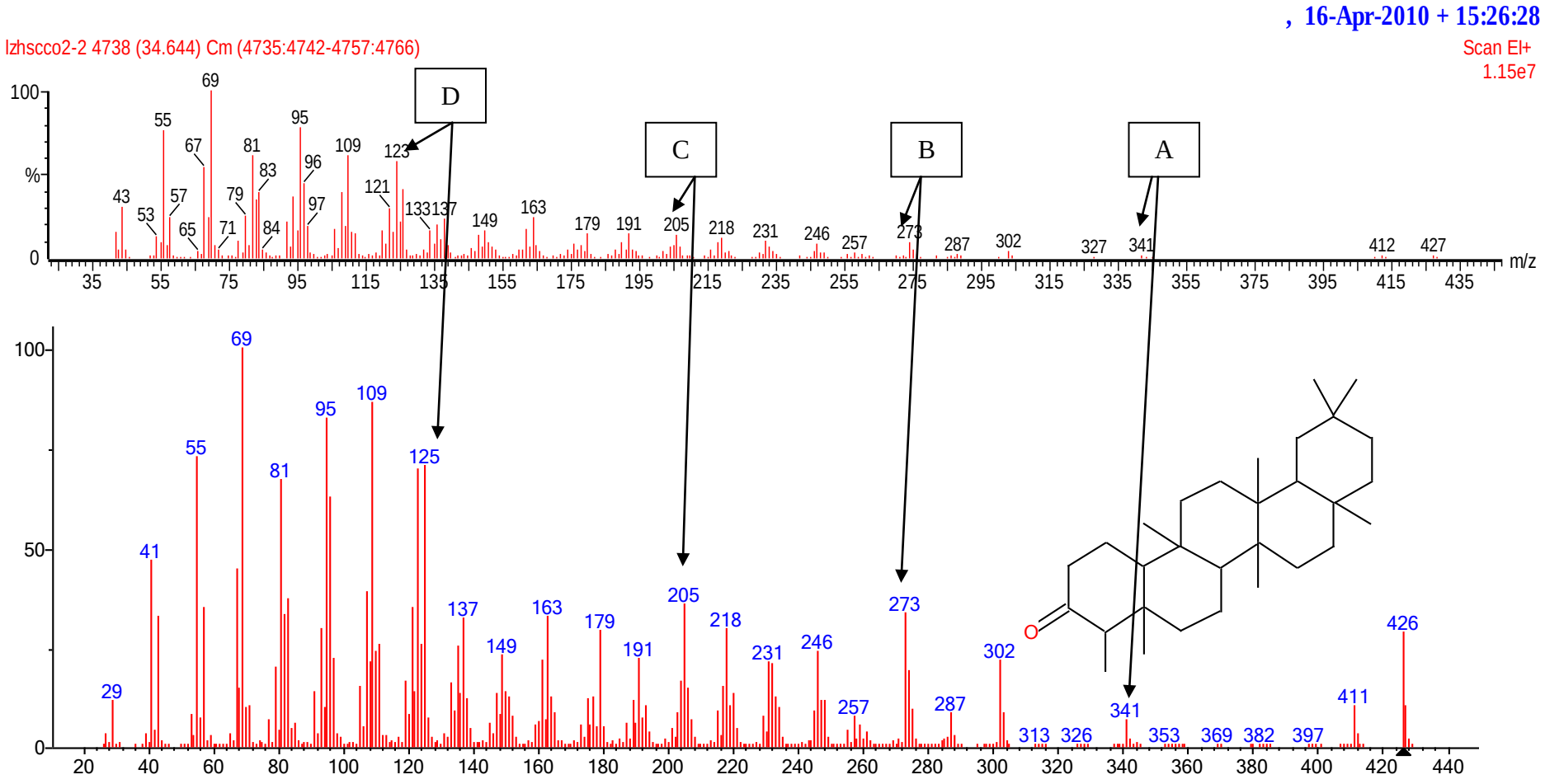



Figure 2.18: The mass spectrum of the peak at 34.63min in fraction 2 compared with standard spectrum of friedelin

13,27-cycloursan-3-one ($C_{30}H_{48}O$, MW 424) was found in both fraction 2 and fraction 3. The peaks present at 33.28 min in fraction 2 and 33.39 min in fraction 3 have the identical mass spectrum and were identified as 13,27-cycloursan-3-one after compared their mass spectrum with standard spectrum obtained from NIST library. 13,27-cycloursan-3-one exhibited a characteristic fragmentation pattern as m/z 409, 205 and 138. Figure 2.20 shows the mass spectrum of the peak at 33.28 min in fraction 2 with the standard spectrum of 13,27-cycloursan-3-one.

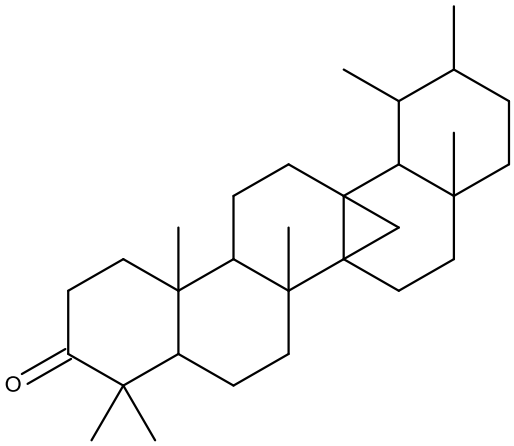



Figure 2.19: Chemical structure of 13,27-cycloursan-3-one

Cyclopropyl ursane-type triterpenoid are very rarely found in narual plant. It's only been reported once that this compound has been discovered in African tree *Phyllanthus polyanthus*,[75] however, it's been synthesised by Barton & de Mayo in 1953.[76] The fragmentation mechanism of 13,27-cycloursan-3-one is not fully understood. The forming of ion at m/z 205 was speculated due to a cleavage in ring C. Therefore, the idenfication of 13,27- cycloursan-3-one is a tentative identification based on the direct comparison between the mass spectrum of the peak and standard spectrum. The quantity of 13,27-cycloursan-3-one was found to be 91 μg/g in fraction 2 and 108 μg/g in fraction 3. Therefore the total concentration of 13,27-cycloursan-3-one in heather supercritical $CO_2$ extract is 199 μg/g. At the moment, no literature about any biological activity of this triterpenoid compound has been published.

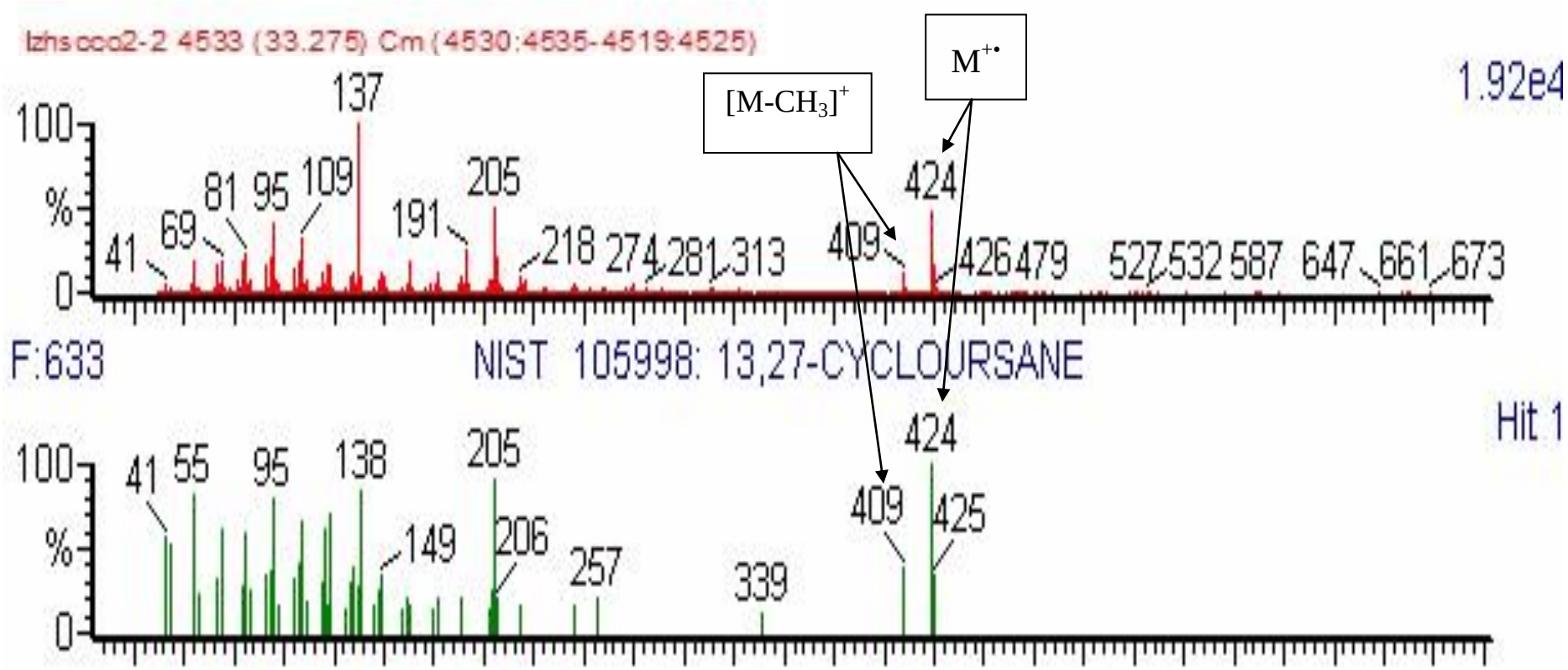
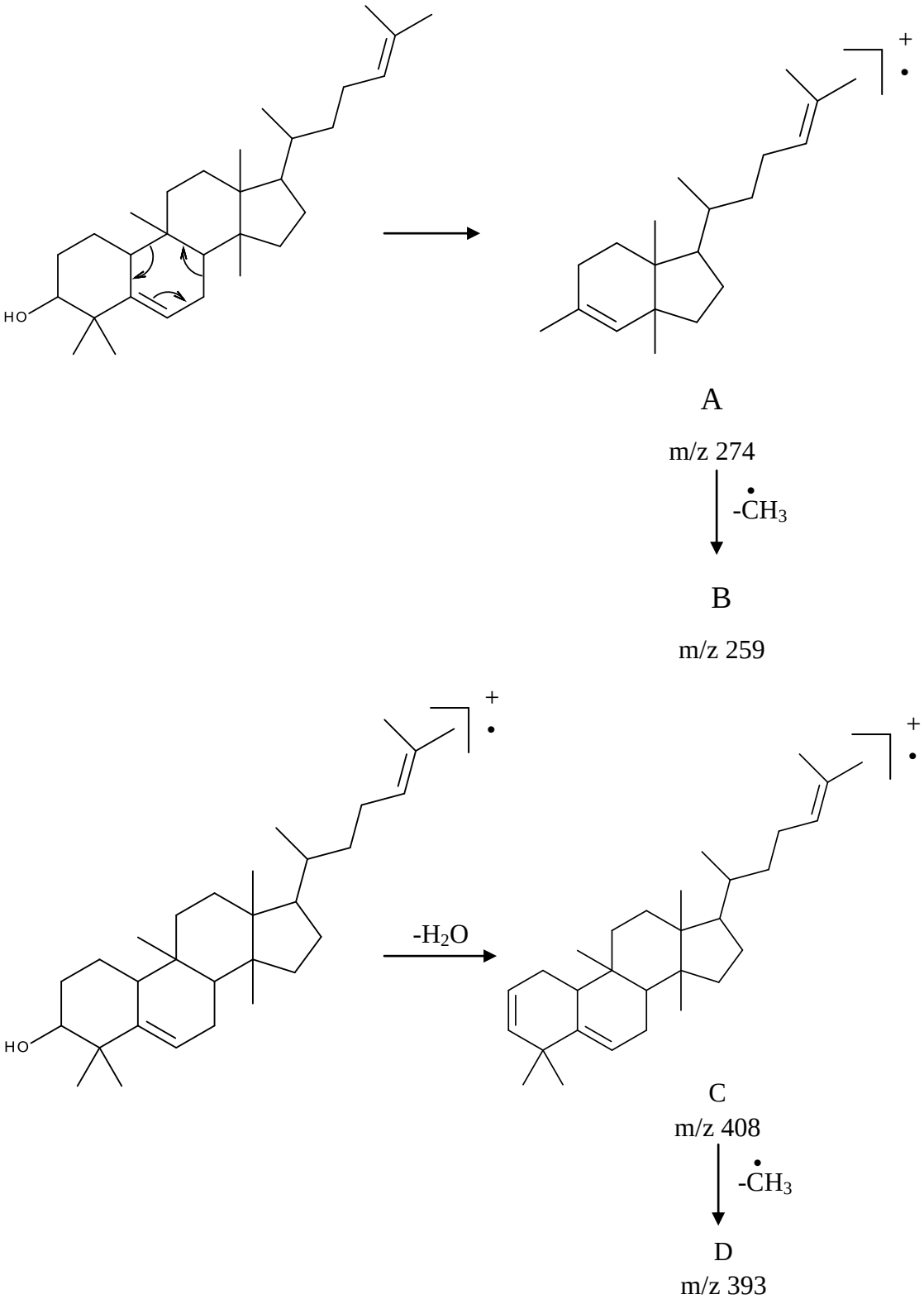



Figure 2.20: The mass spectrum of the peak at 33.28min in fraction 2 compared with standard spectrum of 13,27-cycloursan-3-one

9-methyl-19-Norlanosta-5,24-dien-3-ol ($C_{30}H_{50}O$, MW 426) was detected in fraction 3 at 33.60 min. Due to the standard mass spectrum in NIST library is incomplete (no ion below m/z 100 was recorded), the mass spectrum were not matched perfectly, therefore the identification is only a tentative identification based on the direct comparison between the mass spectrum of the peak and standard spectrum of 9-methyl-19-Norlanosta-5,24-dien-3-ol. 9-methyl-19-Norlanosta-5,24-dien-3-ol is also called as boeticol or (+)-antiquol B, and it's been reported to be found in *Euphorbia boetica*.[77] This previous research paper also recorded the NMR and GC-MS spectrum of this compound. Boeticol yielded a series of ions as m/z 426, 408, 393, 313, 295, 274, 259, 231, 205, 163, 134, 123, 121, 107, 95, 81 and 69. Since the research paper did not present the spectrum of boeticol, figure 2.21 indicates a proposed based on the distribution of fragments according to the author. Figure 2.22 shows the incomplete standard mass spectrum of 9-methyl-19-Norlanosta-5,24-dien-3-ol from NIST; library. The proposed fragmentation mechanism of this compound was also discussed in scheme 2.5. The concentration of this triterpenoid was measured as 80 μg/g in dry plant.




Scheme 2.5: Proposed mechanism for fragmentation of 9-methyl-19-Norlanosta-5,24-dien-3-ol

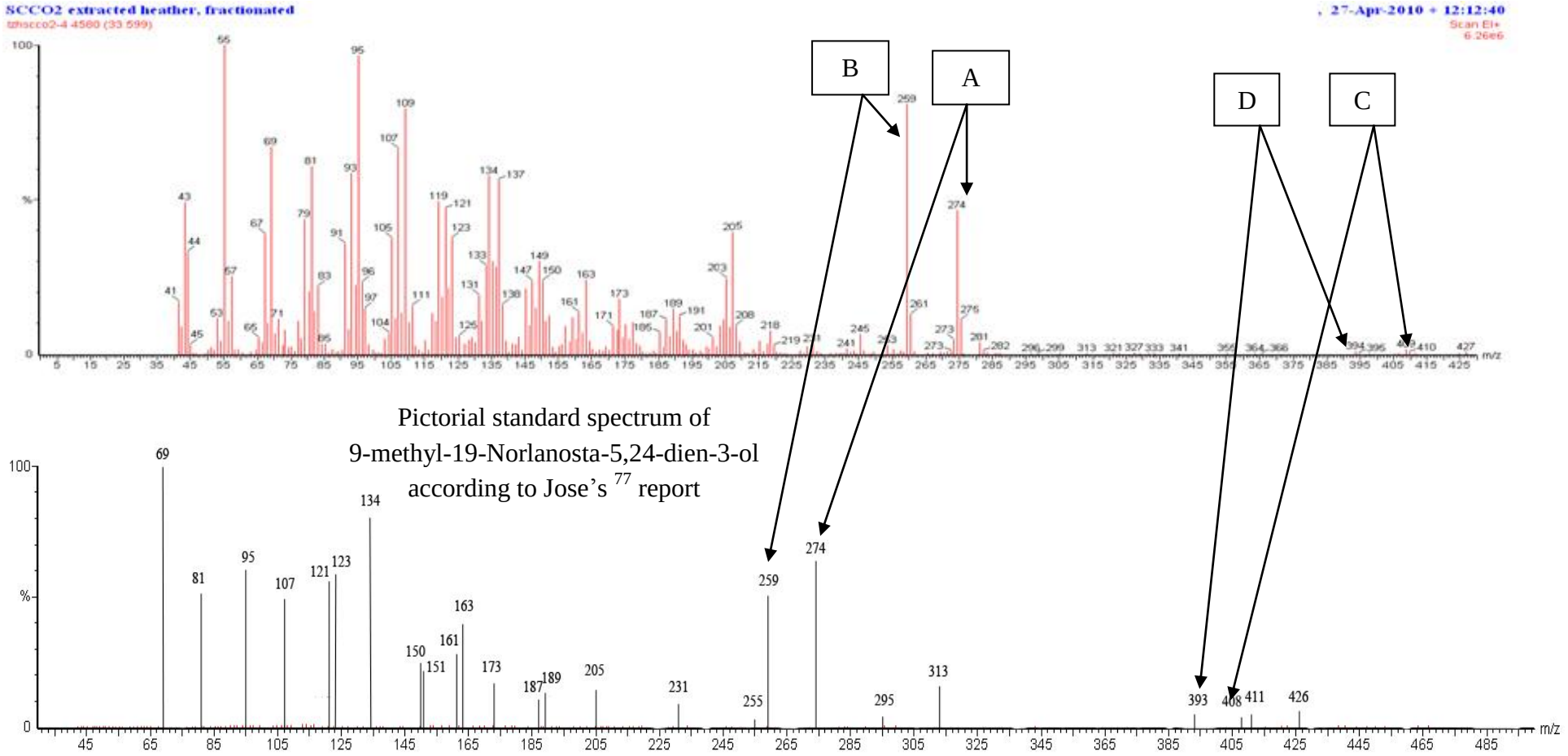



Figure 2.21: The mass spectrum of the peak at 33.60 min in fraction 3 compared with standard spectrum of 9-methyl-19-Norlanosta-5,24-dien-3-ol in previous research[77]

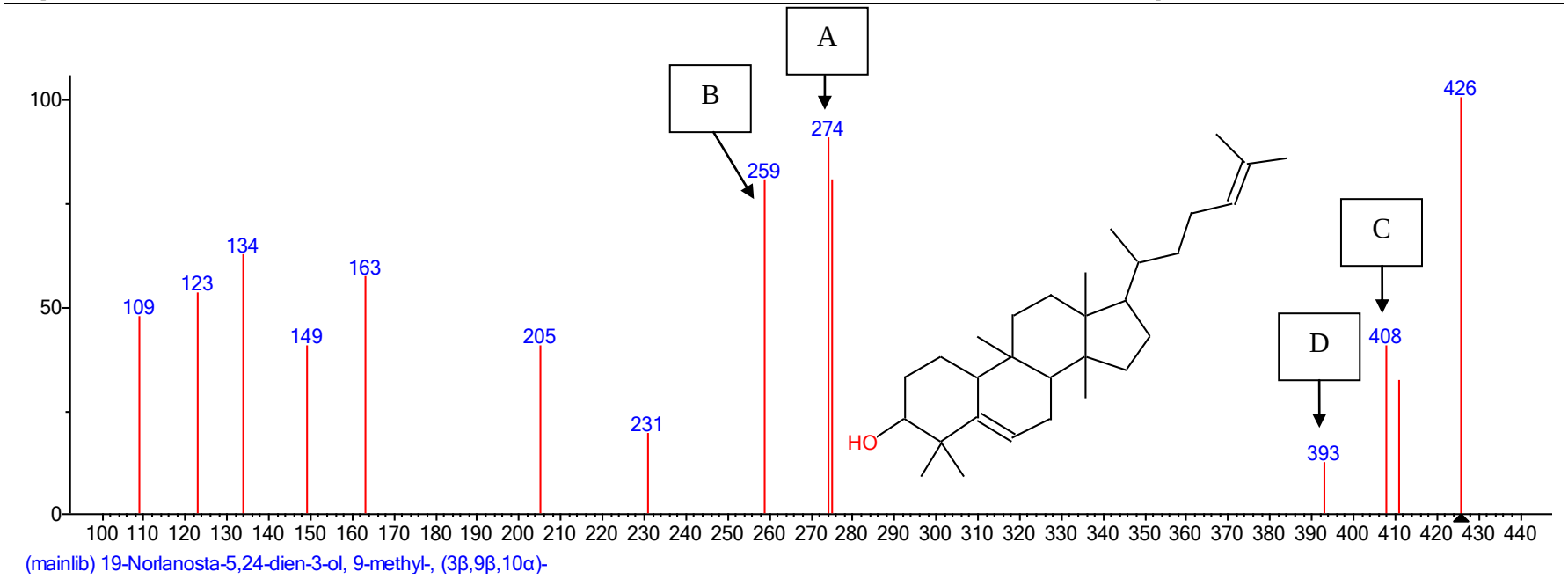
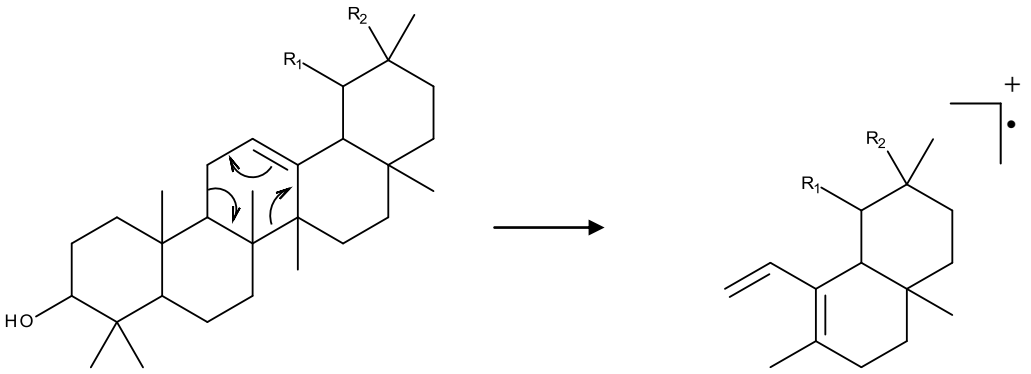



Figure 2.22: The tandard mass spectrum of 9-methyl-19-Norlanosta-5,24-dien-3-ol in Nist library

α-amyrin and β-amyrin ($C_{30}H_{50}O$, MW 426) are very important triterpenoid compounds in heather. In previous research, β-amyrin has been reported to have the highest concentration among all identified triterpenoid compounds in heather.[2] In this study, α-amyrin and β-amyrin are present in fraction 3 to fraction 5 consistently and afford the major constituent of heather total triterpenoids. The peaks at 33.52 min in fraction 3, 32.97 min in fraction 4 and 32.97 min in fraction 5 are identified as α-amyrin. The peaks at 33.17 min in fraction 3, 32.64 min in fraction 4 and 32.62 min in fraction 5 are identified as β-amyrin. Since the spectrum of α-amyrin and β-amyrin are very similar, Kovats index was also applied to help identify peaks besides comparing spectrum directly. According to the standard KI value data published by NIST Mass Spectrometry Data Centre, β-amyrin has lower KI value than α-amyrin on a DB-5 column. Therefore, the peak with shorter retention time was identified as β-amyrin, and the one with longer retention time was identified as α-amyrin. α-amyrin and β-amyrin both have a fragmentation pattern of m/z 218 and 203.




α-amyrin: $R_1$=$CH_3$ $R_2$=H
β-amyrin: $R_1$=H $R_2$=$CH_3$

α-amyrin A1: $R_1$=$CH_3$ $R_2$=H
β-amyrin A2: $R_1$=H $R_2$=$CH_3$
m/z 218

$-\dot{C}H_3$

B

m/z 203

Scheme 2.6: Proposed mechanism for fragmentation of α-amyrin and β-amyrin

A typical retro Diels-Alder collapse of ring C happens during α-amyrin and β-amyrin fragmentation and affords characteristic ions A1 and A2 at m/z 218 and further loss of a methyl group yields ion m/z 203 (Scheme 2.6). Figure 2.23 and 2.24 show the standard spectrum of these two compounds with the spectrum of corresponding peaks.

The concentration of α-amyrin was calculated as 131 μg/g in fraction 3, 494 μg/g in fraction 4 and 68 μg/g in fraction 5. That makes a total concentration of 693 μg/g in heather dry plant. The concentration of β-amyrin was calculated as 46 μg/g in fraction 3, 221 μg/g in fraction 4 and 25 μg/g in fraction 5. That makes a total concentration of 292 μg/g in dry heather plant.

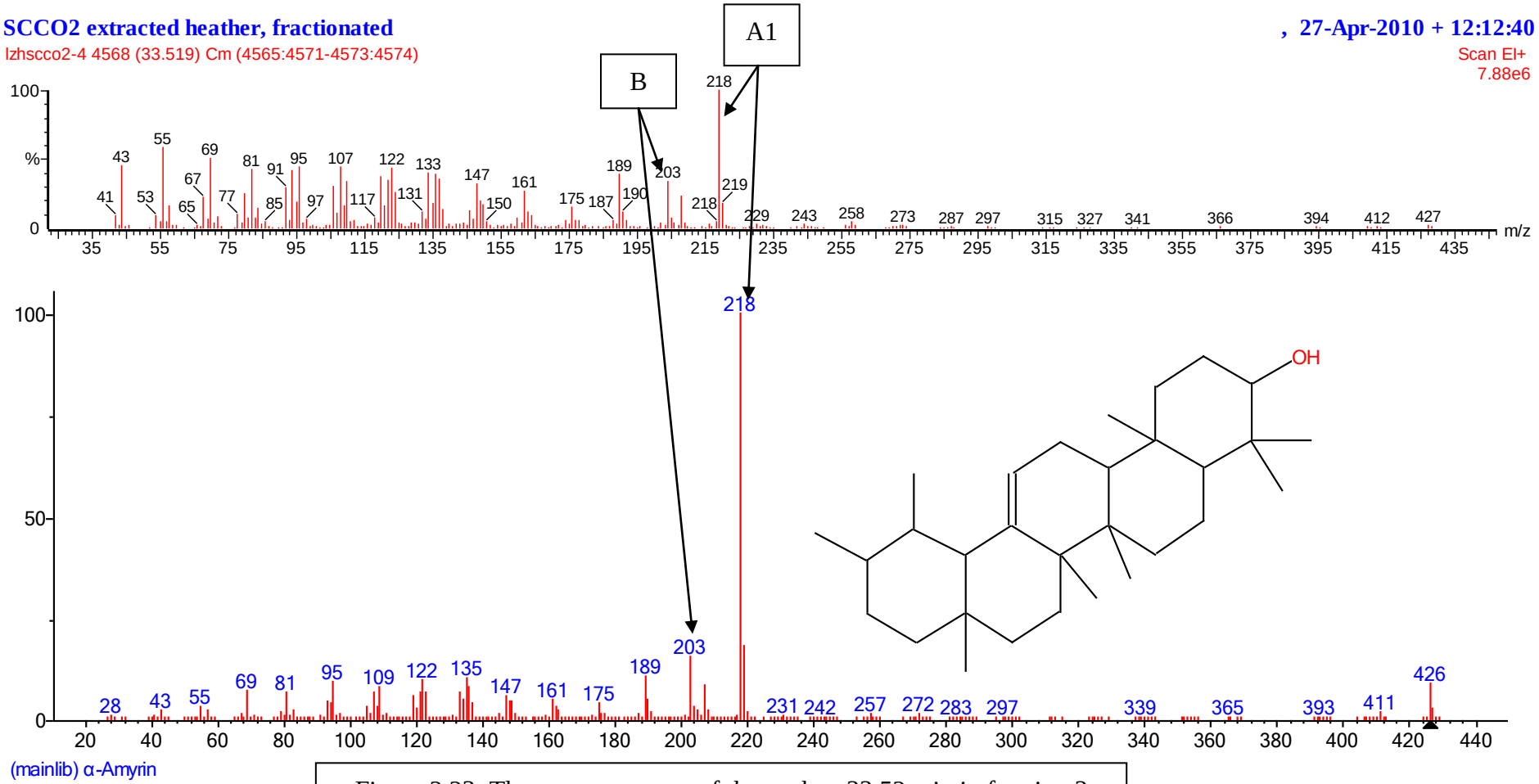



Figure 2.23: The mass spectrum of the peak at 33.52 min in fraction 3 compared with standard spectrum of α-amyrin

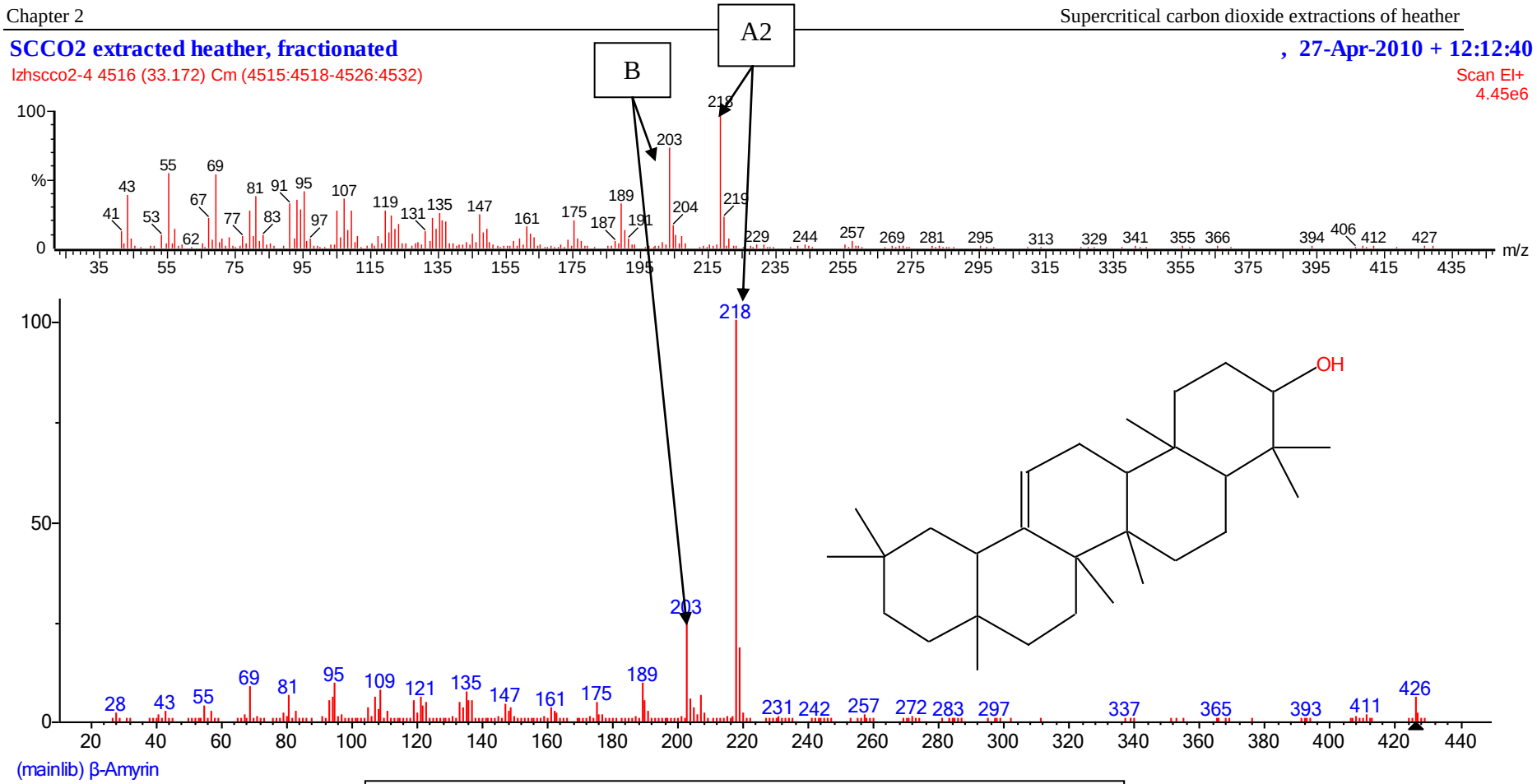



Figure 2.24: The mass spectrum of the peak at 33.17 min in fraction 3 compared with standard spectrum of β-amyrin

α-amyrin and β-amyrin are both well researched in respect to their biological activities. A mixture of α-amyrin and β-amyrin extracted from the Brazilian medicinal herb *Protium hetaphyllum* was tested for its ability to inhibit aggregation of human platelets.[78] The results showed that α-amyrin and β-amyrin significantly inhibited platelet aggregation by 40%, 64%, and 60% in the assay carried out with ADP as agonist, at the doses of 100, 150, and 200 μM respectively. Several derivatives of α-amyrin and β-amyrin were also reported for their anti-fungal activities.[79] α-amyrin and β-amyrin formate and α-amyrin and β-amyrin acetate showed significant inhibition against *Candida* species, α-amyrin and β-amyrin formate also inhibited the adhesion ability of *Candida albicans* to human epithelial cells.

2.3.1.5 Composition of triterpenoid compounds in heather supercritical $CO_2$ extract

The triterpenoid profile distribution demonstrated α-amyrin is the dominant compound within heather supercritical carbon dioxide extract. α-amyrin makes up 27.74% of total triterpenoid extracted by supercritical $CO_2$, and its concentration was recorded as 693 μg/g dry plant. Friedelin contributes the second highest total concentration at 389 μg/g dry plant and this makes up 15.57% of total triterpenoid in this extract. The total concentration of these 9 identified triterpenoid compounds is 2499 μg/g.

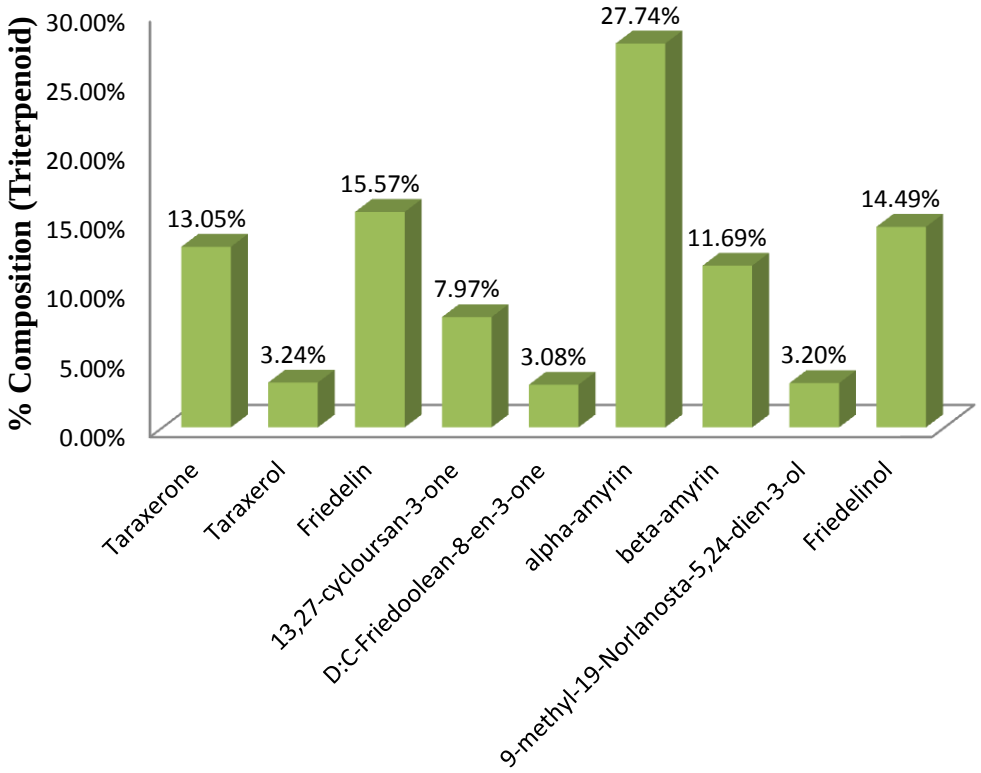



Figure 2.25: Triterpenoid distribution of heather supercritical $CO_2$ extract

### 2.3.2 Fractionation of heather supercritical $CO_2$ with ethanol entrainer extract

The same methods were used for column chromatography and thin layer chromatography to separate this extract and they are stated in chapter 7.3.3 and 7.3.4. Totally 75 fractions were collected from the column and they are combined based on their spot positions revealed by TLC. Eventually, 11 combined fractions were obtained, and the weight of each fraction was recorded after evaporating the solvent using a rotary evaporator. GC-MS samples were then made up in dichloromethane for each fraction at a concentration of 20 mg/ml.

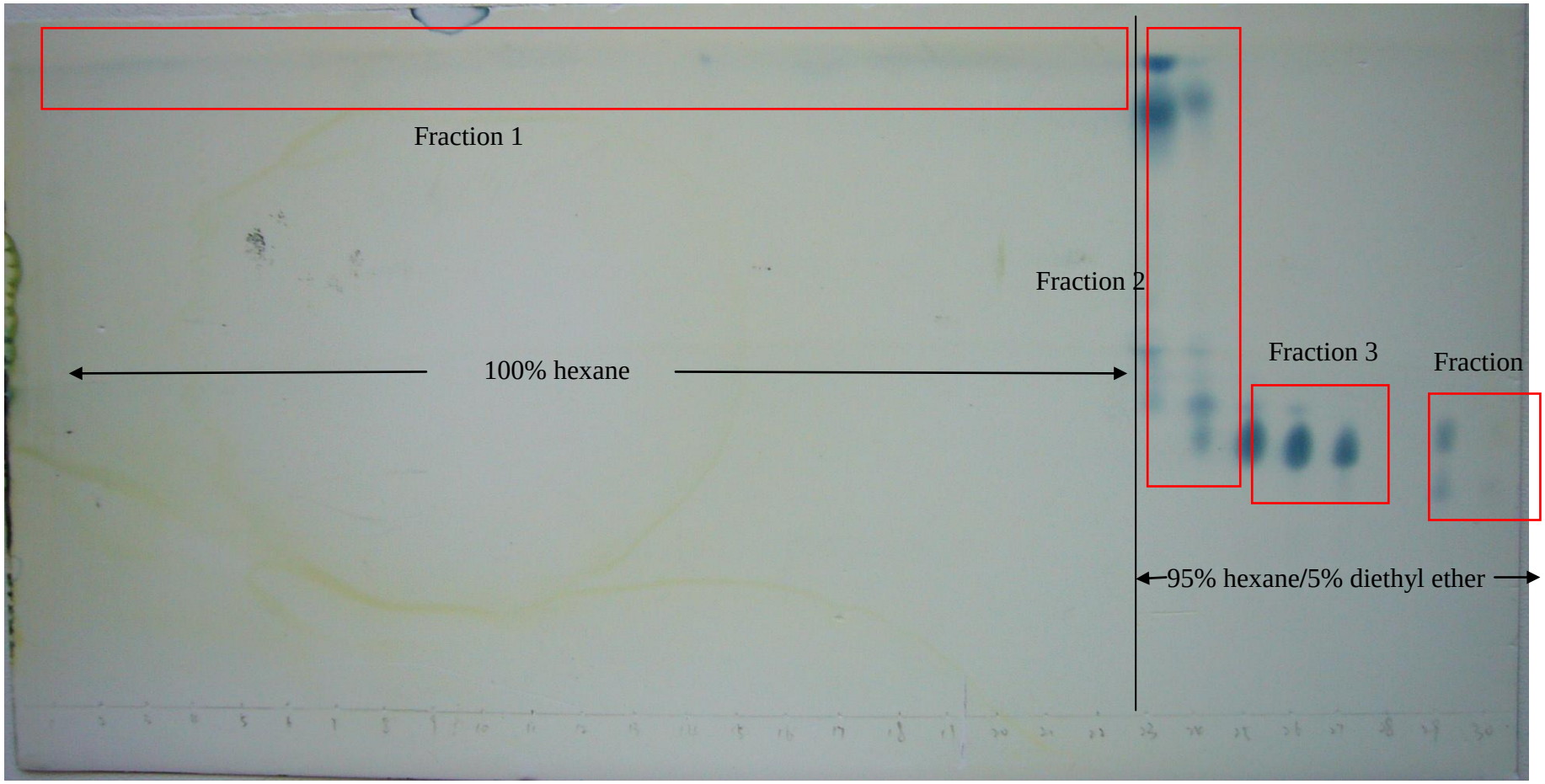



Figure 2.26: TLC plate (1) with spots revealed for supercritical $CO_2$ with entrainer extract

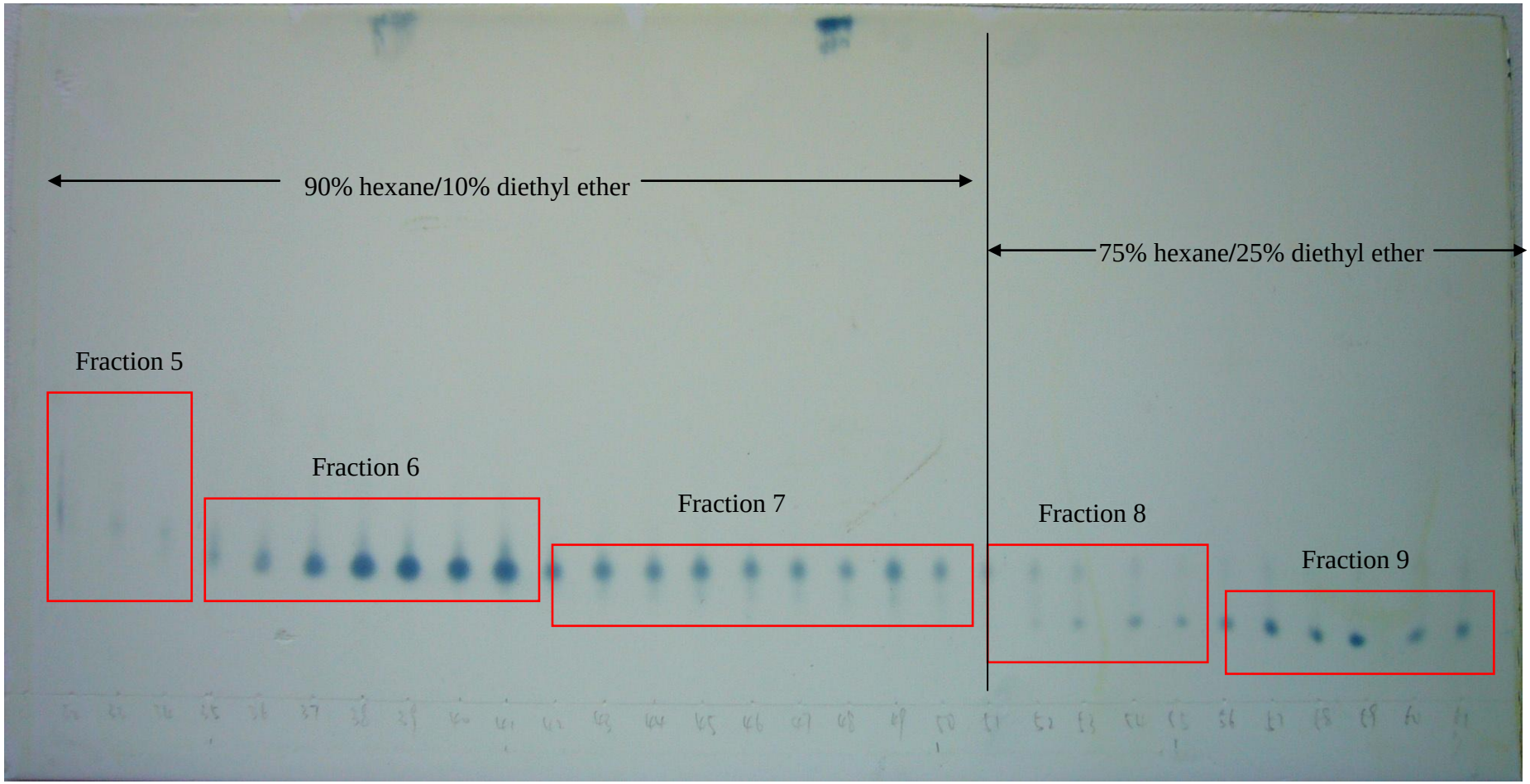



Figure 2.27: TLC plate (2) with spots revealed for supercritical $CO_2$ with entrainer extract

2.3.2.1 Identification of alkanes in heather supercritical $CO_2$ with entrainer extract hexane fraction

The hexane eluent was collected from the column and combined as one fraction and weight 32.8 mg. This fraction 1 was then made up a GC sample at 20 mg/ml. Gas chromatogram of this fraction was shown in figure 2.28.

As found in fraction 1 of supercritical $CO_2$ extract, all major peaks in this fraction are identified as primary alkanes as they all have identical spectra with hydrocarbon standards. The same range of chain length C21 to C35 were identified. The dominant peaks appeared at 31.29 min and 32.81 min were identified as hentriacontane and tritriacontane after comparison of their retention times with C12-C60 hydrocarbon standards. Odd numbered alkanes still express greater absolute peak areas than even number alkanes in this fraction.

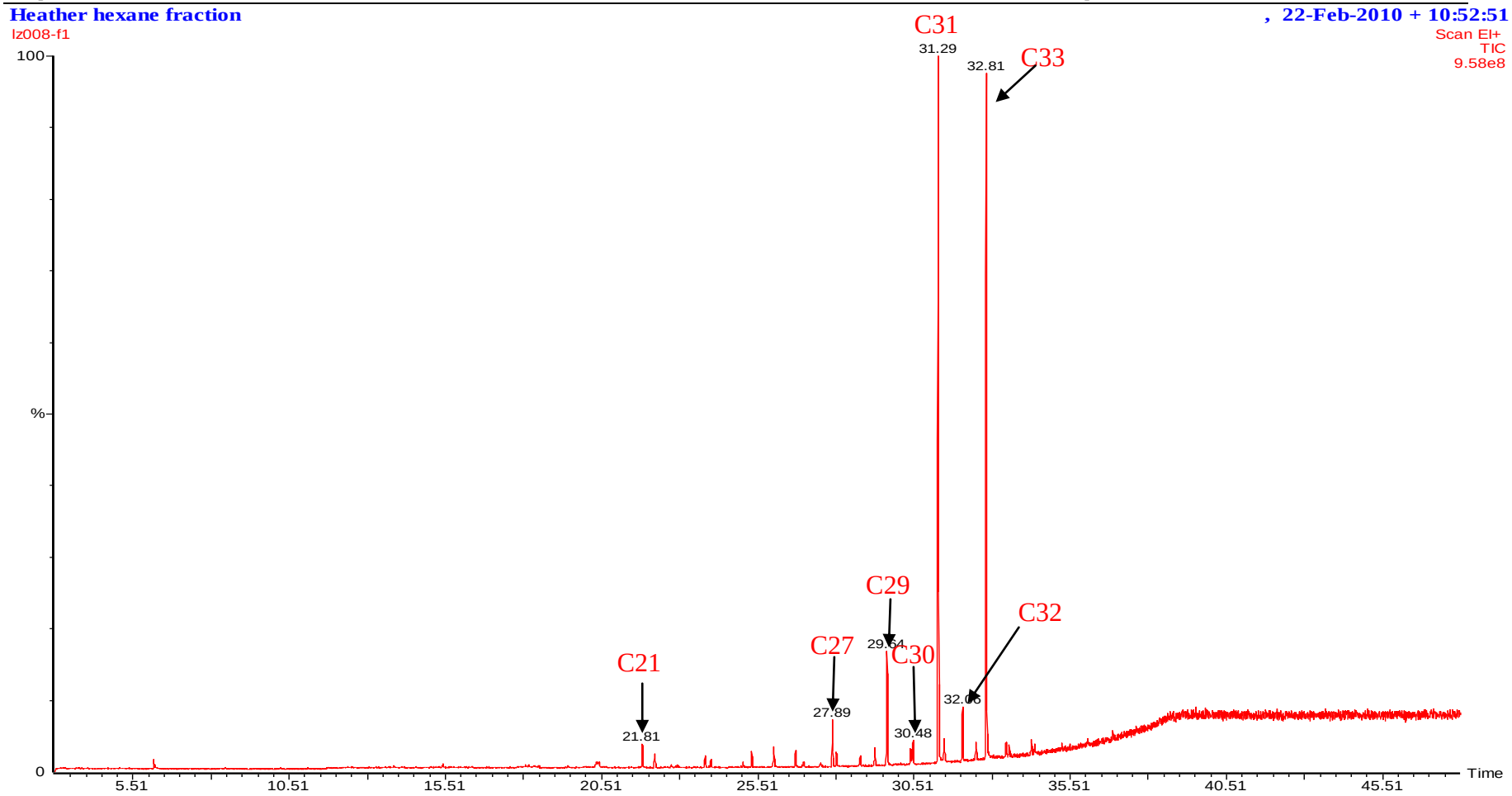



Figure 2.28: Gas chromatogram of fraction 1 of heather supercritical $CO_2$ with ethanol entrainer extract

2.3.2.2 Identification of new triterpenoid compounds in heather supercritical $CO_2$ with ethanol entrainer extract fractions

Triterpenoid compounds are found in fractions 2 to fraction 6 of heather supercritical $CO_2$ with entrainer extract. The weight and triterpenoid components found in each fraction are listed in table 2.3. All the triterpenoid compounds identified in this extract already present in previous supercritical $CO_2$ extract.

Table 2.3: Triterpenoid compounds found in heather supercritical $CO_2$ with ethanol entrainer extract fractions

| | Weight | Triterpenoids identified | Concentration of each triterpenoid (dry plant) |
|---|---|---|---|
| Fraction 2 | 36.8mg | Taraxerone<br>13,27-cycloursan-3-one | 91 μg/g<br>42 μg/g |
| Fraction 3 | 32.4mg | Taraxerone<br>Friedelin<br>13,27-cycloursan-3-one | 136 μg/g<br>157 μg/g<br>25 μg/g |
| Fraction 4 | 21.6mg | Taraxerol<br>13,27-cycloursan-3-one<br>D-friedooleanan-3-ol | 84 μg/g<br>76 μg/g<br>122 μg/g |
| Fraction 5 | 37.8mg | α-amyrin<br>β-amyrin | 227 μg/g<br>121 μg/g |
| Fraction 6 | 62.6mg | α-amyrin<br>β-amyrin | 64 μg/g<br>47 μg/g |

2.3.2.3 Composition of triterpenoid compounds in heather supercritical $CO_2$ with ethanol entrainer extract

α-amyrin is the dominant compound within heather supercritical $CO_2$ with ethanol entrainer extract as well as the supercritical extract without entrainer. However, in this extract, the concentration of each individual triterpenoid compound seems more

even compared with supercritical $CO_2$ extract. α-amyrin composes 24.41% of total triterpenoid in this extract, when taraxerone becomes the second greatest component and makes up 19.04% of total triterpenoid. The total concentration of these 7 identified triterpenoid compounds is 1192 μg/g.

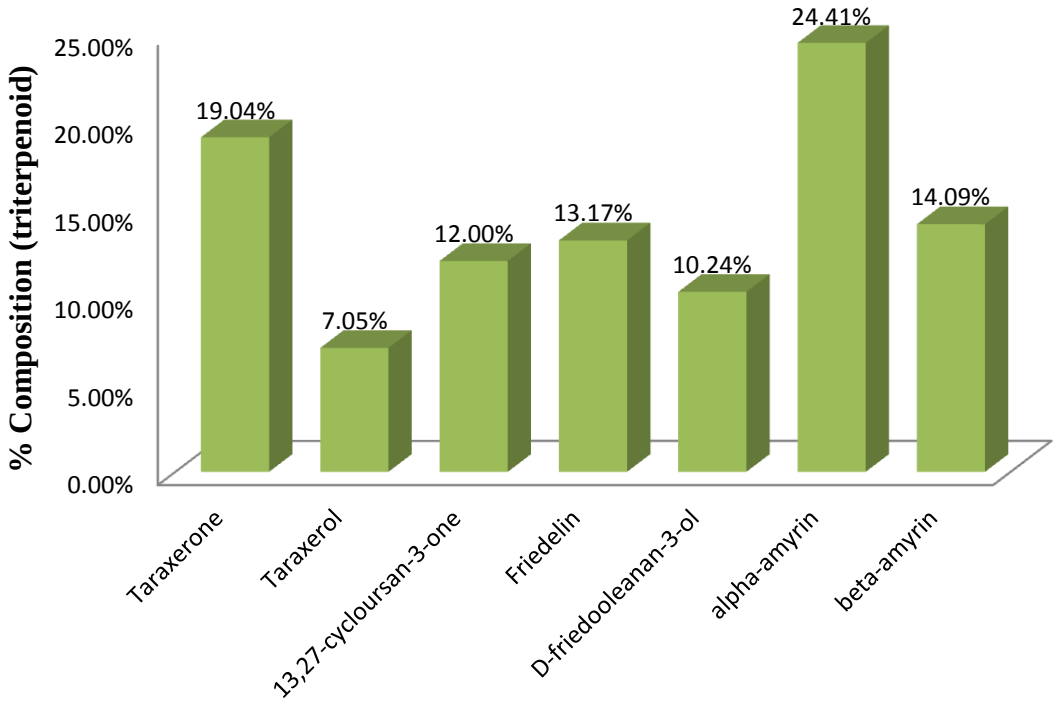



Figure 2.29: Triterpenoid distribution of heather supercritical $CO_2$ with ethanol entrainer extract

## 2.4 Conclusion and further remarks

Heather samples picked in different seasons were extracted using supercritical $CO_2$ and supercritical $CO_2$ with ethanol entrainer. The crude extract yield from each extraction was recorded and evaluated. It was found that summer heather picked in August flowering season exhibited the greatest yield in both supercritical $CO_2$ and supercritical $CO_2$ with entrainer extractions (3.5% and 4.5% dry plant). Therefore, August heather extracts were selected as research object in further studies.

Column chromatography was then applied to help identify triterpenoid compound in heather. Heather supercritical $CO_2$ and supercritical $CO_2$ with entrainer extracts were fractionated with silica columns and analysed by GC-MS for their chemical composition. Totally 9 triterpenoid compounds were identified from heather supercritical $CO_2$ and supercritical $CO_2$ with entrainer extracts. Among these 9 triterpenoids, 5 of them were previously unreported in heather. Taraxerone and taraxerol which have been proved to only be present in heather root were also detected in these heather aerial part extracts. Quantification of each individual triterpenoid was also accomplished by using oleanolic acid as a standard. Several novel triterpenoid compounds found in heather have already been studied with regard to their biological activities and some of them were proven to have good potential as pharmaceutical drugs or drug precursors. However, a few of them seem have never been researched before and very little literature can be found about these new triterpenoids. Therefore, further work is required to fully study these triterpenoids for their potential biological activities. Moreover, the identification of some triterpenoid compounds such as 13,27-cyclousran-3-one was not conclusive as the identification of these compounds was only tentative based on the best match of their spectrum with corresponding standard library data. Therefore, the evidence of identification of these compounds is not strong enough from this data alone and further supporting evidence such as NMR data is required to give a more precise identification.

The distribution of each individual triterpenoid was also evaluated in this study. In heather supercritical $CO_2$ extract, α-amyrin was demonstrated to be the dominant component and made up 27.74% of total triterpenoid extracted by supercritical $CO_2$ and has a concentration of 693 μg/g in dry plant. Friedelin contributes the second highest total concentration at 389 μg/g dry plant and make up 15.57% of total triterpenoid. The total concentration of the 9 identified triterpenoid compounds in heather supercritical $CO_2$ extract is 2499 μg/g. In heather supercritical $CO_2$ with ethanol entrainer extract, the distribution of each identified triterpenoid compound seems more even compared with supercritical $CO_2$ extract. The dominant triterpenoid was still found to be α-amyrin at a concentration of 291 μg/g. It composes 24.41% of total triterpenoid in this extract, when taraxerone becomes the second greatest component and makes up 19.04% of total triterpenoid. The total concentration of the 7 identified triterpenoid compounds in heather supercritical $CO_2$ with ethanol entrainer extract is 1192 μg/g. This change in distribution can be explained by the greater solubility of the more polar triterpenoid alcohols in the supercritical $CO_2$ with entrainer.

Compared to the total triterpenoid result of Pancost,[65] the total identified triterpenoid content in this study is much lower. However, Pancost and co-workers extracted *Calluna vulgaris* in dichloromethane and methanol mixed solvent, and they didn't state when their heather raw material is harvested. Andrew Hunt has done hexane extractions and supercritical carbon dioxide extractions of heather, and his result indicated that the concentration of heather total triterpenoid compounds is 2793 μg/g dry plant in hexane extract.[2] In supercritical $CO_2$ extracts of heather, this figure ranges from 1652 μg/g to 2944 μg/g dry plant depend on the extraction conditions. Therefore, the results obtained in this study strongly corroborate with previous result, and the total triterpenoid content of seasonal heather supercritical carbon dioxide extracts determined by UV spectrophotometry will be discussed in detail in next chapter.

# *Seasonal variation of total triterpenoids compounds in heather*

# Chapter 3

## 3.1 Seasonal variation of total triterpenoid compounds in heather

It has been reported that *Calluna vulgaris* contains high levels of total triterpenoid compounds.[65] Pancost and co-workers previously reported that the total triterpenoid content can reach 65100 μg/g dry plant in heather stems and leaves and Hunt[2] also indicated that the total triterpenoid content in *Calluna vulgaris* hexane extract is 4301 μg/g dry plant. Supercritical carbon dioxide extractions of heather were also carried out by Hunt,[2] and a total triterpenoid content from heather varied from 1652 μg/g to 2944 μg/g dry plant depending on extraction conditions. However, the seasonal variation of total triterpenoid compounds has never been reported before. Therefore, in this study, the quantification of total triterpenoid in heather, alongside with the seasonal variation of total triterpenoid within *Calluna vulgaris* were investigated in detail.

## 3.2 Water content variation of heather

Water content of *Calluna vulgaris* was examined in samples from October 2007 to August 2008 (Figure 3.1). The lowest water content was found to be 10.52% by total weight in autumn heather sample (10/2007). This figure rose up to 13.75% in winter heather (01/2008), and then the increasing trend continues until it reaches a maximum in summer flowering heather (08/2008) at 15.72%. Spring heather (03/2008) also has fairly high total water content at 15.47% which is only slightly lower than that of August heather.

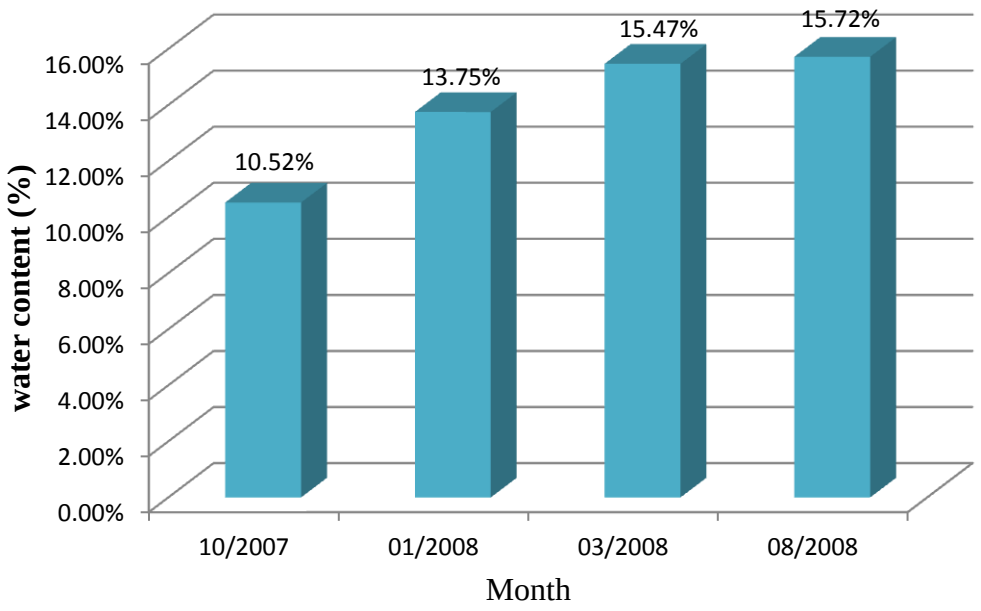



Figure 3.1: Seasonal variation in water content of *Calluna vulgaris*

## 3.3 Total extract yield of comparison between supercritical $CO_2$ and hexane

Since supercritical carbon dioxide has been described to have extracting ability similar to that of hexane,[2] traditional hexane Soxhlet extractions were also applied to give a comparison on yield and triterpenoids composition in heather. The hexane soxhlet extractions were carried out using the identical heather samples as raw material and were extracted for 4 hours. The crude yield comparison of heather hexane extractions and corresponding supercritical $CO_2$ extractions in both dry plant and fresh plant are shown in figure 3.2 and 3.3.

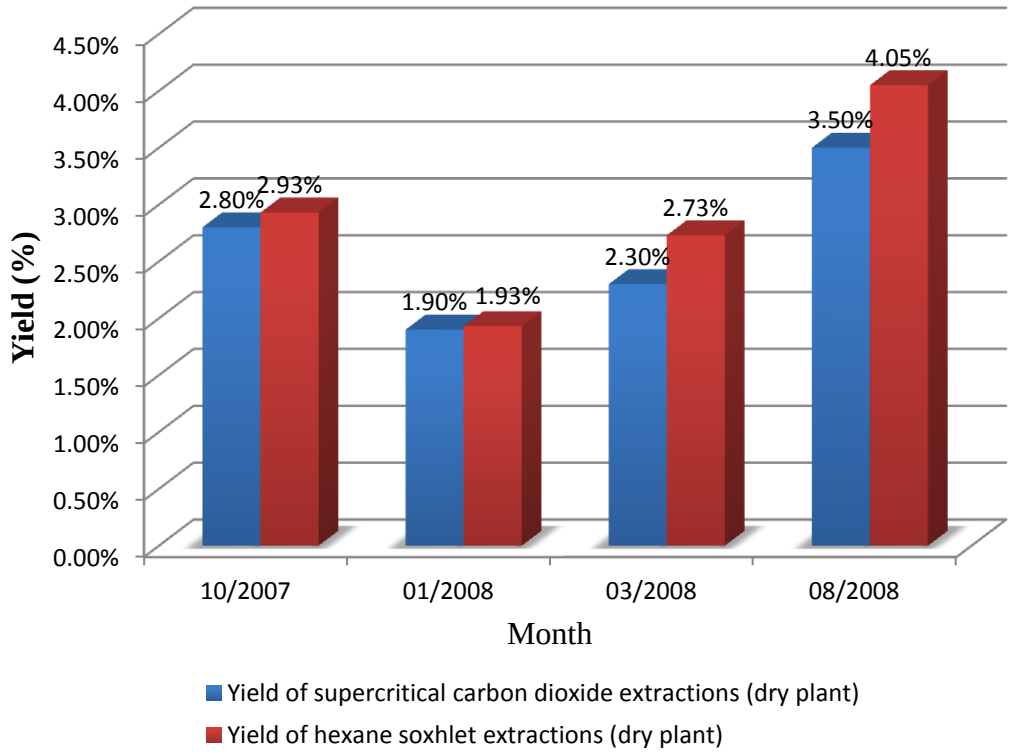



Figure 3.2: Comparison of hexane extractions and supercritical extractions in dry plant

The figure shows that the yields of heather hexane soxhlet extractions are very similar to that of corresponding supercritical $CO_2$ extractions with differences within 0.45% in dry plant and 0.46% in fresh plant. It is obvious that for supercritical $CO_2$ extractions, summer flowering sample picked in August 2008 exhibited the highest crude yield in both dry plant (3.50%) and fresh plant (2.95%) and January winter heather, on the contrast, showed the lowest yield in dry plant (1.90%) and fresh plant (1.64%). The seasonal yield variation of heather hexane extractions is identical to that of supercritical $CO_2$ extractions with the greatest yield apparent in summer and the lowest in winter. These results also corroborate previous work with heather hexane extract yield and seasonal variation.

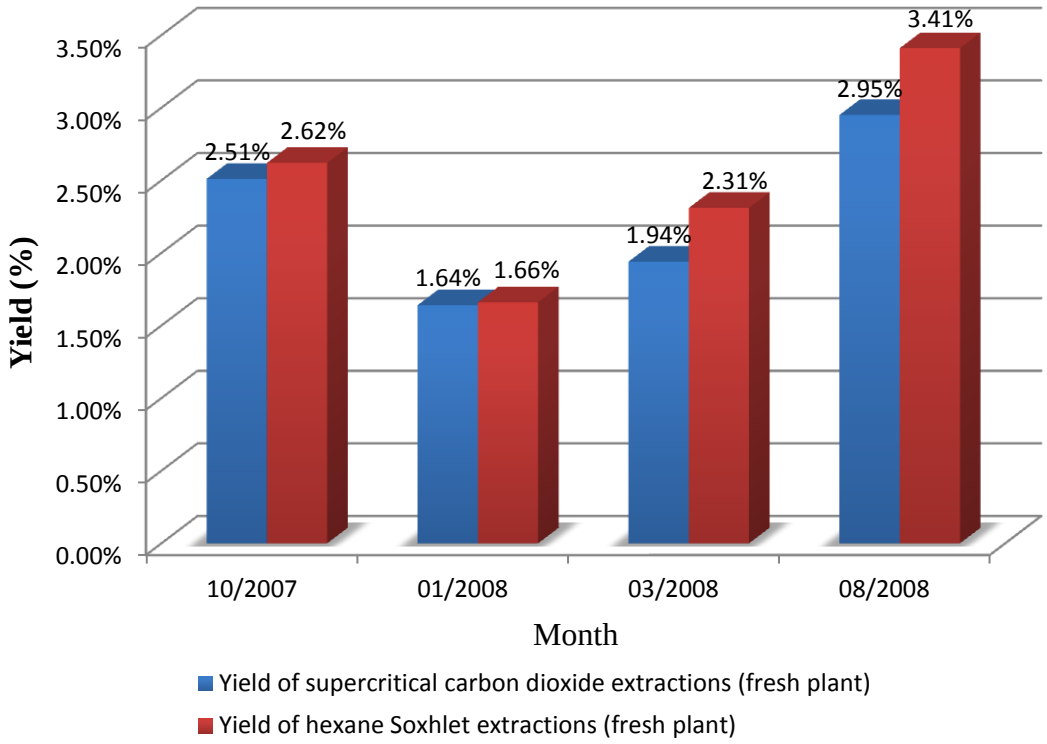



Figure 3.3: Comparison of hexane extractions and supercritical extractions in fresh plant

## 3.4 Seasonal variation of total triterpenoid content in heather

The quantification of total triterpenoid compounds was carried out with the application of oleanolic acid as a standard. Vanillin-perchloric acid was applied as colouring reagent to determine the content of total triterpenoids (method described in 7.3.7). Standard solutions containing 0, 20, 40, 60, 80, 100 and 120 µg of oleanolic acid were made up to create a calibration curve. The total triterpenoid content was calculated as µg/g dried weight of plant material using the following linear equation based on the calibration curve:

$$Y=0.0049X - 0.0111$$

where Y is the absorbance and X is weight of oleanolic acid equivalent. Figure 3.4 shows the standard curve of oleanolic acid and the regressive equation. A correlative coefficient of 0.9981was obtained from this curve.

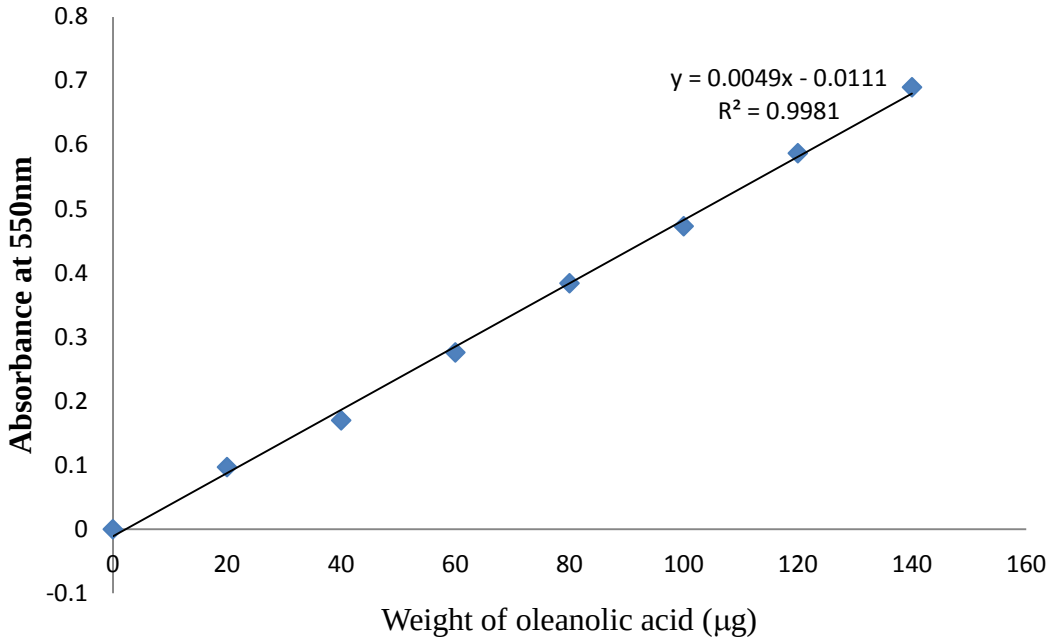



Figure 3.4: The standard curve of oleanolic acid for heather total triterpenoid quantification

Table 3.1 shows the calculated total triterpenoid content of each supercritical carbon dioxide extract. Figure 3.5 and 3.6 shows the seasonal variation trend of total triterpenoid content in these four heather samples in both dry plant and fresh plant.

Table 3.1: Calculated total triterpenoid content in each heather supercritical extract

| Sample date | Total triterpenoid in supercritical $CO_2$ extract (dry plant) | Total triterpenoid in supercritical $CO_2$ with ethanol entrainer extract (dry plant) |
|---|---|---|
| **10/2007** | 8500 μg/g | 8650 μg/g |
| **01/2008** | 4200 μg/g | 4300 μg/g |
| **03/2008** | 11200 μg/g | 12000 μg/g |
| **08/2008** | 15400 μg/g | 16000 μg/g |

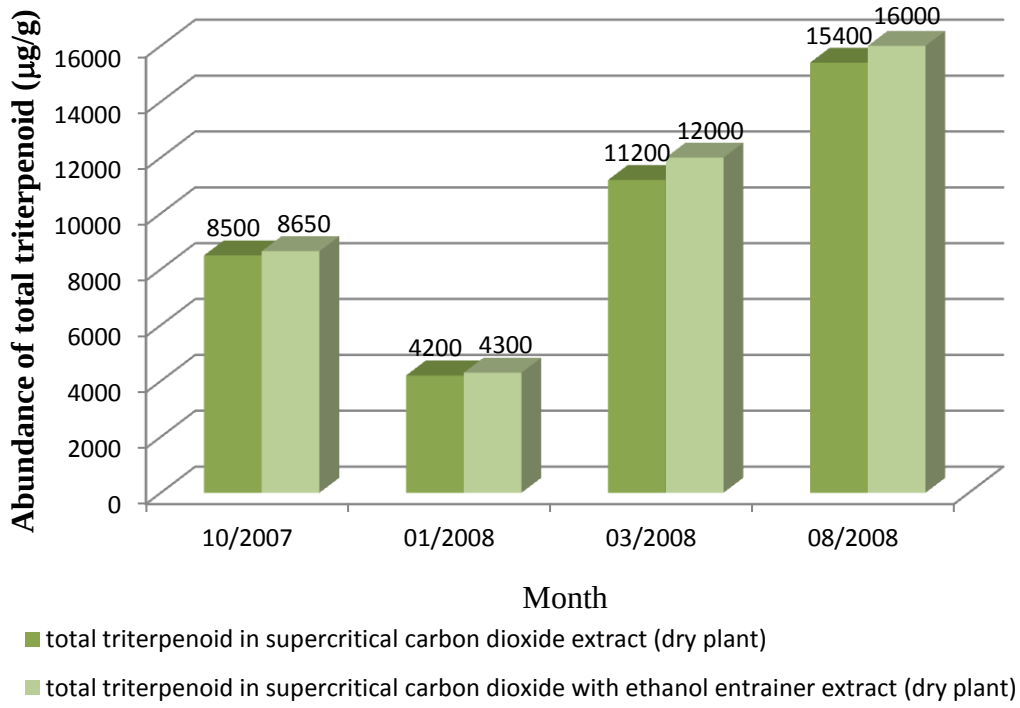



Figure 3.5: Total triterpenoids in supercritical $CO_2$ and supercritical $CO_2$ with ethanol entrainer extracts (dry plant)

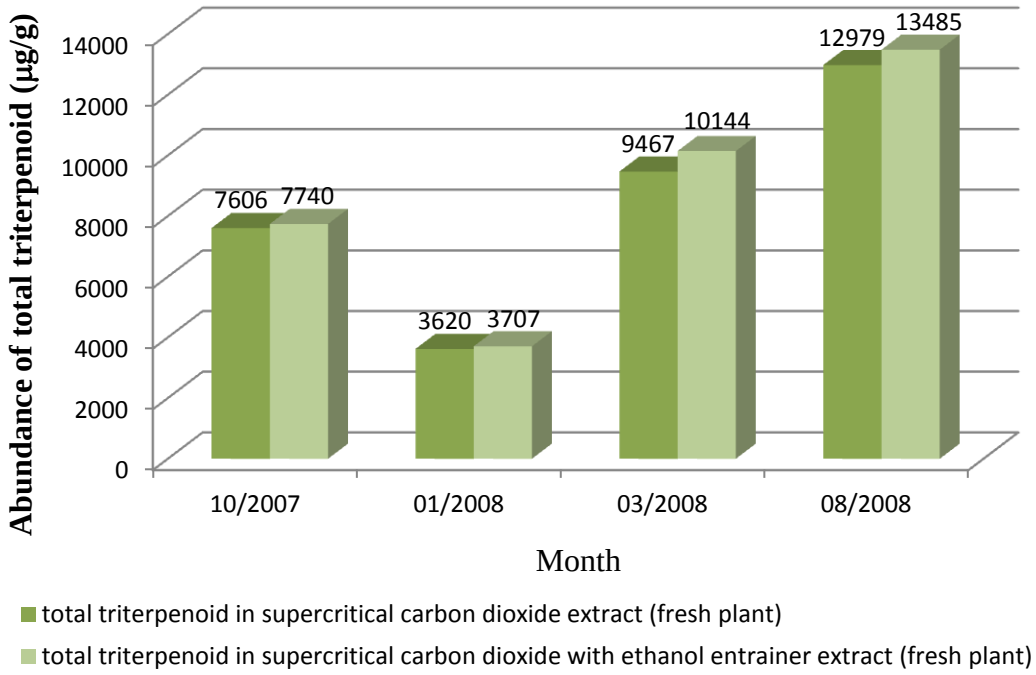



Figure 3.6: Total triterpenoids in supercritical $CO_2$ and supercritical $CO_2$ with ethanol entrainer extracts (fresh plant)

Generally, the total triterpenoid content in supercritical $CO_2$ with entrainer extracts are slightly higher than corresponding supercritical $CO_2$ extracts. The lowest total triterpenoid content was found in winter heather picked in January 2007, which is 4200 µg/g by dry weight and 3620 µg/g by fresh weight in supercritical $CO_2$ extract, and 4300 µg/g dry plant and 3707 µg/g fresh plant in supercritical $CO_2$ with entrainer extract. Thereafter, the total triterpenoid in heather increased dramatically and reached 11200 µg/g dry plant (9467 µg/g in fresh plant) in supercritical $CO_2$ extract and 12000 µg/g dry plant (10144 µg/g in fresh plant) in supercritical $CO_2$ with entrainer extract. Then the total triterpenoid content keeps increasing until it reached the highest amount at 15400 µg/g in dry plant (12979 µg/g fresh plant) in August summer heather supercritical $CO_2$ extract and 16000 µg/g (13485 µg/g fresh plant) in supercritical $CO_2$ with entrainer extract. This triterpenoid content makes up 1.6% of total dry weight and 1.35% of fresh weight of summer heather. In autumn, the total triterpenoid falls down to 8500 µg/g dry plant (7606 µg/g fresh plant) in supercritical $CO_2$ extract and 8650 µg/g dry plant (7740 µg/g fresh plant) in supercritical with ethanol extract.

The seasonal variation trend of total triterpenoid content is exact the same with that of heather crude extracting yield. The greatest amount of triterpenoid content was found in summer flowering heather and the poorest was detected in winter heather.

## 3.5 Comparison of total triterpenoid content extracted by supercritical carbon dioxide and hexane

The total triterpenoid content in supercritical carbon dioxide extracts were also compared with that of hexane extracts due to their similar extracting ability. Figure 3.7 and 3.8 showed the seasonal variation of total triterpenoid content in these two series of extracts.

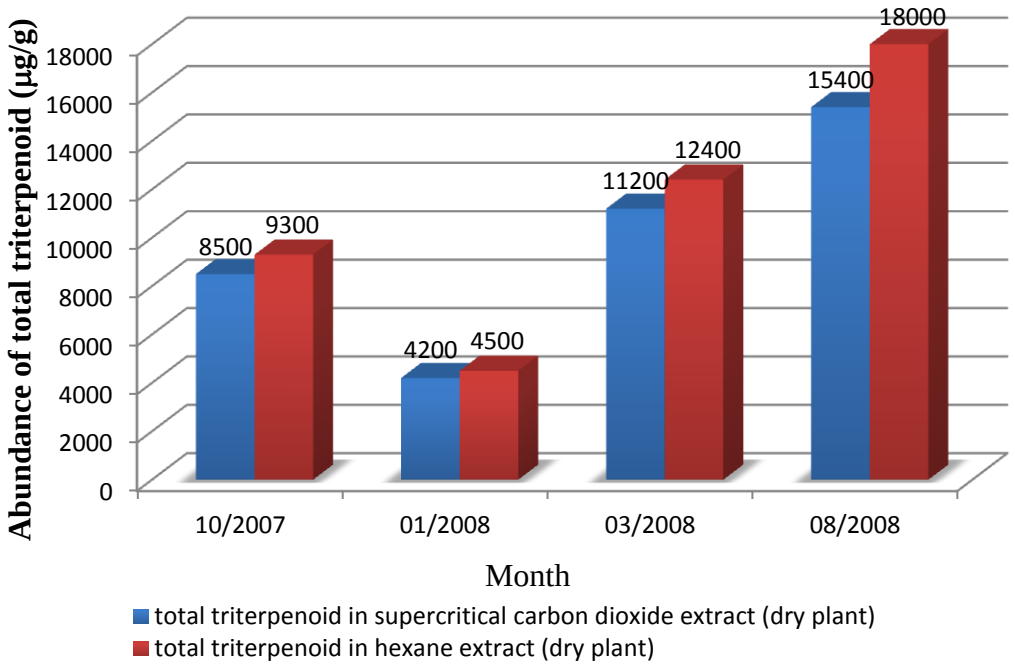



Figure 3.7: Total triterpenoids in supercritical $CO_2$ and heather hexane extracts (dry plant)

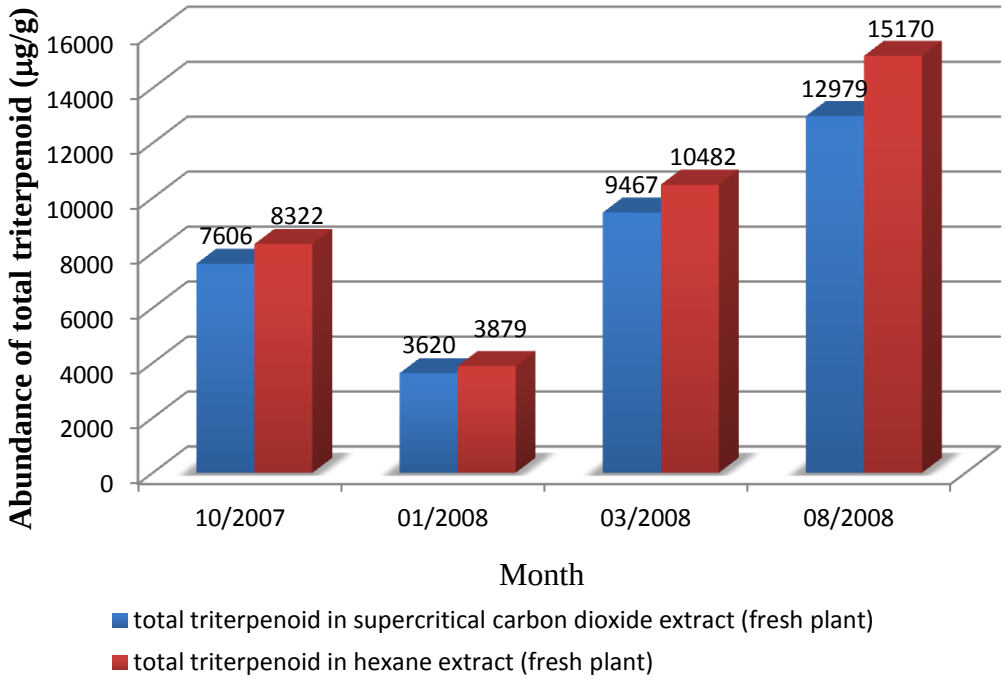



Figure 3.8: Total triterpenoids in supercritical $CO_2$ and heather hexane extracts (fresh plant)

From the data shown in figures 3.7 and 3.8 it is clear that the seasonal variation of total triterpenoid in hexane extract is identical with that in supercritical $CO_2$ extract. The lowest total triterpenoid content was also found in winter heather and the greatest in summer flowering heather. It was also indicated that supercritical $CO_2$ extracts contain at least 90% of total triterpenoid compared with that of hexane extract. This means supercritical $CO_2$ at 35 MPa and 50 $^{o}$C has almost the same extracting ability as hexane with respect to triterpenoid compounds. However a previous study indicated that supercritical $CO_2$ is able to extract higher concentrations of triterpenoid than hexane under conditions of higher pressure and temperature (6000 psi 43MPa) and 100 $^{o}$C),[2] therefore, future studies can be directed at achieving a better yield of total triterpenoid content in supercritical $CO_2$ extract by varying supercritical extraction conditions.

## 3.6 Optimal harvest and extraction time

According to the total triterpenoid content quantification, the greatest concentration of total triterpenoid is present in *Calluna vulgaris* during August. This would be naturally the preferred month for harvesting. However, there are several problems about harvesting heather at this time of the year. The grouse shooting season starts on 12$^{th}$ August every year[2] and this is a major source of revenue for North York Moor National Park and landowners. Harvesting during August will seriously disturb this sport. August is also the biggest tourist season in the moor, tourism will be greatly affected if the harvesting takes place in August. Therefore, August would not be the ideal time to harvest heather when taking these factors into account. Meanwhile, the burning of heather takes place in spring every year since it is restricted by law that burning can only take place between 1$^{st}$ October and 15$^{th}$ April.[2] In March heather sample, total triterpenoid content was evaluated as 11200 μg/g dry plant in supercritical $CO_2$ extract and 12400 μg/g dry plant in hexane extract. The total triterpenoid content in March is approximately 88% of that of August heather sample. Therefore, if harvesting can take place in March instead of burning, the heather will already contain almost 90% of the highest triterpenoid content in the year. A large amount of triterpenoid can be extracted if large-scale harvesting of heather can be achieved at this time of the year.

## 3.7 Conclusion and further remarks

Seasonal variation of total triterpenoid compounds of *Calluna vulgaris* supercritical carbon dioxide extracts was highlighted in this work. The greatest total triterpenoid content was found in August flowering heather, and the lowest was obtained in January winter heather. The total triterpenoids in supercritical $CO_2$ with ethanol entrainer extracts were slight higher than that of supercritical $CO_2$ extracts. Hexane extracts also showed slightly higher concentrations of total triterpenoids compared to

supercritical $CO_2$ extracts. However, by varying extraction conditions, it is expected that supercritical $CO_2$ can extract greater concentrations of triterpenoids than hexane. Therefore, future work will be done to enhance the extraction of triterpenoids by using advanced supercritical $CO_2$ extraction conditions.

The optimal harvesting time of heather was also discussed in this chapter. Although August heather contains the highest level of total triterpenoid, there are difficulties of harvesting heather in large-scale at this time of the year. Due to grouse shooting and tourism taking place in August, it is suggested that March would be a good alternative time to harvest heather since it already contains a high level of total triterpenoid compounds. Burning of heather is originally taking place in spring every year, so if harvesting can take place in March instead of burning, the heather will already contain almost 90% of the highest triterpenoid content in the year. A large amount of triterpenoid can be extracted if large-scale harvesting of heather can be achieved at this time of the year.

# *Extraction and quantification of heather total phenolic compounds*

# Chapter 4

## 4.1 Extraction and quantification of heather phenolic compounds

Phenolic compounds are commonly found in both edible and non-edible plants and are known as one of the largest and most widespread groups of plant secondary compounds.[80] They have been reported to have multiple biological effects, including antimicrobial activity and antioxidant activity[40,43] and phenolic acids have also been shown to inhibit the in-vitro growth of an assortment of fungal genera.[81] Other workers have shown that single groups of plant phenolics and flavonoids have applications as antibiotics, anti-ulcer and anti-inflammatory agents.[17] Corrales and co-workers[43] evaluated the antimicrobial activity of total phenolic compounds in grape seed extract. They observed that the growths of Gram-positive food-borne pathogens were inhibited by phenolic compound, while Gram-negatives were not inhibited.

Previous work with heather demonstrated a high total phenolic content and antioxidant activity throughout the year.[2] A strong correlation was discovered between phenolic content and antioxidant activity.[2] Heather was found to have the strongest antioxidant activity in August, a highest total phenolic content was also detected in this summer flowering sample. The temporary accumulation of phenolic content in heather during the flowering stage may be due to the use of these compounds to attract pollinating insects.[2]

In this chapter, a seasonal variation of total phenolic compound in heather was examined and phenolic samples at various concentrations were made up for antimicrobial assay. Qualitative and quantitative test of phenolic antimicrobial activity were accomplished using several Gram-positive and Gram-negative microorganisms.

## 4.2 Seasonal variation of total phenolic compound in heather

Heather whole plant sample picked in October 2007, January 2008, March 2008 and August 2008 were selected as representative of heather in every season. Heather flower sample picked in October 2007 was selected as representative of heather flower. All the raw material was extracted by ethanol follow the method stated in 7.3.5.1. Total phenolic content of each extract was determined using Folin-Ciocalteau reagent (method described in 7.3.6). Gallic acid was used as a standard for calibration curve (0-120 μg/ml). The total phenolic content was reported as gallic acid equivalents (GAE)/g dried weight of plant material using the following linear equation based on the calibration curve;

$$Y=0.0094X + 0.0056$$

where Y is the absorbance and X is gallic acid equivalent μg/ml. Figure 4.1 shows the standard curve of gallic acid and the regressive equation. A correlative coefficient of 0.9996 was obtained from this curve.

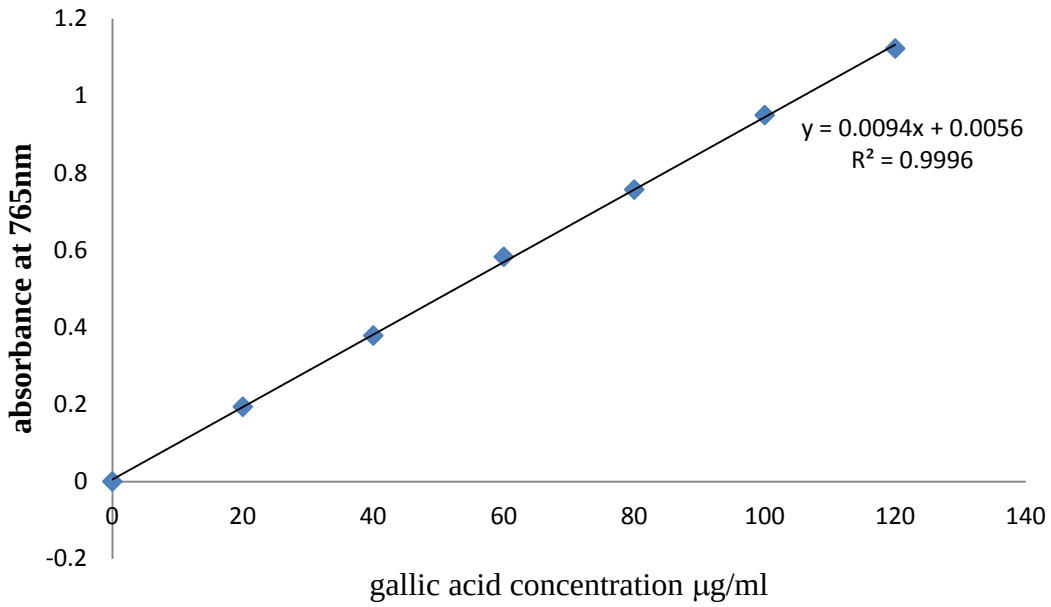



Figure 4.1 The standard curve of gallic acid for heather total phenolics quantification

Table 4.1 listed the examined yield, absorbance at 765 nm and calculated total phenolic content of each extract. Figure 5.2 shows the seasonal variation trend of total phenolic content in these five samples.

Table 4.1 Extract yield and total phenolic content in each heather sample

| Sample date | Yield (%) | Total phenolic content in dry plant |
|---|---|---|
| **October 2007** | 18.77% | 2728 mg/100g |
| **January 2008** | 21.97% | 3750 mg/100g |
| **March 2008** | 21.42% | 5163 mg/100g |
| **August 2008** | 19.27% | 4201 mg/100g |
| **October2007 (flowers)** | 15.88% | 2130 mg/100g |

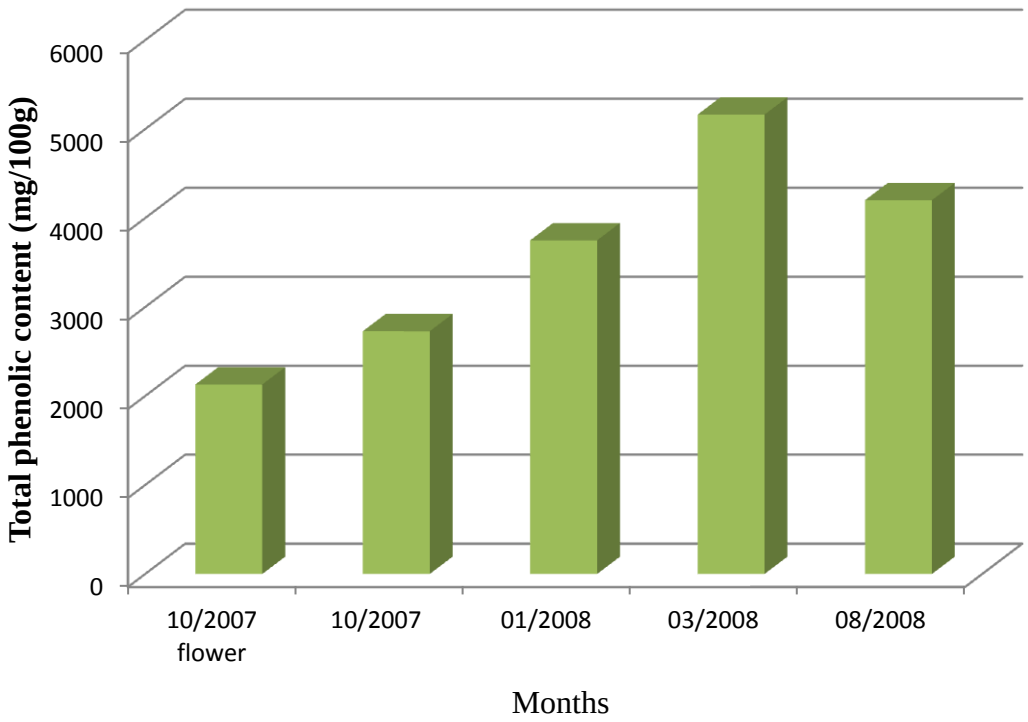



Figure 4.2 Seasonal variation of total phenolic content in heather

The lowest total phenolic content was found in heather flowers, which is 2130 mg/100g dry weight. Heather whole plant seem to have the lowest total phenolics in

winter and then keep rising until reach a peak in spring sample at 5163 mg/100g dry plant. Afterwards the total phenolic content drops slightly to 4201 mg/100g dry plant in summer when it is flowering. The seasonal variation trend is generally very similar to the previous study, although the greatest total phenolic content in this case was detected in March sample rather than August.

## 4.3 Qualitative antimicrobial assay using heather phenolic extracts

Heather sample picked in different seasons (October 2007, January 2008, March 2008 and August 2008) were extracted by ethanol follow the method stated in chapter 7.3.5.1. The crude extracts were then tested for their qualitative antimicrobial activity on both Gram-positive and Gram-negative bacteria. Paper discs are employed as reservoirs of heather phenolic extract. Agar plates and bacteria inoculums were generously provided by department of biology, University of York.

The initial bacteria inoculums were first analysed by a UV spectrophotometer at 500 nm, then the degree of dilution was decided by comparing the absorbance with a graph of viability for the particular strain. A final concentration of $5\times10^6$ cfu/ml was prepared for each bacterial suspension.

*Staphylococcus aureus* was selected as representative of Gram-positive cocci, and *Escherichia coli* k12 was selected as representative of Gram-negative bacteria. Each bacterial suspension in right concentration was spread evenly by a cotton swab onto a sterilized agar plate. Afterwards, sterilized paper disc were dipped into each heather phenolic extract until the paper disc is saturated. Each paper disc was then placed on to inoculated agar plate and was pressed until the surface of the disc is fully in contact with the plate. Since the heather raw materials were extracted by water/ethanol mixture, deionised water and anhydrous ethanol were also tested as controls. After

24-hour incubation at 37 $^{o}$C, inhibition area diameters were recorded and the plates were photographed.

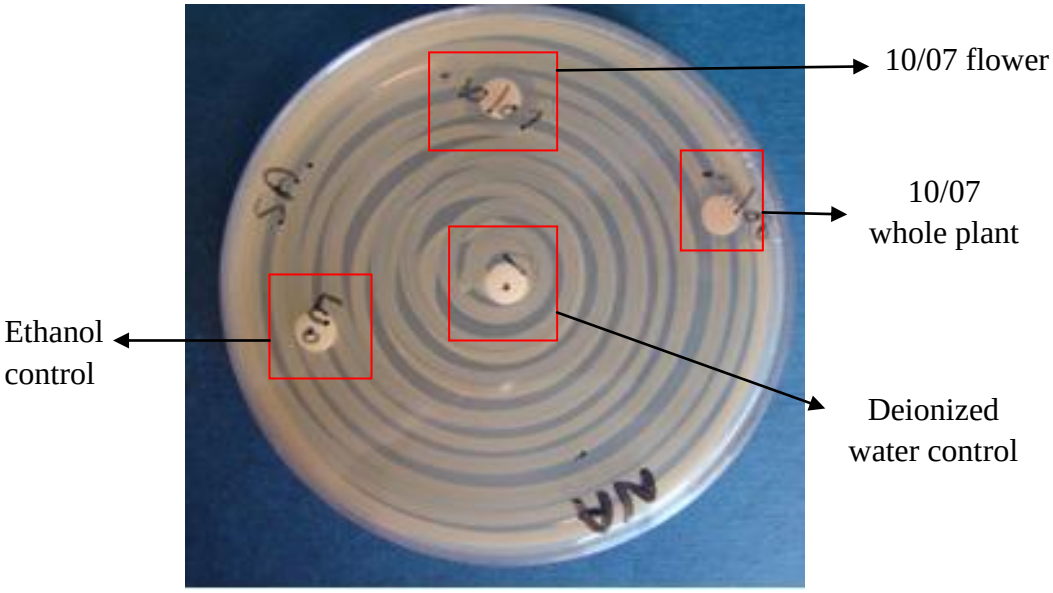



Figure 4.3: Effect of 10/07 flower, 10/07 whole plant, ethanol control and water control on *S. aureus*

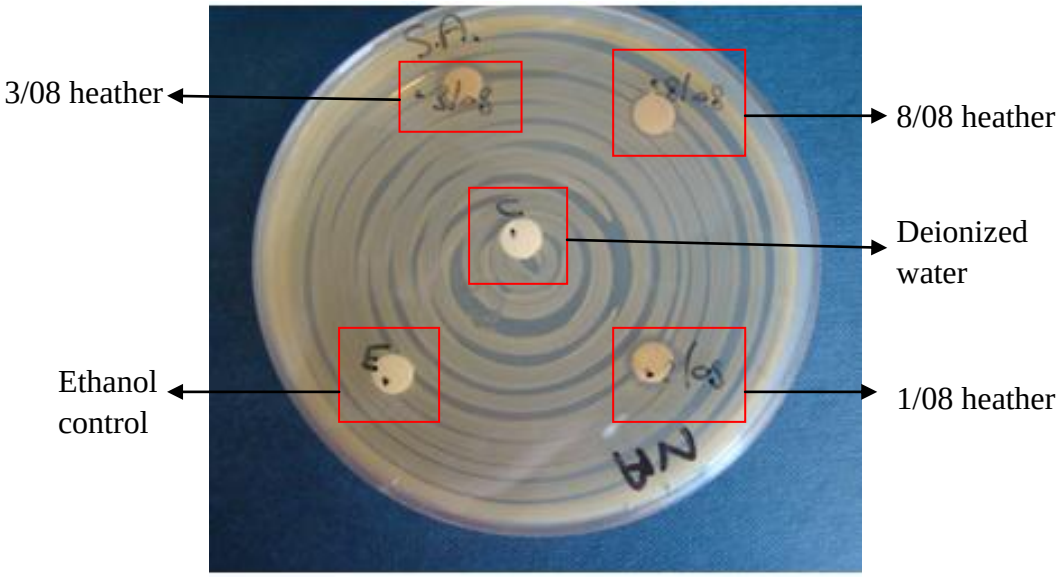



Figure 4.4: Effect of 1/08, 3/08, 8/08, ethanol control and water control on *S. aureus*

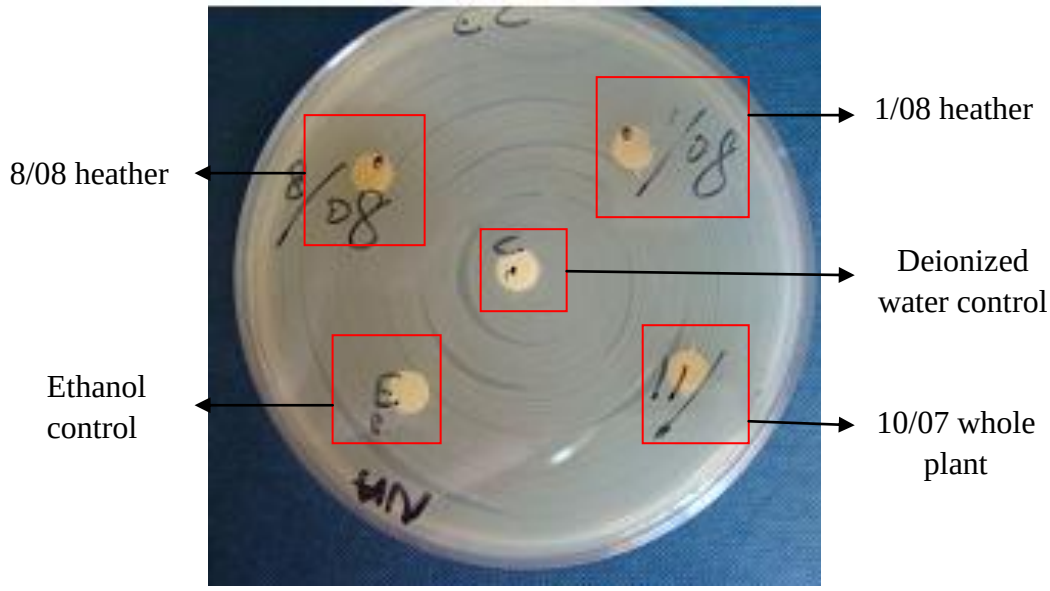



Figure 4.5: Effect of 1/08, 8/08, 10/07whole plant, ethanol control and water control on *E. coli*

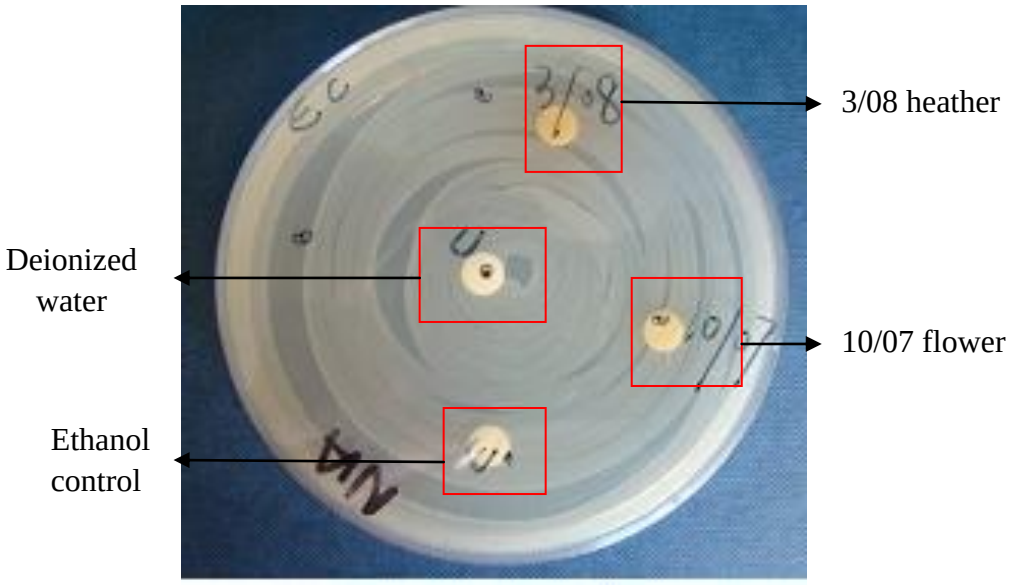



Figure 4.6: Effect of 10/07 flower, 3/08, ethanol control and water control on *E. coli*

Table 5.2 listed the antimicrobial activity of each heather ethanolic extract evaluated by their inhibition zone diameter. From the result, it is clear that all the extracts are effective to *S. aureus* and have no inhibition to *E. coli*. This corroborates the finding obtained in previous research[3] that phenolic compounds inhibit Gram-positive bacteria but not Gram-negative. Among all the extracts, heather flower exhibited strongest antibacterial effect with *S. aureus* and its inhibition zone is 6.0 mm. October whole plant extract was found to have second greatest bacteria inhibition zone which is 4.6 mm. Winter heather 1/08 sample and spring heather 3/08 sample both showed weak antibacterial effect and have an inhibition zone of 3 mm. Flowering summer heather 8/08 seemed to have the weakest inhibition to *S. aureus* and only exhibited a inhibition zone of 1.5 mm.

Table 4.2: Antibacterial activity of heather phenolic extracts against common Gram-positive and Gram-negative bacteria

| | **Inhibition zone (diameter in mm): 0 (-), 1–3 (+), 4–6 (++), 7–10 (+++).** | | | | |
|---|---|---|---|---|---|
| | 10/07 flower | 10/07 whole plant | 1/08 | 3/08 | 8/08 |
| ***S. aureus*** | ++ | ++ | + | + | + |
| ***E. coli*** | – | – | – | – | – |
| **Water control** | – | – | – | – | – |
| **Ethanol control** | – | – | – | – | – |

A seasonal variation tendency of antimicrobial activity was showed in figure 4.7. The trend is a clear reduction from winter to summer. In general, the seasonal variation of antimicrobial activity is surprisingly in complete contrast with that of the total phenolic content. Heather flower, which contains the lowest total phenolics, showed

the strongest antimicrobial activity in this qualitative test. On the other hand, summer heather sample which have the second highest total phenolic content, exhibited the weakest antimicrobial activity. The inhibition zone of heather flower was approximately twice of that of March heather which contains the highest amount of total phenolic compound.

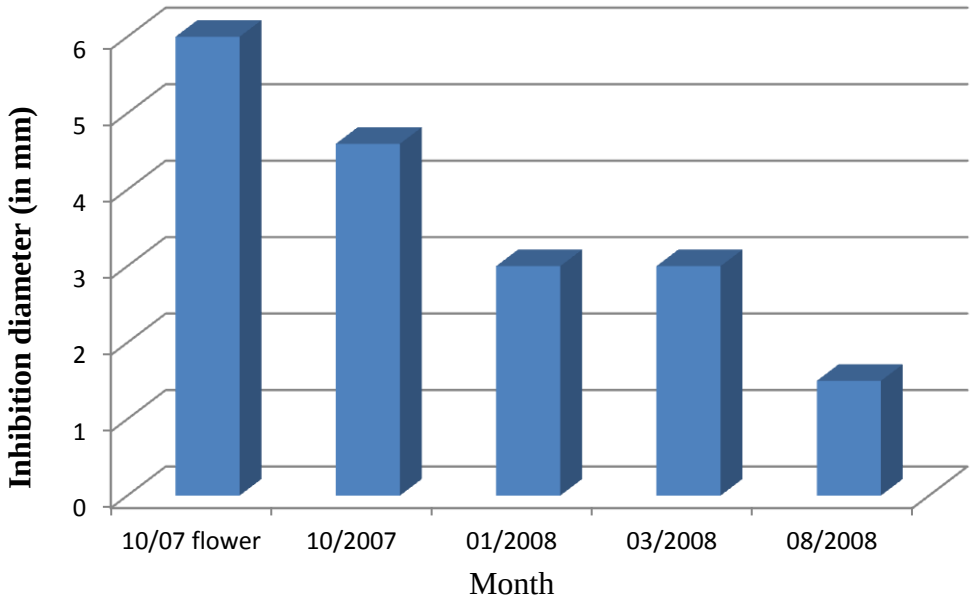



Figure 4.7: Seasonal variation of antimicrobial activity of heather phenolic extracts

When Kahkonen and co-workers[2] evaluated the antioxidant activity of 92 plant ethanolic extracts containing phenolic compound, they found that the antioxidant activity does not necessarily correlate with high amounts of phenolics, and that is why both phenolic content and antioxidant activity information must be discussed when evaluating the antioxidant potential of an extract. Similarly, the antimicrobial activity does not necessarily correlate with total phenolic content in heather. However, the reason for why the variation tendency of antimicrobial activity is in contrast to that of total phenolics content still need more investigation since the composition of total phenolics in heather was not analysed in this study. Further work is required to

identify the phenolic compounds present and the antimicrobial activity for each constituent needs to be evaluated.

## 4.4 Quantitative antimicrobial test of heather flower phenolic extract

Since heather phenolic extracts are proved to be only effective against Gram-positive bacteria, a quantitative antimicrobial test was carried out using *S. aureus*. Heather flower was selected as extraction material due to its strongest antimicrobial activity as shown in qualitative assay. Heather flower ethanolic extract was prepared following the method described in 7.3.5.2. *Staphylococcus aureus* strain was provided by Department of Biology, University of York. The initial bacteria inoculum was diluted by the same method mentioned in qualitative test. A final concentration of $5\times10^6$ cfu/ml was prepared for *S. aureus* bacterial suspension.

Since phenolic compounds are weak acid, there is a possibility that the pH value of the culture environment may affect its antimicrobial activity. Thus, nutrient broths in different pH were prepared by adjusting the original broth (pH 7) using citric acid. Double concentrated nutrient broth was made up by adding 26 g nutrient broth powder (obtained from Fluka) into 1 L distilled water and mixed well. Then the broth solution was autoclaved at 120 $^oC$ for 15 min prior to use. Nutrient broth at pH 4, 5, 6, 7 and 8 were prepared.

A 96 multiwell culture plate was employed in this study. Figure 4.8 shows the image of the plate. It contains twelve columns times eight rows of small wells, each one has a capacity of approximately 200 μl.

At the start of the antimicrobial activity assay of heather flower phenolic extract, each well on the plant was filled up with 100 μl double concentrated broth, 2 μl bacteria inoculum and 100 μl heather flower phenolic extract. Deionized water and anhydrous

2% ethanol water solution were also applied as controls. The plate was incubated at 37 $^{o}$C for 24 hours, and then its turbidity was measured by a microbiophotometer at 580 nm.

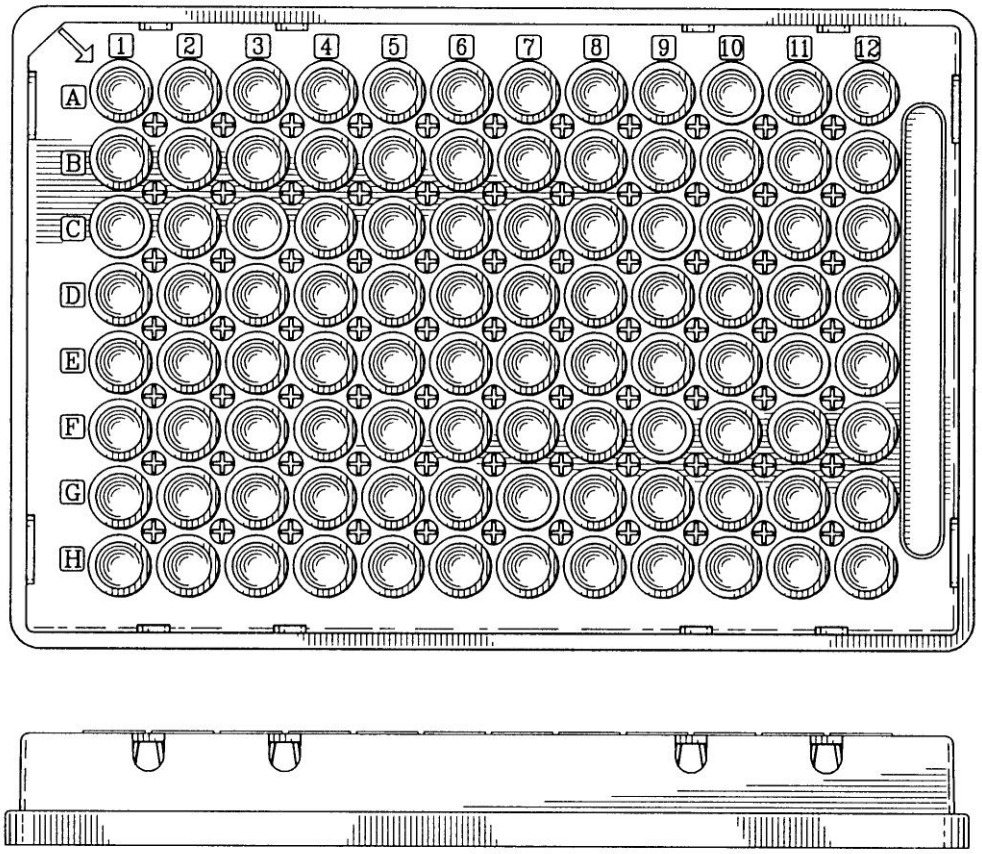



Figure 4.8: Round bottomed 96 multi-well culture plate

However, an unexpected situation occurred after incubation. The phenolic compound in heather flower reacted with the protein content in the broth and produced precipitate. Thus the reading of each well does not precisely reflect the growth of bacteria. To solve the problem, 10 ul solution was transferred from each well and spotted onto sterilized agar plate to give a visible result of the bacteria growth first. Further action will be taken to quantify the inhibition if any bacteria colonies are

revealed on this spotted agar plate. The agar plate was incubated at 37 $^{o}$C for another 24 hours.

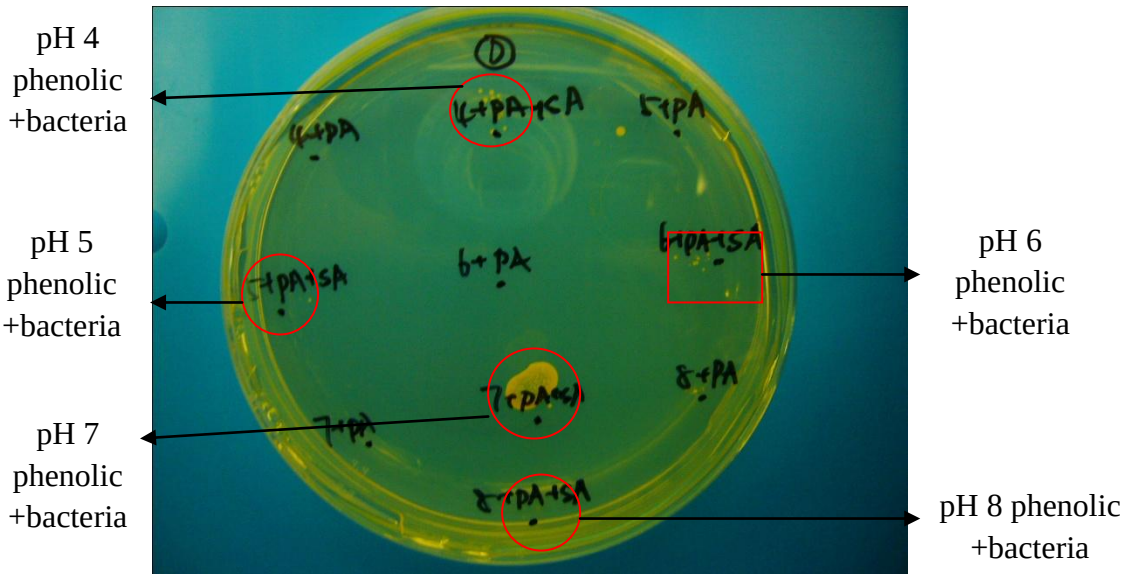



Figure 4.9: The bacteria colonies of pH 4-pH 8 heather phenolic extracts

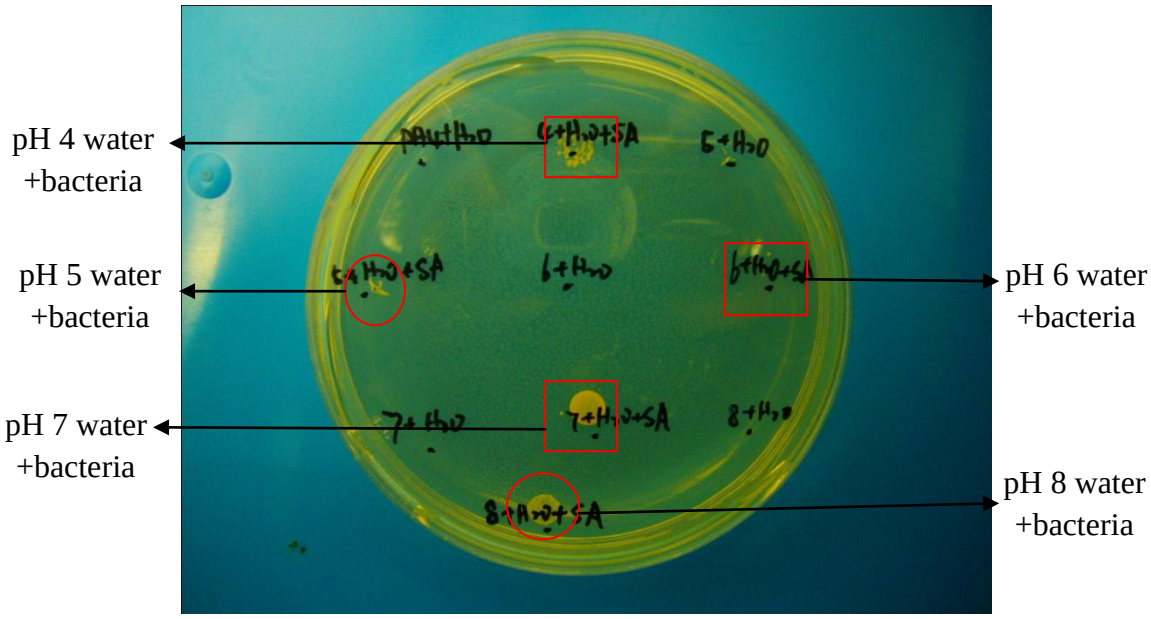



Figure 4.10: The bacteria colonies of pH 4-pH 8 water control

Figure 4.9 and 4.10 show the spotted agar plates of heather phenolic extracts and water control at different pH values with *S. aureus* inoculum. Normal colonies are only revealed in pH 7 in both water control and phenolic solution. This indicate that the absence of bacteria colonies in other pH value culture is because the bacteria itself cannot tolerate lower or higher pH environment, rather than the effect of phenolic antibacterial property. Therefore, another Gram-positive bacteria strain with better pH tolerance should be selected to replace *S. aureus*.

To address this situation, *lactobacillus* was used as experimental subject instead of *S. aureus*. *Lactobacillus brevis* DSM1267 was supplied by Department of Biology, University of York, and the bacteria suspension was diluted to a final concentration of $5\times10^6$ cfu/ml prior to use. Double concentrated MRS broth (obtained from Fluka) was prepared by adding 102 g broth powder into 1L deionized water and then autoclaved at 120 $^oC$ for 15 min. Moreover, to solve the problem of precipitation, the heather flower phenolic extract was diluted to contain 1000 ppm and 100 ppm total phenolics after dilution and prepared follow the method stated in 7.3.5.2. As with the previous experiment, a 96 multiwell culture plate was used and each well was filled up with 100 μl double concentrated MRS broth, 2 μl lactobacillus inoculum and 100 μl 1000 ppm (or 100 ppm) heather flower phenolic extract. 1% ethanol solution, 0.1%ethanol solution was also employed as controls. The plate was then incubated at 30 $^oC$ for 36 hours. The turbidity of each well was then measured by a microbiophotometer at 485 nm.

Table 4.3 and 4.4 shows the plate arrangement and the final reading of turbidity of each well. Table 4.5 shows the average turbidity readings of each column of quadruplicate samples and the inhibit effect of heather flower phenolic extract in 1000 ppm and 100 ppm. The inhibition percentage was calculated based on the bacteria growth difference in water control and in phenolic extracts.

Table 4.3: The arrangement of the plate

| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 |
|---|---|---|---|---|---|---|---|---|
| | P1000=1000 ppm phenolic solution | | P100=100 ppm phenolic solution | | EtOH=1% ethanol control | | EtOH=0.1% ethanol control | |
| A | MRS+P1000 | MRS+P1000 +lactobacillus | MRS+P100 | MRS+P100 +lactobacillus | MRS+EtOH | MRS+EtOH +lactobacillus | MRS+EtOH | MRS+EtOH +lactobacillus |
| B | MRS+P1000 | MRS+P1000 +lactobacillus | MRS+P100 | MRS+P100 +lactobacillus | MRS+EtOH | MRS+EtOH +lactobacillus | MRS+EtOH | MRS+EtOH +lactobacillus |
| C | MRS+P1000 | MRS+P1000 +lactobacillus | MRS+P100 | MRS+P100 +lactobacillus | MRS+EtOH | MRS+EtOH +lactobacillus | MRS+EtOH | MRS+EtOH +lactobacillus |
| D | MRS+P1000 | MRS+P1000 +lactobacillus | MRS+P100 | MRS+P100 +lactobacillus | MRS+EtOH | MRS+EtOH +lactobacillus | MRS+EtOH | MRS+EtOH +lactobacillus |
| | Water control (deionized water instead of phenolic in wells) | | | | | | | |
| E | MRS+$H_2O$ | MRS+$H_2O$ +lactobacillus | MRS+$H_2O$ | MRS+$H_2O$ +lactobacillus | | | | |
| F | MRS+$H_2O$ | MRS+$H_2O$ +lactobacillus | MRS+$H_2O$ | MRS+$H_2O$ +lactobacillus | | | | |
| G | MRS+$H_2O$ | MRS+$H_2O$ +lactobacillus | MRS+$H_2O$ | MRS+$H_2O$ +lactobacillus | | | | |
| H | MRS+$H_2O$ | MRS+$H_2O$ +lactobacillus | MRS+$H_2O$ | MRS+$H_2O$ +lactobacillus | | | | |

Table 4.4 Turbidity readings at 485nm of each well after incubation

| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 |
|---|---|---|---|---|---|---|---|---|
| | P1000=1000 ppm phenolic solution | | P100=100 ppm phenolic solution | | EtOH=1% ethanol control | | EtOH=0.1% ethanol control | |
| A | 0.485 | 0.917 | 0.338 | 0.881 | 0.584 | 0.891 | 0.465 | 1.201 |
| B | 0.598 | 0.897 | 0.476 | 0.804 | 0.505 | 0.858 | 0.473 | 1.066 |
| C | 0.492 | 0.912 | 0.448 | 0.774 | 0.342 | 0.868 | 0.368 | 0.929 |
| D | 0.366 | 0.912 | 0.347 | 0.786 | 0.412 | 0.888 | 0.627 | 0.927 |
| | Water control (deionized water instead of phenolic in wells) | | | | | | | |
| E | 0.422 | 0.836 | 0.358 | 0.800 | | | | |
| F | 0.444 | 0.814 | 0.472 | 0.832 | | | | |
| G | 0.380 | 0.911 | 0.342 | 0.784 | | | | |
| H | 0.361 | 0.928 | 0.477 | 1.032 | | | | |

Table 4.5 Average turbidity readings of each column and calculated inhibition effect of phenolic extracts

| | Average turbidity at 485nm | | | | Inhibition |
|---|---|---|---|---|---|
| | **A**: broth + phenolic extract without bacteria after incubation (blank readings) | **B**: broth+phenolic extract with bacteria inoculums after incubation | Total growth: **B-A** | P1000=1000 ppm phenolic solution P100=100 ppm phenolic solution | |
| 1000ppm phenolic | 0.48525 | 0.90950 | 0.42425 | P1000 effect **-0.04625** | **9.83%** |
| Broth with water control | 0.40175 | 0.87225 | 0.47050 | | |
| | | | | | |
| 100ppm phenolic | 0.40225 | 0.81125 | 0.40900 | P100 effect **-0.04075** | **9.06%** |
| Broth with water control | 0.41225 | 0.86200 | 0.44975 | | |
| | | | | | |
| 1%EtOH control | 0.44500 | 0.89013 | 0.44513 | | 0% |
| 0.1%EtOH control | 0.47163 | 0.98663 | 0.51400 | | |

After 36 hours incubation, 1000 ppm heather phenolic extract and 100 ppm phenolic extract inhibited 9.83% and 9.06% of total growth of lactobacillus respectively. These inhibition figures are very low compared to other commonly used antimicrobial agents. Therefore, this indicated that heather flower phenolic extract at 1000 ppm and 100 ppm both have very weak antimicrobial activity and are not sufficient to be applied as antimicrobial agent even at these fairly high concentrations. However, since the composition of heather flower phenolic were not analysed in this study, it is worth being investigated further to identify the constituent of heather flower total phenolics. Thus, any key components which express noticeable antibacterial activity can be purified and evaluated for their quantified antimicrobial effect to raise the possibility of heather being used as an herbal antibacterial.

## 4.5 Conclusion and further remarks

Heather plant picked in October 2007, January 2008, March 2008 and August 2008 were selected as representative of heather in every season. Heather flower picked in October 2007 was selected as representative of heather flower. Each heather raw material was extracted by ethanol/water solution and was then evaluated for their total phenolic content by using UV spectrophotometer. Gallic acid was employed as a standard in this quantification and the total phenolic content was reported as gallic acid equivalents (GAE)/g dried weight of plant material. A seasonal variation trend was obtained and a clear increasing of total phenolic content in heather from autumn to spring was showed. Heather flower is proved to have the lowest total phenolic content which is 2130 mg/100g dry plant, and spring heather contains the highest total phenolic content at 5163 mg/100g dry plant.

However, when the same heather ethanolic extracts were evaluated for their qualitative antimicrobial activity, an obvious contrast was observed between phenolic content and inhibition of gram positive bacteria. Paper disc qualitative antimicrobial

test was used and the inhibiting effect of each heather extract was estimated by inhibition zone diameter. Heather phenolic extract was proved to be only effective on Gram-positive bacteria which confirmed the results obtained in previous studies. Heather flower with the lowest total phenolic content showed the largest inhibition zone, and summer heather which contains the second highest total phenolic content showed the weakest antimicrobial ability. This phenomenon proves that the antimicrobial activity does not necessarily correlate with high content of total phenolics. Therefore, further investigation is required to identify the composition of heather total phenolic to give a better explanation.

Heather flower phenolic extract was then analysed for its quantitative antimicrobial activity. Multiwell culture plate was employed and *S. aureu*s was selected as experimental subject first. Since phenolic content is weak acid, different pH broths were prepared to estimated the effect of pH value on phenolic antimicrobial activity. However, after 24 hours incubation, the phenolic content was found to have reacted with the protein in the broth and generated precipitate that made the turbidity reading less precise. To solve the problem, 10 μl solution from each well was spotted to sterilized agar plate to give visible bacteria colonies before further quantification. After another 24 hours incubation at 37 $^{o}$C, *S. aureus* was found only survive in pH 7 broth in both water control and phenolic solution. A more tolerant Gram-positive organism was selected to give better growth in lower pH conditions.

*Lactobacillus brevis* was then applied instead of *S. aureus*, and the precipitation problem was resolved by diluting heather phenolic content to 1000 ppm and 100 ppm. 96 multiwell culture plates were again used and the plate was incubated at 30 $^{o}$C for 36 hours after inoculation. The turbidity of each well was then measured by a microbiophotometer at 485 nm. After calculation, heather phenolic extract inhibited 9.83% of total bacteria growth at 1000 ppm and 9.06% at 100 ppm. These figures are very low compared with commonly used antimicrobial agent. To conclude, heather flower phenolic extract did not show strong antimicrobial activity at 1000 ppm and

100 ppm. However, since the composition of heather flower phenolics was not analysed in this study, it is worth being investigated further to identify the constituent of heather flower total phenolics. Key components with better antibacterial activity can be purified and evaluated for their quantified antimicrobial effect to raise the possibility of heather being used as herbal antibacterial.

# *Liquid and supercritical carbon dioxide extractions of bracken*

# Chapter 5

## 5.1 Liquid $CO_2$ and supercritical $CO_2$ extractions of bracken

The application of supercritical fluid extraction especially liquid and supercritical carbon dioxide is getting more and more attention in the field of natural product research. This environmentally benign extraction technique offers extraction yields that are comparable with those achieved by conventional solvent extraction. Meantime, the remarkable selectivity of liquid and supercritical $CO_2$ has unique superiority and makes this technique a perfect separating method. Moreover, in contrast with organic solvents, carbon dioxide is non-toxic, non-flammable, non-corrosive, cheap and readily available in large quantities with high purity.[82] Since the critical pressure (72.8 bar) and critical temperature (31 $^{o}$C) of carbon dioxide is easily accessible, liquid and supercritical $CO_2$ are really ideal solvent for natural compound extraction.[83]

To achieve a comprehensive scan of the compounds present in bracken, liquid $CO_2$ extraction was employed along with supercritical $CO_2$ extraction. Moreover, to enhance to solvent power of the supercritical $CO_2$, the use of ethanol entrainer was also introduced in this work. Thus, a standard bracken extraction scheme is first, liquid $CO_2$ extraction, followed by supercritical $CO_2$ extraction and finally supercritical $CO_2$ with ethanol entrainer. To the best of my knowledge, liquid and supercritical $CO_2$ extractions have never carried on bracken plant before. Bracken was normally either extracted by organic solvent or water due to the solubility of ptaquiloside in aqueous solution. Therefore, this work is of great meaning to develop and probe a new field of knowledge of the plant. By analysing each extract obtained from these consecutive processes, a panorama of bracken chemical compounds could be revealed.

Samples in different bracken life cycle steps are picked from the same site (Kilburn White Horse, Sutton Bank, Grid reference SE 514 813) in different months of a year.

The first crozier sample was picked in 17th April 2009 and 19th May 2010, followed by the 2nd step sample picked in 21st May 2009, 3rd sample picked in 27th July 2009 and 4th sample picked in 12th October 2009. Unfortunately, the sample picked in April 2009 was misidentified and found to be lady fern rather than bracken fern, all the data and information for bracken crozier was obtained from May 2010 sample. All the plant samples were dried in room temperature and milled to a particle size of ≤2 mm.

## 5.2 Liquid and supercritical carbon dioxide extraction of bracken crozier

### 5.2.1 Liquid $CO_2$ extraction of bracken crozier

It was reported that the carcinogenicity of young bracken shoots was shown to be much stronger than that of mature plant.[6] Due to the remarkable distinction of bracken crozier, liquid $CO_2$ extraction, along with supercritical $CO_2$ extraction was employed in bracken crozier analysis to give a more comprehensive understanding of its chemical content. The conditions for bracken liquid carbon dioxide extraction were set to 5 $^{o}$C and 65 bar and a $CO_2$ flow-rate of 40 g/min. An initial yield of 0.60% was obtained from this extraction. The whole liquid $CO_2$ extraction lasted for 6 hours, and the extract was recovered from the separator by washing out with dichloromethane then concentrated by removing the solvent using a rotary evaporator. It seems liquid $CO_2$ is able to extract more moisture from the raw material than supercritical $CO_2$ since the extract was moister than the waxy extract obtained by supercritical extraction. A sample with a concentration of 20 mg/ml of crude extract was made up for GC-MS analysis.

#### 5.2.1.1 Identification of pterosin B

Figure 5.1 shows the gas chromatogram of bracken liquid $CO_2$ extract and also highlights the composition. It is well known that ptaquiloside presents in bracken young frond by a very high amount, but the quantification of ptaquiloside is really difficult due to its nature of instability. Therefore pterosin B, known as the most common final metabolite of ptaquiloside, is usually used as a representative compound in ptaquiloside quantification. Pterosin B ($C_{14}H_{18}O_2$, MW 218) has a basic indanone structure, and can be generated directly from ptaquiloside decomposition. In bracken liquid $CO_2$ extract, pterosin B appeared at 21.02 min and its identification was achieved by GC-MS analysis.

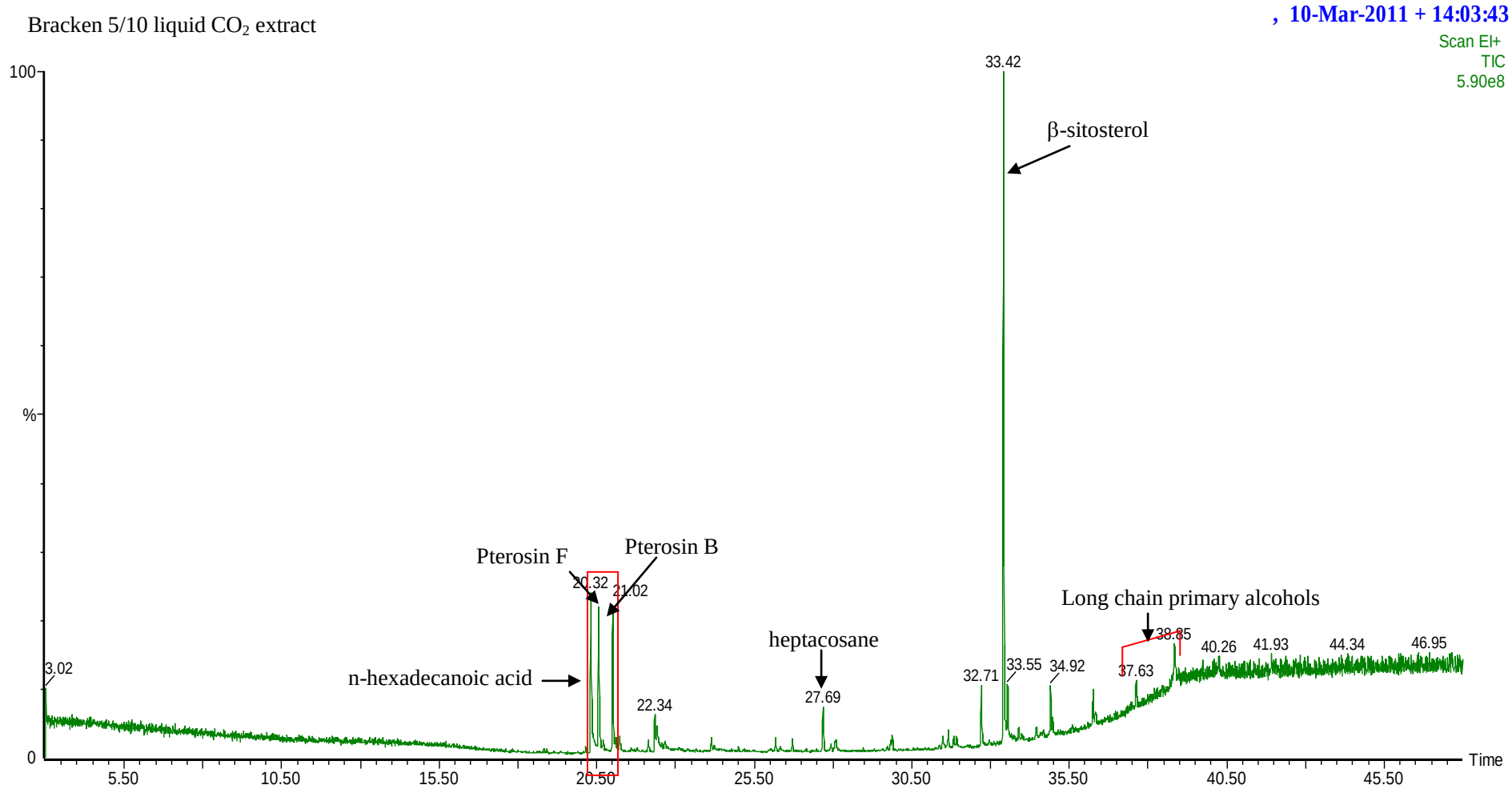



Figure 5.1 Gas chromatogram of bracken crozier liquid $CO_2$ extract

Figure 5.2 shows the mass spectrum of pterosin B. Major ions are observed at m/z 187, 203 and 218. Benzylic cleavage of the side chain is an important process that gives rise to the ion at m/z 187, and the abundance of m/z 203 is due to the loss of methyl group. These characteristic ions lend support to the previous spectral data obtained by Bonadies and co-workers.[84]

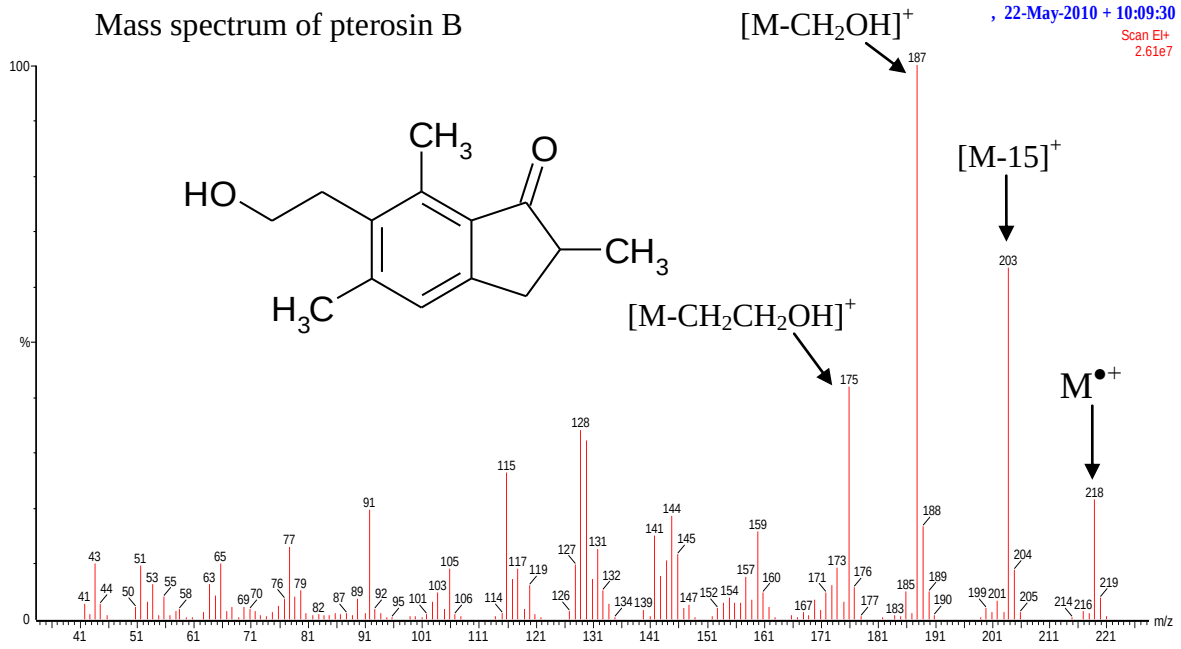
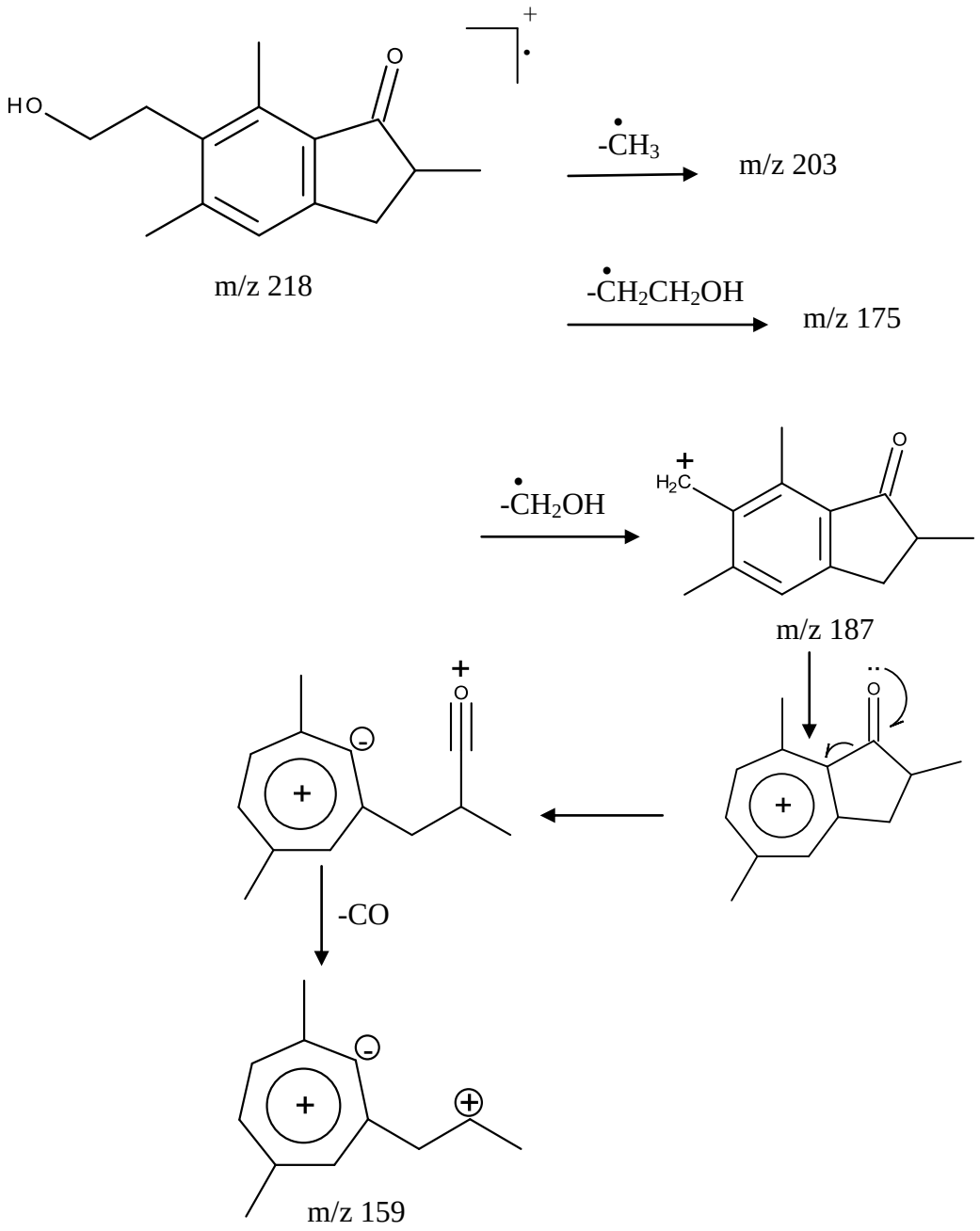



Figure 5.2 Mass spectrum of pterosin B

**Summary of GC/MS data for compounds III-VIII**

| | GC retention times and MS ions | | |
|---|---|---|---|
| | GC (min) | GC/MS (min) | m/z (abundance) |
| **Pterosin B (III)** | 7.89 | 12.66 | 218(42); 203(85); 187(100) |

Figure 5.3 GC-MS data of pterosin B published by Bonadies[84]




Scheme 5.1: Proposed mechanism for fragmentation of pterosin B

Since the conditions of liquid $CO_2$ extraction were quite mild, the pterosin B present in this extract is more likely to be in the plant originally prior to the extraction. As mentioned before, pterosin B is usually used as a representative of ptaquiloside. But there is a consideration that the transformation of ptaquiloside to pterosin B would already occur in the frond when alive since ptaquiloside is known to degrade into pterosin B under heat, acid or base. Therefore, the identification of ptaquiloside using pterosin B may not really reflect the presence of ptaquiloside in the plant matrix. This discovery of pterosin B in liquid $CO_2$ extract confirms this supposition. The presence of pterosin B in this extract means that the decomposition of ptaquiloside already exists during the early growing stage of the frond, and the quantity of pterosin B after experimental conversion is a summation of this compound present originally in the plant and pterosin B converted from ptaquiloside during the experiment.

#### 5.2.1.2 Identification of pterosin F

Along with pterosin B, pterosin F ($C_{14}H_{17}OCl$ MW 236.5) is also detected in this extract. Pterosin F has a very similar structure with pterosin B, only with chlorine instead of the hydroxyl on the side chain. Pterosin F peak exist at 20.57 min, same as pterosin B, its identification is also achieved by GC-MS analysis. Figure 5.4 shows the mass spectrum of pterosin F.

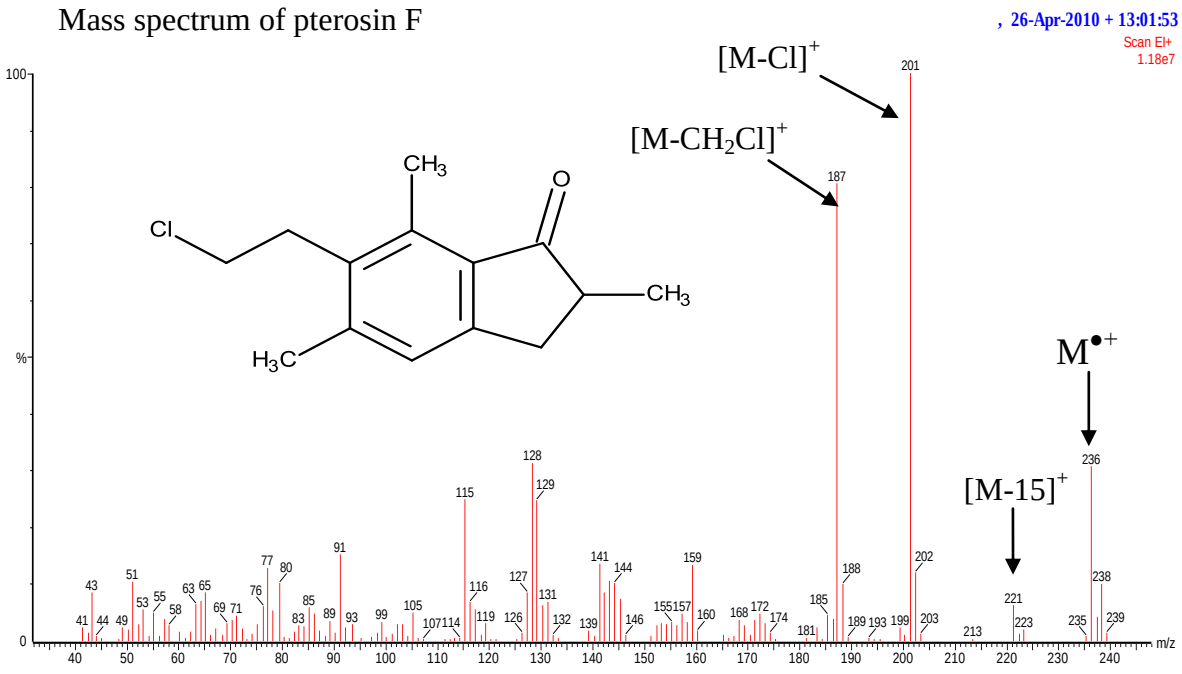



Figure 5.4 Mass spectrum of pterosin F

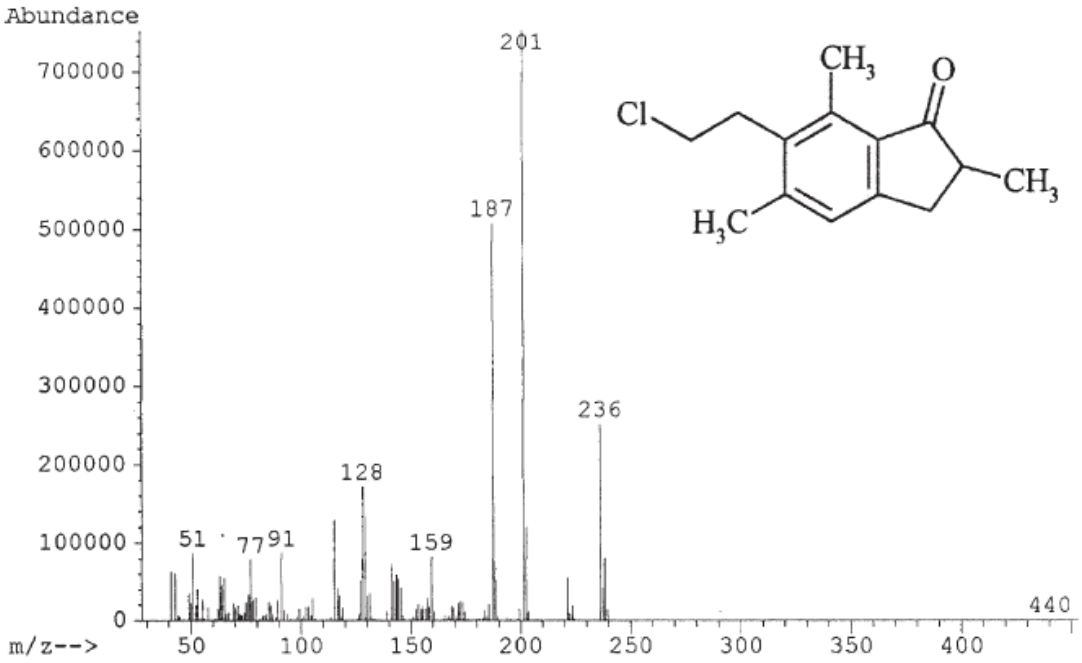
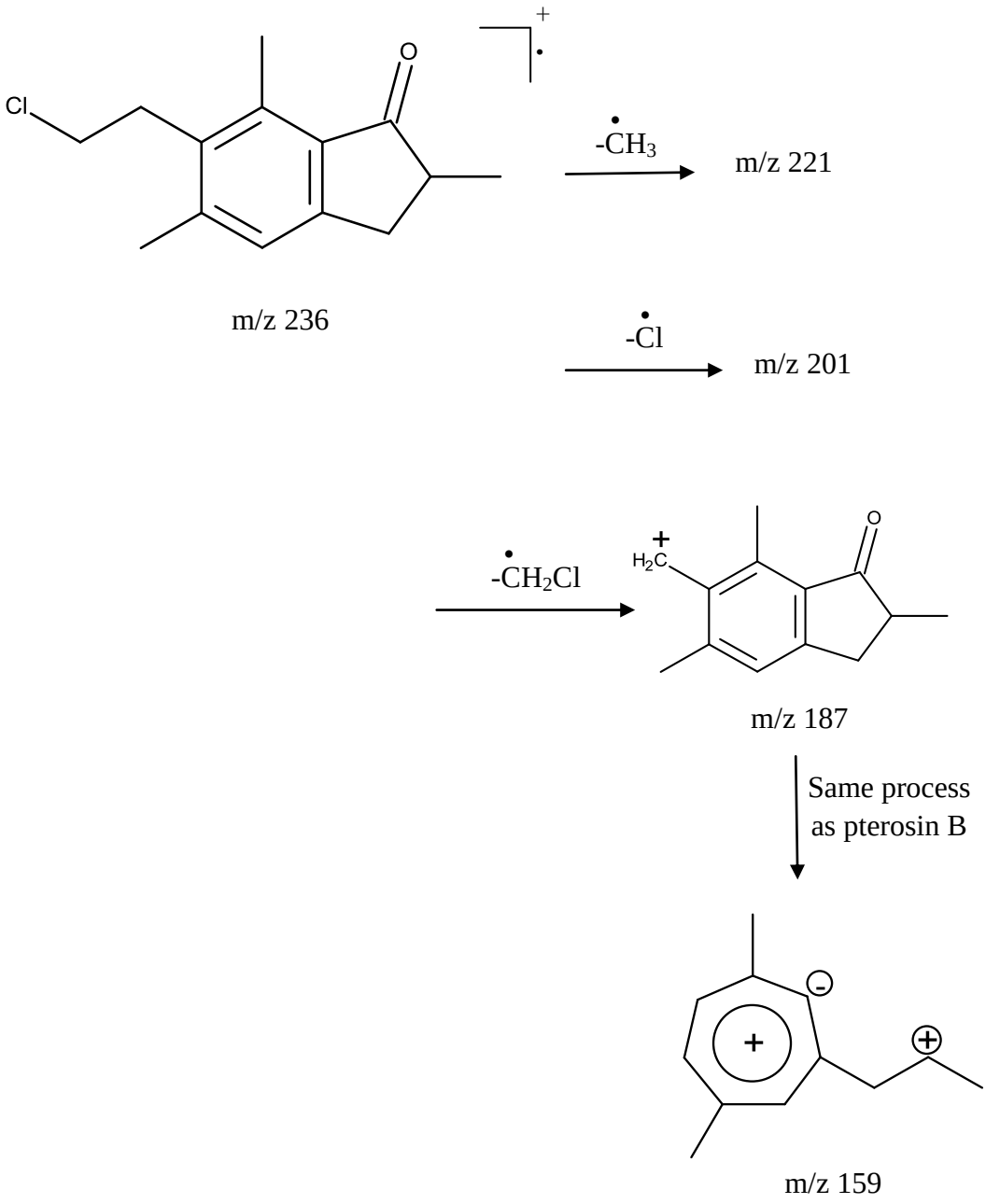



Figure 5.5 Mass spectra of pterosin F published by Bonadies[84]




Scheme 5.2: Proposed mechanism for fragmentation of pterosin F

Characteristic ions were found at m/z 187, 201 and 236. As seen in pterosin B, benzylic cleavage of the side chain is the main reason for the ion at m/z 187. The loss of the chlorine from the side chain affords the ion at m/z 201. According to Bonadies and co-workers, this ion only dominates when the substituent on the side chain can be

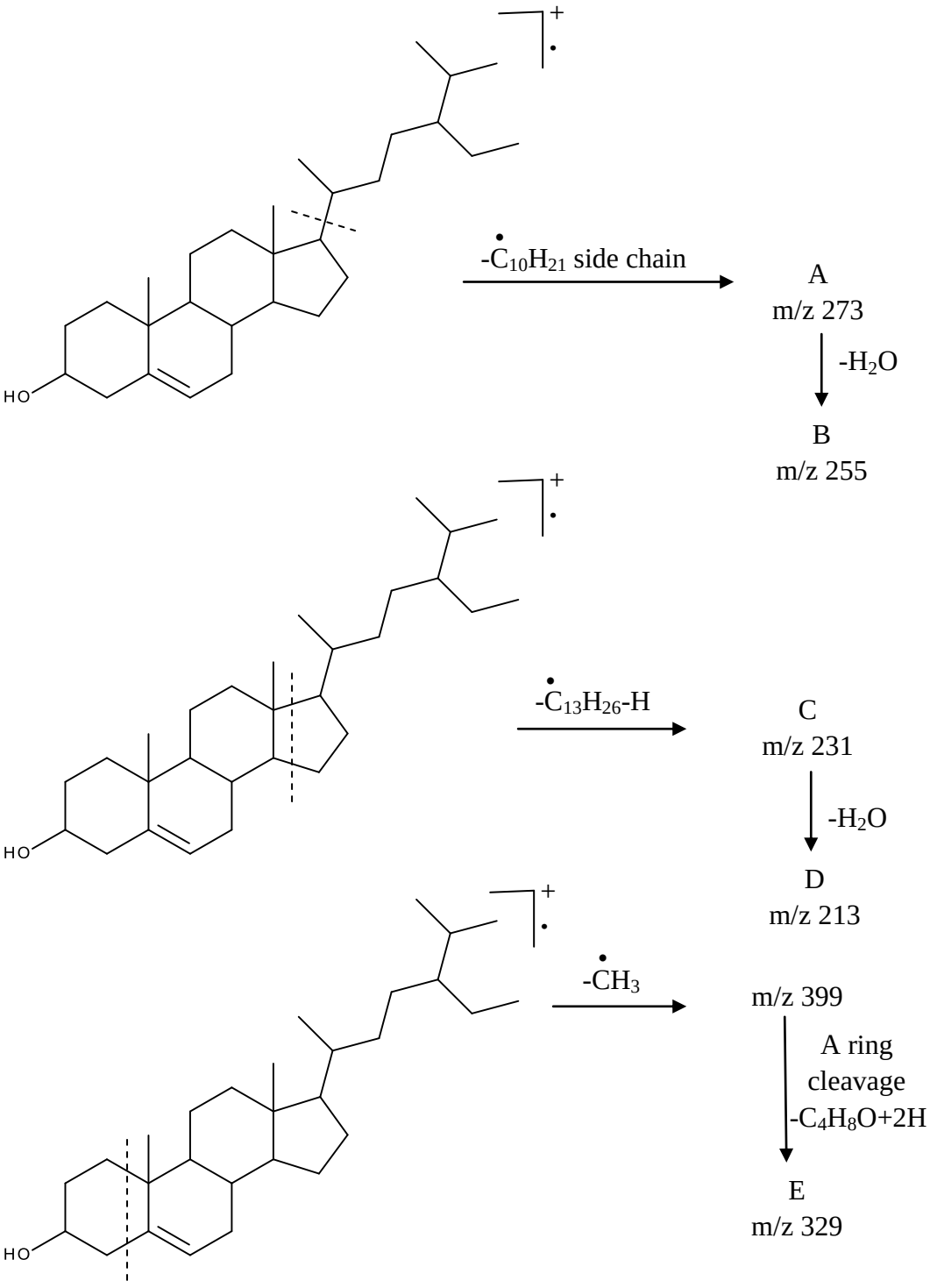
released as low-energy radical. In the case of pterosin B, this mechanism is not favorable, thus the loss of methyl group become more significant and affords an ion at m/z 203.[84] The molecular ion falls at m/z 236, and the ratio between m/z 236 and m/z 238 precisely reflects the relative abundance of chlorine isotopes ($^{35}Cl$:$^{37}Cl$ 3:1). Scheme 5.2 shows the proposed mechanism for fragmentation of pterosin F.

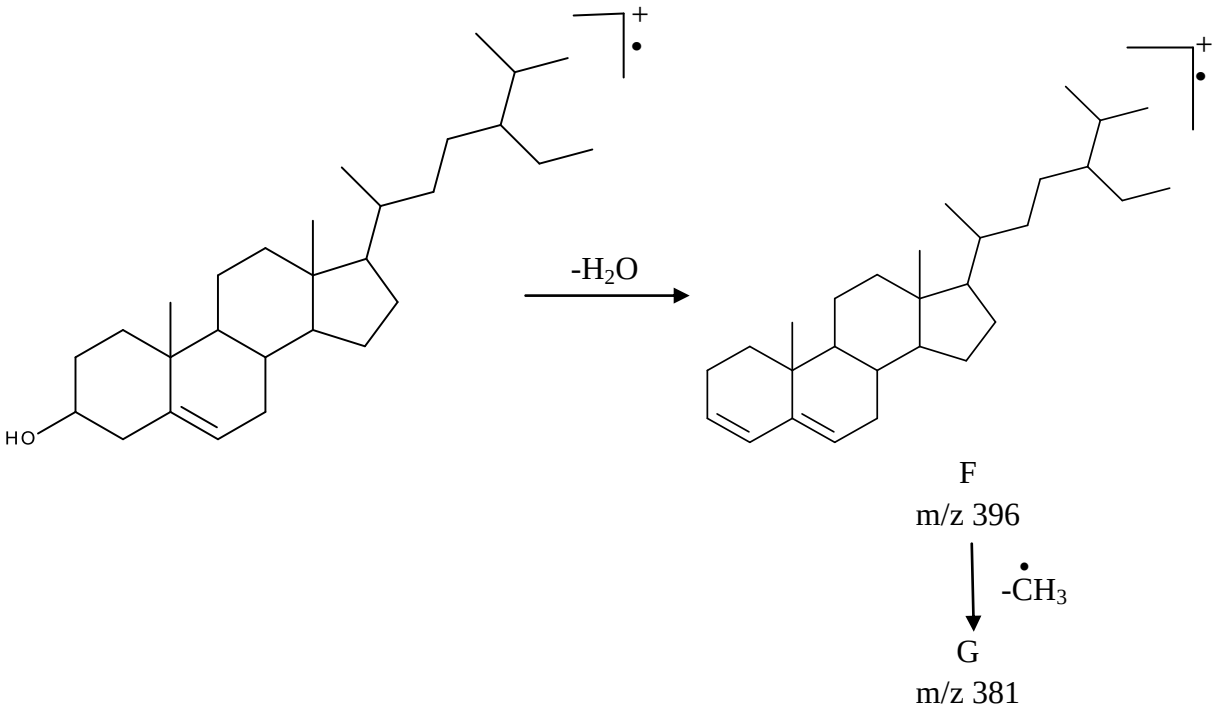
The presence of pterosin F in this extract also indicates that it already exists in the frond prior to the extraction. In previous studies, pterosin F was found in methanol extract of young leaves.[85,86] However, it was never reported whether pterosin F is present in the frond alone or as a metabolic product from a related precursor. As suggested by Saito, pterosin B results from light-induced decomposition of ptaquiloside.[87] Therefore, it is conceivable that ptaquiloside is a relocalizable source of pterosin B in the frond. Meanwhile, another indanone compound, pterosin A, exists in bracken and was suggested to have its own ptaquiloside-like precursor which was isolated from another fern species; although this precursor named dennstoside A has not been discovered in bracken fern.[88] In the case of pterosin F, any precursor or transformation mechanism is currently unknown. Further study is required to determine the source of pterosin F in bracken.

### 5.2.1.3 Identification of other major compounds found in bracken crozier liquid $CO_2$ extract

Some other compounds are also found in this liquid $CO_2$ extract. β-sitosterol($C_{29}H_{50}O$ MW 414) is a widely distributed phytosterol that is found in many plant species. It's well known for its blood cholesterol lowering effect. β-sitosterol limits the amount of cholesterol entering the body by inhibiting cholesterol absorption in the intestines, therefore decreasing the levels of cholesterol in the body. It is also helpful with benign prostatic hyperplasia (BPH), due to its anti-inflammatory effects and its ability to improve urinary symptoms and flow.[89] The β-sitosterol peak appears at 33.42 min,

and its quantification was also achieved by using stigmasterol as a standard. Figure 5.6 shows the standard spectra of β-sitosterol and spectra of peak at 33.42 min.






Scheme 5.2: Proposed mechanism for fragmentation of β-sitosterol

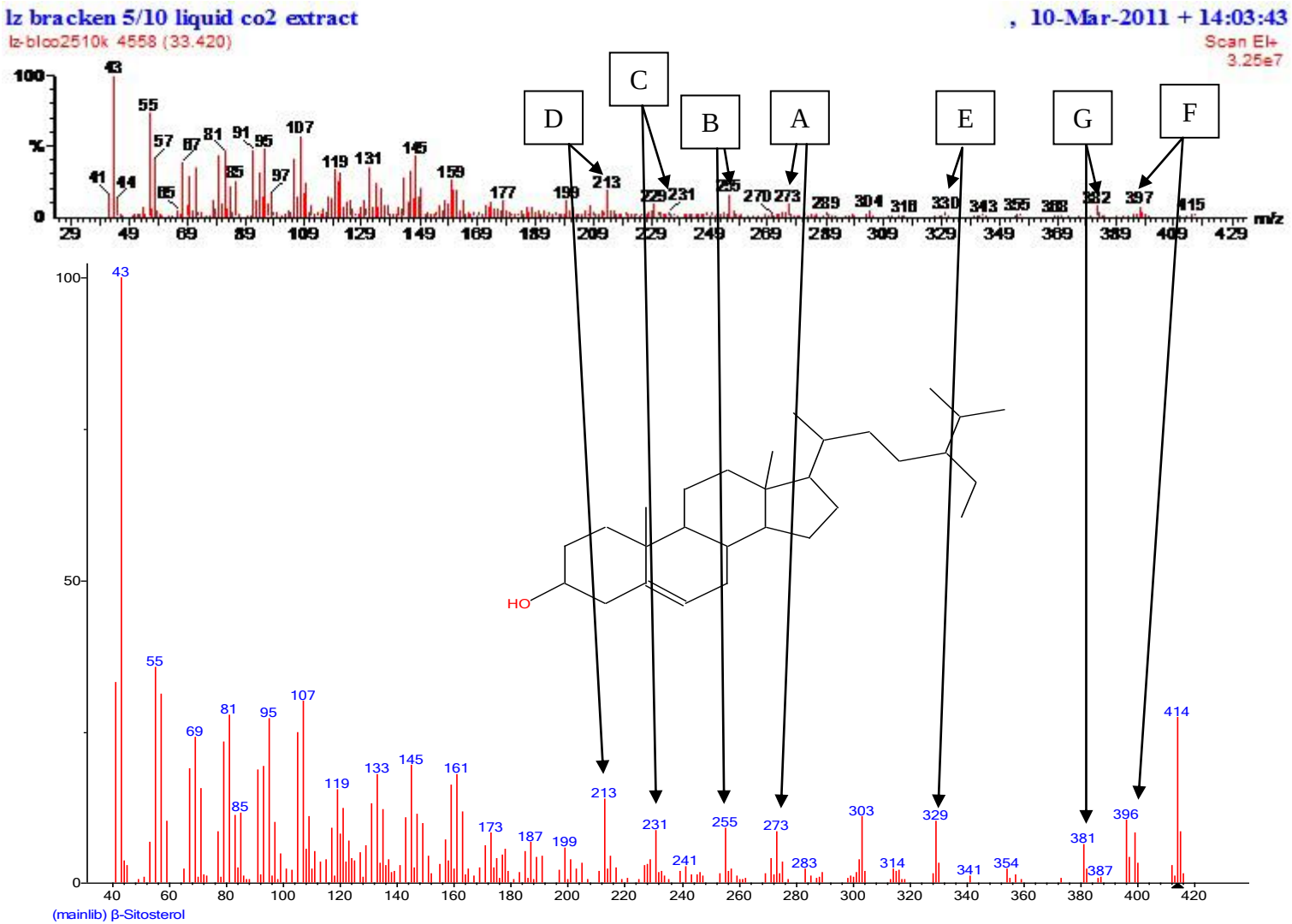
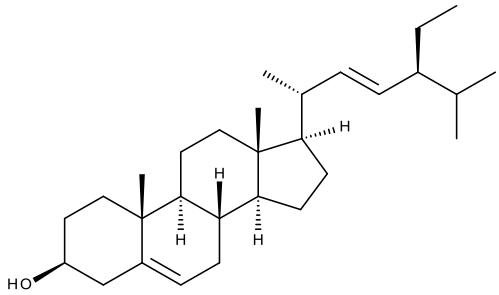


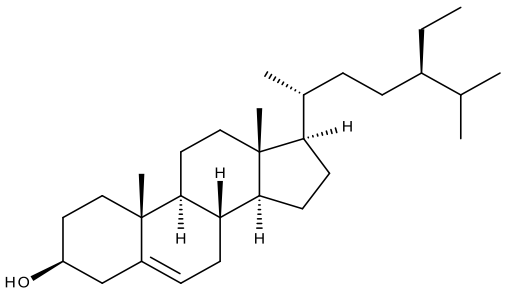

Figure 5.6 Mass spectra of β-sitosterol and the peak with retention time at 33.42min

Stigmasterol($C_{29}H_{48}O$, MW 412) is another phytosterol with a structure very similar to that of β-sitosterol. Thus it can be used as a standard for β-sitosterol quantification since their response factors would be very similar. Figure 5.7 shows the chemical structure of stigmasterol and β-sitosterol.

Standard solutions of stigmasterol in 0.6, 1.0, 1.5, 1.9, 2.5 and 3mg/ml concentrations were made up and analysed by GC. The absolute areas were recorded and a standard curve was determined using these area data. Figure 5.8 shows the standard curve of stigmasterol and the regressive equation. A correlative coefficient of 0.994 was obtained from this curve.




stigmasterol




β-sitosterol

Figure 5.7 Chemical structures of stigmasterol and β-sitosterol

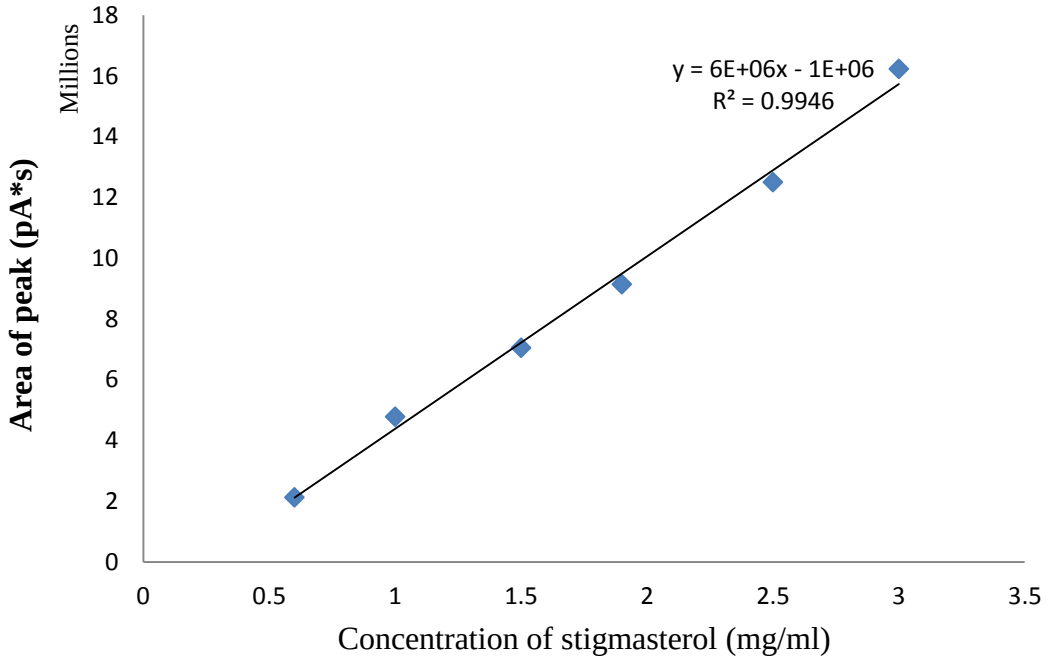



Figure 5.8 The standard curve of stigmasterol for β-sitosterol quantification

The β-sitosterol content was then calculated from the equation by using its absolute peak area. The quantity of it was found to be 1217 μg/g dry plant and 324 μg/g fresh plant. Its seasonal variation was also examined in later chapter.

Some long chain primary alcohols were also found in this extract. The two peaks with retention time of 37.63 min and 38.85 min both showed very similar spectra to a long chain primary alcohol standard (1-triacontanol $C_{30}H_{62}O$). Figure 5.9 shows the spectra comparison between the two peaks and 1-triacontanol standard.

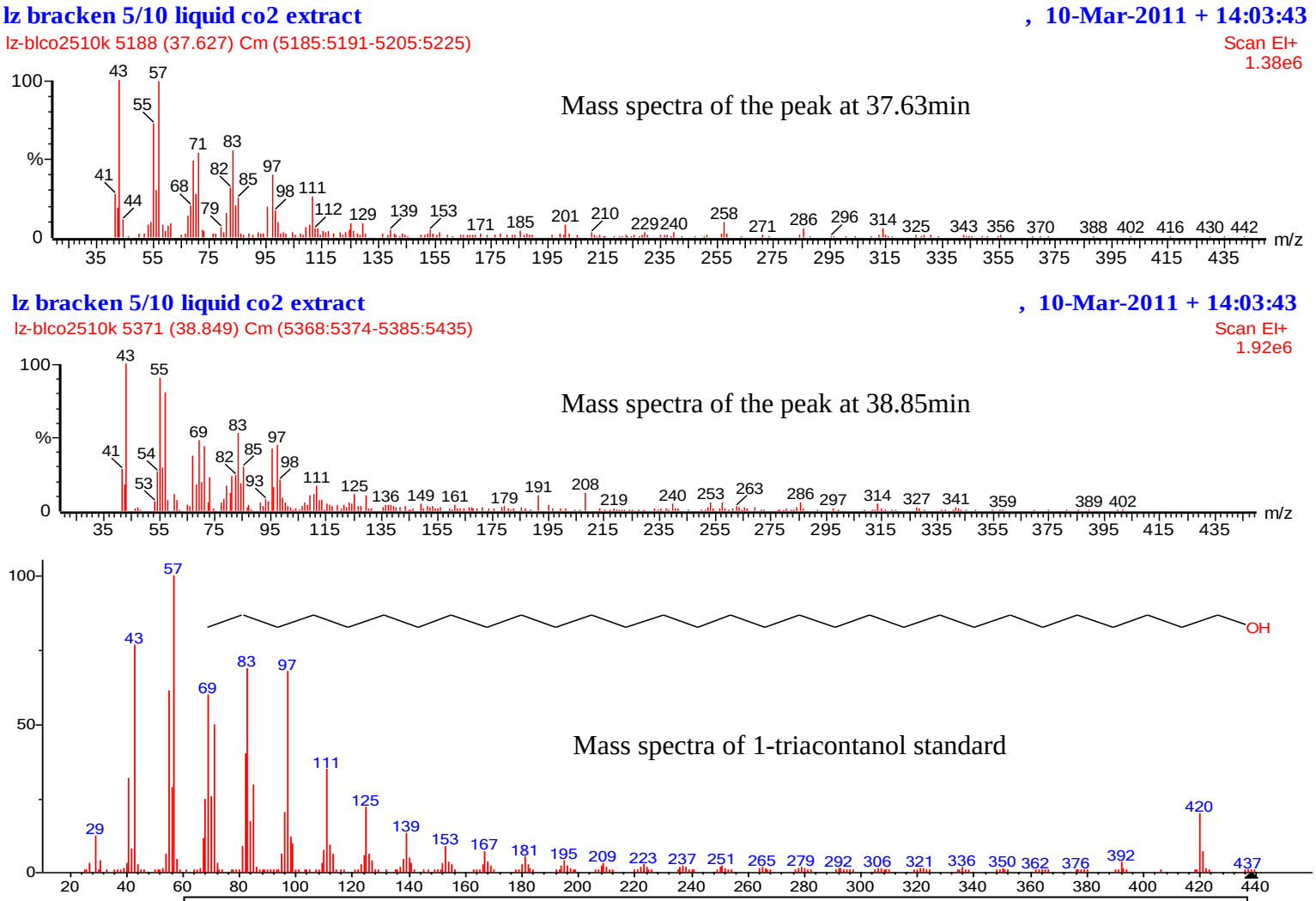



Figure 5.9 Mass spectra of the two peaks in liquid $CO_2$ extract and 1-triacontanol standard

The three spectra are all very similar in respect of the key fragment ions, indicating that these are a homologous series of long chain primary alcohols. No molecular ion is present to indicate the chain length; however, the Kovats index of each peak shows that they are separated by two $CH_2$ units (200 Kovats units). Table 5.1 shows the published Kovat index of 1- heptatriacontanol and 1-nonatriacontanol with KI value calculated for the two peaks. The chain length is 37 carbons for the peak at 37.63 min based on the calculated KI value relative to estimated standard KI data, and for the peak at 38.85 min the chain length was determined to be 39 carbons. Estimated standard KI value was calculated based on the standard KI data of heptacosanol and triacontanol published by Nist Mass Spectrometry Data Centre.

Table 5.1 Kovats index calculated for the two peaks
and comparison with published data

| Retention time | Possible compound | Molecular formula and weight | KI value calculated | Estimated standard KI value |
|---|---|---|---|---|
| 37.6min | 1-heptatriacontanol | $C_{37}H_{76}O$ MW 536 | 3977 | 3942 |
| 38.85min | 1-nonatriacontanol | $C_{39}H_{80}O$ MW 564 | 4165 | 4142 |

### 5.2.2 Supercritical carbon dioxide extractions of bracken crozier

Bracken crozier supercritical $CO_2$ extraction was carried out at 50 $^oC$, 350 bar and a flow-rate of 40 g $CO_2$/minute for 4 hours. The extract was recovered by washing out the separator with dichloromethane and then concentrated by evaporating the solvent using a rotary evaporator. A light green waxy extract was obtained with a crude yield of 0.68%. A sample was made up in dichloromethane at a concentration of 20 mg/ml of crude extract for GC-MS analysis.

Figure 5.10 shows the gas chromatogram of bracken crozier supercritical $CO_2$ extract. Pterosin B and pterosin F appear once again in this extract at 20.40 min and 19.94 min. The mass spectra of pterosin B and F are identical to those of the corresponding peaks in liquid $CO_2$ extract and published spectra.

β-sitosterol is also present in this extract and is identified as the peak with retention time of 32.64 min. The quantification of β-sitosterol in this extract was also achieved by using stigmasterol as a standard. The quantity of β-sitosterol was calculated as 754 μg/g dry plant and 201 μg/g fresh plant.

The chromatogram indicates clearly that more long chain primary alcohols were extracted using supercritical $CO_2$. In this extract, alcohols with more carbon numbers were extracted along with the two peaks that have been identified in 5.2.1.3. The peaks at 36.87 min and 38.07 min in this extract have identical spectra and Kovats index to the heptatriacontanol and nonatriacontanol that were identified in the liquid $CO_2$ extract. (The retention time of these two peaks has shifted ahead since the column of GC-MS has been removed and reinstalled between analyses)

Figure 5.11 shows the spectra of the series of alcohol peaks and 1-triacontanol standard. As can see from the figure, the series of peaks all have nearly identical spectra with 1-triacontanol standard with molecular ion missing. Thus, Kovats index was applied again to help identify the chain length.

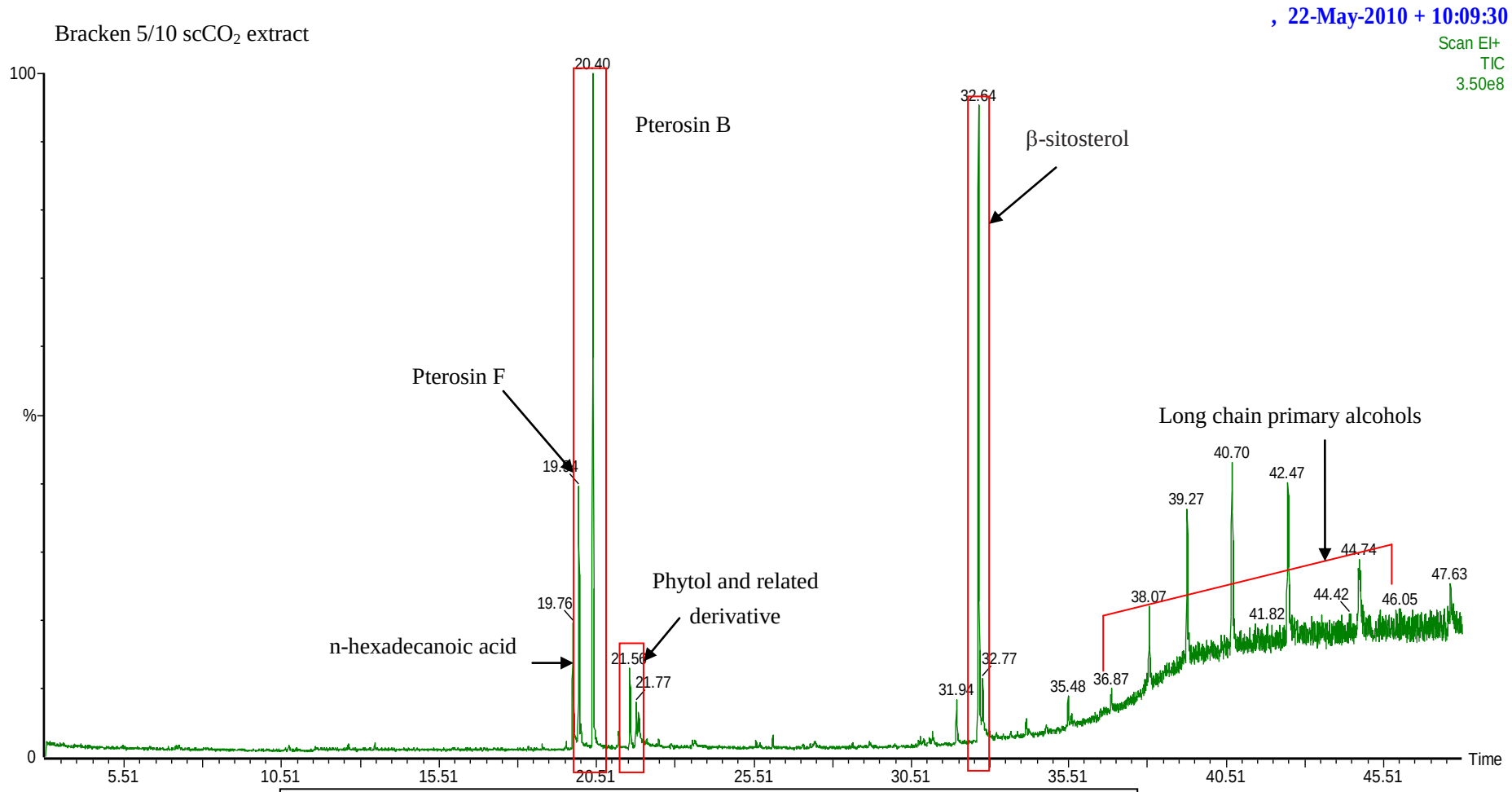
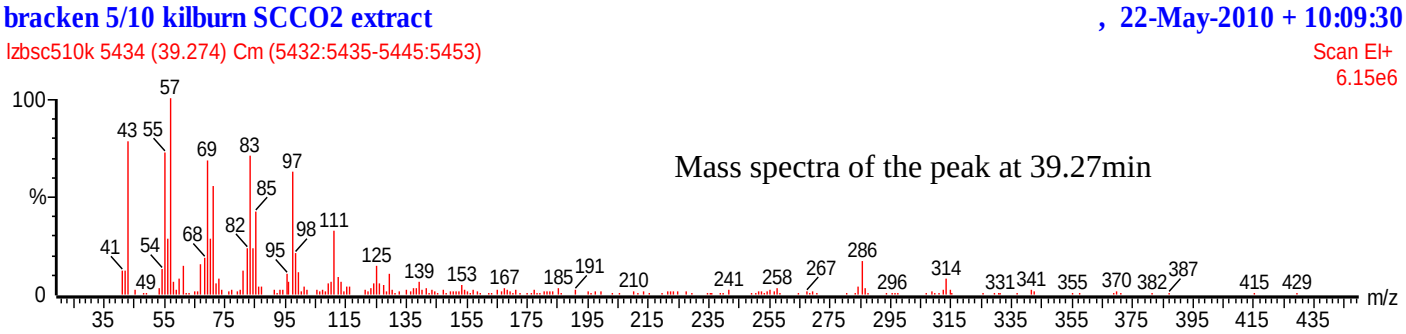
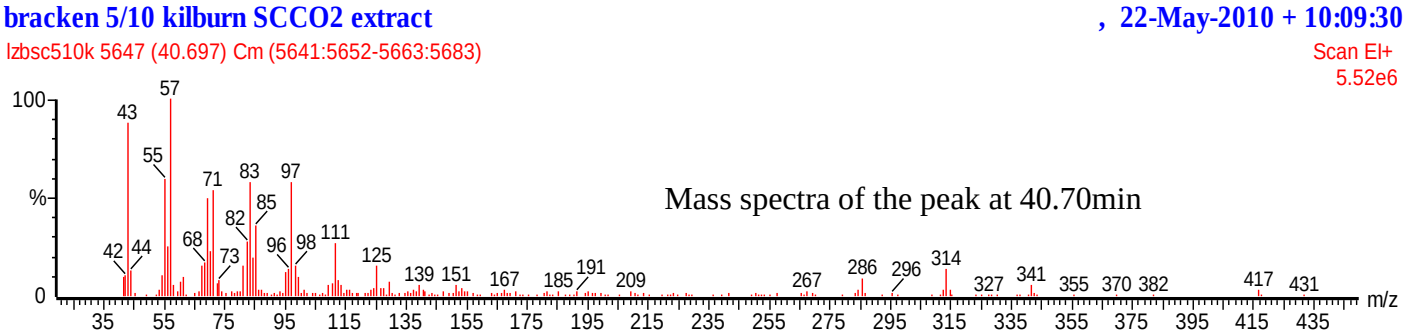
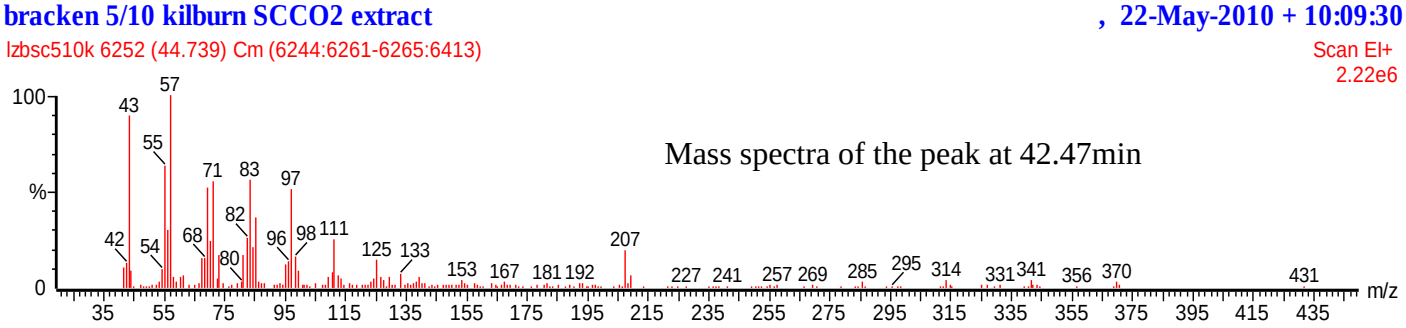



Figure 5.10 Gas chromatogram of bracken crozier supercritical $CO_2$ extract


bracken 5/10 kilburn SCCO2 extract , 22-May-2010 + 10:09:30
lzbsc510k 5434 (39.274) Cm (5432:5435-5445:5453)
Scan EI+
6.15e6
Mass spectra of the peak at 39.27min
m/z


bracken 5/10 kilburn SCCO2 extract , 22-May-2010 + 10:09:30
lzbsc510k 5647 (40.697) Cm (5641:5652-5663:5683)
Scan EI+
5.52e6
Mass spectra of the peak at 40.70min
m/z


bracken 5/10 kilburn SCCO2 extract , 22-May-2010 + 10:09:30
lzbsc510k 6252 (44.739) Cm (6244:6261-6265:6413)
Scan EI+
2.22e6
Mass spectra of the peak at 42.47min
m/z

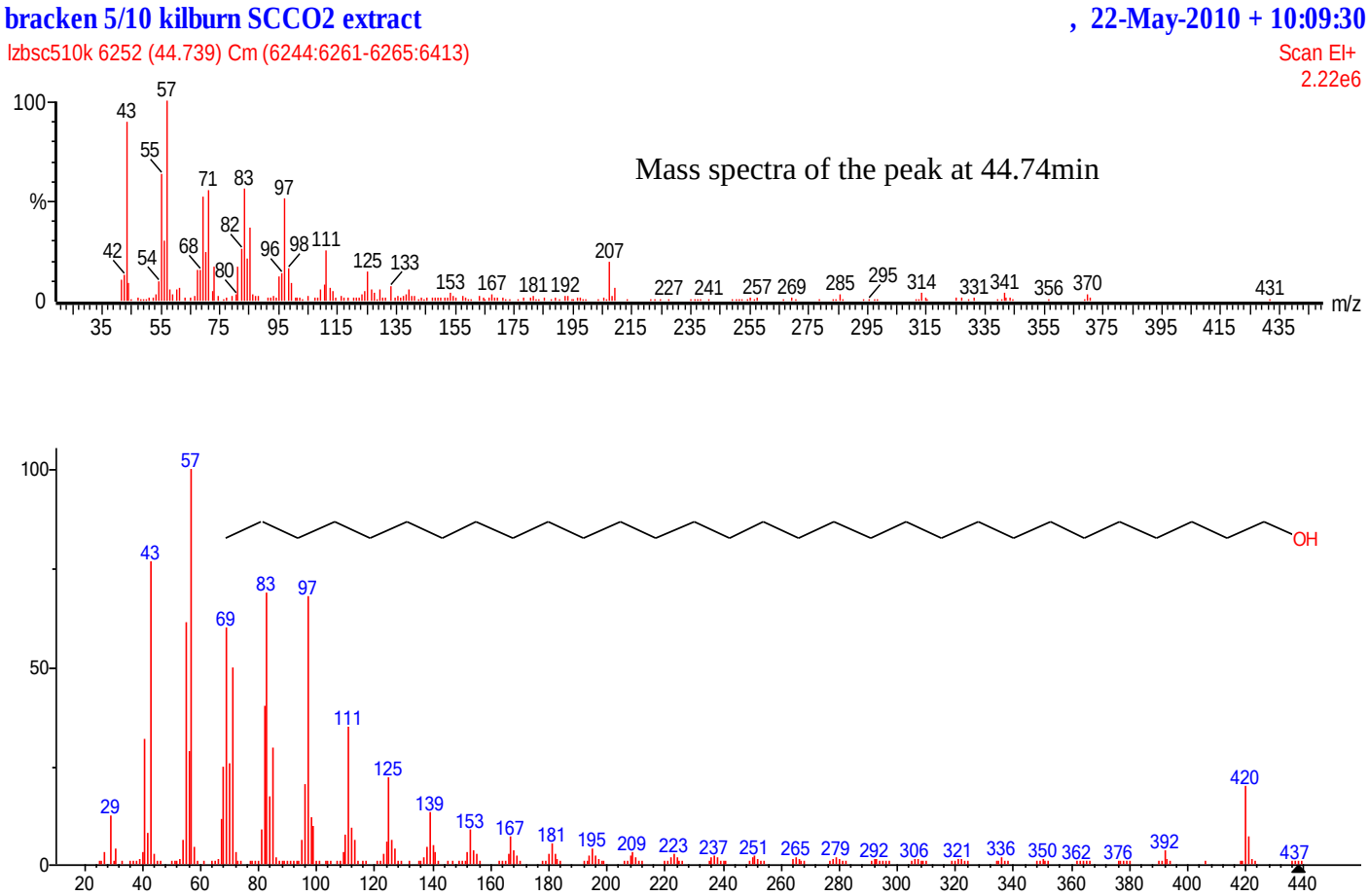



Figure 5.11Mass spectra of long chain alcohol peaks in supercritical $CO_2$ extract and 1-triacontanol standard

Table 5.2 shows the published or estimated Kovats index of standard alcohols and KI value calculated for the peak series. Estimated standard KI value was calculated based on the standard KI data of heptacosanol and triacontanol published by Nist Mass Spectrometry Data Centre. The Kovats index of each peak shows that they are separated by two $CH_2$ unit (200 Kovats units), and all the peaks have a difference within 32 Kovats unit with their corresponding standard. The variance between calculated KI and estimated standard KI may due to the difference of temperature conditions applied in gas chromatography programs and some of this variance can be explained by the transition from temperature programmed conditions to isothermal at this part of the chromatogram. The peaks are identified to be 1-hentetracontanol, 1-tritetracontanol, 1-pentatetracontanol and 1-heptatetracontanol. The presence of longer chain alcohols in the supercritical extract indicates that the extracting ability of $scCO_2$ is substantially higher than liquid $CO_2$. With higher temperature and pressure, supercritical $CO_2$ is able to extract molecules with greater polarity and higher molecular weight. This result indicates that the selectivity of $CO_2$ is dependent on operating conditions.

Table 5.2 Calculated KI value of alcohol peak series
and corresponding standard KI value

| Retention time | Possible compound | Molecular formula and weight | KI value calculated | Estimated standard KI value |
|---|---|---|---|---|
| 39.27min | 1-hentetracontanol | $C_{41}H_{84}O$ MW 592 | 4374 | 4342 |
| 40.70min | 1-tritetracontanol | $C_{43}H_{88}O$ MW 620 | 4535 | 4542 |
| 42.47min | 1-pentatetracontanol | $C_{45}H_{92}O$ MW 648 | 4727 | 4742 |
| 44.74min | 1-heptatetracontanol | $C_{47}H_{96}O$ MW 676 | 4970 | 4942 |

### 5.2.3 Supercritical carbon dioxide extraction of bracken crozier with ethanol entrainer

Bracken crozier was extracted using supercritical $CO_2$ and ethanol entrainer under conditions of 50 $^oC$, 350 bar for 4 hours and a $CO_2$ flow-rate of 40 g/min. Entrainer flow rate was controlled at 10% of $CO_2$ flow rate i.e. 4 ml/min. The extract was washed out of the separator using dichloromethane and then concentrated by evaporating the solvent using a rotary evaporator. A dark green waxy extract was obtained with a crude yield of 0.84%. A sample was made up in ethanol at a concentration of 20 mg/ml of crude extract for GC-MS analysis.

Figure 5.12 shows the gas chromatogram of bracken crozier extracted by supercritical $CO_2$ with ethanol entrainer. Basically, the pattern is very similar to that of liquid $CO_2$ extract and supercritical $CO_2$ extract. Major peaks like pterosin B and β-sitosterol still exist in the extract. However, another important compound pterosin F does not appear in this extract anymore and this may due to it having been extracted completely in previous extractions. The quantity of β-sitosterol in this extract was calculated as 702 μg/g dry plant and 187 μg/g fresh plant. Several new peaks appear in this extract due to the greater polarity of sc$CO_2$ with ethanol entrainer. Some additional branched unsaturated alcohols related to phytol were detected in this extract, and further sitosterol derivatives also eluted immediately prior to the β-sitosterol peak.

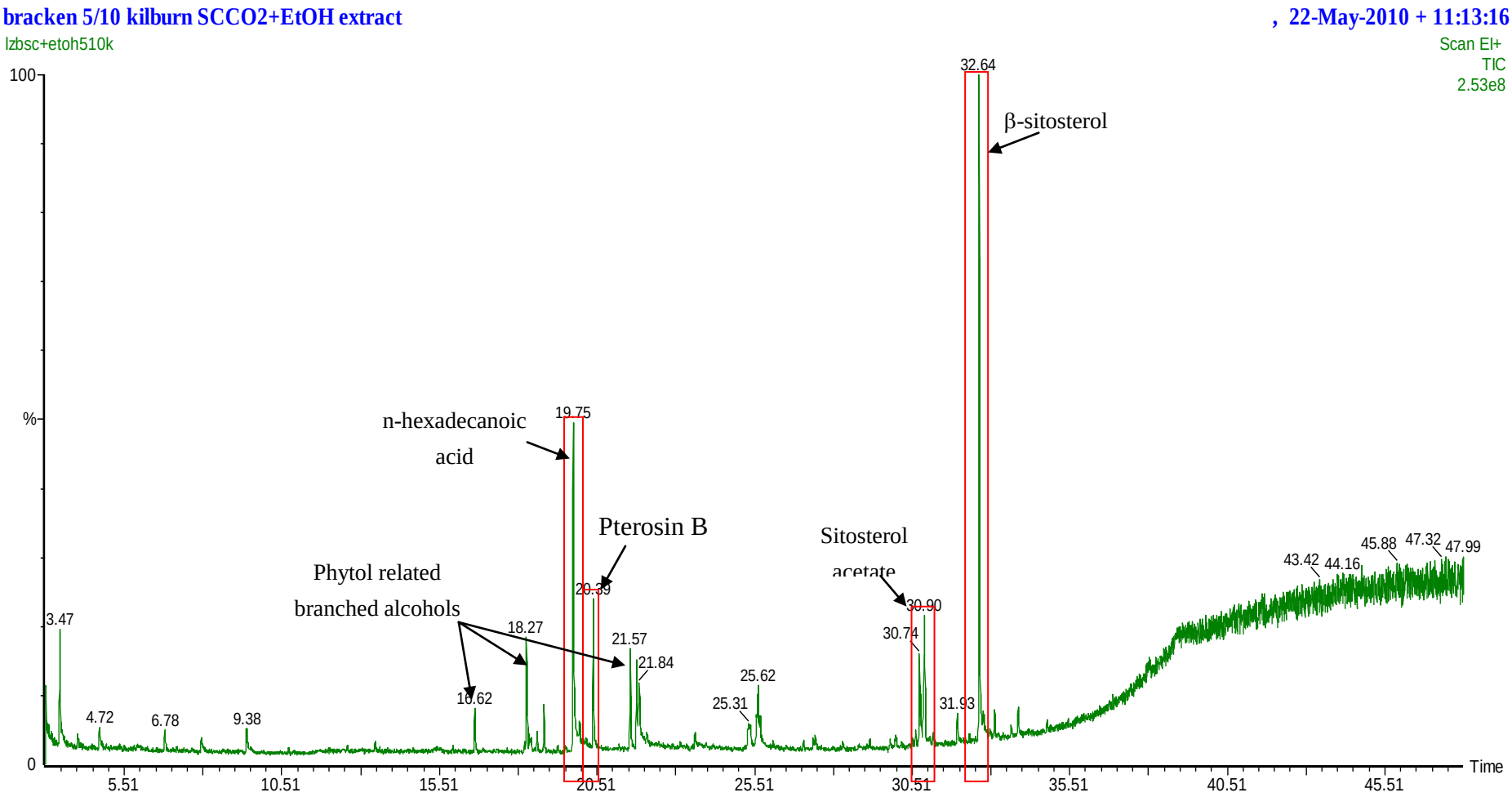



Figure 5.12 Gas chromatogram of bracken crozier supercritical $CO_2$ with ethanol extract

When comparing the gas chromatogram of all three bracken crozier extracts, it seems clear that pterosin B has higher abundance compared to pterosin F. Pterosin B afforded greater peak areas than pterosin F in every chromatogram. Moreover, pterosin B presents consistently throughout the extracts, meanwhile pterosin F does not appear in the last extract anymore. Due to the scarcity of pterosin standards, quantification of pterosin B and pterosin F was not carried out, thus the comparison of their quantity in bracken crozier cannot be achieved precisely. However, a seasonal variation of pterosins in different life stage samples was demonstrated by relative level comparison with an internal standard and this is described in a later chapter.

## 5.3 Supercritical carbon dioxide extraction of bracken at different growth stages

### 5.3.1 Supercritical $CO_2$ extractions of bracken samples at different growth stages

Bracken samples harvested in May 09, July 09 and October 09 were air dried and extracted by supercritical carbon dioxide under a condition of 35 MPa and 50 $^{o}$C for four hours. After the extraction, the extract was recovered from the separator by washing out with dichloromethane then concentrated by removing the solvent using a rotary evaporator. Light green waxy extracts were obtained with crude yields varies from 1.31% to 1.84% dry plant and 0.39% to 1.02% in fresh plant. Samples of each extract were made up at a concentration of 20 mg/ml for GC-MS analysis.

Table 5.3 lists the extracted material and yield of each extraction. The figures clearly stated that the total yield in both dry plant and fresh plant varies in a fairly wide range with the seasons. The seasonal variation of bracken extraction yield will be discussed in more detail in a later chapter.

Table 5.3 The extract yields of supercritical carbon dioxide extractions.

| Material | Harvest date | Extraction conditions | Weight of extract | Yield (dry plant) | Yield (fresh plant) |
|---|---|---|---|---|---|
| Dry bracken whole plant 90g | May 2009 | 35 MPa, 50 $^{o}$C, 4 hrs | 1.18 g | 1.31% | 0.39% |
| Dry bracken whole plant 70g | July 2009 | 35 MPa, 50 $^{o}$C, 4 hrs | 1.14 g | 1.63% | 0.76% |
| Dry bracken whole plant 70g | October 2009 | 35 MPa, 50 $^{o}$C, 4 hrs | 1.29 g | 1.84% | 1.02% |

Figure 5.13 shows the gas chromatogram of bracken supercritical $CO_2$ extract in different life stages. The pattern of the May 09 sample is almost the same with that of bracken crozier. Most interesting peaks such as pterosin B, pterosin F and β-sitosterol all still present in this extract. As was observed in the crozier supercritical $CO_2$ extract, pterosin B is present at a fairly high concentration and afforded a peak of the greatest absolute area in this extract. Pterosin F also exhibited a relative high content in May 09 bracken, its peak area achieved approximately 30% of that of pterosin B. β-sitosterol is also one of the major peaks in this extract, its quantity was calculated as 1074 μg/g dry plant and 318 μg/g fresh plant by using stigmasterol as standard.

The chromatogram of July bracken sample is quite different from earlier ones. As the season changes, the chemical composition in the plant varies significantly. The most interesting change is in this fully matured frond, pterosin B and pterosin F exist at very low concentration compared to younger frond. The peak area of pterosin B in this mature bracken is only about 3% of that in May sample when the GC samples are made in the same concentration. Meanwhile, pterosin F peak is not even detectable in this extract. Moreover, when looking at the chromatogram of October sample, both pterosin B and pterosin F have disappeared. No peak within the time range of 19.87 min to 21.68 min is detected in this senescing frond. Apparently, there is a clear seasonal dependent reduction of pterosin compounds in bracken plant. The seasonal variation of pterosins in bracken will be discussed in detail in later chapter.

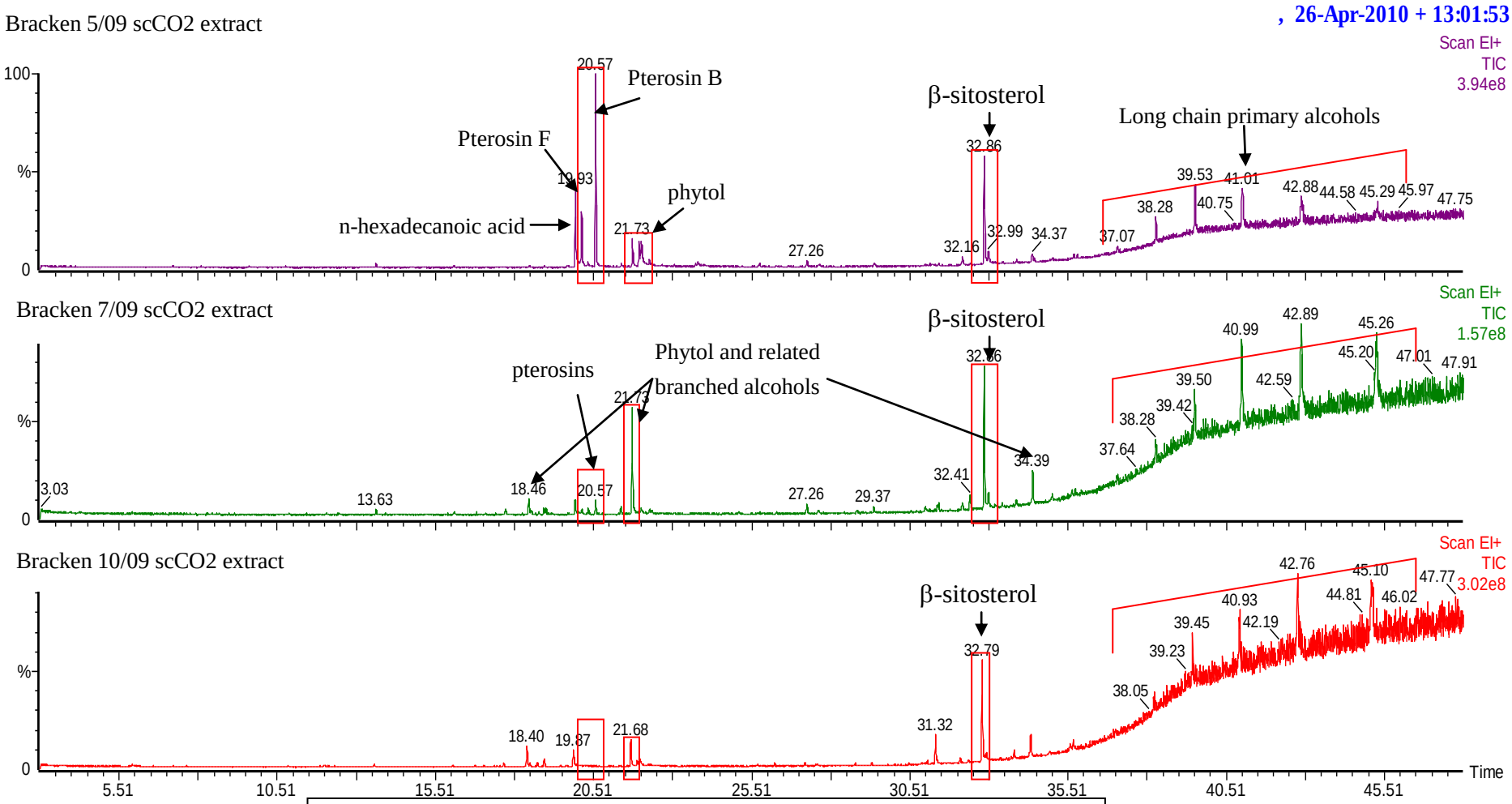



Figure 5.13: Gas chromatogram of bracken supercritical $CO_2$ extracts

In the chromatogram of July sample, phytol and other two related branched unsaturated alcohols with retention time of 18.48 min, 21.73 min and 34.39 min become noticeable. Particularly, phytol peak afforded the second greatest absolute area in this chromatogram. Meanwhile, β-sitosterol still presented as a major peak. Its quantity was calculated as 660 μg/g in dry plant and 309 μg/g in fresh plant. Apart from this, C37-C47 long chain primary alcohols are also more prominent in this extract. Their spectra and Kovats index values are all identical with standard and previous calculation.

As mentioned before, pterosin B and pterosin F no longer appear in October bracken extract. However, other major peaks such as β-sitosterol and long chain alcohols still exist in this extract. β-sitosterol was found to have a content of 1132 μg/g dry plant and 625 μg/g fresh plant in this over-matured frond.

To conclude, β-sitosterol and C37-C47 long chain primary alcohols present persistently in bracken supercritical $CO_2$ extract at every growth stage. The seasonal variation trend of β-sitosterol in both dry plant and fresh plant will be discussed in detail later. The amount of Pterosin B and pterosin F in bracken plant seems to decrease considerably as the season progresses. The supercritical $CO_2$ extract of younger frond (May10 and May 09 samples) contain relatively higher level of pterosin than mature frond (July 09 sample) and senescing fronds no longer contain any pterosin compounds.

### 5.3.2 Supercritical $CO_2$ extractions of bracken with ethanol entrainer at different growth stages

Bracken samples harvested in May 09, July 09 and October 09 were air dried as before and extracted using supercritical carbon dioxide under a condition of 35 MPa and 50 $^{o}$C for four hours. The residue from this extraction was then extracted using supercritical carbon dioxide at 35 MPa and 50 $^{o}$C with the addition of ethanol as an

entrainer. The co-solvent flow rate was set to 4 ml/min to make it 10% of the flow rate of $CO_2$. After the extractions, the extract were recovered from the separator by washing out with dichloromethane then concentrated by removing the solvent using a rotary evaporator. Dark green waxy extract were obtained with crude yields varies from 0.74% to 0.90% dry plant and 0.27% to 0.47% in fresh plant. Samples of each extract were made up at a concentration of 20 mg/ml for GC-MS analysis.

Table 5.4 The extract yields of supercritical carbon dioxide extractions with ethanol entrainer

| Material | Harvest date | Extracting conditions | Weight of extract | Yield (dry plant) | Yield (fresh plant) |
|---|---|---|---|---|---|
| Dry bracken whole plant 90 g | May 2009 | 35 MPa, 50 $^{o}$C, 4 hrs EtOH 4 ml/min | 0.82 g | 0.90% | 0.27% |
| Dry bracken whole plant 70 g | July 2009 | 35 MPa, 50 $^{o}$C, 4 hrs EtOH 4 ml/min | 0.52 g | 0.74% | 0.35% |
| Dry bracken whole plant 70 g | October 2009 | 35 MPa, 50 $^{o}$C, 4 hrs EtOH 4 ml/min | 0.60 g | 0.86% | 0.47% |

Table 5.4 shows the extracted material and yield of each extraction with ethanol. Compared to the yields of supercritical $CO_2$ extract, the yields of extractions with ethanol entrainer fluctuate within a relative limited range in both dry plant and fresh plant. As previously stated, the seasonal variation of bracken extraction yield will be discussed in more detail in a later chapter.

Figure 5.14 shows the gas chromatogram of each supercritical and EtOH extract with the composition highlighted. The pattern of May 09 bracken sample is very similar to that of crozier extract. Almost every peak that is present in crozier supercritical $CO_2$

ethanol extract still exists in this more mature frond. Pterosin B remains at a high concentration is this extract and afforded a major peak with the second greatest peak area. Pterosin F peak in this extract is very weak compared to pterosin B, this may due to the majority of this compound has been extracted out during previous supercritical $CO_2$ extraction. β-sitosterol is still one of the major peaks in this extract; its quantity was calculated as 509 μg/g dry plant and 153 μg/g fresh plant. As seen with the crozier extract, n-hexadecanoic acid and phytol with related branched alcohols were still found in this May 09 sample. Long chain primary alcohols were no longer detectable in this extract possibly because they were fully extracted in previous supercritical $CO_2$ extraction.

The chromatogram of July 09 and October 09 samples are similar in composition. Pterosin B and pterosin F were non-existent in these mature and over-mature fronds and instead of β-sitosterol, phytol and related branched unsaturated alcohols become the major peaks in these extracts. The quantity of β-sitosterol was calculated as 118 μg/g dry plant and 55 μg/g fresh plant in fully matured frond, and 181 μg/g dry plant and 100 μg/g fresh plant in senescing frond. C45 and C47 long chain primary alcohols also present in this extract. However, their peaks are very weak and barely visible in the chromatogram.

In conclusion, in supercritical $CO_2$ with entrainer extracts, pterosin compounds also exhibited a clear reduction with the seasons. Pterosin B and pterosin F disappeared in the frond when it is growing towards its maturity. β-sitosterol consecutively exists in bracken frond in every life stage. Its quantity was all recorded and its variation tendency will be discussed in later chapter.

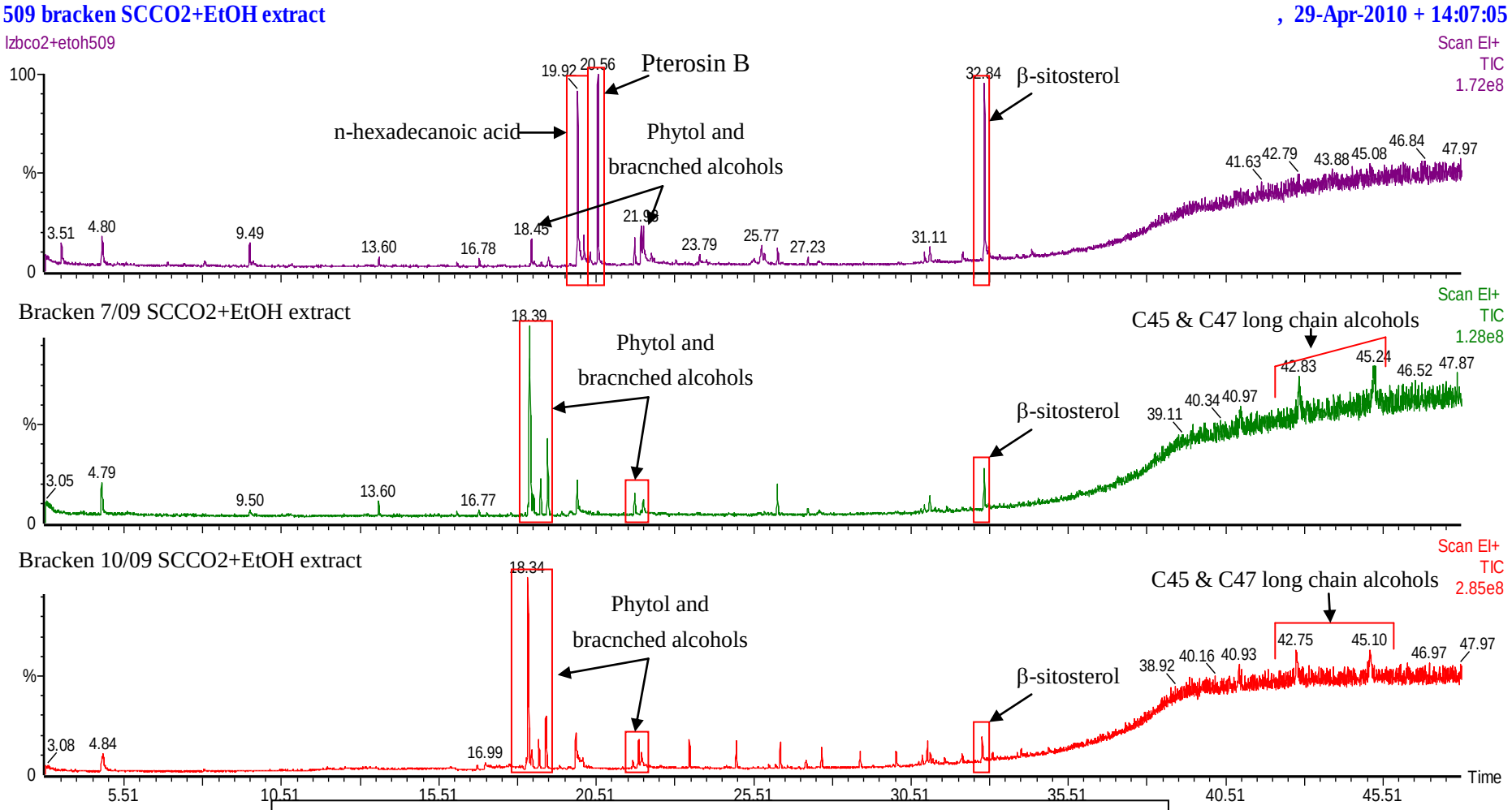



Figure 5.14: Gas chromatogram of bracken supercritical $CO_2$ with EtOH extracts

## 5.4 Investigation of pterosin precursor in lady fern (*Athyrium filix-femina*) by using supercritical fluid chromatography

A sample of Lady Fern at crozier stage was harvested in April 2009 from Kilburn White Horse (Sutton Bank, Grid reference SE 514 813) and was misidentified as bracken fern. This lady fern sample was dried and pre-treated using the same method as bracken fern. However, when the lady fern ethanol extract (extracted using method in chapter 7.4.3) was analysed by GC-MS, an interesting peak was discovered and was worthy of further studies in detail.

Figure 5.15 shows the chromatogram of lady fern ethanol extract. A peak with retention time of 10.08 min was identified as 2,3-dihydrobenzofuran by matching its mass spectra with NIST library standard spectra which has a very similar chemical structure with indanone. Besides, the peak at 22.00 min was guessed to be methyl lathodoratin which has a similar structure with pterosin B. Moreover, the characteristic ions of methyl lathodoratin were found to be m/z 220, 205 which is very similar to that of pterosin B (m/z 218, 203). Therefore, there is a conjecture that although lady fern is not carcinogenic, there might be some form of ptaquiloside related precursor present in lady fern and afforded this indanone-like metabolite. To have a better understanding of the chemical similarity between lady fern and bracken fern, supercritical fluid chromatography was employed due to its rapid analysis and lower detection limit in ethanol extract compared to GC-MS.

Lady fern and bracken fern crozier (picked in May 2010) were extracted by ethanol using the method described in section 7.4.3. The crude ethanol extracts were analysed by supercritical fluid chromatograph directly using the method stated in 7.4.4. Figure 5.16 shows the supercritical chromatogram of bracken fern overlaid with lady fern and with some mutual peaks and characteristic peak highlighted. The major peak in bracken ethanol extract presented at 2.8 min and was postulated to be pterosin B since

it was the major peak in corresponding GC-MS sample. Two mutual peaks are also observed at 5.0 min and 6.3 min.

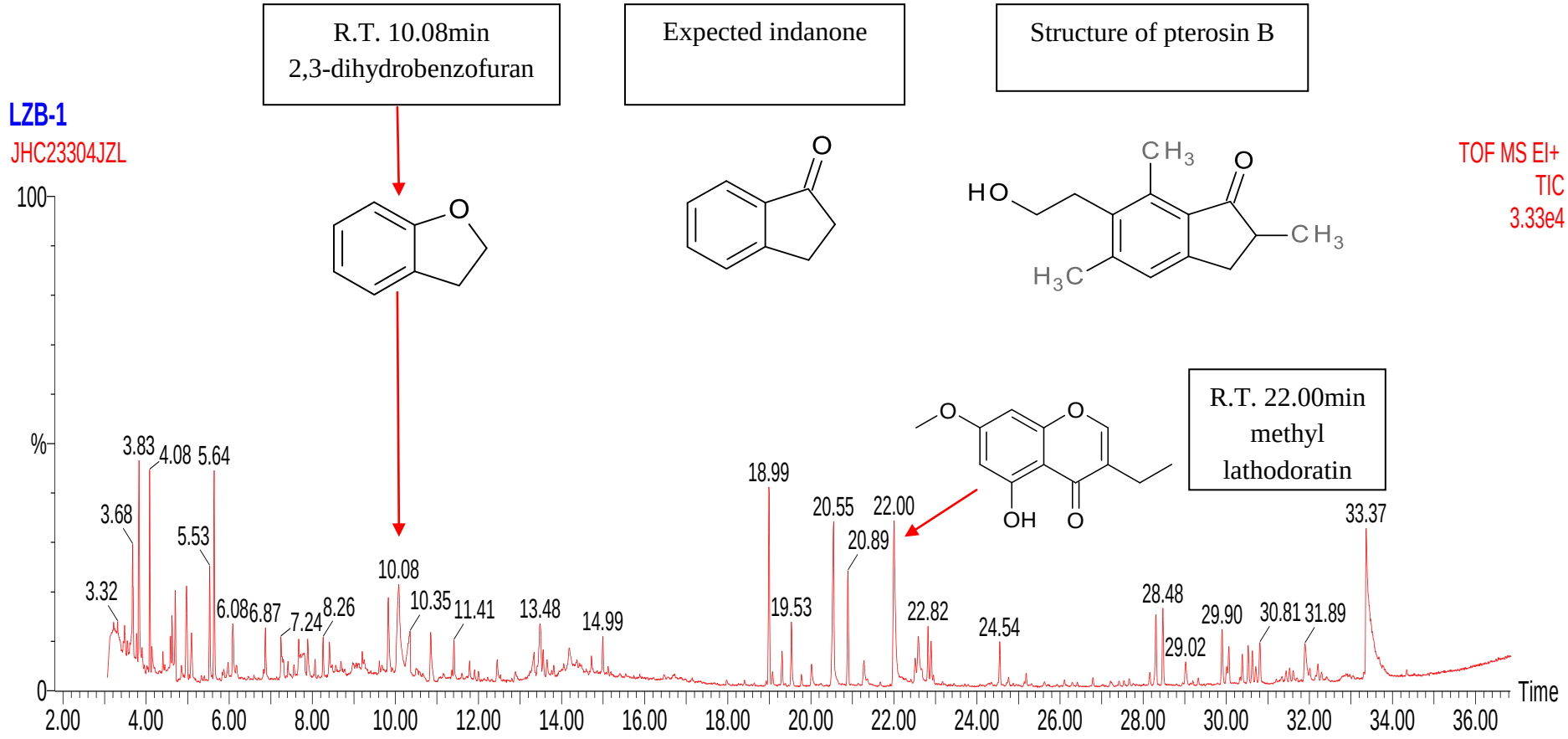



Figure 5.15: Gas chromatogram of lady fern ethanol extract

At the moment, standard pterosin B is obtained by converting standard ptaquiloside in alkaline and acidic conditions. Standard ptaquiloside itself was first extracted by water from bracken fern, and then its isolation was accomplished by a series of extreme complicated purification process. Since one of the major advantage of preparative SFC is it combines rapid analysis with precise fractionation, thus it may be able to make pterosin B isolation much more efficient and simple.

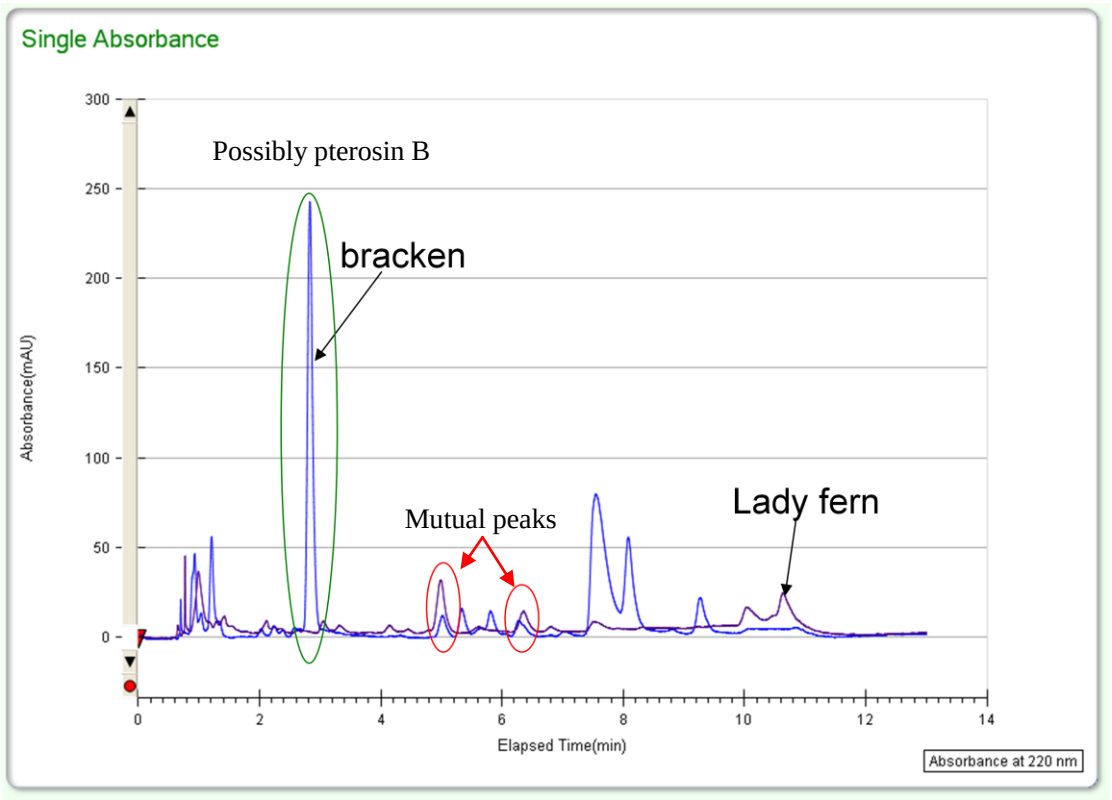
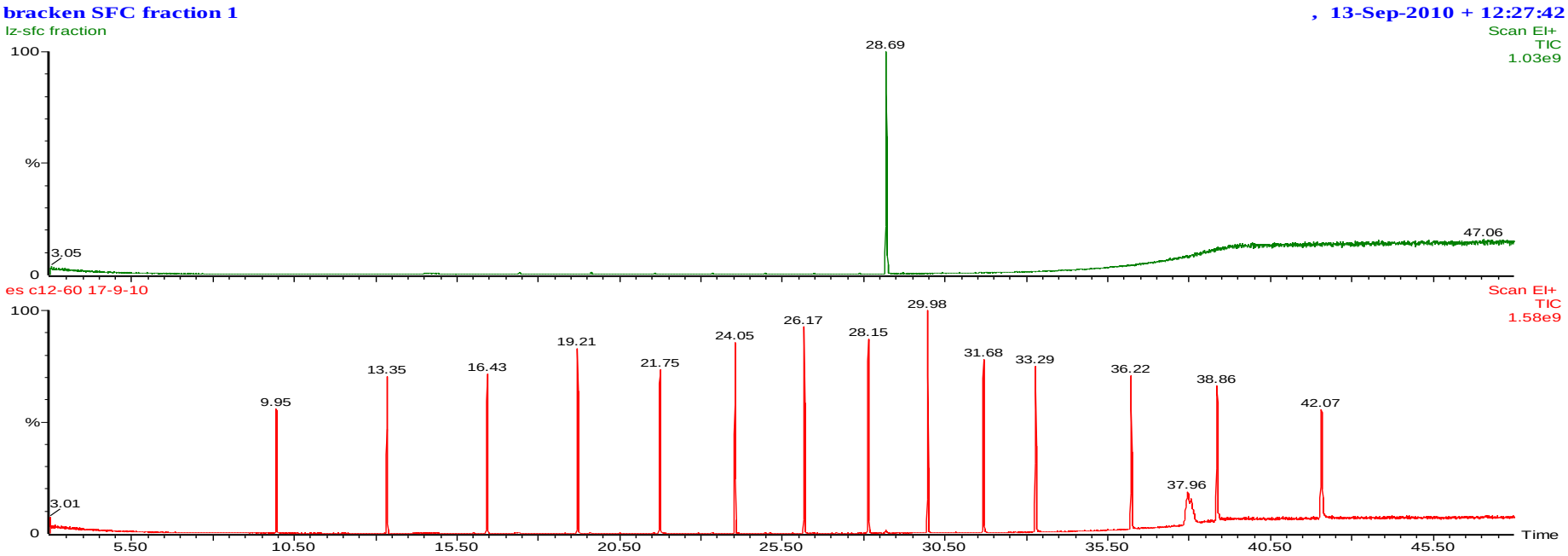



Figure 5.16: Supercritical chromatogram of bracken fern and lady fern ethanol extracts

Since there is a high possibility that the peak at 2.8 min in bracken extract is pterosin B, the fractionation technique was applied to isolate this peak so that it could be used as pterosin B standard in future research. The fractionation was accomplished by Waters Investigator SFC system and a fraction between 2.6 min to 3.0 min was collected. A light green compound was obtained and was analysed by GC-MS for identification.

Figure 5.17 shows the gas chromatogram and mass spectra of the peak at 2.8 min in bracken extract and the retention time comparison with C12-60 hydrocarbon standard. The mass spectra of this compound indicates clearly that it is an alkane, and after comparing its retention time with C12-60 hydrocarbon standard, the number of carbon was confirmed to be 27 since the peak falls between hexacosane and octacosane. Therefore, the major peak of bracken ethanol extract observed in supercritical chromatogram was identified to be heptacosane rather than pterosin B.

Because the Waters Investigator SFC system was only available from one month before this project finished, many further investigations on bracken and lady fern mutual peaks and further pterosin B isolation were not accomplished because of time constraints. Although the pterosin B searching in bracken fern ethanol extract wasn't achieved, the success of fractionation demonstrate the technique itself could be a powerful technology for target compound purification and isolation. Further research is required to reveal the chemical similarity between lady fern and bracken fern, also further work in pterosin B fractionation by preparative SFC is needed as well.



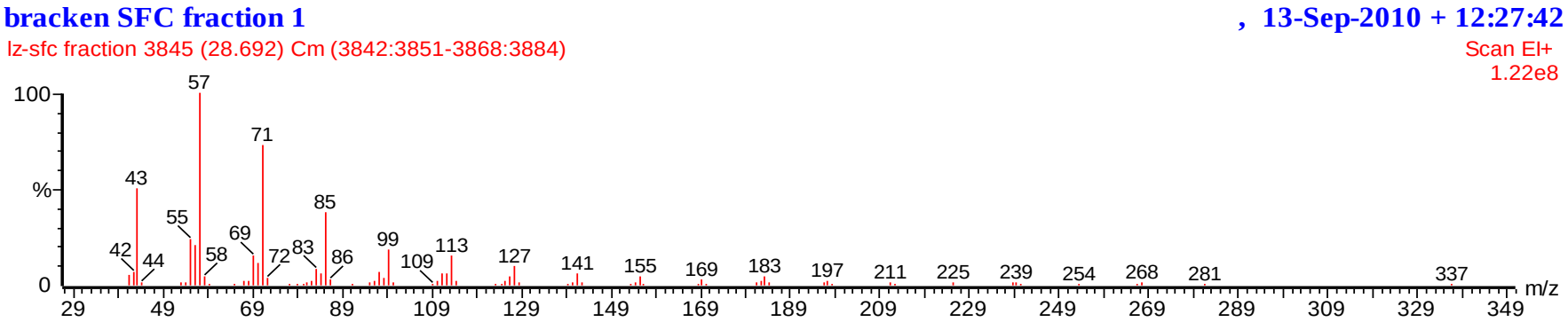




Figure 5.17 Gas chromatogram and mass spectra of the fraction and gas chromatogram of C12-60 alkane standard

## 5.5 Conclusion and further remarks

Bracken crozier (May 2010 sample) was extracted by liquid $CO_2$, supercritical $CO_2$ and supercritical $CO_2$ with ethanol entrainer sequentially. Pterosin B and pterosin F were discovered in bracken liquid $CO_2$ extract and were identified by comparing their mass spectra with published standard spectra. Their existence possibly means that these compounds are present in bracken crozier natually prior to the extraction although the source of pterosin F in bracken frond still needs further investigation. β-sitosterol was also detected in crozier liquid $CO_2$ extract, and its quantity was calculated to be 1217 μg/g dry plant and 324 μg/g fresh plant by using stigmasterol as a standard due to their structure similarity. C37 and C39 long chain primary alcohols are also present in this extract and were identified by comparing their spectra and calculated Kovats index with published standard data.

In bracken crozier supercritical $CO_2$ extract, all these peaks listed are still present with some new peaks appearing due to the change of extracting conditions. Phytol and some related branched unsaturated alcohols were discovered along with more long chain primary alcohols. C37-C47 odd number carbon alcohols were found and this demonstrates that the extracting ability of sc$CO_2$ is significantly higher and it is now able to extract compounds with much higher molecular weight than liquid $CO_2$. β-sitosterol was found to be 754 μg/g dry plant and 201 μg/g fresh plant in this extract.

Pterosin F and all long chain alcohols were absent in bracken supercritical $CO_2$ with ethanol entrainer extract. This may due to these compounds have been completely extracted in previous extractions. However, major peaks such as pterosin B and β-sitosterol were still present in this extract. β-sitosterol has a concentration of 702 μg/g dry plant and 187 μg/g fresh plant in this extract.

Bracken fronds in three more mature life stages were also extracted by supercritical $CO_2$ and supercritical $CO_2$ with ethanol entrainer. The reduction of pterosin compounds with seasons is very obvious. Pterosin B and pterosin F are present in May 09 frond as major peaks. When it gets fully matured in July, pterosin B is barely detectable and pterosin F is totally non-existent. In October senescing bracken, both pterosin B and pterosin F have disappeared. The changing tendency of pterosins in bracken was discussed in detail in a later chapter.

β-sitosterol presents consistently in bracken of every life stage. It was found to be 1074 μg/g dry plant and 318 μg/g fresh plant in May 09 sample, 660 μg/g dry plant and 309 μg/g fresh plant in July 09 sample and 1132 μg/g dry plant and 625 μg/g fresh plant in over-matured October sample. Besides this, C37-C47 long chain primary alcohols also present persistently in bracken supercritical $CO_2$ extract of every life stage. The seasonal variation of β-sitosterol will be discussed in later chapters.

In bracken supercritical $CO_2$ with ethanol entrainer extracts, pterosin B is present with a high peak area and is the major peak in May pre-mature frond. However, compared with pterosin B, pterosin F peak is very weak in this extract. As season changes, both pterosins disappear as the bracken grows towards its maturity. A further discussion on seasonal variation of pterosins will be brought out in a later chapter.

In contrast, phytol and related branched alcohols become noticeable in fully matured bracken and afford major peaks in the July and October bracken extract. Two largest long chain alcohols (C45 and C47) were also found in July and October bracken. β-sitosterol exists persistently in bracken supercritical $CO_2$ with entrainer extracts. It was found to be 509 μg/g dry plant and 153 μg/g fresh plant in May sample, 118 μg/g dry plant and 55 μg/g fresh plant in July frond, and 181 μg/g dry plant and 100 μg/g fresh plant in senescing October frond. Its seasonal variation trend will be discussed in detail in later chapter.

In bracken crozier sequential carbon dioxide extractions, the raw material has been extracted by liquid $CO_2$, supercritical $CO_2$ and supercritical $CO_2$ with ethanol entrainer continuously for 14 hours. Pterosin B was found in the extracts obtained in every step and pterosin F was found in the first two extracts. The reason for their consistent existence may be their insufficient solubility in liquid $CO_2$ and supercritical $CO_2$. Since pterosin B and F do not have enough solubility in liquid $CO_2$, after 6 hours liquid $CO_2$ extraction, there was still some residual pterosins left in the material. Therefore, in the subsequent supercritical $CO_2$ extraction, more pterosins were extracted due to their enhanced solubility in supercritical $CO_2$. Similarly, pterosin B was still present in supercritical $CO_2$ with entrainer extract due to its insufficient solubility in supercritical $CO_2$, when pterosin F is completely extracted in the second step. Therefore, it is clear that compared to liquid $CO_2$, supercritical $CO_2$ is preferable to give a maximum extracting ability and achieve higher yield despite a reduction in selectivity. When considering a full scan of plant chemical composition, supercritical $CO_2$ extraction would be a better choice than liquid $CO_2$ extraction.

Finally, supercritical fluid chromatography was applied to isolate pterosin B and to study the chemical similarity between lady fern and bracken fern. Two mutual peaks were found in lady fern and bracken fern ethanol extracts. Meanwhile, a characteristic peak of bracken ethanol extract was successfully fractionated by semi-preparative SFC system. However, this fraction was identified as heptacosane by GC-MS. Due to the time constraints, further isolation and identification of mutual peaks and pterosin B was not accomplished. Apparently, if time allows, more study will be carried out to achieve this incomplete research.

# *Seasonal variation of pterosins and other major compounds in bracken*

# Chapter 6

## 6.1 Seasonal variation in ptaquiloside derivatives pterosins and other major compounds

Bracken fern (*Pteridium aquilinum*) is a widely distributed species that dominates the vegetation of large areas in many UK upland regions. Bracken is a perennial fern, it's also the only higher plant known to cause cancer naturally in animals. The content of the major toxin and carcinogen ptaquiloside varies widely throughout the seasons. It was reported that the carcinogenicity of young bracken shoots was shown to be stronger than that of mature plants.[6] During bracken's life cycle, the crozier (Figure 6.1) contains the highest concentration of ptaquiloside. This is due to its self-protecting mechanism against herbivores.[90]

Figure 6.1: The crozier step in bracken vegetation process

It is reported that the concentration of ptaquiloside in the frond decreases over time.[91] When the crozier start to stretch and generate the first pair of leaves (Figure 6.2), the ptaquiloside is still present at fairly high concentration. As the growth cycle

progresses, ptaquiloside starts to reduce dramatically and end up with a low level in mature (Figure 6.3) and dying plants (Figure 6.4).

Figure 6.2: 2nd step in bracken's life cycle-with first pair of leaves

Figure 6.3: 3rd step in bracken's life cycle-mature plant

Figure 6.4: 4th step in bracken's life cycle-dying

Therefore, samples in different bracken life cycle steps are picked from the same site (Kilburn White Horse, Sutton Bank, Grid reference SE 514 813) in different months of a year. The first crozier sample was picked in 17th April 2009 and 19th May 2010,

followed by the 2nd step sample picked in 21st May 2009, 3rd sample picked in 27th July 2009 and 4th sample picked in 12th October 2009. Unfortunately, the sample picked in April 2009 was misidentified and found to be lady fern rather than bracken fern, all the data and information for bracken crozier was obtained from May 2010 sample. In this chapter, seasonal variation of pterosins and some other compounds in bracken will be examined and will demonstrate a clear trend of concentration of ptaquiloside related compounds.

## 6.2 Water content variation of bracken

Water content of bracken fern was examined in samples at different life cycle steps from April to October 2009 (Figure 6.5). The highest water content was found to be 73.40% by total weight in bracken crozier sample (05/2010). When the plant has the first pair of leaves, the water content dropped slightly to 70.40% but this trend accelerated as the plant becomes more mature. It decreased sharply by almost 20% within 2 months and reached a figure of 53.20% in July 2009. Finally, the decrease in water content continued until it reached 44.80% in over-matured frond.

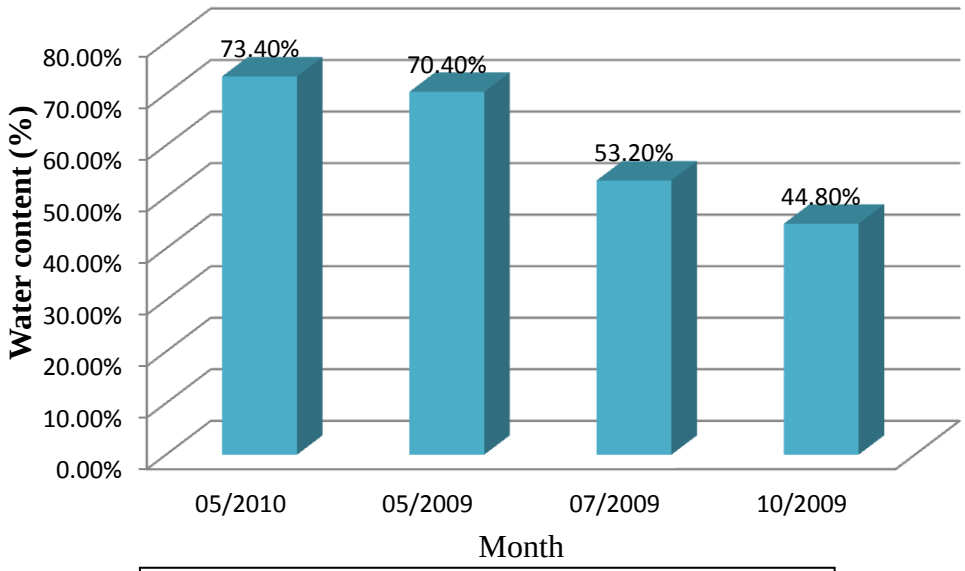



Figure 6.5: Seasonal variation in water content of bracken fern

## 6.3 Variation in total extract yield of bracken over a life cycle

Extract yields of supercritical $CO_2$ extractions with and without ethanol entrainer are listed below (Table 6.1). Dry bracken samples were extracted using supercritical $CO_2$ at 350 MPa and 50 $^oC$ for 4hrs on the first day, and were then extracted with the addition of ethanol as a co-solvent at 10% of the $CO_2$ flow under the same conditions on the following day. Two yields were recorded respectively and a total yield was made up by adding these two together. Figure 6.6 shows the trend in yield change throughout the year.

Table 6.1 Extract yield of supercritical carbon dioxide extractions

| | Material | Extracting conditions | Yield | Total yield |
|---|---|---|---|---|
| 1 | Dry bracken 05/10 whole plant 90g | 35 MPa, 50 $^oC$, 4 hrs | 1.42% | 2.19% |
| | Dry bracken 05/10 whole plant 90g | 35 MPa, 50 $^oC$, 4 hrs + EtOH | 0.77% | |
| 2 | Dry bracken 05/09 whole plant 90g | 35 MPa, 50 $^oC$, 4 hrs | 1.31% | 2.22% |
| | Dry bracken 05/09 whole plant 90g | 35 MPa, 50 $^oC$, 4 hrs + EtOH | 0.90% | |
| 3 | Dry bracken 07/09 whole plant 70g | 35 MPa, 50 $^oC$, 4 hrs | 1.63% | 2.37% |
| | Dry bracken 07/09 whole plant 70g | 35 MPa, 50 $^oC$, 4 hrs + EtOH | 0.74% | |
| 4 | Dry bracken 10/09 whole plant 70g | 35 MPa, 50 $^oC$, 4 hrs | 1.84% | 2.70% |
| | Dry bracken 10/09 whole plant 70g | 35 MPa, 50 $^oC$, 4 hrs + EtOH | 0.86% | |

The total yield was found to vary from 2.19% to 2.70%. The highest supercritical $CO_2$ extract yield and total yield were both obtained in October 2009, with a figure of 1.84%

and 2.70% respectively. However, the highest $CO_2$ with entrainer extract yield was obtained in May 2009 with a level of 0.90%. The supercritical $CO_2$ and total yield from dried bracken increase over time, until they reach a peak in the senescing plant. However, the yields of $CO_2$ with entrainer remained stable over the season, with some fluctuation within very limited range.

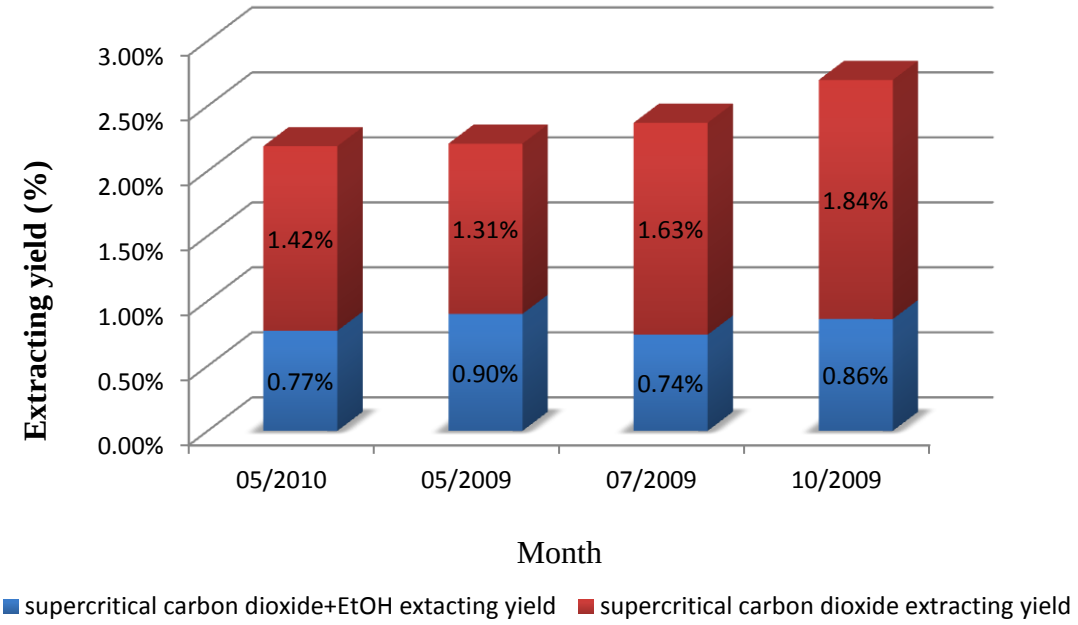



Figure 6.6 Seasonal variation in supercritical extraction yield and total yield of dry plant

Taking into account the water content, the total yield and individual extract yields were also examined in fresh plant material. Figure 6.7 shows the seasonal variation of total yield and different supercritical extract yield from fresh plant material. Overall, the changing tendency of fresh plant extracting yield is exact the same with dry plant. The supercritical $CO_2$ yield increases remarkably over the season and reach the highest amount in October at 1.02%. In contrast, the increasing rate of $CO_2$ with entrainer yield is much milder. The yield is at its lowest point in the crozier extraction at 0.20% and then rises until it reaches a peak in October. The trend of increase of

fresh plant material is even more significant than dry plant. The differences between yields are more obvious when water content is taken into account.

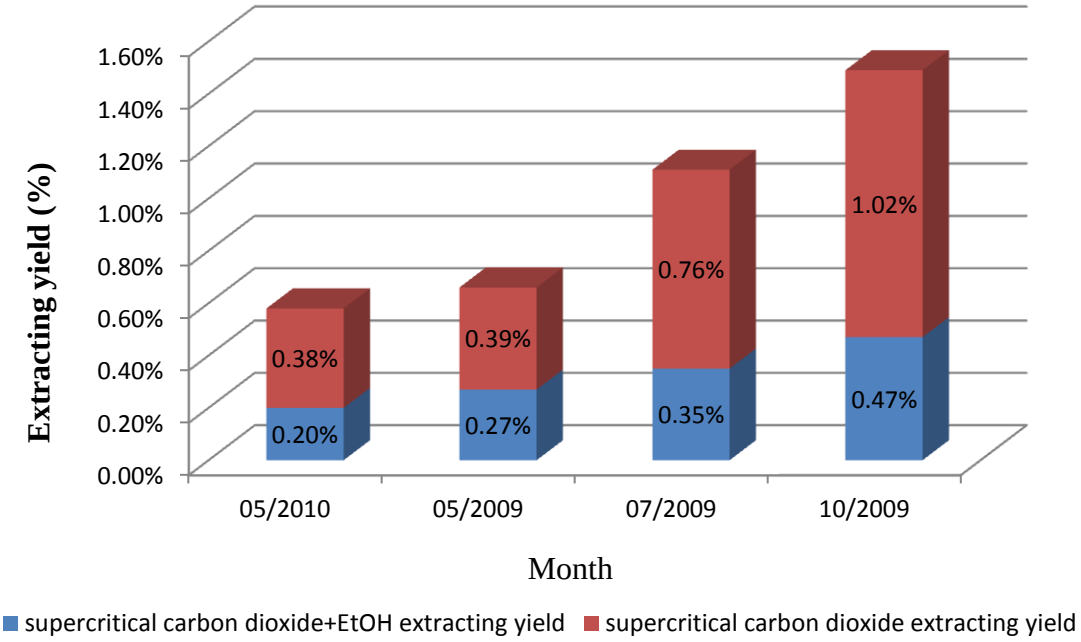



Figure 6.7 Seasonal variation in supercritical extract yield and total yield of fresh bracken

## 6.4 Seasonal variation of pterosins in bracken

As mentioned before, pterosin B and pterosins F are naturally occurring pterosins in bracken supercritical $CO_2$ extract. It was reported before that when the frond approaches towards its maturity, the tender tissues of the crozier become harder and less attractive for herbivore. Therefore compounds that protect the crozier such as ptaquiloside and pterosins will become no longer necessary in the organism at a same high concentration. They are either metabolized or transferred to water or soil etc.[2]

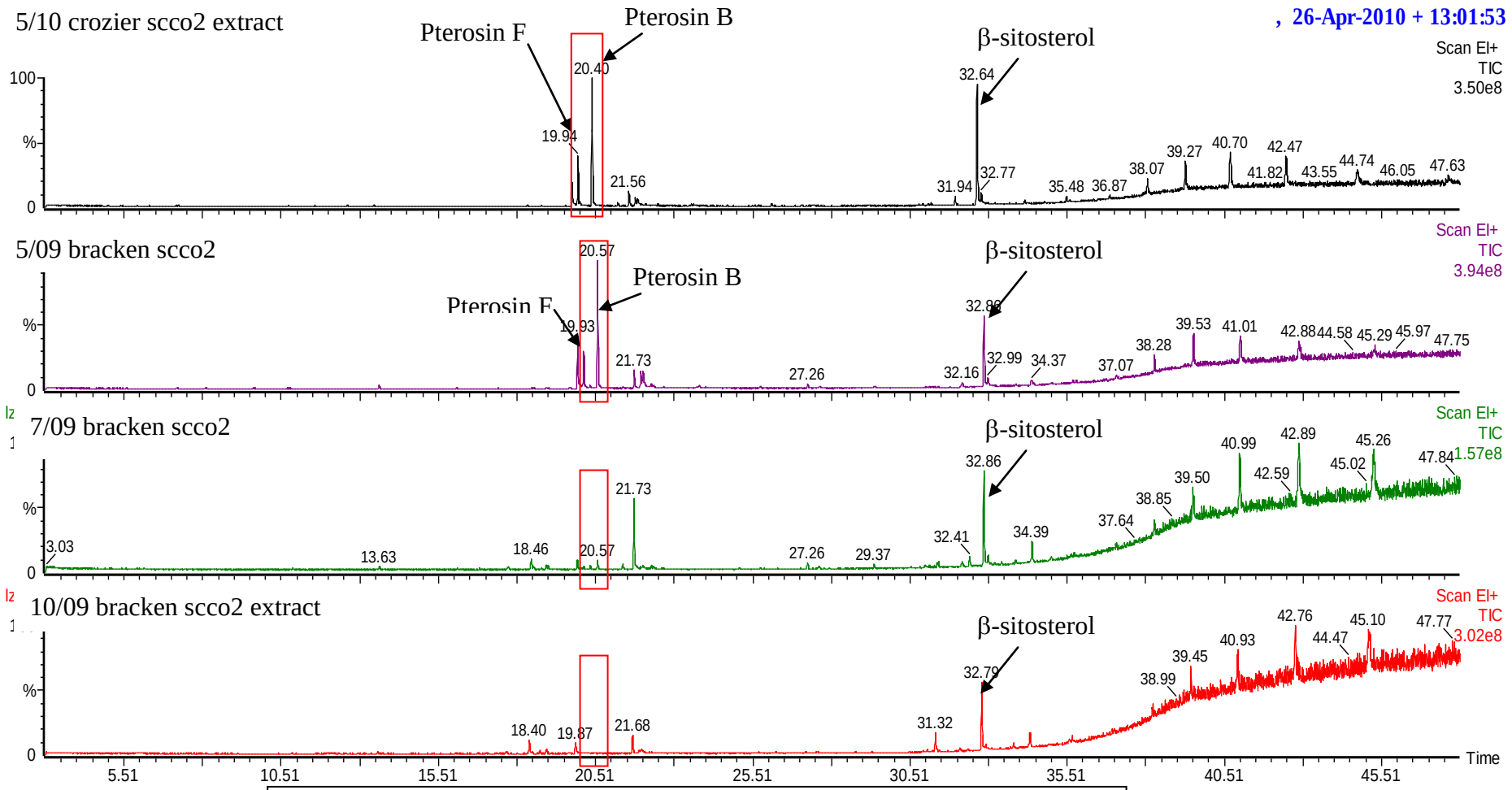



Figure 6.8 Original GC traces of seasonal bracken supercritical $CO_2$ extract

Figure 6.8 shows the original GC traces of bracken supercritical $CO_2$ extract samples. As can see from these traces, in early bracken samples (crozier and May 09 samples), pterosin B is present at a very high concentration as the peak is the major peak in these two traces. Meanwhile, pterosin F is also present at a relatively high concentration and its peak is the third highest peak in the chromatogram. After that, in July sample, pterosin B and pterosin F decreased dramatically and are only present at very low concentration. Finally, as the plant enters senescence in October, pterosin B and pterosin F scarcely exist and are even not able to be detected by GC-MS. It is apparent from these results that young fronds contains the highest concentration of pterosins, then after the frond advance towards mature, pterosins will reduce sharply and finally disappear in fully matured plant. This changing tendency strongly corroborates previews studies and makes the result even more convincing.

Due to lack of pterosin standards, the quantification of pterosins could not be carried out. Therefore, to confirm the GC analysis, a direct comparison of the relative level of pterosins and octadecane internal standard in peak height/area was done. Figure 6.9 shows the GC traces of different bracken samples with octadecane as internal standard at a consistent concentration of 1mg/ml. Since the concentration of octadecane is identical in every single sample, comparing the relative peak height/area between octadecane and pterosins, the trend of seasonal variation will be even more obvious. As can see from the diagram, in crozier sample (trace 1, 5/10), the level of octadecane and pterosin B peaks are almost the same with octadecane slightly higher than pterosin B. Meanwhile, pterosin F peak is about half height of octadecane. Thereafter, in the second sample, peak height of PtB falls to about 2/3 of octadecane and PtF only present at a 1/3 height of octadecane. As the plant becomes more mature, PtB and PtF are only present at very low concentration and barely exist in senescing plant since their peaks are hardly detected by GC. The direction of change based on the relative level comparison of pterosins and octadecane again confirmed the previous conclusions.

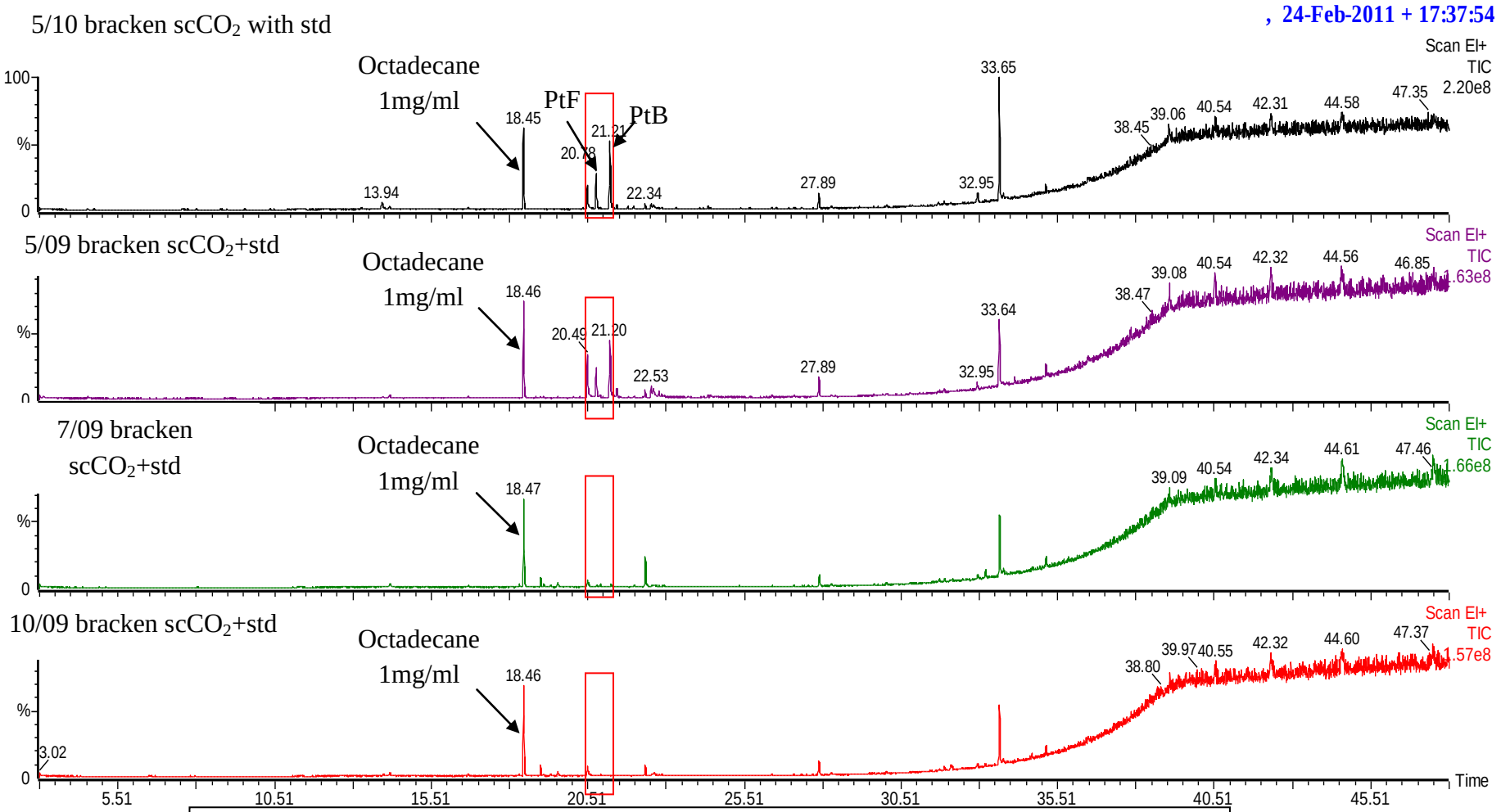



Figure 6.9 GC traces of seasonal bracken supercritical $CO_2$ extract with octadecane standard

Apart from the seasonal variation of pterosins, another interesting phenomenon can be observed from the two figures of GC traces. In figure 6.8, when the samples were freshly extracted, pterosins especially pterosin B used to exist as the highest peak in the extract. Particularly in the second sample, the peak height of pterosin B is approximately twice that of β-sitosterol. That indicated in the early stages of growing, pterosins are always the major compound in the frond. However, when the very same extracts were analysed again nine months later with internal standard by GC at the same initial concentration, the situation changed greatly. The peak height of pterosin peaks falls by 1/3 or even half and are much shorter than β-sitosterol. That means pterosins have degraded drastically during storage. It is well known that ptaquiloside has a very unstable nature. It can readily decompose in both acidic and alkaline conditions. It is generally acknowledged that pterosin B is much more stable compared to ptaquiloside and it is the main final metabolite of ptaquiloside decomposition. This discovery of pterosin B degradation draws attention to stability as an issue, and a further investigation is needed to ascertain the mechanism of this degradation.

## 6.5 Seasonal variation of β-sitosterol

β-sitosterol is one of the main compounds in bracken supercritical $CO_2$ extract. It appears in bracken samples of every growth stage at a high concentration. To the best of my knowledge, the seasonal variation of sitosterol in bracken has never been researched before. Since β-sitosterol possess some impressive biological properties such as blood cholesterol lowering effect, it is necessary to have some further investigation on seasonal variation of it in bracken. Because of the high content of β-sitosterol, bracken could be a potential source of this high value chemical. Therefore quantification of β-sitosterol in bracken is significant.

The quantification of β-sitosterol was carried out with the application of stigmasterol as a standard. Standard solutions of stigmasterol in different concentrations were made up and analysed by GC. The absolute areas were recorded and a standard curve was determined using these area data. Figure 6.10 shows the standard curve of stigmasterol and the regressive equation. A correlative coefficient of 0.994 was obtained from this curve.

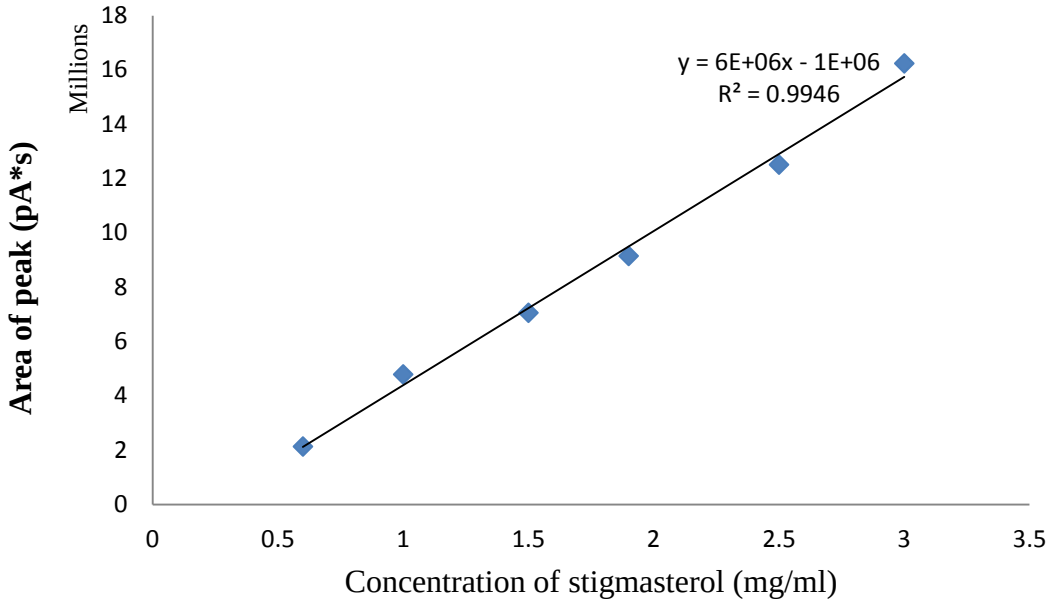



Figure 6.10 The standard curve of stigmasterol for β-sitosterol quantification

β-sitosterol content of bracken samples extracted by supercritical $CO_2$ and supercritical $CO_2$ with ethanol entrainer were examined respectively. The total β-sitosterol content in bracken dry plant was also determined by adding these two figures together. Figure 6.10 shows the β-sitosterol content in every extract and the variation tendency throughout the year.

The pattern of change in concentration of β-sitosterol is not similar to that of any previous compounds. The greatest total concentration of β-sitosterol, 2176 μg/g dry plant was obtained in the first crozier sample. Then the β-sitosterol content declined

significantly to 1583 μg/g in second stage sample and this trend continued and reached the lowest point in June with a figure of 778 μg/g. However, the content of β-sitosterol finally rebounds in over-mature plant with a level of 1312 μg/g dry plant. It is clear that the variation in level of β-sitosterol is similar to that of pterosins before the plant gets over-mature. The abundance of β-sitosterol is higher in the early stages of growing, and then reduces gradually over time. Unlike the pterosins, β-sitosterol finally rises up in dying plant and the mechanism behind this needs further investigation.

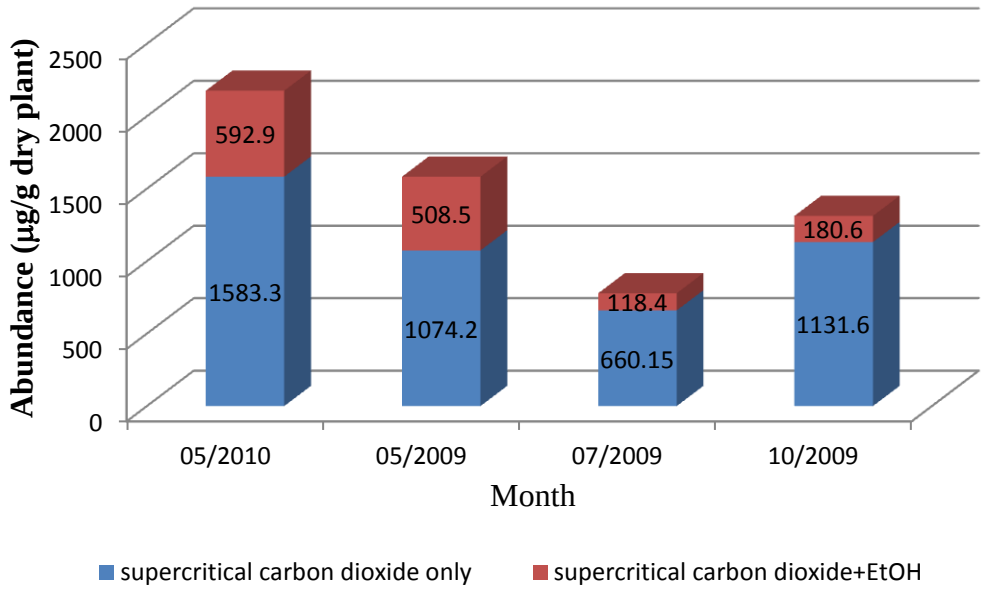



Figure 6.11 Seasonal variation of β-sitosterol in bracken dry plant

Quantitative variation of β-sitosterol in fresh plant material was also examined. Figure 6.12 shows the seasonal variation of β-sitosterol in fresh bracken. The downward trend in earlier samples remains the same with dry plant. 579 μg/g β-sitosterol was found in crozier fresh sample, at the same time 469 μg/g and 364 μg/g was found in May and July bracken respectively. Surprisingly, when water content is taken into account, the greatest total concentration of β-sitosterol was obtained in October sample rather than the crozier. A total of 724 μg/g β-sitosterol was found in senescing

plants and this sample also contains the highest concentration of β-sitosterol in $CO_2$ with entrainer extract. In summary the abundance of β-sitosterol in fresh plant is high in the crozier then reduces gradually over time, later in the growing season the β-sitosterol content increases dramatically and shows the highest abundance in senescing plants. As mentioned before, the mechanism behind this rebound needs further investigation.

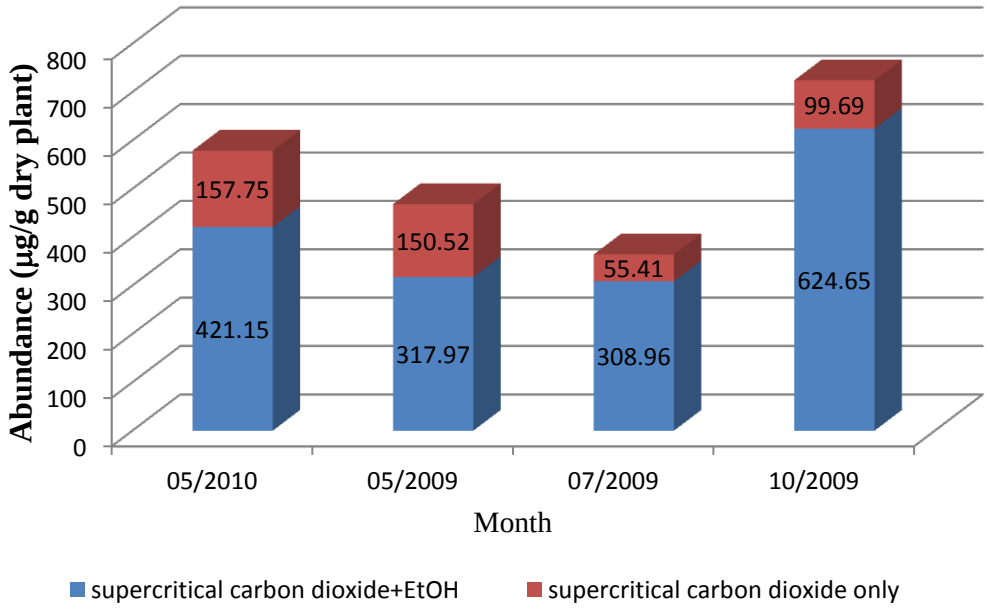



Figure 6.12 Seasonal variation of β-sitosterol in bracken fresh plant

## 6.6 Pterosins variation caused by geographical difference

In order to understand the influence of geographical location on bracken pterosins content, additional bracken samples was picked from Penrallt in North Wales in May 2010. Since bracken crozier contains the highest concentration of pterosins, these samples picked at the same time of the year should indicate the impact of the location difference at a time when the pterosins are at their highest. The Welsh bracken sample

was extracted using supercritical $CO_2$ under identical conditions to the Yorkshire samples and analysed by GC-MS.

Figure 6.13 shows the chromatogram of bracken extract from the different locations. Due to the lack of pterosin standards, quantification was not accomplished. However, in the integrated chromatogram, β-sitosterol has a very similar absolute area in both samples (within 1.17% difference) and since the two samples are prepared at the same initial concentration (20 mg/ml) the levels can be seen relative to the concentration of β-sitosterol.

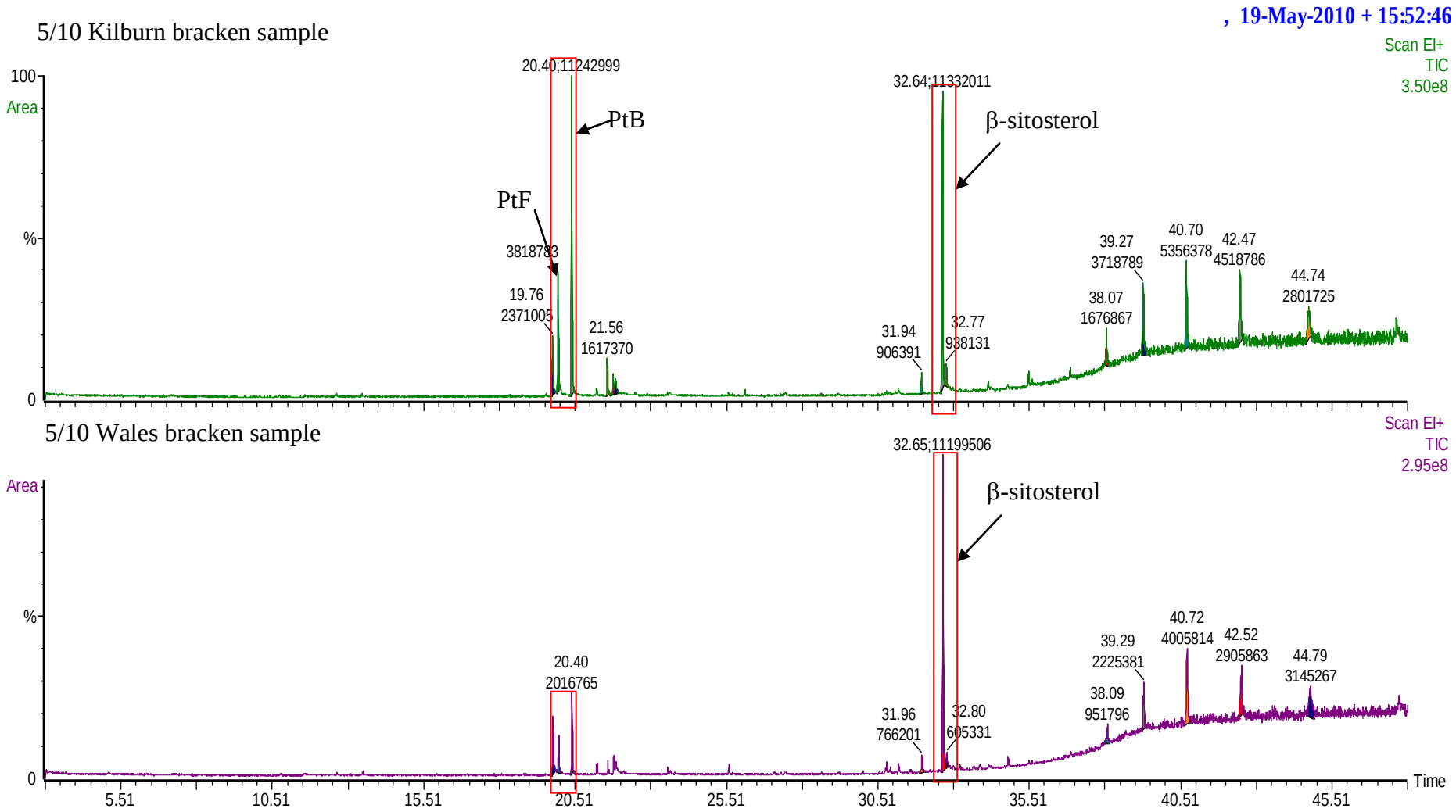



Figure 6.13 Comparison of Kilburn and Wales bracken supercritical $CO_2$ extract

As can be seen from the first trace of Kilburn sample, pterosin B is present at a very high concentration, approximately the same as that of β-sitosterol. Meanwhile, pterosin F also exists at a fairly high concentration and its peak area was slightly more than one third of pterosin B. It is clear that in this sample, pterosins, particularly pterosin B, are the major compounds in this extract along with β-sitosterol. On the other hand, in Welsh bracken sample is completely different. Pterosin B peak has less than one-fifth area of β-sitosterol, and pterosin F has less than one-tenth area of β-sitosterol. The quantity difference of pterosins in Kilburn and Welsh bracken samples are very obvious. Welsh bracken contains less than 40% of total pterosins compared to Kilburn bracken. This distinct difference may be due to different pH value of the soil in Kilburn and Wales.

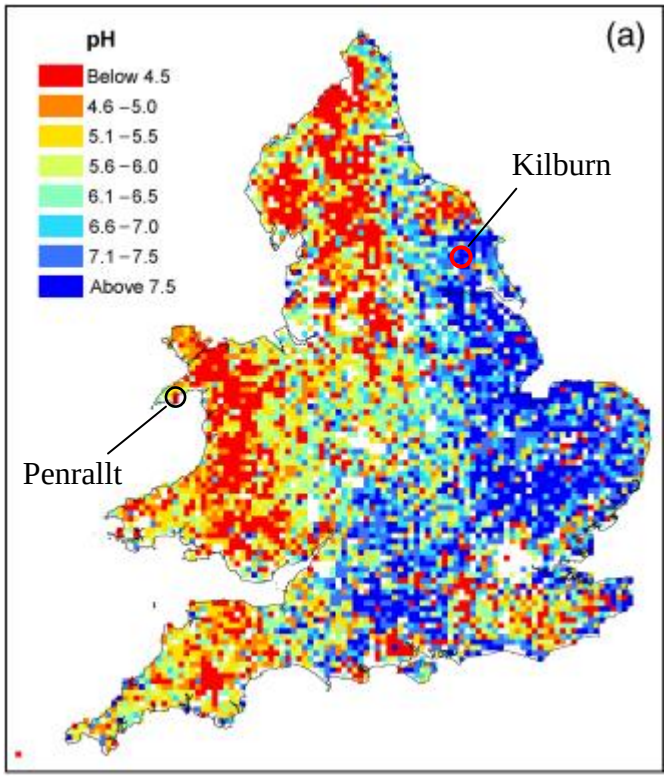



Figure 6.14 Map of soil pH in the UK[92]

According to the map of soil pH in the UK supplied by National Soil Resources Institute,[92] the soil pH value around North Yorkshire Moore is quite different from Wales. In most land around York and North Yorkshire Moore, the top 15cm of soil is neutral or alkaline (pH 6.6-7.5) and the White Horse at Kilburn where the samples were collected is immediately below a limestone cliff. Conversely, in Wales, most of the area has strong to moderate acid soil (pH below 4.5-6.5) and on the Lleyn peninsula lime is regularly added to arable land to increase the naturally low pH. In consideration of the ptaquiloside decomposition pathway, pH value variation between these two sites may be the most likely explanation. Since the ptaquiloside will more rapidly degrade in alkaline conditions than in acid, their lifetime in Kilburn bracken will be shorter than in the Welsh sample. According to Natori group,[87] the half-live of ptaquiloside in aqueous solution is 5 days for pH 4.0 and 23 min for pH 10.0. This means alkaline condition is favorable to ptaquiloside decomposition. Thus the alkaline soil of Kilburn may accelerate the ptaquiloside degradation and result in the increasing in the final metabolites pterosin B and pterosin F. The pterosins content in the supercritical $CO_2$ extract may come from three sources: first, pterosins that originally appear in the frond; second, pterosins degraded from ptaquiloside prior to extraction and third, pterosins generated from ptaquiloside degradation during the extraction (because of the acidic extracting conditions). In this case, apparently because of the basic soil, in Kilburn bracken, much more ptaquiloside is converted to pterosins thus it is more likely that the second scenario is correct and can explain why Kilburn bracken contains higher levels of pterosins compared to the Welsh sample. However, because standards were unavailable, quantification of pterosins from each source in both bracken samples cannot be accomplished. Therefore, further work is required to quantify exactly the level of pterosins from each source when standards are available.

## 6.7 Conclusion and further remarks

Seasonal variation of some major compounds of bracken supercritical $CO_2$ extract was highlighted in this work. A relative-level comparison between pterosins and internal standard was applied to mitigate the lack of standards. Pterosins variation largely reflected previous studies. Pterosins are present at very high concentration early in the growth cycle but reduce dramatically when the plant approaches senescence. Barely no pterosins can be detected from over-mature samples, and the reason behind this variation may be a self-protecting mechanism. Degradation in pterosins was also found during this work. Pterosins are significantly reduced when repeat analysis was carried out on samples after nine months. Further work is required to find out the mechanism behind this decomposition.

Seasonal variation of another important compound β-sitosterol is also demonstrated in this work. Quantitative results from each bracken sample were obtained and the changing trend is obvious. Bracken crozier contains β-sitosterol at 579 μg/g in the fresh plant material and this declined gradually until it reached the lowest point at 364 μg/g in fully mature bracken. Later in the growth cycle concentration increased and finally reached 724 μg/g in over-mature fronds.

It is also discovered that geographical difference can affect the pterosins content. Bracken samples from Kilburn and Wales picked at the same time period of the year were extracted and compared. The result indicated Kilburn samples contains almost three times as much pterosins as the Welsh sample. This could due to the pH value variation between these two places. Generally speaking, the soil pH value is lower in the area of harvest in Wales than at Kilburn in NorthYorkshire. Since ptaquiloside is more unstable in alkaline condition than in acidic condition, more ptaquiloside in Kilburn bracken may be converted to pterosins prior to harvest and extraction. Therefore, higher levels of pterosins were obtained from Kilburn bracken than Welsh

bracken after the extraction. This work indicates that geographic location indeed has a remarkable influence in plant chemical composition.

# *Materials, methods and experimental procedures*

# Chapter 7

## 7.1 Materials and reagents

Liquid carbon dioxide (99.99%) and bulk liquid nitrogen were purchased from BOC Ltd. All analytical and HPLC grade solvents, ethanol, methanol, acetone, dichloromethane, toluene, ethyl acetate, diethyl ether, and acetonitrile were obtained from Fisher Scientific.

Phenolic acid standards, gallic acid, (+)-catechin, (-)-epicatechin and chlorogenic acid, Folin-Ciocalteu's phenol reagent 2N, triterpenoid standards such as ursolic acid 90%, oleanolic acid 97% and lupeol 98% were all purchased from Sigma-Aldrich.

For retention time calculations alkane standard $C_{12}$-$C_{60}$ was purchased from Sigma-Aldrich and the $C_{21}$-$C_{40}$ standard solution was purchased from Fluka.

Samples of *Calluna vulgaris* were collected from the North Yorkshire Moor National Park, North Yorkshire regularly from October 2007 to November 2008 with the consent of North York Moor National Park Authority. Samples of *Pteridium aquilinum* were collected from Kilburn, North Yorkshire (Grid reference SE 514 813) in April, May, July, October 2009 and May 2010 and from Penrallt, Gwynedd (Grid reference SH 370 355) in May 2010.

## 7.2 Harvesting and milling process of heather and bracken

### 7.2.1 Harvesting and preparation of heather material

The heather was cut with garden scissors at 5 cm above the ground and was bagged in paper sacks for transportation. The raw material was air-dried in a fume hood at room temperature for 5 days and then ground using a Glen Creston Ltd. hammer mill fitted

with a 2.5 mm screen. The processed material was kept in glass sample bottles and stored at -18 $^{o}$C prior to extraction.

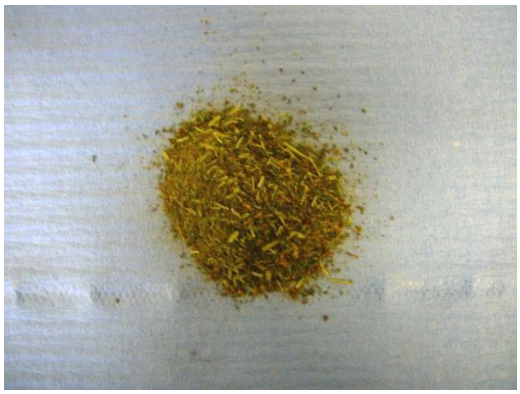


Figure 7.1: Milled whole heather

Flowering *Calluna vulgaris* was collected in August 2008 and the flowers were separated manually. The separated flowers were kept in glass sample bottles and stored at -18 $^{o}$C prior to extraction.

Figure 7.2: Flowering heather

Figure 7.3: Separated heather flowers

### 7.2.2 Harvesting and preparation of bracken

Previous research has indicated that the very young frond of bracken, known as the crozier, contains the highest level of carcinogen compared with other life cycle steps. Samples were collected in April, May, July and October from Kilburn, North Yorkshire (Grid reference SE 514 813) to obtain bracken at different growth stages so that the change in ptaquiloside and pterosins could be determined throughout the year. The bracken was cut by hand at a height of less than 2cm above ground level and was bagged in paper sacks for transportation. The raw material was air-dried in a fume hood at room temperature for 2 weeks and then milled using a Glen Creston Ltd. hammer mill fitted with a 2.5 mm screen. The processed material was kept in glass sample bottles and stored at -18 $^{o}$C prior to extraction. Because of the toxicity of the plant material protective clothing and gloves were worn at all stages of harvest and processing.

### 7.2.3 Water content of heather

Whole unmilled plant was dipped into liquid nitrogen then transferred to a one litre freeze drying vessel and placed in an ice chest at -30 $^{o}$C for several hours. Thereafter freeze drying was operated overnight. A Heto DW1 lyophiliser manufacture by Heto-Holten A/S was used and the temperature was kept at -100 $^{o}$C. After drying, the material was then weighed again in order to calculate the water loss. The water content was calculated using the following equation:

$$\% \text{ Water content} = [(\text{Initial mass} - \text{Mass after drying}) / \text{Initial mass}] \times 100$$

## 7.3 Extraction and fractionation of heather

### 7.3.1 Soxhlet extractions of heather

20 g of ground heather was placed in a Soxhlet thimble and placed into a Soxhlet extractor. A 1 litre round bottomed flask was connected to the Soxhlet extractor together with a double-wall condenser. 400 ml hexane (or ethanol) was used as extraction solvent during the process. The solution was heated under reflux for 6 hours. The reaction solution was cooled then filtered and the solvent was evaporated using a Buchi R-200 rotary evaporator. The crude extract was then weighed and the yield was calculated.

### 7.3.2 Supercritical carbon dioxide extraction of heather

Supercritical $CO_2$ extraction was carried out by using a Thar Supercritical Fluid Extraction 500 system (Thar technologies). Extraction was carried in a 500 ml extractor at 35 MPa and 50 $^{o}$C for 4hrs with a $CO_2$ flow rate of 40 g/min. After the extraction was complete the extract was washed out of the separator using dichloromethane and the solvent was removed by rotary evaporator to obtain the yield. This was a necessary step as the extract yield was very low.

The supercritical $CO_2$ extractions of heather with an ethanol entrainer was carried out under the same condition (35 MPa, 50 $^{o}$C) with ethanol added at a flow rate of 4 ml/min (10% of $CO_2$ flow rate). The ethanol solution was released regularly from the separator and combined at the end of the extraction before being evaporated to obtain the yield.

### 7.3.3 Fractionation of crude heather extract using column chromatography

200 mg of crude extract was dissolved in 10 ml dichloromethane in a round bottom flask with a small amount of Kieselgel silica gel. The dichloromethane was then evaporated using a rotary evaporator in order to coat the extract onto the silica gel. The silica gel and sample mixture was then transferred on top of a 20 cm x 1 cm slurry packed column of Kieselgel 60 silica gel. The upper surface of the column was covered with an approximately 2 mm layer of sand. The column was then eluted with hexane, hexane/diethyl ether (90/10, v/v), hexane/diethyl ether (4/1, v/v), hexane/diethyl ether (1/1, v/v), hexane/diethyl ether (1/4, v/v) and diethyl ether continuously. The eluent was collected and analysed by TLC. Fractions are combined based on their spot positions revealed by TLC and were analysed by GC and GC-MS later.

### 7.3.4 Thin layer chromatography (TLC) of crude heather extract and fractions obtained after column chromatography

20mg of crude extract was dissolved in 1ml diethyl ether. A spot of the extract solution was placed approximately 1 cm from the bottom of the TLC plate using a micropipette. Using the method of Yamashiro,[93] the plate was developed using hexane and toluene sequentially to full height and then a solvent mixture (hexane/diethyl ether/acetic acid, 70/30/1 v/v) was allowed to reach the half height of the plate. The plate was dried in air for several minutes and then placed under UV light to identify the position of the components. Alternatively the plate was dipped into a staining solution (10% w/w phosphomolybdic acid in ethanol), and dried in an oven at 120 $^{o}$C for 2 minutes.

For the eluent obtained following column chromatography, the fractions were spotted onto the plate without any diluting or concentrating. The fractions were combined based on their spot positions revealed by TLC and were analysed by GC and GC-MS.

### 7.3.5 Anti-microbial assays using heather extracts

7.3.5.1 Sample preparation for paper disc antimicrobial test (qualitative test)

Ground heather (200 mg) was added to 2 ml ethanol/water (80:20) and stirred for 2 hours at room temperature. The mixture was centrifuged at 3000 rpm for 15 min and the supernatant was decanted. Residual plant material was extracted again under identical conditions and supernatants were combined. Solvent was removed using a rotary evaporator.

7.3.5.2 Preparation for multi-well plate antimicrobial test (quantitative test)

Ground heather (1 g) was added to 10 ml ethanol/water (80:20) and stirred for 4 hours at room temperature. The supernatant was filtered and collected, and then the solvent was removed by a rotary evaporator. After the yield calculated, the solid residue was dissolved in 2% ethanol water solution to give a concentration of 2 mg/ml. The solution was sterilised using a Corning® syringe filter before adding to the 96-well cell culture plate.

### 7.3.6 UV analysis of heather total phenolic content

Analysis of total phenolic content was carried out using Folin-Ciocalteu reagent and absorbance measured at 765 nm using a Jasco V-550 UV/Vis spectrophotometer according to the method of Andrew L. Waterhouse.[94] A calibration curve was produced using 0, 20, 40, 60, 80, 100 and 120 μg/ml of gallic acid made up in methanol and used as standards. 10μl of each standard solution was added to a 10 ml volumetric flask containing 1 ml Folin-Ciocalteu reagent and 5 ml 7.5% $Na_2CO_3$ water solution. Deionised water was then added to each volumetric flask to make up the solutions to 10 ml. The solutions were incubated for 1 hour in the dark after mixing well and then the absorption measured at 765 nm. Linear calibrations were

produced by plotting absorption against concentration. Using the samples prepared in 7.3.5.2, 10 μl of each heather ethanol/water extract was added to a 10 ml volumetric flask containing 1 ml Folin-Ciocalteu reagent and 5 ml 7.5% $Na_2CO_3$ water solution. Deionised water was then added to each volumetric flask to make up the solutions to 10 ml. The solutions were incubated for 1 hour in the dark after mixing well and then the absorption measured at 765 nm. The total phenolic concentration of each heather extract solution sample was then determined based on the calibration curve and expressed in mg gallic acid equivalents (GAE) $g^{-1}$.

### 7.3.7 UV analysis of heather total triterpenoid content

Analysis of total triterpenoid content was carried out using Vanillin-perchloric acid as coloring reagent and absorbance measured at 550 nm using a Jasco V-550 UV/Vis spectrophotometer according to the method of W. Wei.[95] 10 mg oleanolic acid was dissolved in 50 ml methanol, then 0.00, 0.10, 0.20, 0.30, 0.40, 0.50, 0.60, 0.70 ml of standard solution was transferred to 8 testing tubes and the methanol was evaporated in a water bath. 0.3 ml 5% vanillin acetic acid solution and 1 ml perchloric acid were added to each testing tube before heating these testing tubes in water bath at 70 $^oC$ for 25 min. The solution was cooled in ice water bath after heating and then 10.0 ml acetic acid was added to each testing tube. Absorbance of each solution was then measured at 550 nm and recorded to create a standard curve. Using the samples prepared in 7.3.1 and 7.3.2, 10 mg of each heather supercritical $CO_2$ extract (or hexane extract) were dissolved in 1ml dichloromethane in testing tubes. The solvent was then evaporated in a water bath. Same 0.3 ml 5% vanillin acetic acid solution and 1ml perchloric acid were added to each extract before heating these testing tubes in water bath at 70 $^oC$ for 25 min. The solutions were cooled in ice water bath after heating and then 10.0ml acetic acid was added to each testing tube. Absorbance of each extract was then measured at 550 nm and recorded for further calculation of total triterpenoid.

### 7.3.8 High temperature-Gas chromatography mass spectrometry (HT-GCMS) of heather extracts

Gas chromatography-mass spectrometry analysis was carried out using a Perkin Elmer Clarus 500 chromatograph coupled with a Perkin Elmer Clarus 560S mass spectrometer. A ZB5-HT (Phenomenex) capillary column (30 m x 0.25 mm I.D x 0.25 μm film thickness, max temperature 430 $^{o}$C) was used with constant carrier gas flow rate of 1ml/min helium. Initial oven temperature was maintained at 60 $^{o}$C for 1 minute, and then ramped at a rate of 8 $^{o}$C/min to 360 $^{o}$C. The oven temperature was then held at 360 $^{o}$C for 10 minutes before cooling. The injector was maintained at 350 $^{o}$C and the split ratio used was 10:1. Spectra were recorded in electron impact (EI) mode at 70 eV scanning from 40-1200 amu in 1 second. The source temperature and quadruple temperature were maintained at 350 $^{o}$C. Peaks from the extracts were initially identified by searching the NIST library (2008). Then some compounds were further identified by comparing their retention time and spectrum with standard material, the others were identified by comparing spectrum with published data of previous researches. Fragmentation mechanisms were also studied to help confirm the identification.

## 7.4 Extraction and fractionation of bracken

### 7.4.1 Supercritical carbon dioxide extraction of bracken fern

Supercritical $CO_2$ extractions were carried out using a Thar SFE 500 system. A 3-step extraction scheme was be used for bracken extraction. Liquid $CO_2$, supercritical $CO_2$ and then supercritical $CO_2$ plus co-solvent were used sequentially to extract molecules of increasing polarity.

Firstly, the bracken sample was extracted using liquid $CO_2$. Liquid $CO_2$ extraction of bracken (200g) was carried out at a pressure of 6.5 MPa and a temperature of 5 $^oC$ for 6 hours. $CO_2$ flow rate was set to 40 g/min. The extract was washed out of the evaporator using dichloromethane and then dried using a rotary evaporator to obtain a yield. The residue from the first extract remained in the extractor and was extracted using supercritical $CO_2$ thereafter. The conditions of the first supercritical $CO_2$ extraction were 35 MPa, 50 $^oC$ and a flow rate of 40g $CO_2$/min for 4 hours. The extract was again washed out of the evaporator using dichloromethane and then dried using a rotary evaporator to obtain a yield.

Finally, the residue from the second extraction was extracted using supercritical $CO_2$ and ethanol as a co-solvent. The conditions of the second supercritical $CO_2$ extraction were 35 MPa, 50 $^oC$ and a flow rate of 40 g $CO_2$/min for 4 hours with addition of ethanol at 10% of the $CO_2$ flow, i.e. 4ml/min. During the extraction, the ethanol extract solution was bled regularly from the evaporator to prevent overfilling. The extract solution was finally combined and evaporated to dryness to afford a yield.

### 7.4.2 High temperature-Gas chromatography mass spectrometry (HT-GCMS) method for bracken analysis

Samples prepared according to 7.4.1 were diluted at 20mg/ml dichloromethane for GC-MS analysis. Gas chromatography-mass spectrometry analysis was carried out using a Perkin Elmer Clarus 500 chromatograph coupled with a Perkin Elmer Clarus 560S mass spectrometer. A ZB5-HT (Phenomenex) capillary column (30 m x 0.25 mm I.D x 0.25 μm film thickness, max temperature 430 $^oC$) was used with constant carrier gas flow rate of 1 ml/min helium. Initial oven temperature was maintained at 60 $^oC$ for 1 minute, and then ramped at a rate of 8 $^oC$/min to 360 $^oC$. The oven temperature was then held at 360 $^oC$ for 10 minutes before cooling. The injector was maintained at 350 $^oC$ and the split ratio used was 10:1. Spectra were recorded in electron impact (EI) mode at 70 eV scanning from 40-1200 amu in 1 second. The

source temperature and quadruple temperature were maintained at 350 $^{o}$C. Peaks from the extracts were initially identified by searching the NIST library (2008). Then some compounds were further identified by comparing their retention time and spectrum with standard material, the others were identified by comparing spectrum with published data of previous researches. Fragmentation mechanisms were also studied to help confirm the identification.

### 7.4.3 Methanol extraction of bracken for supercritical fluid chromatography

1 g of bracken powder was added to a sample bottle containing 5 ml of methanol. This solution was then stirred for 4 hours at room temperature prior to centrifugation at 3000 rpm for 5 minutes. The supernatant was decanted and transferred to a GC vial for supercritical fluid chromatography.

### 7.4.4 Supercritical Fluid Chromatography of bracken extracts

An Investigator SFC system (Waters) coupled with integrated 2998 Photo Diode Array detector and 2424 Evaporative Light Scattering detector was used to analyse the bracken sample obtained in 7.4.3. A constant flow of 4ml/min supercritical $CO_2$ along with methanol as organic modifier were used as the mobile phase. The system was fitted with a Viridis (Waters) SFC silica column (25 cm×4.6 mm×5 μm). Analysis of the bracken extracts was carried using a gradient of increasing levels of methanol in $CO_2$. The percentage methanol was initially 10% rising from 10% to 25% in 8 minutes and then holding at 25% for 1 minute. The methanol content was then reduced back to 10% in 1 minute. The oven temperature was kept at 40 $^{o}$C and back pressure was set to 15.0 MPa. Compounds were identified by direct comparison of retention time with standard compounds.

# *Conclusion and further work*

# Chapter 8

# 8.1 Concluding remarks

## 8.1.1 General background

Heather (*Calluna vulgaris)* and bracken fern (*Pteridium aquilinum*) are two widely distributed species which dominate the vegetation of large areas in many UK upland regions particularly the North Yorkshire Moors. The North York Moors National Park contains the largest patch of continuous heather moorland (50,100 ha) in England, representing over 10% of the country's resource. Heather is dominant on dry heath vegetation which covers 26,500 ha (52.9%) and forms the main land cover on the western, southern and central moors. Heather is also abundant on the wet heath (8,700 ha, 17.4%), which occurs on moister soils. Meanwhile, the total area of bracken fern is estimated at 12,300 ha (24.6%) in North York Moors National Park and this figure is increasing every year since bracken is seriously invading heather-dominated areas.

Currently, burning is the predominant method of managing moorland vegetation especially heather. The most noticeable value of heather is as bird habitat for breeding and feeding. To suit the bird's different phases of growth, moorland needs to be burnt to various heights to maximise the food availability since certain invertebrate species are only associated with recently burnt or cut areas. On the other hand, bracken is a major weed problem in Britain since its expansion is causing the loss of large areas of semi-natural vegetation. Its vigorous growth makes it difficult to control and its dense canopy shades out other species, reducing a site's grazing or forestry potential. Bracken can be controlled by herbicide use or mechanical means. At the moment, the use of the herbicide Asulam (methyl *N*-[(4-aminophenyl)sulfonyl] carbamate) accounts for most attempts at bracken control. It has a low toxicity for animals and affects few other plants; however it still has some disadvantages such as high cost and spray drift which can affect populations of other ferns. More seriously, Asulam spray can cause sudden large-scale hydrological change that can lead to soil erosion before

colonisation by other species. For all these reasons, if any valuable compounds with potential commercial application opportunities can be extracted from these two currently wasted resources, harvesting can take place regularly at a specific time of the year instead of burning and spraying herbicide. Within this study, heather and bracken are demonstrated to be very good resources of some valuable chemicals which can potentially be applied as herbal medicine, pharmaceutical drugs or drug precursors.

### 8.1.2 Conclusions from heather study

Aerial parts of heather were harvested in October 2007, January 2008, March 2008 and August 2008 from Hole of Horcum, North York Moors National Park (Grid reference SE 84558 93633) to be representative of heather in different seasons. Heather samples were extracted using supercritical $CO_2$ and supercritical $CO_2$ with ethanol entrainer. The highest yields in both dry plant and fresh plant were all obtained in August flowering sample. Since supercritical carbon dioxide has been described to have extracting ability similar to that of hexane, traditional hexane Soxhlet extractions were also applied to give a yield comparison in heather. The general yield variation trend of hexane extraction was the exactly the same as seen with supercritical $CO_2$ extraction. August heather expressed highest yield and winter heather has the lowest. Since it was also reported that triterpenoid content is also higher in summer flowering heather, August heather supercritical $CO_2$ and supercritical $CO_2$ with ethanol entrainer extracts were fractionated by column chromatography and fully analysed for their triterpenoid composition. Several triterpenoids such as Friedelin and 13,27-cycloursan-3-one were discovered in these supercritical $CO_2$ extracts. The quantification of these new triterpenoids was also accomplished by using oleanolic acid as a standard. Taraxerol and taraxerone which have not been previously reported in heather leaves and stems were also found in heather aerial part supercritical $CO_2$ extracts. As with other triterpenoids, the quantification of taraxerol and taraxerone were also achieved by using oleanolic acid

as standard. Taraxerol and taraxerone are both reported to have several biological activities such anti-cancer and anti-inflammatory. Taraxerone showed in vitro anti-leishmanial activity against promastigotes of *Leishmania donovani* and anti-tumour activity on K562 leukemic cell line. Taraxerol was reported to have significant activity against dermatophytes *T. rubrum* and *T. mentagrophytes* with MIC values between 6.2 and 25 μg/ml, additionally, taraxerol also express moderate activity against *Aspergillus niger*.

Seasonal variation of total triterpenoid compounds in heather supercritical $CO_2$ and supercritical $CO_2$ with ethanol entrainer extracts were also evaluated in this study. The highest total triterpenoids content was found in August heather with 15400 μg/g dry plant and 12979 μg/g fresh plant. March heather contains the second highest amount of total triterpenoids at 11200 μg/g in dry plant and 9467 μg/g fresh plant. Winter heather was found to have the lowest triterpenoid content. January heather only obtains 4200 μg/g total triterpenoids in dry plant and 3620 μg/g in fresh plant. October heather contains 8500 μg/g triterpenoids in dry plant and 7606 μg/g in fresh plant. Heather total triterpenoids increase from winter to summer, then decrease again in the autumn. Summer flowering heather was found to have the greatest amount of total triterpenoid. The same trend was shown in heather supercritical $CO_2$ with ethanol entrainer extracts. However, these extracts were found to have slightly higher total triterpenoid than supercritical $CO_2$ extracts. Heather samples were also extracted using hexane to give a comparison of total triterpenoid content with supercritical $CO_2$ extracts. The seasonal variation of triterpenoid in hexane extracts was exactly the same as seen with supercritical $CO_2$ extracts. The greatest total triterpenoid content was still found in August heather which is 17000 μg/g dry plant and 14328 μg/g fresh plant.

The burning of heather takes place in spring every year since it is restricted by law that burning can only take place between 1$^{st}$ October and 15$^{th}$ April. In March heather sample, total triterpenoid content was evaluated as 11200 μg/g dry plant in

supercritical $CO_2$ extract and 12400 μg/g dry plant in hexane extract. The total triterpenoid content in March is approximately 88% of that of August heather sample. Therefore, if harvesting can take place in March instead of burning, the frond will already contain almost 90% of the highest triterpenoid content in the year. A large amount of triterpenoid can be extracted if large-scale harvesting of heather can be achieved at this time of the year.

Total phenolic content of heather was also analysed in this study. The lowest total phenolic content was found in heather flowers, which is 2130 mg/100g dry plant. Heather whole plant seem to have the lowest total phenolics in autumn and then keep rising until reach a peak in spring sample at 5163 mg/100g dry plant. Afterwards the total phenolic content drops slightly to 4201 mg/100g dry plant in summer when it is flowering. Heather phenolic extracts were then evaluated for their anti-microbial activity. Qualitative results showed that the seasonal variation of antimicrobial activity of heather is in clear contrast to that of total phenolic content. This result suggested that the antimicrobial activity does not necessarily correlate with total phenolic content in heather. Quantitative tests were then carried out on phenolic extracts at concentrations of 1000 ppm and 100 ppm. However, 1000 ppm heather phenolic extract and 100 ppm phenolic extract only inhibited 9.83% and 9.06% of total growth of lactobacillus respectively. These inhibition figures are very low compared to other commonly used antimicrobial agents. Therefore, heather phenolic extract at 1000 ppm and 100 ppm both have very weak antimicrobial activity and are not sufficient to be applied as an antimicrobial agent even at these fairly high concentrations.

### 8.1.3 Conclusions from bracken study

Samples in different bracken growth cycle stages were picked from the same site (Kilburn White Horse, Sutton Bank, Grid reference SE 514 813) in different months of a year. The first crozier sample was picked in 17$^{th}$ April 2009 and 19$^{th}$ May 2010,

followed by the 2nd step sample picked on 21st May 2009, 3rd sample picked in 27th July 2009 and 4th sample picked on 12th October 2009. It was reported that the carcinogenicity of young bracken shoots is much stronger than that of mature plant. Due to this remarkable distinction of bracken crozier, liquid $CO_2$ extraction, along with supercritical $CO_2$ extraction was employed in bracken crozier analysis to give a more comprehensive understanding of its chemical content. Thus bracken crozier has been extracted by liquid $CO_2$, supercritical $CO_2$ and supercritical $CO_2$ with ethanol entrainer sequentially. Two naturally occurring pterosins, pterosin B and pterosin F, were successfully identified in bracken crozier liquid $CO_2$ extract by comparing their mass spectrum with published standard data. Other major components such as β-sitosterol and long chain primary alcohols were also detected in this extract at fairly high concentration. All these identified components are still present in following supercritical $CO_2$ extract. More long chain alcohols with higher chain length were also identified in supercritical $CO_2$ extract, and their existence indicates that the extraction ability of sc$CO_2$ is substantially higher than liquid $CO_2$. With higher temperature and pressure, supercritical $CO_2$ is able to extract molecules with greater polarity and higher molecular weight. This result indicates that the selectivity of $CO_2$ depending on operating conditions is achievable and successful. In bracken crozier supercritical $CO_2$ with ethanol entrainer extract, major peaks including pterosin B and β-sitosterol were still present, however pterosin F and long chain primary alcohols are no longer present in this extract. Several new peaks appear in this extract due to the greater polarity of sc$CO_2$ with ethanol entrainer. Some additional branched unsaturated alcohols related to phytol were detected in this extract, and further sitosterol derivatives also eluted immediately prior to the β-sitosterol peak.

Bracken samples harvested in May 09, July 09 and October 09 were extracted by supercritical $CO_2$ and supercritical $CO_2$ with ethanol entrainer sequentially. Most interesting peaks such as pterosin B, pterosin F and β-sitosterol all still present in May bracken sample at very high concentration, however, the chemical composition in the plant varied significantly after July. The most interesting change is in this fully

matured frond, pterosin B exist at very low concentration compared to younger frond, and pterosin F was not even detectable. Both pterosin B and pterosin F no longer appear in October senescing bracken extract. In both bracken supercritical $CO_2$ extracts and supercritical $CO_2$ with entrainer extracts, pterosin compounds exhibited a clear reduction as the seasons progressed. Pterosin B and pterosin F disappeared in the frond when it is growing towards its maturity; however β-sitosterol is present in bracken frond at every growth stage.

Seasonal variation of pterosins compounds and other components are also evaluated in this study. The highest total water content of bracken was found in crozier at 73.40%, and then it decreased progressively during the growth cycle until it reached the lowest point in October senescing bracken at 44.80%. The total yields of supercritical carbon dioxide extract in both dry plant and fresh plant increases from crozier to over-matured plant. Due to lack of pterosin standards, the quantification of pterosin compounds was not accomplished. However, octadecane was introduced as an internal standard to give a direct comparison of the relative level of pterosins and octadecane in peak height/area. Result shows that pterosin B and pterosin F decreased dramatically when plant grows towards its maturity. Geographical difference was also found to cause significant variation in the level of pterosin compounds. Bracken crozier samples picked from Penrallt in North Wales in May 2010 was extracted using supercritical $CO_2$ under identical conditions. It was found that Welsh bracken contains much less pterosin B and pterosin F compared to Yorkshire sample. This distinct difference may be due to different pH value of the soil in Kilburn and Wales.

Recently, pterosins are discovered to have anti-diabetic and anti-obesity activities. Totally 84 different pterosins or pterosin related compounds were synthesized or extracted from plants and have been tested in vitro and in vivo for their anti-diabetic and anti-obesity activities. 15 of them expressed very strong biological activities against type 1 and type 2 diabetes and obesity, and among these 15 pterosins, four of them are naturally occurring in bracken fern and some other natural plant materials.

These pterosin compounds were indicated to significantly lower blood glucose levels in STZ-induced diabetic mice, and also dramatically enhanced the insulin sensitivity and glucose consumption in vitro. The pterosin compounds may activate AMPK (AMP-activated protein kinase), which in turn regulates insulin regulation of carbohydrate and fatty acid metabolism. These compounds can therefore be considered as potential anti-diabetic and anti-obesity agents. Further results also indicated that these pteorsin compounds can significantly reduce serum lipids and exhibited anti-obesity effects in high fat-diet fed mice.

Nowadays, most pterosin compounds are synthesized through a series of complex processes. For example, a typical method to synthesize pterosin F is mixing pterosin B and triphenylphosphine in carbon tetrachloride and reflux for 3 hours. The starting material pterosin B was obtained from pterosin O by demethylation with 48% hydrobromic acid in acetic acid. Moreover, pterosin O was afforded through catalytic hydrogenation with 5% Pd on carbon in ethyl acetate from an intermediate indandione, and this key intermediate was formed through a Friedel-Crafts bisacylation of the methyl ether of 2-(2,6-dimethylpheny1) ethanol with methylmalonyl chloride. The synthesis processes are very complicated and will generate large amounts of chemical waste and solid waste. However, in my study, pterosin F and pterosin B were demonstrated to be naturally occurring in bracken liquid $CO_2$ and supercritical $CO_2$ extracts at fairly high concentration. Therefore, it is clear that naturally occurring valuable pterosins can be extracted from bracken by using environmental benign technologies rather than synthesized through complex processes. This study will not only add value to this troublesome natural plant, but will also make some contribution to the green chemistry application in this research field.

To the best of my knowledge, bracken frond has never been extracted by liquid or supercritical carbon dioxide before. Previous common extraction methods of bracken were limited to water extraction or methanolic extraction. In my study, liquid $CO_2$ and supercritical $CO_2$ were indicated to be capable to extract at least two natural pterosins

from bracken crozier and pre-mature frond with high concentration. Although pterosin B and F were not identified to have certain anti-diabetic or anti-obesity activities in previous research, these pterosin compounds, especially pterosin F, have the possibility to be used as precursors to other more effective pterosins because of its active chlorinated side chain.

To conclude, heather and bracken can both be good potential resources of herbal medicine or pharmaceutical drugs and precursors. Heather was proved to have high content of triterpenoid compounds which have been reported for their biological activities such as anticancer and liver protecting property. Bracken was proved to contain high content of natural occurring pterosin B and pterosin F which can be converted to other pterosins which have been proven for their anti-diabetic and anti-obesity activities. All these high value target chemicals can be extracted by environmental benign liquid carbon dioxide or supercritical dioxide.

## 8.2 Future work and publications

Heather flower supercritical $CO_2$ extract and supercritical $CO_2$ with ethanol entrainer extracts were fractionated by column chromatography and 14 or 11 final fractions were obtained respectively, however, only a few of them which contain triterpenoid compounds were selected to be fully analysed. Further analysis can be carried out on the more polar fractions to look for more interesting components with valuable biological activities.

It was clear that bracken crozier and pre-mature frond contains the highest amount of pterosin compounds. If young bracken frond can be harvested regularly, it will be able to afford larger scale of liquid and supercritical $CO_2$ extractions. Therefore higher amount of pterosin compounds especially pterosin F which is more likely to be applied as a pharmaceutical precursor can be extracted. Thereafter, an isolation and

purification of pterosin compounds can be achieved by supercritical fluid chromatography which is a proven purification application used in the pharmaceutical industry. Pure pterosin compounds can be then analysed individually for biological activities, or converted to other pterosins which have been proven to be anti-diabetic and anti-obesity.

This study has revealed new aspects of the chemistry of heather and bracken as well as demonstrating the commercial potential for harvesting this material. It is intended that the identification of new triterpenoids in heather and the extraction of bracken using liquid and supercritical $CO_2$ will be published as two separate papers in peer reviewed journals.

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