DIAGENESIS OF TRICYCLIC DITERPENOIDS USED AS BIOMARKERS IN AN ANOXIC LAKE

By

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ABSTRACT

Diterpenes with a tricyclic skeleton and two other hydrocarbons, perylene and 6-methyl hexadecane were extracted from anoxic sediments of Powell Lake, B.C. The aim of the project was to establish the precursor to product relationships between biosynthesized resin acids of the abietane skeleton from trees and the defunctionalized and aromatized compounds found in these sediments. A Douglas Fir (*Pseudotsuga menziesii*) from U.B.C. Research Forest, Maple Ridge provided the resin acids for the comparison to the sedimentary diterpenes. Perylene and 6-methyl hexadecane were selected as they are known to be of different origin than the resin acids and were assumed to show a different isotopic signature from the C_3-plant acids when analyzed by Stable Carbon Isotope Mass Spectrometry (SCIMS).

A range of values had been estimated previously for the various groups of compounds and it was expected that the resin acids would show $\delta^{13}C$ values of –21 to –33‰ (plus an additional –8‰ for lipids). The dehydroabietic, abietic, and pimaric acids extracted from the Douglas Fir wood had a $\delta^{13}C_{PDB}$ of –29.5±0.3‰ as expected. However, the degraded diterpenes extracted from the lake sediments showed a range of values from dehydroabietane, $\delta^{13}C_{PDB}$ –22.7±0.4‰ to retene, $\delta^{13}C_{PDB}$ –24.5 ±0.3‰. It was estimated that the loss of CH_3-groups at C-4 and C-10 and COOH at C-4 should generate a degradation product that would be up to 2‰ lighter than the precursor compound, since these groups were heavier in the original acetyl-CoA used in the biosynthesis of these compounds. It was, furthermore, thought that a small isotope effect might be associated with the degradation of these acids. However, the degraded compounds from the sediments were heavier than the original acids and it is concluded that dehydroabietane may be a residual product produced by the conifer trees, with an original heavier isotopic signature than the resin acids. The lighter fraction of the diterpenoids is suggested to have been totally degraded to short chain acids and it is the partially degraded heavier fraction of the original acids that are observed in the sediments, showing an isotope signature less negative than the resin acids.

The $\delta^{13}C$ values for 6-methyl hexadecane (known to be a cyanobacterial product) given in the literature are quite varied, depending upon source of CO_2. In the sediments this compound has a $\delta^{13}C_{PDB}$ value of –25.1±0.1‰ which compares well with values for whole cell material from freshwater, eutrophic lake plankton ($\delta^{13}C$ = –26 to –30‰) and with the range of whole cyanobacterial mat material ($\delta^{13}C_{PDB}$ = –16 to –32‰).

Perylene is an ubiquitous compound in both terrestrial and marine environments but its origin is unknown. It is believed that in Powell Lake sediments the origin is most likely fungal—a reduced fungal pigment, cercosporin or perylene-quinone. The $\delta^{13}C_{PDB}$ value of –26.0±0.7‰ reflects the terrestrial source of the carbon used and shows the expected lighter isotopic signature. It is unlikely that perylene is of anthropogenic origin as its signature should be lighter, less than –28‰.

Based on their $\delta^{13}C$ values it was established: (1) that the diterpenes in the sediments and their acid precursors with an abietane skeleton belong to the same family of compounds; (2) that they are microbially degraded showing slight isotope effects; (3) that no definite route of degradation could be seen, but the more oxidized and/or reduced diterpenes generally have lighter isotopic signatures; (4) that aromatization and defunctionalization take place rapidly

and early during diagenesis limiting their use as biomarkers in the assessment of thermal maturation; and (5) that single organic compounds defined by their isotopic signatures could be used as tracers in environmental geochemistry studies.

For perylene and 6-methyl hexadecane it was shown that their isotopic ratios reflect the terrestrial origin of their CO_2 (HCO_3^-), lighter than would be expected for either fungal or cyanobacterial whole cell material, but consistent with a terrestrial, freshwater environment.

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1. INTRODUCTION

Diterpenes with tricyclic skeletons have been used as biomarkers in the determination of terrestrial input into sediments. Stable carbon isotope analyses have routinely been performed on bulk sediments from different sedimentary environments, again, to obtain information as to the origin of the preserved organic material. These isotopic values have given only a broad idea about the origin of the material, and this author decided therefore to investigate a specific group of compounds, the resin acids, and to follow the precursor to product relationship between the biosynthesized acids from trees and the degraded compounds extracted from anoxic sediments. The application of stable carbon isotope analysis on single organic compounds was expected to give a better picture of the changes that take place in a specific molecule in a particular sedimentary environment and will help in the further understanding of diagenetic processes. Hayes *et al.* (1987) instigated this kind of thought with the $\delta^{13}C$ analyses of single geoporphyrins from the Eocene Messel shale which showed that the obtained values could be related to the biological origin of the compounds.

1.1. Objectives

Previous work (Barnes and Barnes, (1978, 1983) isolated a suite of reduced and oxidized diterpenes from sediments of Powell Lake, B.C. and showed that aromatic hydrocarbons of the abietane skeleton can form early in diagenesis and prior to thermal maturation (Barnes and Barnes, 1983). Powell Lake consists of oxic and anoxic basins which share an upper layer of oxic fresh water. Sediments from the permanently anoxic bottom waters of South Basin are greatly enriched in both saturated and aromatized diterpenes relative to sediments from West Basin which contains oxygenated water to the sediment surface, but has reduced sediments at depths of a few centimetres.

The objectives of this study are to show:

1) the precursor-product relationship between the higher plant diterpenoid acids and their oxidized or reduced products in the sediment using stable carbon isotopic mass spectrometry (SCIMS);
2) the role microbial alteration plays in their diagenesis; and
3) differences in algal, fungal and higher plant precursors using SCIMS to compare 6-methyl hexadecane and perylene with retene.

1.2. Diterpenoids—their structure, occurrence and distribution.

Diterpenes with varying basic skeletons consisting of 20 carbons occur in plants, fungi, and bacteria. These compounds are secondary metabolites, are enzymatically produced and are frequently degraded by microorganisms. Abietane, palustrane, and pimarane are the most common diterpene skeletons found in higher plants; their concentrations and structures have been elucidated by numerous researchers (Zinkel and Engler, 1977; Ekman, 1979; Rogers *et al.*, 1979; Ohgaku *et al.*, 1984; Simoneit *et al.*, 1986).

Zinkel and Engler (1977) extracted and identified the following acids shown in Figure 1.1 from pinewood extractives and oleoresins. All these acids are biosynthesized (Fig. 1.2) from a common precursor, acetyl-Coenzyme A (acetyl-CoA) (Mann, 1987) into a tricyclic skeleton. It has been shown in laboratory experiments (Weiss and Edwards, 1980) that the pimarane skeleton can be the precursor of the abietane skeleton through a 1,2 shift of the C-17 methyl group. This arrangement has taken place under acid-catalyzed conditions. It has, however, not been confirmed as a step in the biosynthesis of the abietanes.

Another study by Zinkel *et al.* (1985) and Zinkel and Clarke (1985) of the diterpene acid content of *Pinus nigra* and *Pinus resinosa* needles showed a different distribution in wood as compared with needles. In these species labdane rather than abietane diterpenes were the dominant acids in pine needles; depending on their location, there were also differences in the distribution of individual labdanes. In this case, the biosynthesis proceeds with some minor modifications; geranyl-geranyl pyrophosphate is cyclized in a slightly different fashion to give (+)-labdadienyl pyrophosphate which in turn undergoes the allylic rearrangement and hydroxylation shown in Figure 1.3 to form manoyl oxide, epi-manoyl oxide and communic acid.

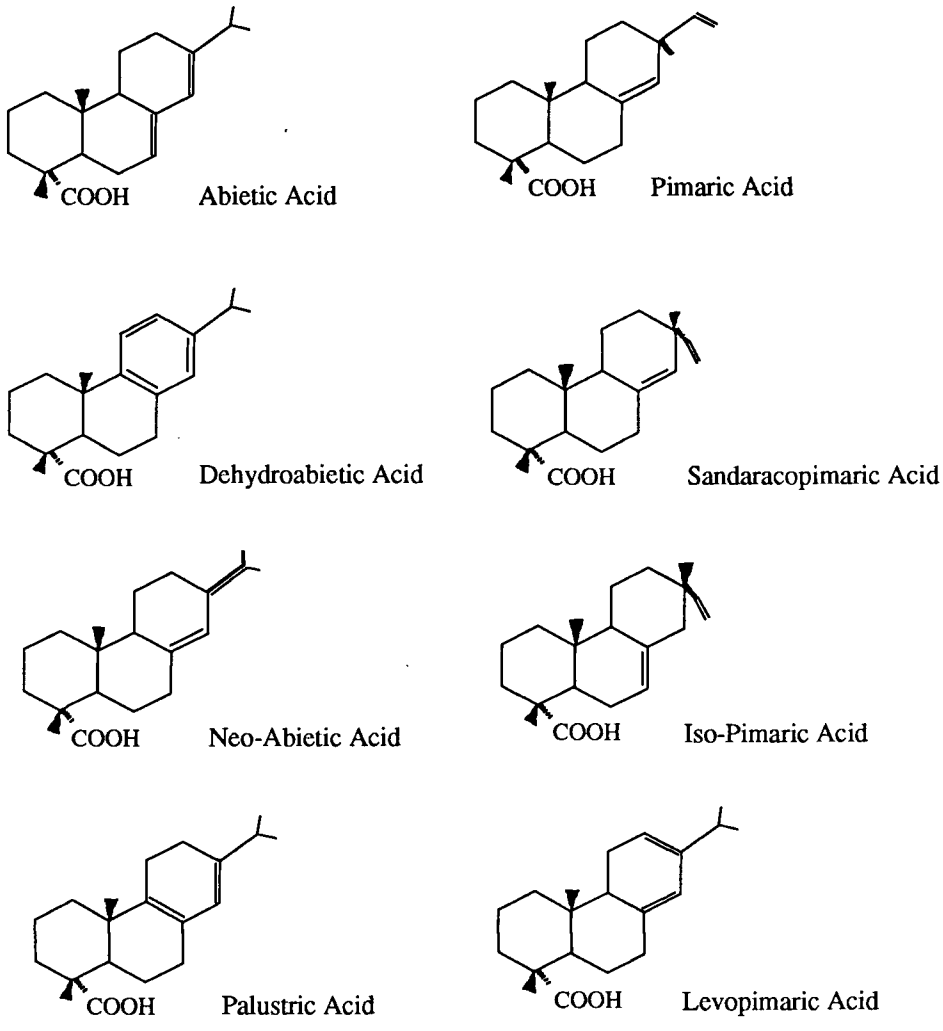


Fig. 1.1. Common diterpenoids—abietane and pimarane skeletons.

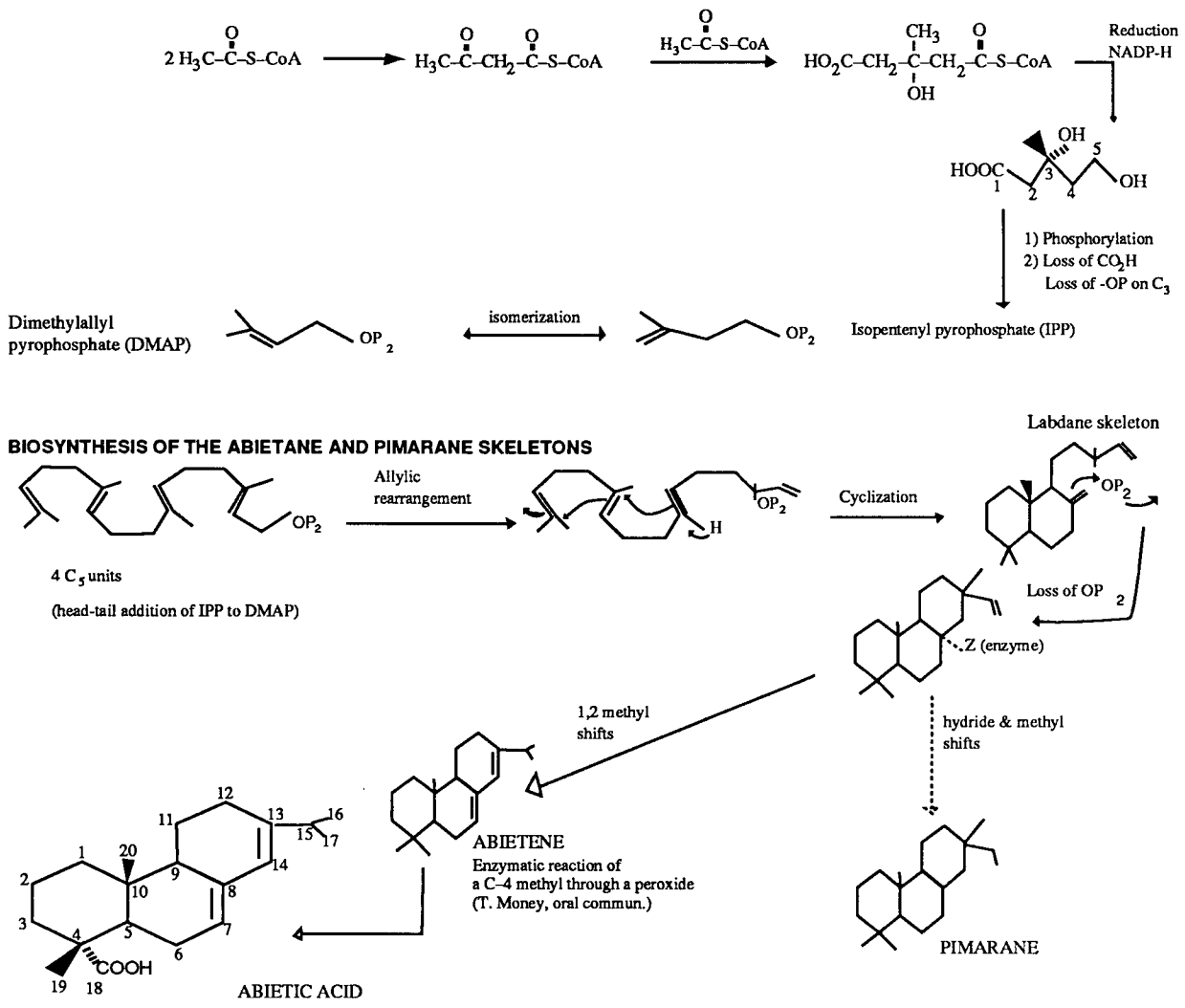
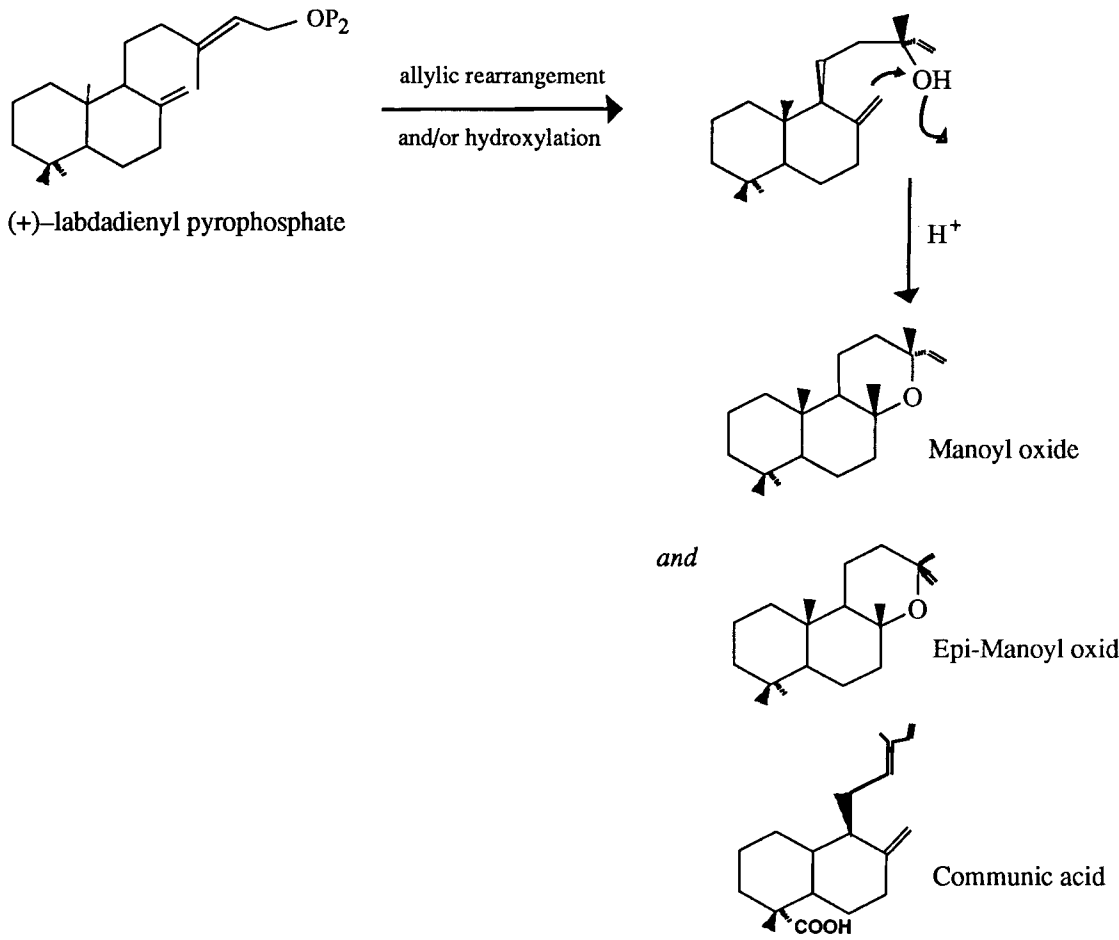


Fig.1.2. Biosynthetic pathway for abietic acid.
The convention used assumes a linear or cyclic structure lies approximately in the plane of the paper; solid triangles at 19 and 20 in abietic acid indicate methyl groups which lie above the plane; the dashed line at C-4 shows an acid group which lies below the plane of the cyclic structure; triangles represent methyl groups; —= represents a —CH_2-CH=CH_2 attached to the ring; —COOH represents a carboxyl group; = represents a double bond.

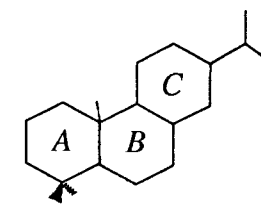


(+)–labdadienyl pyrophosphate

Fig. 1.3. Rearrangement of labdadienyl pyrophosphate to form manoyl oxide, epi-manoyl oxide and communic acid.

It was found that among young trees (less than 5 years old) the concentration of these acids was very low, but that the concentration increased and stabilized when the growth was about 5 years. Among the populations of *Pinus resinosa*, the needle acid composition was fairly consistent and any differences could be attributed to latitudinal provenances; i.e., more southerly trees have lower manoyl oxide/epi-manoyl oxide and higher content of communic acid compared to more northerly growths.

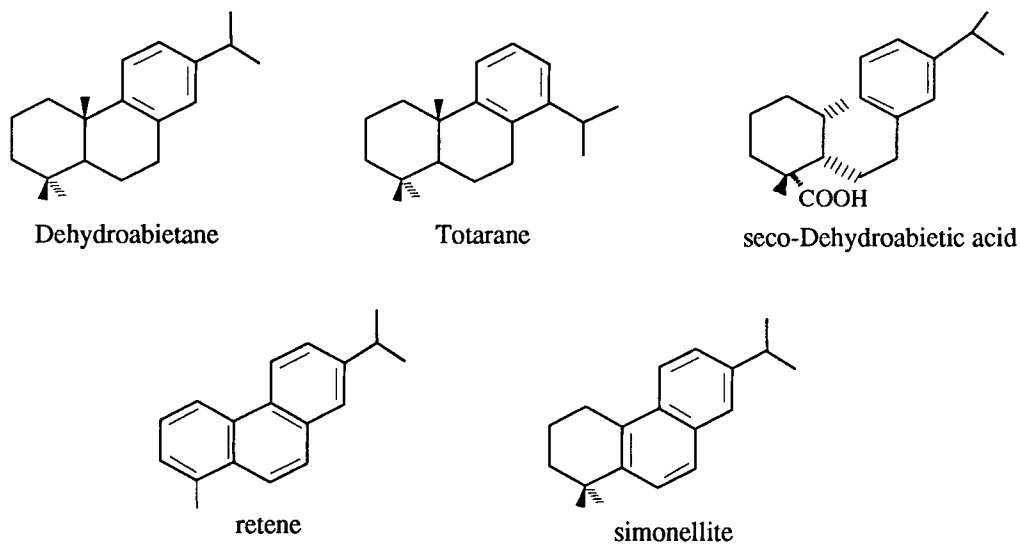
It is well established that higher plants produce resin acids of pimarane, isopimarane, and abietane skeletons. The main concentration is in the xylem and, as just discussed, this changes when needles are examined separately. However, it turns out that mono-aromatic diterpenes are synthesized as well (Weiss and Edwards, 1980). This aromatization almost always involves ring *C* only, although there is no reason why the other rings, *A* and *B*, through a loss or shift of methyl groups, could not be aromatized. The mono-aromatic hydrocarbon, dehydroabietane, has been



identified in wood extractives (Hafizoglu, 1983) and in the calluses of *Cupressaceae* (Ohgaku, 1984). Both abietadiene and the alcohol, abienol, have also been reported in twigs from spruces (Lorbeer and Zelman, 1988).

Two fossil di- and tri-aromatic hydrocarbons, simonellite and retene (Fig. 1.4), have been found in soils (Simoneit *et al.*, 1986) and sediments (Simoneit and Beller, 1985), but not in higher plants. The biosynthetic path for their aromatization is unknown, but abietic acid has been suggested as a precursor (Robinson *et al.*, 1986; Weiss and Edwards, 1980). Ring *C* is always aromatic; to date no naturally occurring tricyclic diterpenoids are known in which only rings *A* and *B* are aromatized. The aromatic compounds that are derived from abietane can be divided into four subclasses:

1) retain skeleton, at least of ring *C*; (dehydroabietane);
2) iso-propyl group is shifted (totarane);
3) cleavage of individual bonds of ring system with retention of all carbon atoms (seco-dehydroabietic acid); and
4) elimination of methyl groups (retene, simonellite).



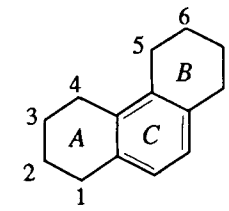
Dehydroabietane Totarane seco-Dehydroabietic acid

retene simonellite

Fig.1.4. Aromatic diterpenes derived from an abietane skeleton.

Microbial degradation takes place rapidly during early diagenesis, and includes both aerobic and anaerobic processes. The aerobic bacteria are the most active and varied; fungal attacks occur as well. Depending upon environmental conditions a small number of the original compounds will survive as *biomarkers* in sediments and petroleum.

Many studies have been done on aerobic bacterial degradation and several have been compiled by Kieslich (1976). It has been shown that non-volatile components can be attacked by *Flavobacterium resinovorum* and *Pseudomonas resinovorum*. These two strains are capable of using these non-volatile substances as their sole C-sources. Several compounds of the octahydro-phenanthrene skeleton were examined; a carboxylic function at C-4 and at least one double bond, especially in ring *C*, were favoured for growth.



When dehydroabietic acid is used as the only carbon source in a cation deficient medium, the compounds shown in Figure 1.5 can be isolated. Not all degradation products are shown, only those with the abietane skeleton.

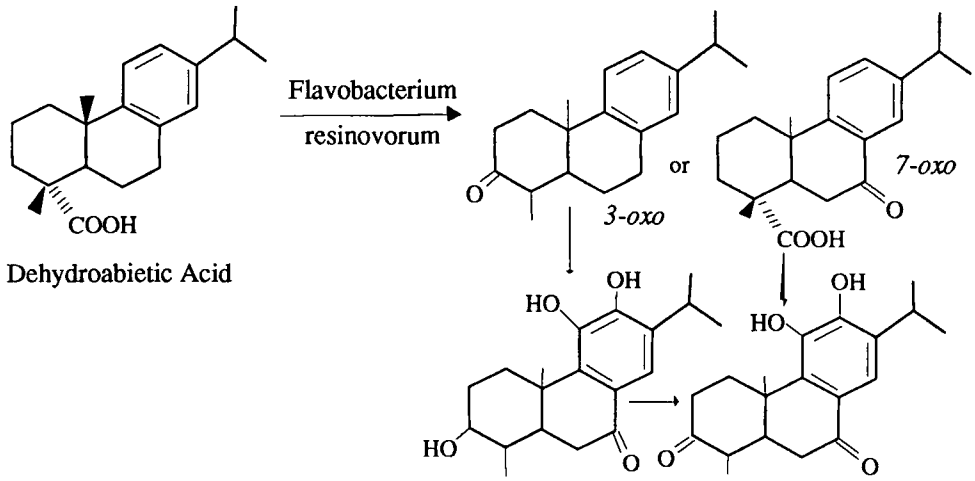


Fig. 1.5. Bacterial alteration products derived from dehydroabietic acid.

Eukaryotic fungal species have also been investigated for their ability to degrade fairly complex organic compounds. Ekman (1979) found various hydroxy- and keto-acids of the abietane skeleton when a spruce was inocculated with *Fomes annosus* (Fig. 1.6).

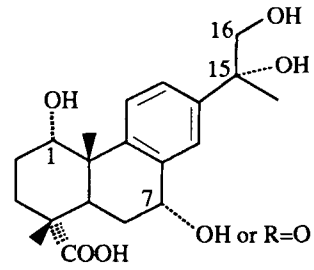


Fig. 1.6. Fungal alteration by *Fomes annosus* of dehydroabietic and abietic acid produces changes at C-1, C-7, C-15, and C-16

Only dehydroabietic acid was stable in the zone of infection by *Fomes annosus*. The acid was not easily hydroxylated due to its aromaticity or it could have been formed in this zone from other resin acids with conjugated double bonds.

Kutney *et al.* (1981, 1982) tried various bacterial and fungal strains in order to examine their ability to biologically detoxify dehydroabietic and abietic acid. *Mortierella isabellina*, a fungus, turned out to be the species that provided the greatest degradation of dehydroabietic acid. This took place through hydroxylation of C-2, C-15, and C-16 (Fig. 1.7).

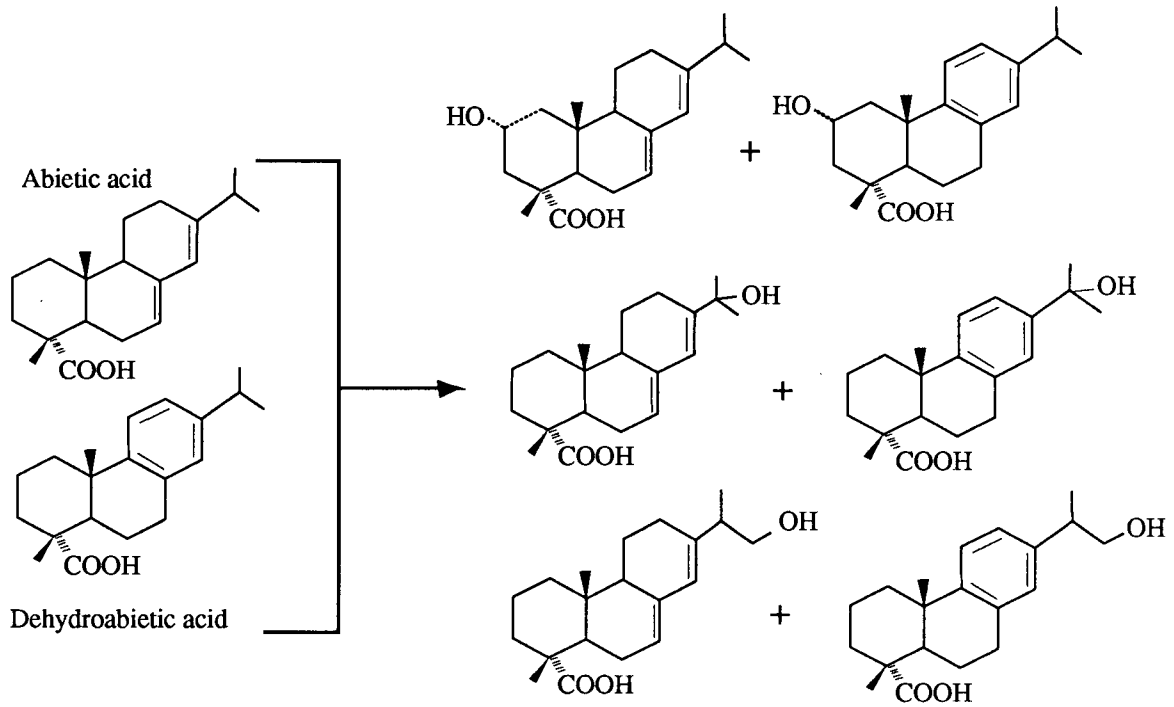


Fig. 1.7. Fungal alteration by *Mortierella isabellina* of abietic and dehydroabietic acid produces changes at C-2, C-15 and C-16.

The steroids (triterpenoids) have been used by a number of researchers to: 1) model sedimentary basins (MacKenzie *et al.*, 1985) and 2) investigate the origin and degradation of steroids (Brassell, 1985; Smith *et al.*,1986; Wakeham, 1987).

On the other hand some cyclic diterpenes retain their biological fingerprints during early diagenesis and have been identified in various oxidizing and reducing environments. They are used as biomarkers for terrestrial source materials and as a measure of transport patterns; however, their use as a measure of catagenesis is somewhat limited by earlier diagenetic changes. Abietane diterpenoids are oxidized and or reduced very early in the degradation process, and as the present study will show, both end-members (fichtelite and retene) are found in sediments which are no

more than 200 years old; therefore, they cannot be used in the determination of degree of thermal maturation of sediments as loss of methyl groups or increases in aromatization can occur prior to catagenesis.

Aromatized cyclic diterpenes of the abietane skeleton and their reduced counterparts have their main use in establishing terrestrial, resinous input into sediments; Simoneit *et al.* (1986) and Simoneit (1986) investigated the diterpenes and their derivatives in plant detritus, ambers, and coals. They have not been found to be produced by other organisms or plants in significant amounts (Weiss and Edwards, 1980; Simoneit, 1986).

1.3. Biogenic fractionation of carbon isotopes: preferential selection of ^{12}C relative to ^{13}C during biosynthesis.

The element carbon consists of a mixture of two stable isotopes, ^{12}C and ^{13}C, and one radioactive nuclide, ^{14}C. The relative abundances of ^{12}C and ^{13}C are 98.9% and 1.1% respectively. Biological material is considerably enriched in ^{12}C while the heavier species ^{13}C is retained in inorganic carbon species such as carbonate, bicarbonate, and carbon dioxide. It has been shown, and it is widely accepted, that most biological carbon isotope fractionations are due to kinetic rather than equilibrium effects (Schidlowski, 1986). The main isotope-discriminating steps are associated with:

1) the diffusion of CO_2 into and out of photosynthetically active plant tissue; and
2) the first carbon-fixing carboxylation reaction by which CO_2 is incorporated in the carboxyl group of an organic acid.

A number of controlled laboratory studies have been undertaken. Earlier studies examined biologically-produced acetic acid and showed the different isotopic values of methyl carbon versus carboxyl carbon (Meinschein *et al.*, 1974; Rinaldi *et al.*, (1974a, 1974b). In Smith *et al.* (1979), the differences between C_3- and C_4-photosynthesis exhibited by plants are highlighted by the apparent differences in the carbon isotopic ratios. Reviews by Schidlowski *et al.* (1983), De Niro (1983), and O'Leary (1981), among others, made it obvious that isotopic values, at natural abundance levels, could be quite useful when trying to establish the various contributions to sediments from marine, terrestrial, microbial and anthropogenic sources. Organic geochemists and petroleum geologists have applied this information to the interpretation of the origin of sediments. Bulk isotopic ratios can be used as guidelines to the origin of biological material found in the sedimentary record (Botello *et al.*, 1980; Arthur *et al.*, 1985; Gearing *et al.*, 1984; Peters *et al.*, 1986; Sackett, 1986).

To date it has not been common to look at individual compounds rather than groups of compounds surviving as *biomarkers* in sediments and petroleum; usually, average values for heterogeneous samples have been measured. This has probably been due to lack of instrumentation which could actually do analysis on microgram-size samples and at the same time give meaningful results as to the natural abundance of isotopes in these samples. Hayes *et al.* (1987) examined individual porphyrins in the Eocene Messel Shale; this study will examine biologically produced abietic and dehydroabietic acids and their degraded products as well as two other compounds, perylene and 6-methyl hexadecane, and use stable carbon isotope mass spectrometry to investigate their origin and degradation pathways (Fig. 1.8).

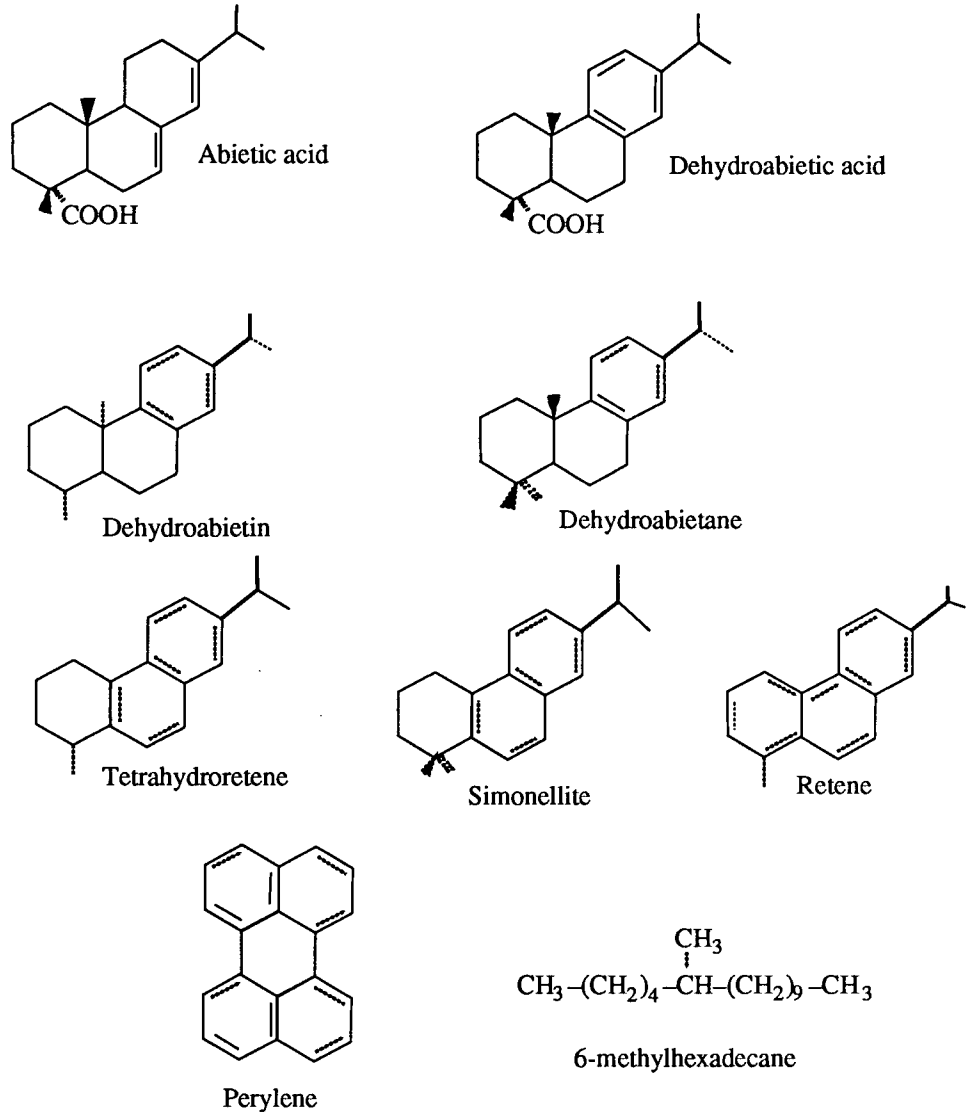


Fig. 1.8. Compounds isolated from the sediments of Powell Lake and used for SCIMS.

Douglas Fir (*Pseudotsuga menziesii*) belongs to the Pinaceae family of woody plants. Most woody and flowering plants are C_3-plants, which is quite important as it determines the expected $^{13}C/^{12}C$ ratios. According to Smith (1976), the C_3-plants are biologically earlier and more primitive; C_4-plants evolved in the Pliocene in response to reduced efficiency of C_3-photosynthesis, due to increased oxygen partial pressures in our recent atmosphere. The C_3-plants as a group have about 10 to 15‰ more negative $\delta^{13}C$ values than the C_4-plants (Kokke *et al.* 1984). These differences are relative to the Peedee belemnite (PDB), which has been used as an universal standard since the 1950's for determining carbon isotopic content in both organic and inorganic materials.

Ribulose bisphosphate carboxylase, the enzyme that fixes CO_2 in C_3-plants discriminates against $^{13}CO_2$ and will give an isotopic value between –21‰ to –33‰ (De Niro, 1983). This value will, of course, depend upon the isotopic value of inorganic carbon available to the plant. Lipids (defined as chloroform-soluble compounds) are further enriched in ^{12}C, with $\delta^{13}C$ values as much as 8‰ less than the whole plant (Park and Epstein, 1961).

Monson and Hayes (1980; 1982a; 1982b) did several studies on the fractionation of carbon isotopes by following the biosynthesis of fatty acids in *Escherichia coli* under controlled conditions. They discovered that the fractionations occur at the earliest stages in: 1) synthesis of acetyl-CoA and 2) whenever there is a branching in the biosynthetic pathway—a two-way path—to generate the necessary 1° and 2° metabolites.

O'Leary (1981) reviewed many of the variables involved when trying to determine the carbon isotopic ratios in plants. One fairly significant observation was that different metabolites of a particular class can have quite different isotopic compositions and that the part of the particular plant one is examining, is also important. This means that leaf versus stem versus root will show differing isotopic values.

In the present study only abietic and dehydroabietic acid were chosen, as the extracted aromatic hydrocarbons from Powell Lake sediments all are of the abietane skeleton; this also avoids any isotopic differences due to compound classes. It was, therefore, expected that the $\delta^{13}C$ values for abietic and dehydroabietic acids would be between –21 to –33‰ (for C_3-plants), plus an additional depletion of as much as 8‰ due to the compounds being lipids (De Niro, 1983; Park and Epstein, 1961). This estimated value will be compared with the values measured for the degraded hydrocarbons isolated from Powell Lake.

The degradation of diterpenoid acids and the appearance of aromatic tricyclic diterpenes (retene, simonellite, tetrahydroretene, dehydroabietane, and dehydroabietin) in sediments are thought to be due to alterations by microorganisms; i.e., bacteria and/or fungi. The tricyclic terpenes are complex molecules for microbes to attack so they

are able to survive with their skeletons intact, contrary to sugars and amino acids, which in most cases are totally oxidized to CO_2 and/or reduced to CH_4 by microbes. It is reasonable to assume that time, temperature, and pressure do not play any important roles in the changes occurring in the diterpenes examined, since the age of the sediments is only about 200 years (Barnes and Barnes, 1983).

Methane in sediments, generated by methanogenic bacteria from CO_2 and water, commonly has $\delta^{13}C$ values as low as –60‰. This can be used to postulate a possible fractionation of about 30 to 40‰ when compared to isotopic values for terrigeneous organic material (about -25‰). If this total fractionation during methanogenesis is averaged over twenty carbons, the estimated effect for a diterpenoid (C_{20} compound) is about -2‰ relative to the original isotopic value. It should then be possible to see a fractionation effect, due to microbial degradation, in the values obtained for the original diterpenes and their degraded products since the microorganisms select the isotopically lighter organic material for growth purposes and leave the heavier fraction behind; thus, the diterpenoid hydrocarbon end-products should reflect a microbial signature overprinting the original conifer signature that is 2‰ lighter for altered products and 2‰ heavier for a residual pool of unaltered hydrocarbons. This can be partly substantiated by data obtained from cell material from photosynthetic bacteria showing the C_3-pathway. Sirevaag *et al.* (1977) and Fuller *et al.* (1961) found that fractionation, in most cases, was similar to or greater than for C_3-plants with values as much as 2‰ lighter than the average for C_3-plants.

Perylene, another compound extracted from the Powell Lake sediments, has a controversial origin. It has been found in almost every type of environment. It is widespread in natural organic-rich accumulations and it is commonly accepted that anoxia in the early stages of sedimentation—during accumulation and stabilization—is a prerequisite for its generation (Louda and Baker, 1984). Perylene precursors are present in marine and terrestrial organic detritus and could be derived either from perylene-quinone pigments of fungi (Allport and Bu'lock, 1958; 1960) or marine microorganisms (Wakeham *et al.*, 1979). The greatest concentrations have been found in anoxic diatomaceous sediments, comparable to those found in Powell Lake. It has also been found as a product of wood combustion (Ramdahl, 1983). Kawamura *et al.* (1987) and Louda and Baker (1984) found that perylene-quinones were reduced *in situ* under anoxic conditions to give perylene. If the assumption, then, is that perylene originates from fungi or a terrestrial plant, it should probably show $\delta^{13}C$ values of –12 to –35‰—similar to algal products (Schidlowski *et al.*, 1983), or ca. –27‰—similar to C_3 plants (O'Leary, 1981). Freshwater algal products should be about 7‰ lighter than marine products.

The branched alkane, 6-methyl hexadecane, was extracted as an example of a precursor known to be specific to cyanobacteria; it was selected with the objective of using SCIMS to substantiate its bacterial origin (Bird and Lynch, 1974). Algae and cyanobacteria synthesize the major varieties of lipids which are also found in the photosynthetic membranes of higher plants (Harwood and Russell, 1984). An estimate for ^{13}C abundance in membrane lipids could be derived from chlorophyll *a* which is also found in the membranes; Hayes *et al.* (1987) published $\delta^{13}C$ data for various chlorophylls found in the Eocene lacustrine Messel Shale. Their $\delta^{13}C$ value for chlorophyll *a* from algae was –21.5 to –22.0‰. These compounds had not undergone thermal diagenesis since the temperature never exceeded 40°C and burial was not deeper than 300 m. Chlorophyll *a* is, of course, from another group of compounds synthesized by a different biosynthetic pathway, but it is reasonable to assume that the $\delta^{13}C$ value for 6-methyl hexadecane is in the range –16 to –22‰. As –16‰ is a value quoted for the whole microbial mat community, and lipids are generally about –8‰ depleted relative to whole cell values (Park and Epstein, 1961; Monson and Hayes, 1982a; Blair *et al.* 1985) one might expect a value closer to –22‰. However, Bogacheva *et al.* (1980) reported $\delta^{13}C$ values for cyanobacteria with an average of –32‰ for waxes and hydrocarbons. It is, therefore, possible that the branched alkane in question is either similar to, or heavier or lighter than the terrestrial diterpene acids. With such a wide range of possible values, its $\delta^{13}C$ value can at this stage only be inferred.

1.4. Summary

Based on data from the literature, a range of $\delta^{13}C$ values can be predicted for comparison with isotopic values found in this study. These are summarized in Table 1.1 for the three groups of compounds examined in this study.

Table 1.1. Expected $\delta^{13}C$ values

COMPOUND CLASS	EXPECTED RANGE OF VALUES
dehydroabietic acid (with trace amounts of abietic acid)	–21 to –33‰ + (–8‰ for lipids)
tricyclic hydrocarbons with abietane skeleton	up to ±2‰ different than parent compound
perylene	–12 to –35‰ (fungal) approx. –27‰ (C_3-plants)
6-methyl hexadecane	–16 to –32‰ (marine to freshwater)

2. GENERAL ANALYTICAL METHODS.

In organic geochemistry, the analysis of organic compounds is a result of successive extraction procedures. It is therefore worthwhile to give a general overview (Fig. 2.1) of what is involved in obtaining single compounds from sediments before these actual procedures are discussed (see chapters 3, 4 and 5).

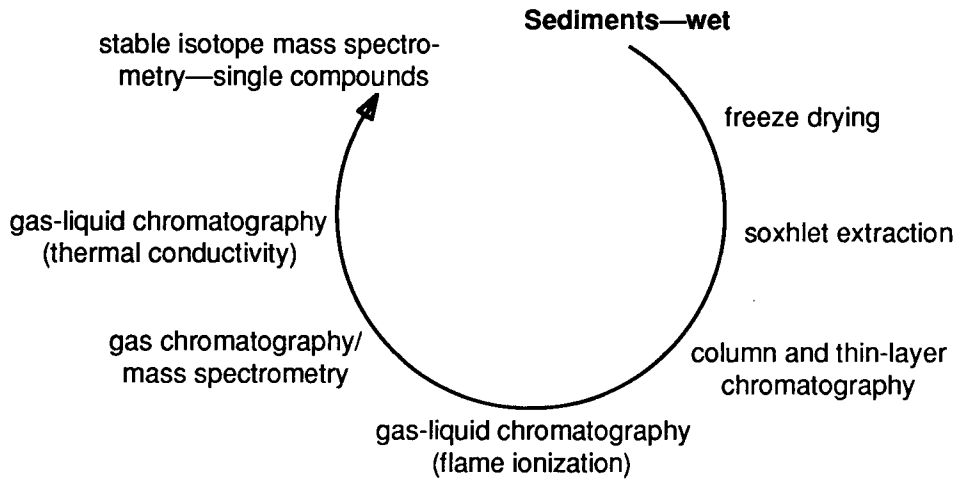


Fig. 2.1. The sequence of steps involved in extraction procedures.

2.1. Sediments—freeze drying

Sediments were cored from Powell Lake, B.C. using a 8.8 cm outside diameter gravity corer and frozen at the time of collection. The entire cores were used in this study and were subsequently freeze dried and sieved into fractions coarser and finer than 0.5 mm. The finer sediments contained the major part of the organic material and were used in the following separations.

2.2. Sonication and soxhlet extraction

Sediments extracted in organic solvents can either be sonicated or soxhlet extracted. Grimalt *et al.* (1984) evaluated these commonly used procedures and found that sonication was suitable for hydrocarbons even if recovery was slightly lower. They also found the main concentrations of hydrocarbons in the finer sediment fractions (<66 μm). Another study by Sportsoel *et al.* (1983) obtained similar results. In both studies, a solvent mixture (polar/non-polar) was used to facilitate the extraction of polar compounds from the sediments. In this study compounds were isolated by both sonication and soxhlet extraction; benzene/methanol (3:2), which forms an azeotrope, was selected as the solvent. Extraction with a solvent system that forms an azeotrope has two advantages: 1) the benzene-

methanol azeotrope boils at a lower temperature than either solvent alone—the solvent can thus be evaporated from the extract at a lower temperature, protecting thermally labile compounds; and 2) a mixed polarity solvent system is more effective in the extraction of a lipid fraction which itself is a mixture of polar and non-polar compounds.

2.3. Column and thin-layer chromatography

Chromatography, discovered and named by the Russian botanist M. Tswett in 1906, is the general name for procedures in which two or more compounds distribute between two phases: (1) **a stationary phase**, which can be a solid or a liquid supported on a solid, and (2) **a mobile phase**, either a gas or a liquid which flows continuously around the stationary phase. Both gas and liquid chromatography can use silica gel as an adsorbent. Silica gel is an irregular, amorphous fine powder with a general formula $SiO_2 \cdot xH_2O$. The general structure of silica gel particles is shown in figure 2.2.

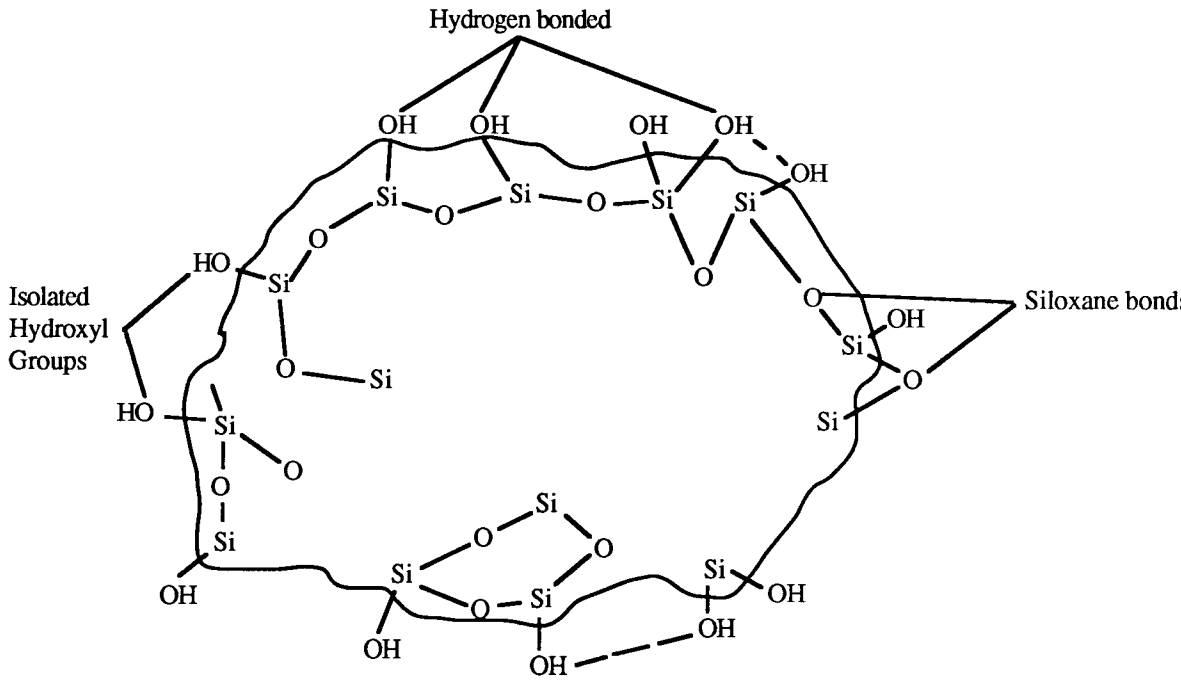


Fig. 2.2. General structure of the surface of silica gel (adapted from Majors, 1978).

Silica gel is slightly acidic and is quite often deactivated with water to decrease its ability to adsorb too well. It will adsorb unsaturated, aromatic, or polar compounds through hydrogen bonding (Majors, 1978). Other attractive forces involved are: (1) dipole-dipole interactions—an attractive force between polar adsorbents and polar solutes; and (2) chemisorption—the formation of a covalent bond or an ion pair between the adsorbent and solute.

In liquid column chromatography, silica gel is packed into a glass column; solute (containing extracted compounds) is added in a minimal amount of a non-polar solvent. The polar and non-polar compounds are eluted from the column by adding a non-polar solvent such as hexane and then increasing the polarity by adding increasing proportions of diethyl ether to the hexane. In this manner saturated and unsaturated hydrocarbons are separated from functionalized more polar compounds like alcohols, esters, and acids.

Thin-layer chromatography (TLC) operates under the same principles. A glass plate is coated with silica gel (using $CaSO_4 \cdot 1/2H_2O$ as binder). Compounds dissolved in a solvent are applied to this layer at one end of the plate, which is left to dry and afterwards placed vertically in a closed container into which a small amount of an appropriate solvent has been placed. Capillary action in the solvent vapour atmosphere draws the solvent up the plate; compounds move with the solvent at rates depending upon whether they are more attracted to the adsorbent (solid phase) or to the solvent (mobile phase).

The modified version of TLC used in this study uses the Chromatotron™, which has a motor driven glass rotor coated with silica gel (Fig. 2.3). Solvent mixtures (as in column chromatography) of increasing polarity are used to elute the compounds. Very good separations can be obtained using centrifugal force in this way and the technique is much faster than column chromatography, which depends only upon gravity to elute the compounds. With this motor driven chromatograph, it is possible to reduce elution time by two thirds.

In order to separate saturated from unsaturated hydrocarbons, silver nitrate was added to the silica gel (Morris, 1962). The silver(I) ions complex with the double bonds of unsaturated hydrocarbons, thereby decreasing their mobility. To reverse this reaction solvents or mixtures of solvents of increasing polarity are added to the plate and the compounds elute with the mobile phase.

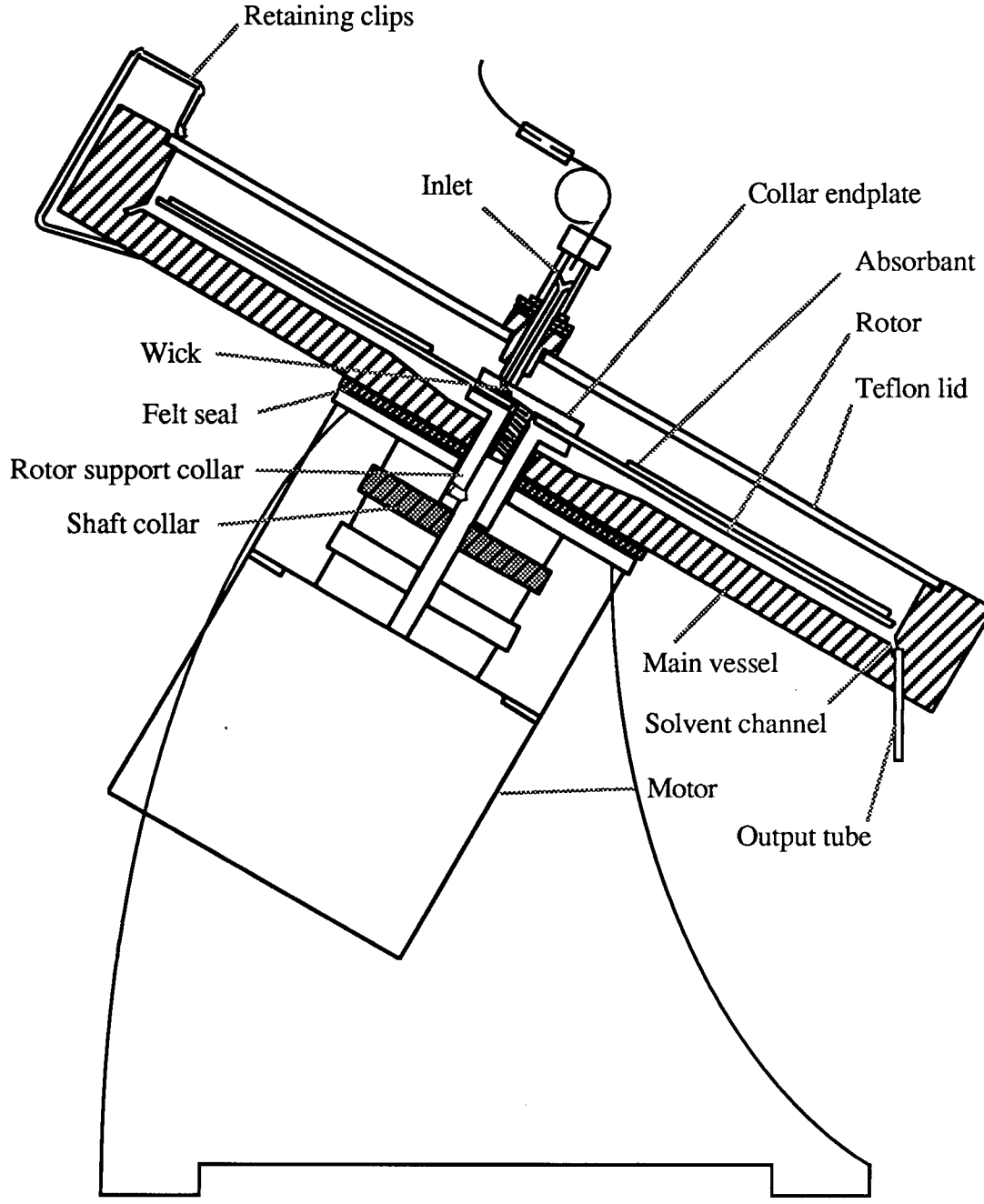


Fig. 2.3. Chromatotron™.

2.4. Gas-liquid chromatography

Gas chromatography started in the 1950's after James and Martin in 1951 published a paper describing its potential (Lochmüller, 1978). In gas-liquid chromatography [GLC] the mobile phase or carrier gas flows through a column packed with a solid support. The stationary phase is a thin film of non-volatile liquid coated on the surface of the support. The mixture of compounds to be separated is introduced through an inlet, through which a carrier gas (He or N_2) also flows. This inlet should be hot enough to flash evaporate the sample. Separation is based upon the differences in the solubilities of compounds in the liquid phase, and upon differences in vapour pressures. Thus, a high solubility gives a low vapor pressure above the liquid phase and vice versa. The choice of stationary phase and temperature required for good separation for a specific mixture depends on the functional groups, structure and molecular weight of the compounds in question.

2.4.1. Flame ionization

With a flame ionization detector, an ignited hydrogen-air flame ionizes the sample components as they elute from the column and ions are collected at electrodes, producing a current. This is used for quantitative GLC and the sample is totally lost unless a pre-detector splitter is used to retrieve part of the sample.

2.4.2. Thermal conductivity

The thermal conductivity cell has two or four filaments. A reference column is needed through which only carrier gas flows since the signal depends on the difference in heat conductivity between sample plus carrier and carrier alone. There will be no signal when the composition of the gas is constant, since the heat loss is constant. Helium is a preferred carrier gas due to its high thermal conductivity; most organic vapours have low thermal conductivities. When a compound in its vapour phase comes through the detector, there will be a difference in heat loss between the two columns, giving the signal. Thermal conductivity detectors are non-destructive; i.e., the separated compounds can be collected for further analyses. They are, however, not suitable for quantitative work since response is usually non-linear (Lochmüller, 1978). Capillary columns with thick coatings were used in this study to increase separation efficiency and loading capacity, the latter ability being quite important when compounds are to be collected from a column (Grob and Grob, 1983).

2.5. Mass spectrometry

Two types of mass spectrometers were used in this study. In the first, a combined gas chromatograph-mass spectrometer (GC/MS), the structure and mass of molecular fragments are determined from the fragmentation pattern of the carbon skeleton; different chemical structures and functional groups have characteristic fragmentation patterns that can be used to deduce the structure and molecular weight of the parent molecule. In the second a C,H,N-analyzer was combined with a stable carbon isotope mass spectrometer (SCIMS) and the relative abundance of isotopes ^{12}C and ^{13}C was determined. The mass spectrometer is used to analyze substances in the gaseous state and separates them according to their molecular masses. The gaseous sample, before it is analyzed, must be ionized, most commonly by bombarding it with electrons emitted from a heated filament. When the sample molecule is struck by an electron with sufficient energy to remove an electron, a molecular ion results:

$$M + e^- \rightarrow M^+ + 2e^-$$

In most applications, only the positive ions are used for analysis. These charged particles, when injected into a magnetic field, will move in a circular orbit of constant radius. This radius is dependent upon the velocity of the particle, its mass, its charge, and the strength of the magnetic field. The ions are separated in the analyzer by their molecular masses and their detection is enhanced by an electron multiplier or a plate collector (Fessenden and Fessenden, 1982; Wolfe, 1984; Howe *et al.*, 1981).

2.5.1. Quadrupole mass spectrometer

A quadrupole, also called a mass filter, consists of four parallel circular rods that irradiate an electrodynamic field. Opposite rods are electrically connected. The ions which enter and can pass through this field depend upon the amplitude of the quadrupole voltages; a range or a single selected ion mass resonates through the filter while others will be pumped away (Wolfe, 1984). This type of mass spectrometer was used to identify individual organic compounds using their patterns of fragmentation.

2.5.2. Electromagnet mass spectrometer

With an electromagnet, the mass spectrometer is used with a fixed voltage and a changeable magnetic field. However, all parameters can be changed in the sense that voltage can be varied and all the collectors can be adjusted before the actual analysis is undertaken. The various instruments have either two or three collectors (spatially separated) which facilitate the simultaneous collection of major and minor isotopes and enhance the precision of

isotope ratio measurements. Masses 44 and 45 ($^{12}CO_2$, $^{13}CO_2$) will each give an ion current, and the ratio of these is then measured so that isotopic ratio is directly related to current ratio (Gross, 1978; Wolfe, 1984).

3. EXPERIMENTAL PROCEDURES AND DATA FOR BULK SEDIMENTS FROM SOUTH BASIN, POWELL LAKE, B.C.

The extraction scheme shown in Table 3.1 outlines the procedures used for sediments which were collected and frozen on site at Powell Lake, B.C. in July, 1981. The samples are bulk cores representing a period of about 200 years of sedimentation.

3.1. Extraction and hydrolysis of South Basin sediments

Sample S.B. 1984-2 was collected July 9, 1981 and frozen on site; on July 6, 1984 it was thawed, acidified to pH 3.5 with 25 ml 6.5 M HCl and freeze dried with a Virtis™ freeze dryer. The freeze dried sample was then extracted with benzene:methanol (3:2) using a solvent-to-sediment ratio of 4:1 to 5:1.

The extract (18.4 g) was collected in three parts, and hydrolyzed as three subfractions; base was added to make solutions up to 0.3 M in KOH; the solution was flushed with N_2 for 10 minutes to remove oxygen and then refluxed for 12 hours. Hydrolysis was assumed to be complete after 12 hours if the residual solutions were alkaline. The yields for all samples from South Basin sediments after extraction and hydrolysis are summarized in Table 3.2.

The thimbles used for the sediments in the soxhlet extractions would quite often overflow with solvent. The very fine grained sediments, which had been ground on a ring grinder after they had been dried and sieved, would not allow the solvent to flow at an appreciable rate. Celite™ (pre-extracted with benzene-methanol) was mixed in with the sediments to alleviate 'clogging' and increase flow rates.

It was more effective to hydrolyze the total extract rather than first trying to extract and separate a free acid fraction from the neutrals. The latter method caused the development of emulsions in which it was quite difficult to separate the organic phase from the H_2O/methanol/benzene phase.

Table 3.1 GENERAL EXTRACTION SCHEME FOR SEDIMENT SAMPLES

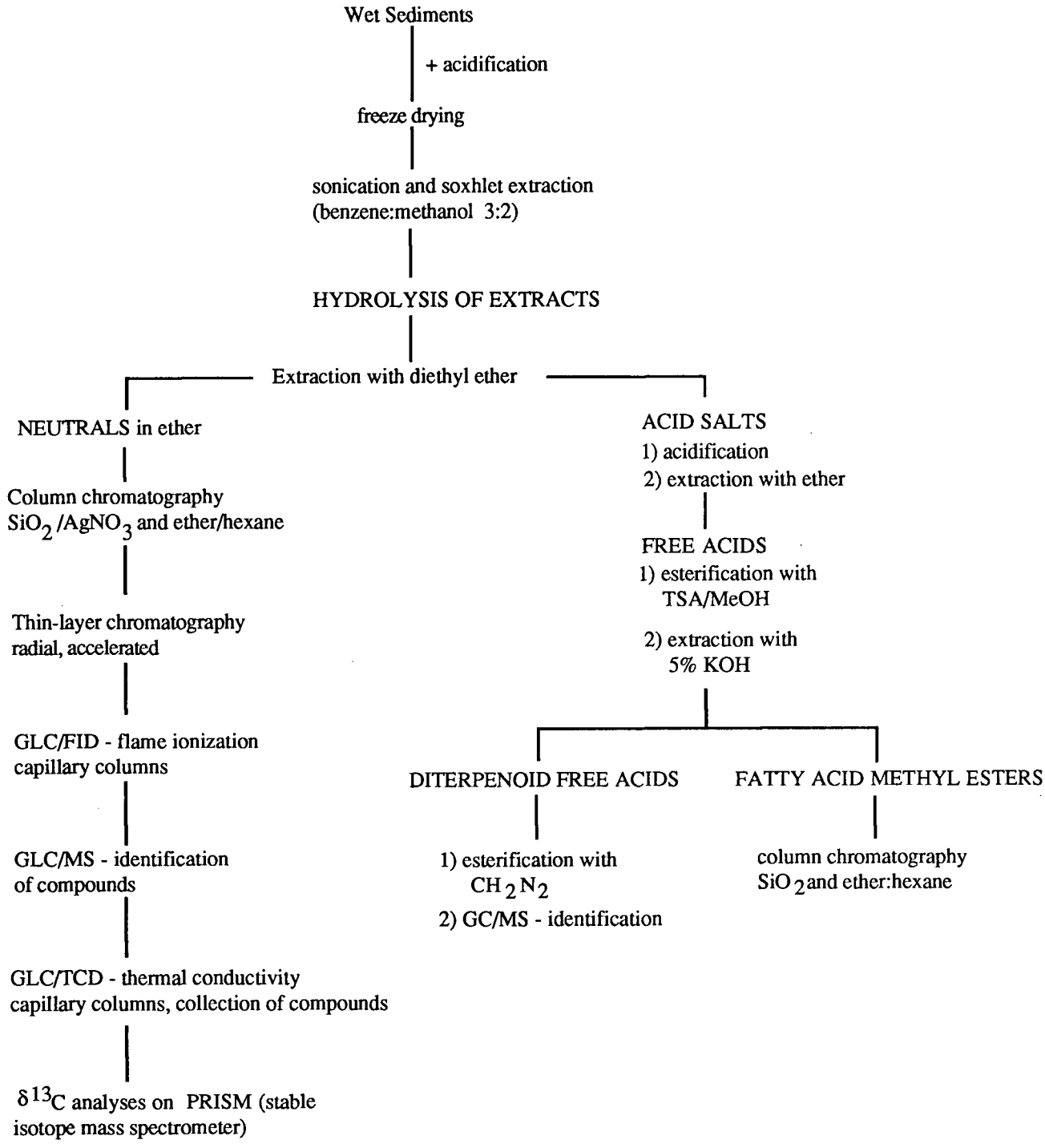


Table 3.2 shows the yields of four different bulk samples from South Basin. In the following procedures, the results for only one set of sediments, S.B. 1984-2, are given, as after completion of all extraction procedures for the neutral compounds, it was obvious that the neutrals from this sample represented the best set of compounds with respect to purity for the stable carbon isotope analysis. It might have been beneficial to have continued clean-up procedures, but since only micrograms or at best one or two milligrams of each compound were obtained in each case, any further procedures would have diminished the yield even further. It was decided to do SCIMS analysis only on S.B. 1984-2.

Table 3.2. Yields after extraction and hydrolysis

BULK SEDIMENTS SOUTH BASIN	S.B. (1984-2)	S.B. (1984-1)	S.B. (1984-3)	S.B. (1986-1)
collected				
wet sediments	1713.7 g	1715.6 g	1437.1 g	1662.7 g
dry sediments	202.7 g	167.2 g	173.7 g	151.8 g
dry sediments				
<0.5mm	199.0 g	162.7 g	161.7 g	150.3 g
after sonication and soxhlet extraction	18.4 g	6.2 g	7.4 g	13.5 g
hydrolysis				
free acids	304.8 mg	420.0 mg	74.2 mg	260.1 mg
	1.7% extr.*	6.87% extr.	1.0% extr.	1.9% extr.
neutrals	171.0 mg	447.5 mg	45.5 mg	288.6 mg
	0.9% extr.*	7.2% extr.	0.6% extr.	2.1% extr.

*% extractable after sonication and soxhlet extraction of sediments

3.2. Chromatography

3.2.1. Column chromatography of S.B. (1984-2), retene, and perylene

Column chromatography was the next procedure (Table 3.1) used on the neutral fraction from S.B. (1984-2) to isolate the diterpene hydrocarbons and alcohols. Four fractions were recovered with a yield of 79%; the details are summarized in Table 3.3.

Table 3.3. Column chromatography of neutrals

EXTRACT: 171 mg in 17 ml hexane

COLUMN: 15 g SiO_2 (ca. 1 g SiO_2 to 10 g of solute) 'prewetted' with H_2O and pre-eluted with 20 ml hexane.

COLLECTED:	Weight
SOLVENT ADDED:	
90 ml hexane (4.5 × column vol.)	33.0 mg
110 ml 1% Et_2O/hexane (5.5 × column volume)	9.0 mg
100 ml 5% Et_2O/hexane (5 0 × column volume)	42.3 mg
80 ml 20% Et_2O/hexane (4 0 × column volume)	51.3 mg
	135.6 mg
135.6 mg/171 mg =	79% yield
RESIDUE: mainly pigments left on column.	21%

Two compounds, retene and perylene, were collected using accelerated thin-layer chromatography on a Chromatotron™; they were then purified on another column prior to mass spectrometry and SCIMS. Both compounds are fluorescent, so it was possible to follow the movement of the sample down the column using a UV lamp (366 nm) (Table 3.4).

Table 3.4. Column chromatography of Retene and Perylene

RETENE: #17 from Chromatotron™

COLUMN: 0.75 g SiO_2 (4% $AgNO_3$ w/w) and pre-eluted with 1.5 ml hexane

COLLECTED: retene in 5% Et_2O/hexane Weight: 2.4 mg

RECOVERY: 2.4 mg/3.18 mg × 100 = 75.5%

PERYLENE: #19→22 from Chromatotron™

COLUMN: 1.5 g SiO_2 (4% $AgNO_3$ w/w) and pre-eluted with 3 ml hexane

COLLECTED: Perylene in 10% Et_2O/hexane Weight: 0.98 mg

RECOVERY: 0.98 mg/ 5.25 mg × 100 = 18.7%

3.2.2. Thin-layer chromatography of S.B. (1984-2)

Only the hexane fraction containing saturated and unsaturated hydrocarbons was used from the original separation of the neutral extract by column chromatography. A mixture of retene, n-C_{21}, and anteiso-C_{18} was used to investigate whether urea adduction or clathrates with molecular sieves could be used to separate the normal, saturated and branched hydrocarbons from unsaturated hydrocarbons and aromatic diterpenes.

UREA ADDUCTION: Normal saturated hydrocarbons and branched saturated hydrocarbons were adducted in a urea clathrate. It was, however, difficult to grow large crystals of urea and to separate them from supernatant.

5 Å MOLECULAR SIEVES: Both retene and n-C_{21} were adsorbed but the saturated and unsaturated hydrocarbons were not separated. These conventional methods may have failed either because of the small sample size used or because the 5 Å molecular sieve did not discriminate the tricyclic retene from n-C_{21}. As a result radial accelerated thin-layer chromatography using a Chromatotron™ was used to isolate hydrocarbon subfractions (Table 3.5).

3.3. Gas-liquid chromatography and quadrupole mass spectrometry

GC-MS was used to identify the separated single organic compounds and to verify their purity. In the case where several of the desired compounds were in the same fraction, it was then necessary to use preparative gas-liquid chromatography with a thermal conductivity detector [GLC-TC] in order to separate and trap them.

Both retene (Fig. 3.1) and perylene (Fig 3.2) were analyzed on a Hewlett-Packard Model 5880 gas chromatograph-mass spectrometer under the following conditions:

Column:	007-methyl silicone, I.D. 0.32 mm, length: 10 m
Mass range:	10→450
Temperature programming:	from 50°C→170°C at 30°C/min.
	from 170°C→275°C at 5°C/min.

The GC-MS ion chromatograms of 6-methyl hexadecane (Fig. 3.3), dehydroabietin and dehydroabietane (Fig. 3.4), and tetrahydroretene and simonellite (Fig. 3.5) show five of the compounds which were later separated and collected as five separated fractions using GLC-TC. Only the relevant peaks in each of the three chromatograms have been identified. Conditions for the GC-MS were similar to those for retene and perylene.

Table 3.5. Thin-layer chromatography of hydrocarbons on a Chromatotron™

EXTRACT: S.B. (1984-2) neutrals (33 mg) in hexane

CHROMATOTRON™: Model 7924., Harrison Research, CA.

PLATE: 1 mm $SiO_2/AgNO_3$—activated at 70°C before use.

Silica Gel PF-254 (Merck) with $CaSO_4 \cdot 1/2H_2O$	50 g
H_2O	100 ml
$AgNO_3$	2 g

FRACTIONS COLLECTED: 1→22

# 1→5	eluted with hexane*
# 6→10	eluted with 1% EtOAc/hexane*
#11→15	eluted with 5% EtOAc/hexane*
#16→20	eluted with 10% EtOAc/hexane*
#21→22	eluted with EtOAc**

CONCENTRATES:	WT. RECOVERED	
#1→4	0.10 mg	
#5→6	8.35 mg	
#7→9	0.39 mg	
#10→13	0.70 mg	
#14	0.14 mg	
#15	0.80 mg	
#16†		
#17	3.18 mg	(retene)
#18	2.86 mg	
#19→22	5.25 mg	(perylene)
	21.77 mg/33 mg × 100 = 66.0% recovery	

*each fraction contained in 4→5 ml of solvent

**contained in 11 ml, followed by a rinse of 33 ml

†contaminated

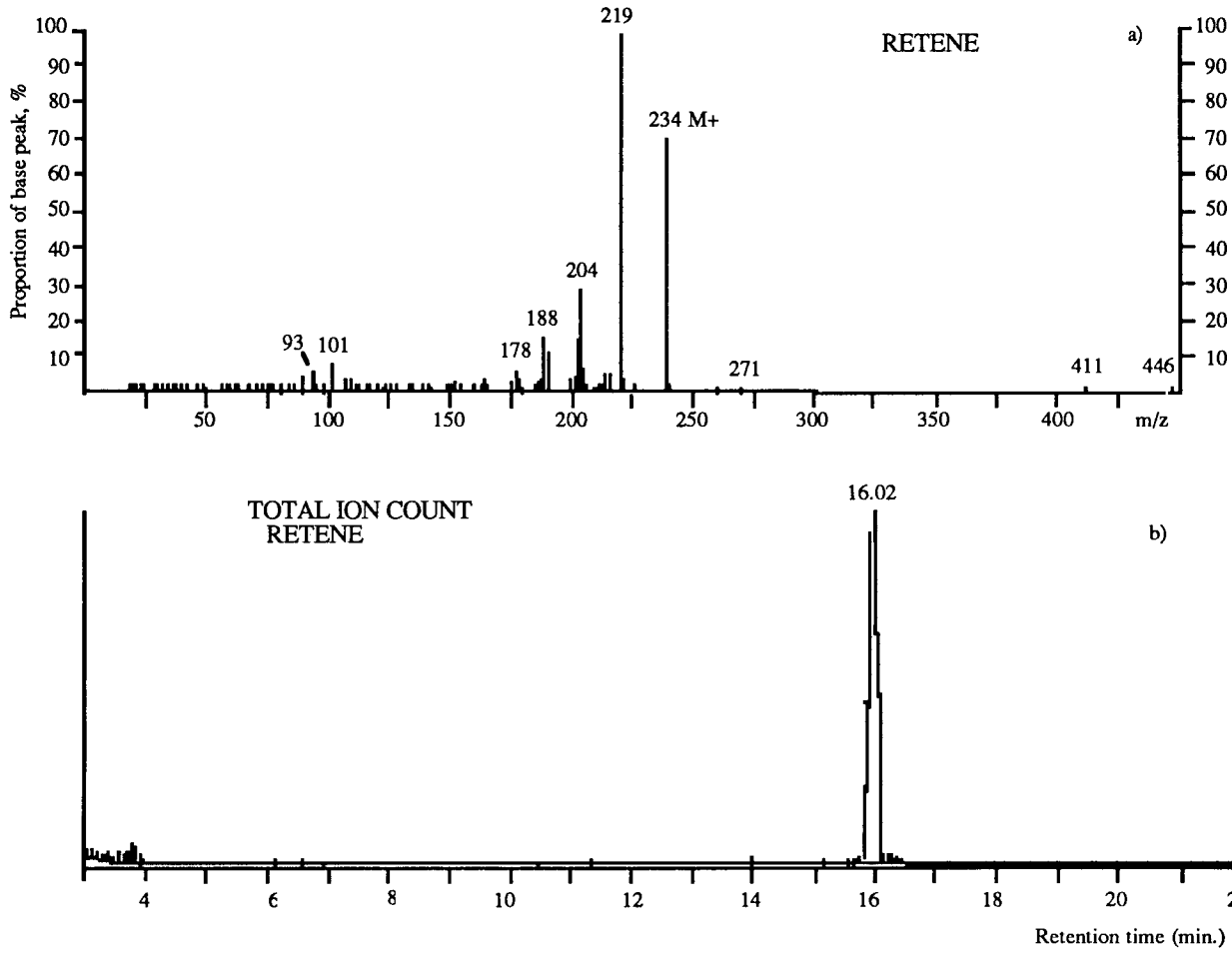


Fig 3.1. a) Mass spectrum of retene: M+234 ($C_{18}H_{18}$); base peak: 219; b) total ion chromatogram of retene

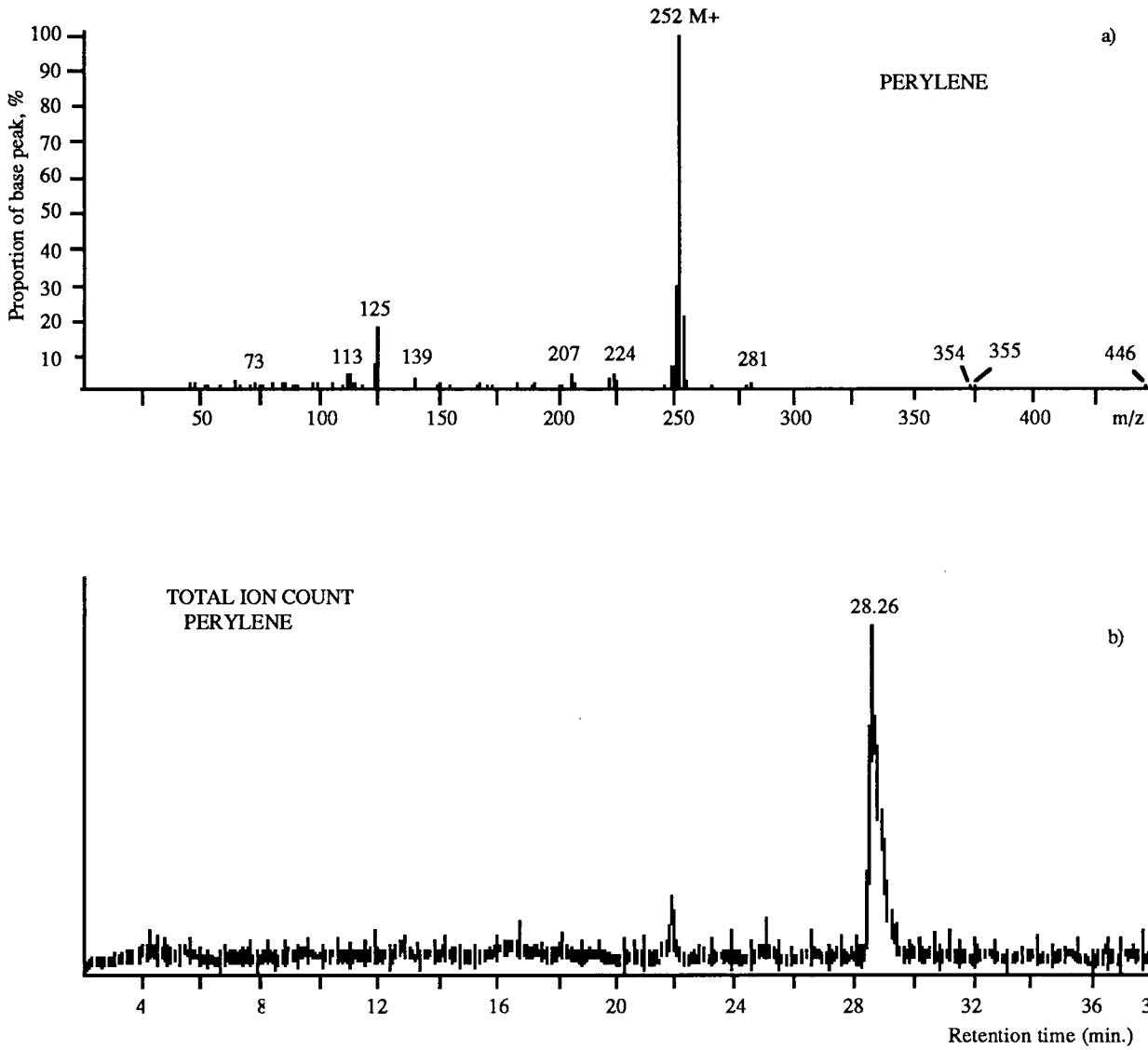


Fig 3.2. a) Mass spectrum of perylene: M+252 ($C_{20}H_{12}$); base peak: 252; b) total ion chromatogram of perylene.

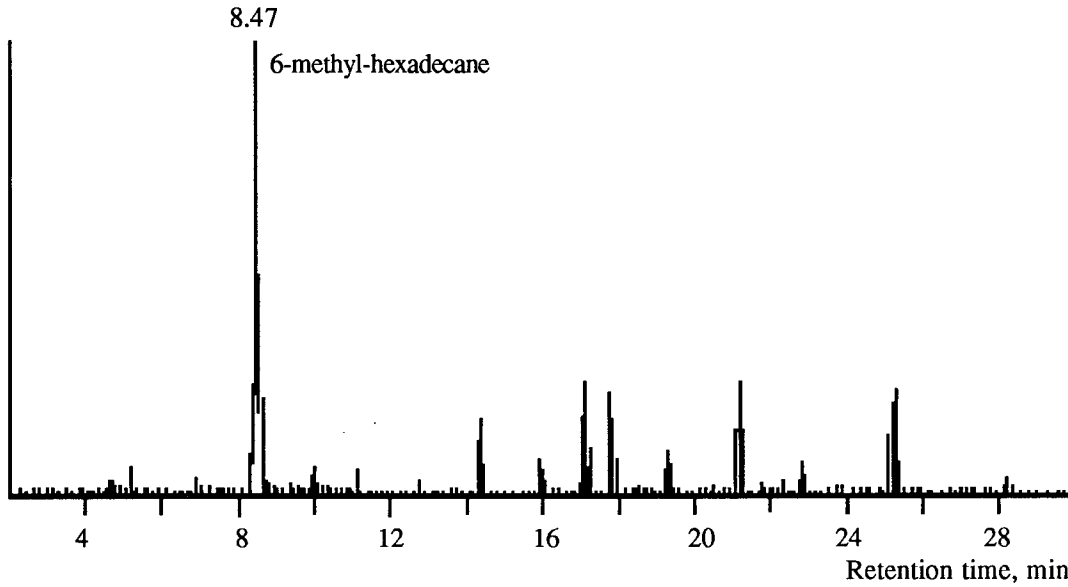


Fig 3.3. Total ion chromatogram of 6-methyl hexadecane.

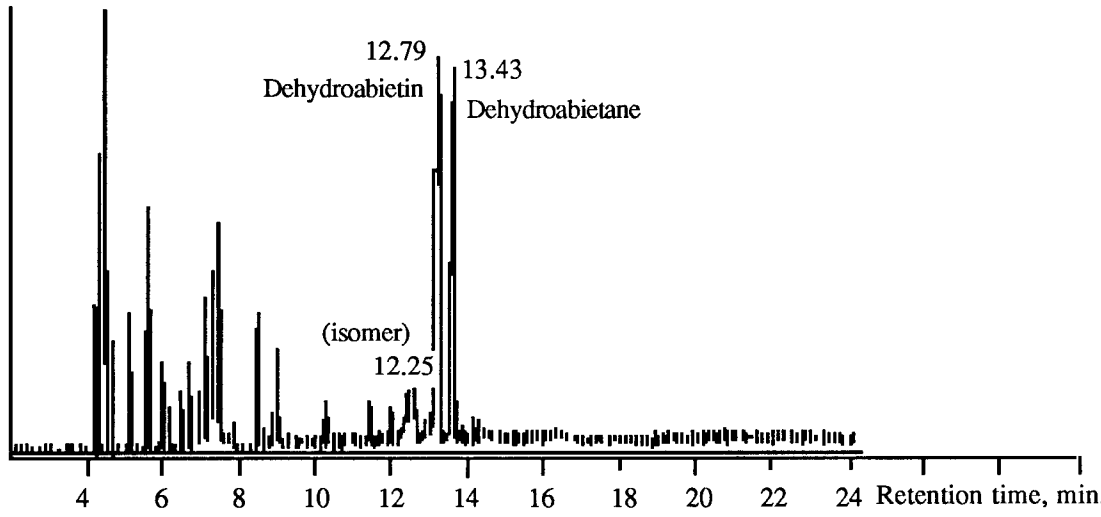


Fig 3.4. Total ion chromatogram of dehydroabietane and dehydroabietin.

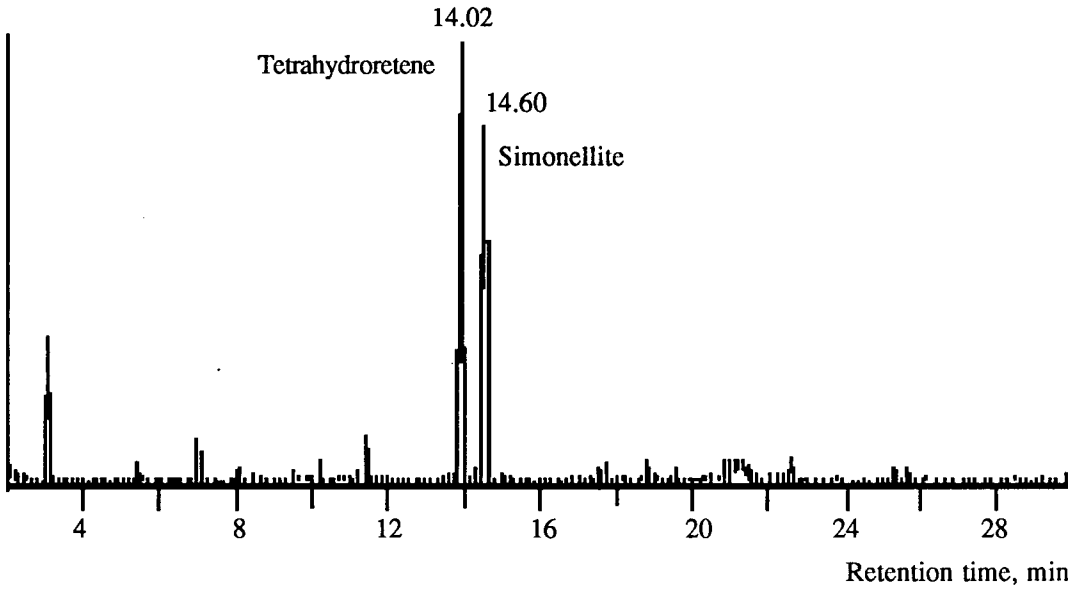


Fig 3.5. Total ion chromatogram of tetrahydroretene and simonellite.

3.4. Gas-liquid chromatography with thermal conductivity detector (GLC-TC)

It had been verified (see Section 3.3.2) that several of the collected fractions from the Chromatotron™ contained the desired compounds, but that further separation and collection were needed. After collection of these compounds, their identity and purity were verified using GC-MS.

3.4.1. GLC-TC of S.B. (1984-2) neutral compounds

Preparatory GLC-TC was used to purify and to collect selected compounds off a Thikote™ capillary column. Conditions and samples are summarized in Table 3.6.

3.4.2. GC-MS Ion chromatograms and mass spectra of 6-methyl hexadecane, dehydroabietin, dehydroabietane, tetrahydroretene, and simonellite

Spectra and total ion plots are shown in Figures 3.6, 3.7, 3.8, 3.9 and 3.10. All the above compounds were analyzed on a Hewlett-Packard Model 5880 gas chromatograph/mass spectrometer. Conditions are summarized in Table 3.7.

Table 3.6. Preparatory GLC-TC

EXTRACT: S.B. (1984-2) neutrals 21.77 mg (total weight) from Chromatotron™

SEPARATE FRACTIONS COLLECTED:	CHROMATOTRON™ #	WT.	WT. after PREP GLC-TC
1) 6-methyl-hexadecane	#5→6	8.35 mg	0.28 mg
2) Dehydroabietane +	#10→13	0.70 mg	0.11 mg
Dehydroabietin	#10→13	0.70 mg	0.08 mg
3) Tetrahydroretene +	#15	0.80 mg	0.31 mg
Simonellite	#15	0.80 mg	0.25 mg

GLC-TC CONDITIONS:

BRIDGE CURRENT	HE FLOW	OVEN TEMP	COLUMN
120 ma	15ml/min	t_0 150°C	5 µm Thikote™ [0.53 mm I.D.] 10 m length
		or	
		t_0 170°C	007-methyl silicone (Quadrex™)

COMPOUND	**TEMPERATURE PROGRAM**		
6-Methyl-hexadecane.	t_0 150°C	5°C/min to	190°C
		10°C/min to	255°C and hold for 7 min.
Dehydroabietin and	t_0 150°C	5°C/min to	180°C isothermal, collect compounds
Dehydroabietane		10°C/min to	255°C
Tetrahydroretene and	t_0 170°C	5°C/min to	190°C isothermal, collect compounds
Simonellite		10°C/min to	255°C

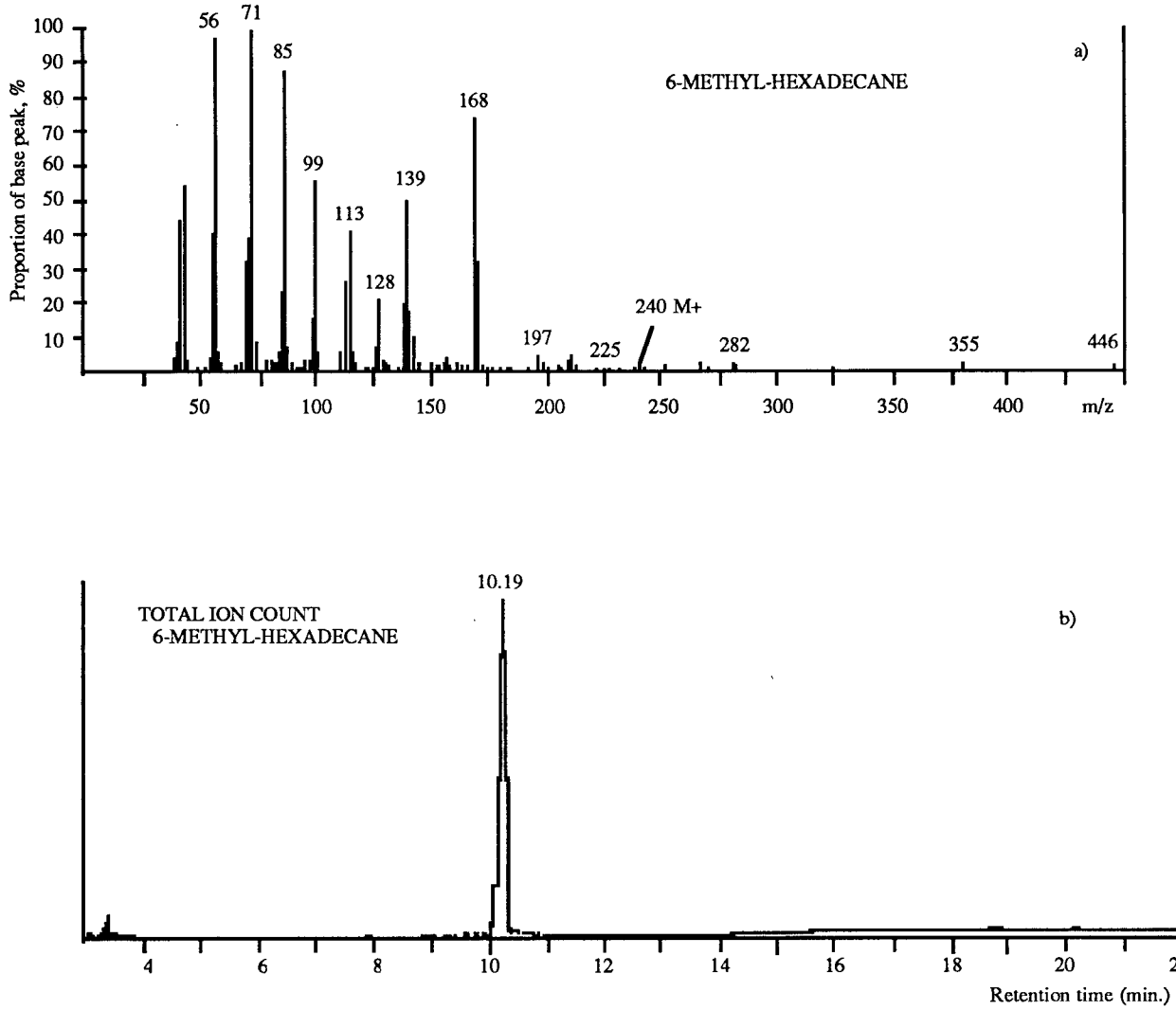


Fig 3.6. a) Mass spectrum of 6-methyl hexadecane: base peak: 71; M^+240 ($C_{17}H_{36}$); M-15: 255; M-C_5H_{12}: 168; M-C_7H_{17}: 139; b) total ion chromatogram of 6-methyl hexadecane

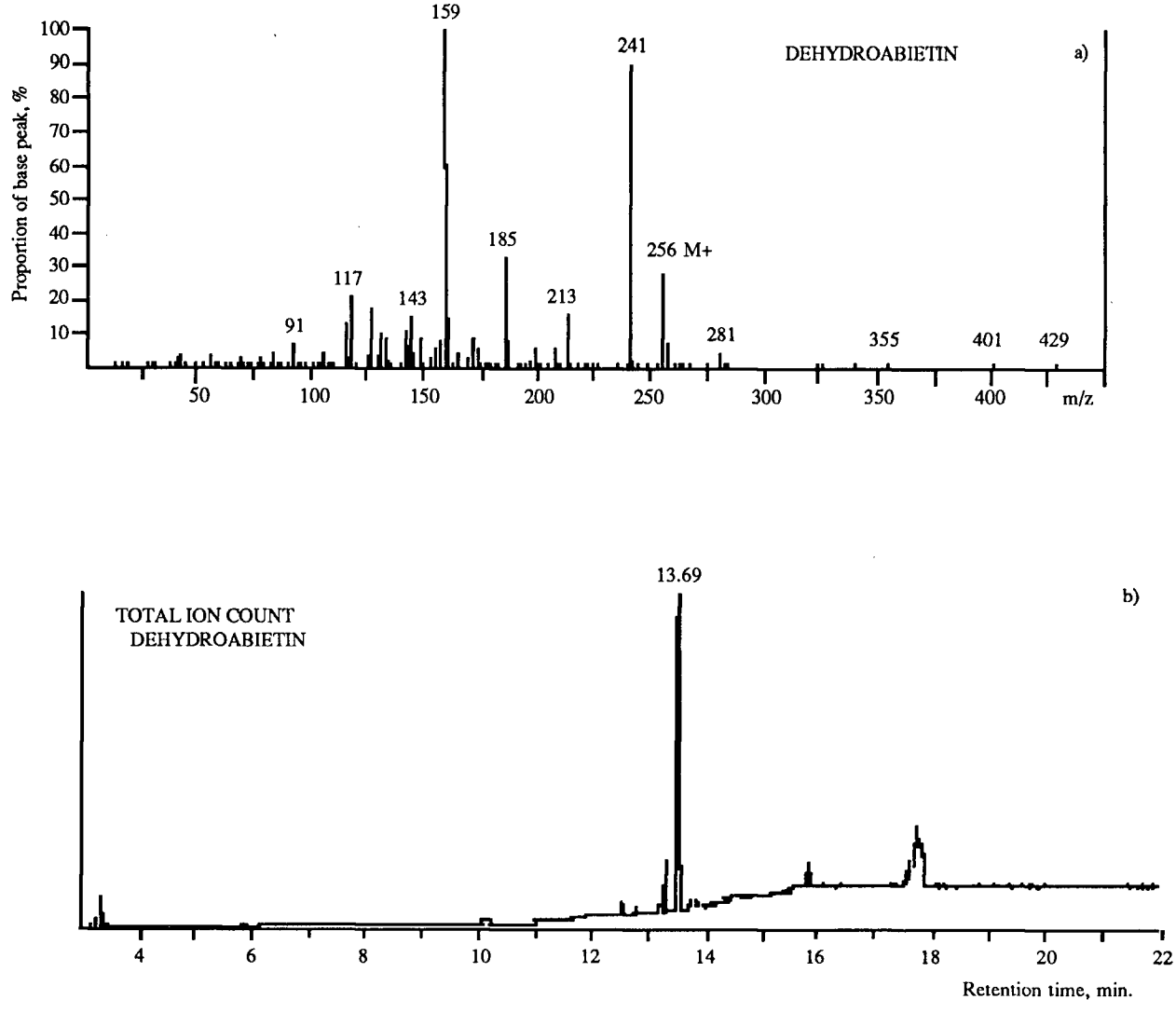


Fig 3.7. a) Mass spectrum of dehydroabietin: base peak: 159; M^+256 ($C_{19}H_{28}$); M-15: 241(CH_3 lost at C-4); b) total ion chromatogram of dehydroabietin

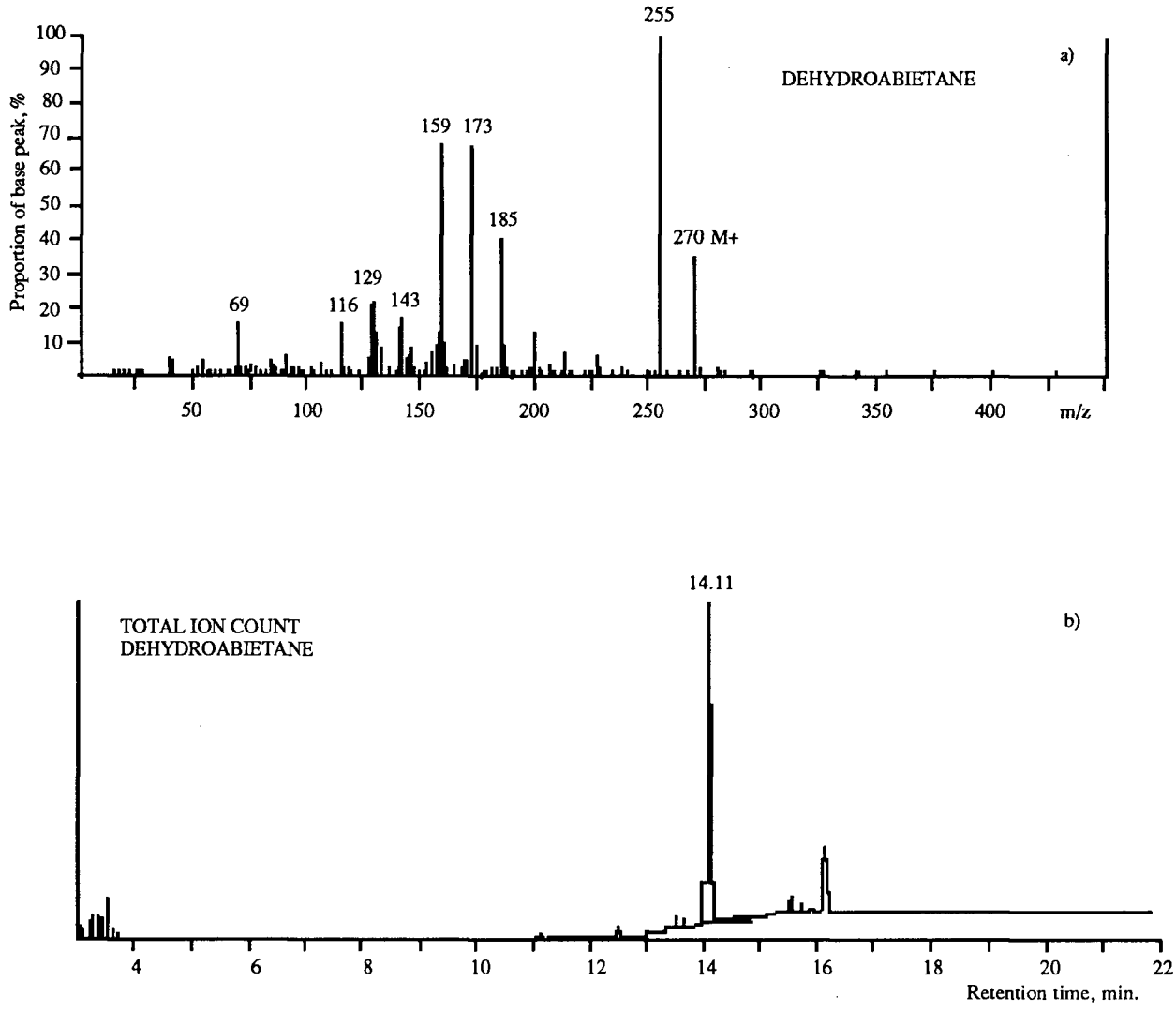


Fig 3.8. a) Mass spectrum of dehydroabietane: base peak: 255; M^+270 ($C_{20}H_{30}$);M-97: 173; M-111: 159; b) total ion chromatogram of dehydroabietane

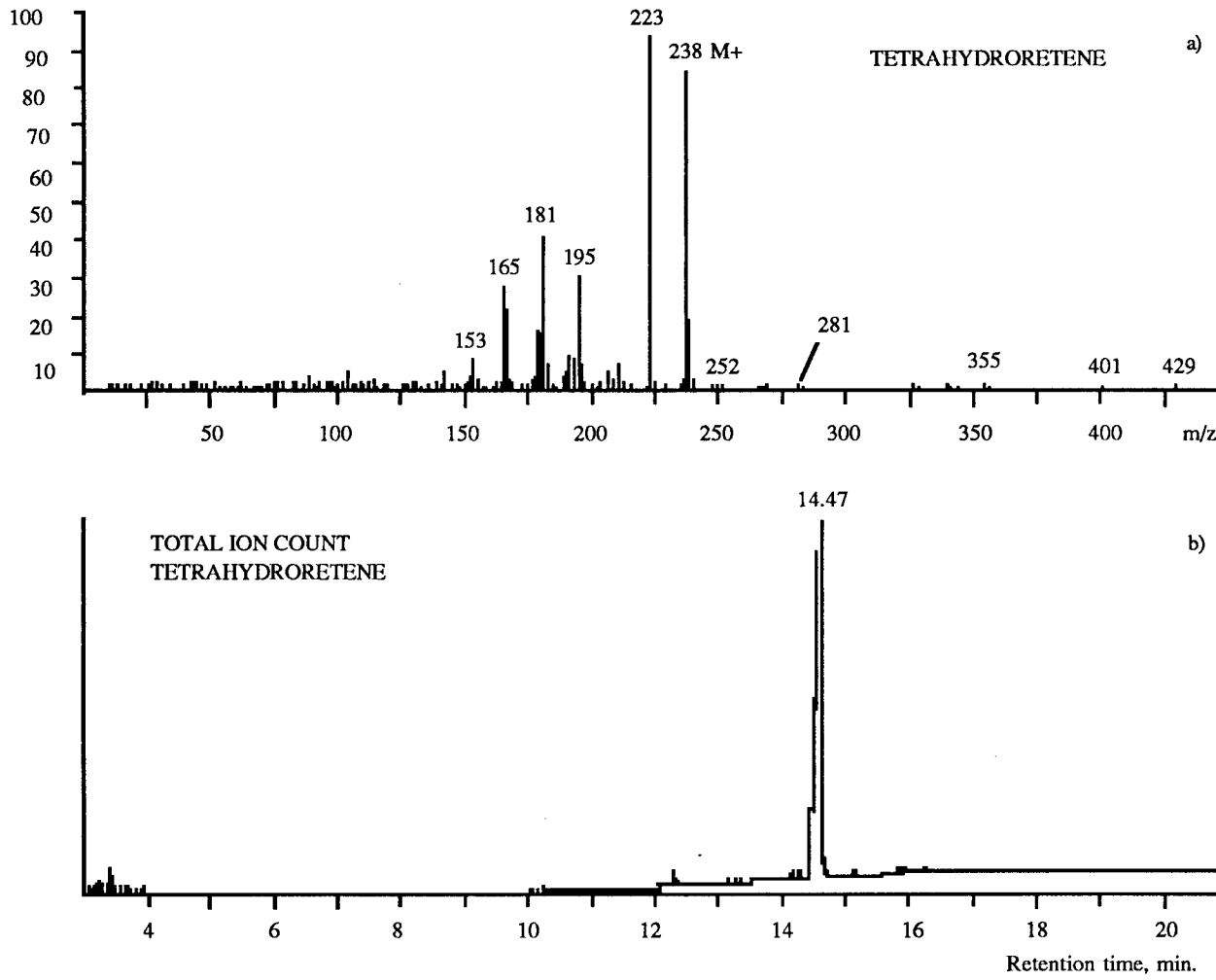


Fig 3.9. a) Mass spectrum of tetrahydroretene: base peak: 223; M^+238 ($C_{18}H_{22}$);M-43: 195; b) total ion chromatogram of tetrahydroretene.

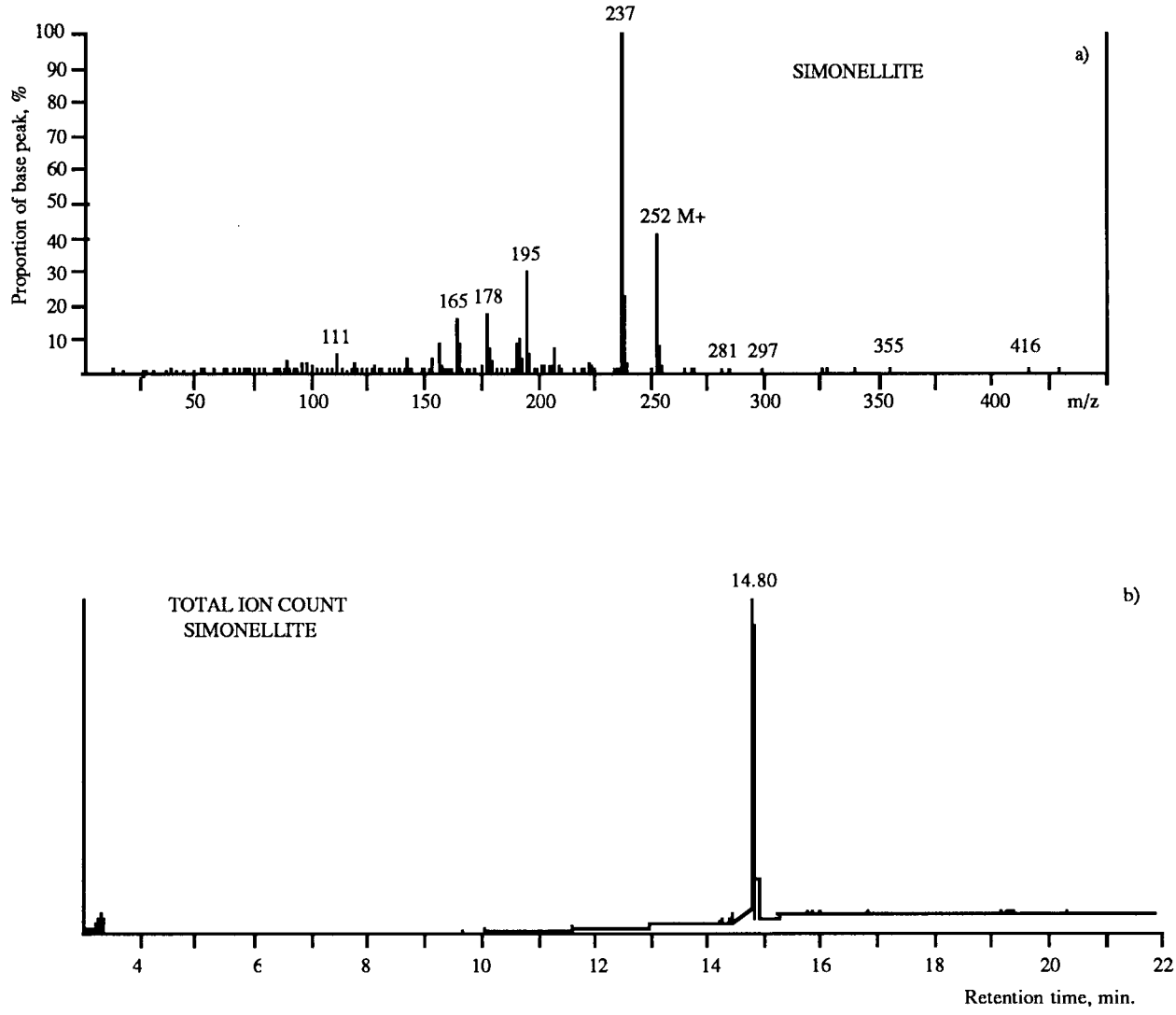


Fig 3.10. a) Mass spectrum of simonellite: base peak: 237; M^+252 ($C_{19}H_{24}$);M-57: 195; b) total ion chromatogram of dehydroabietane

Table 3.7. Conditions for GC-MS of diterpenes and 6-methyl hexadecane

Column: 007-methyl silicone.	I.D.: 0.32 mm.	Length: 10 m
Inlet pressure:	8 psig (He)	
Scanning:	from 3 min.	
Mass range:	10→450	
Temperature program	from 50°→170°C	at 30°C/min.
	from 170°→285°C	at 10°C/min.

3.4.3. Summary of collected compounds for GC-MS and SCIMS

The five hydrocarbons isolated from sediments from South Basin, Powell Lake, B.C., purified and subsequently used to measure $\delta^{13}C$ values are summarized in Table 3.8. All mass spectra were compared to published spectra (Wakeman *et al.*, 1980; Simoneit, 1975; 1977; Gelpi *et al.*, 1970).

Table 3.8. Summary of compounds isolated and collected from S.B. (1984-2)

COMPOUND	WEIGHT
6-methyl-hexadecane	0.28 mg
Dehydroabietin	0.08 mg
Dehydroabietane	0.11 mg
Tetrahydroretene	0.31 mg
Simonellite	0.25mg
Retene	2.40 mg
Perylene	0.98 mg

4. EXPERIMENTAL PROCEDURES AND DATA FOR DOUGLAS FIR

A Douglas Fir (7 years old) was cut down in U.B.C. Research Forest in Maple Ridge on January 21, 1987. It was cut into four pieces, rolled into acetone cleaned aluminum foil and placed in a Coleman™ cooler. Liquid N_2 was poured over the wood and after transportation to the laboratory, the wood was transferred into a freezer. The wood was cut into smaller pieces before freeze drying. After freeze drying it was stored in glass containers before finally being ground into wood-meal (<60 mesh) in a wood grinder.

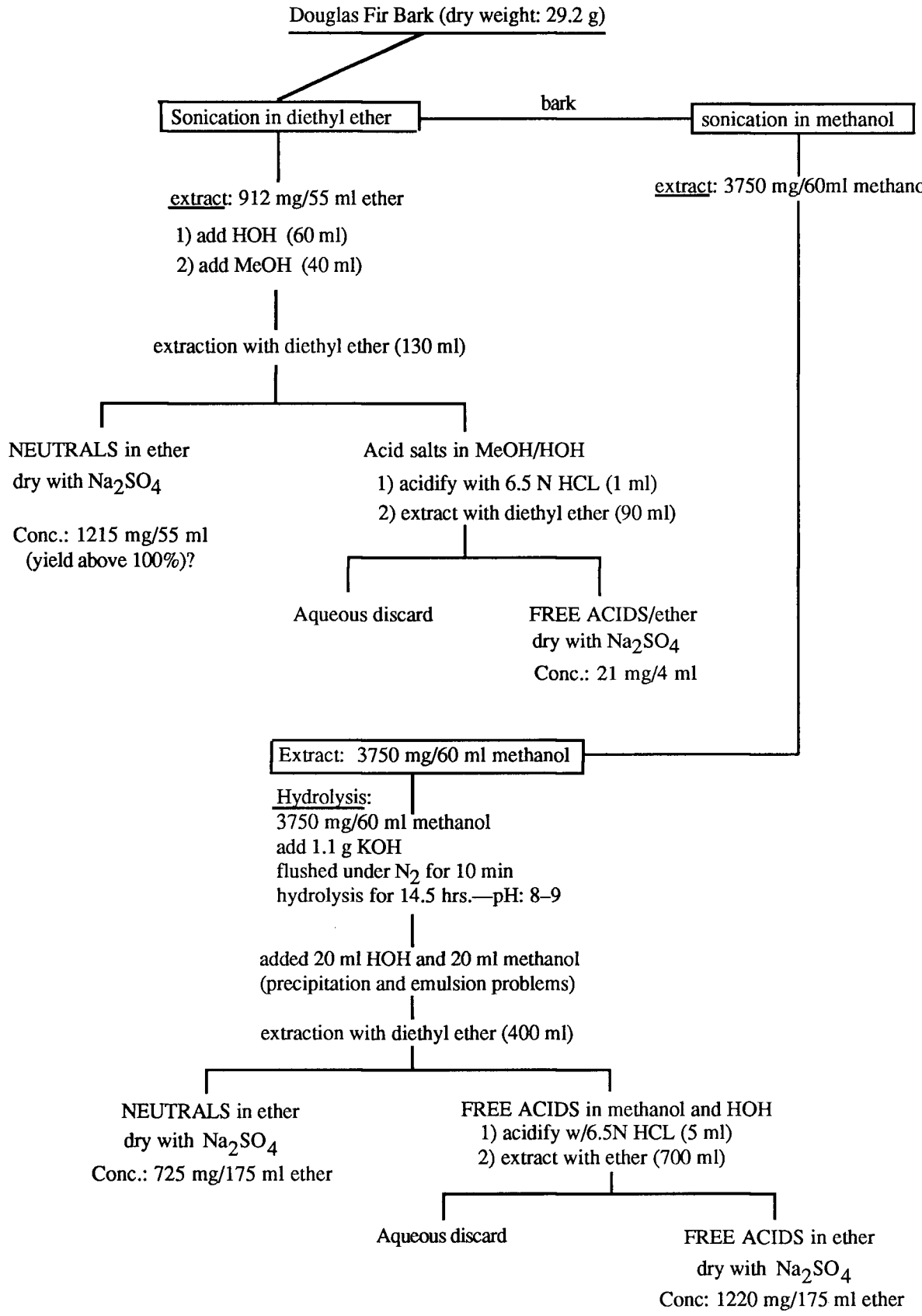
The original intention had been to extract diterpene acids from South Basin sediments in order to have a direct comparison between the $\delta^{13}C$ values of the acids and the $\delta^{13}C$ values of the degraded diterpene hydrocarbons. However, the original extract from S.B. (1984-2) did not contain more than small amounts of dehydroabietic, abietic, and pimaric acids. The coarser fraction (>0.5 mm) did contain an oxidized resin acid, 7-keto-dehydroabietic acid, but, again, not enough for the SCIMS analysis. It was therefore decided to isolate the resin acids from tree bark, to obtain enough material to do the $\delta^{13}C$ analysis; this would provide a reference point, if not a direct one, for the results that would be obtained from the sedimentary diterpene hydrocarbons.

The wood (without the bark) was sonicated first in diethyl ether, then in methanol and extracted into diethyl ether before esterification with toluenesulfonic acid in methanol (TSA/methanol) in order to separate fatty acid esters from diterpene acids. It was assumed that mainly polar compounds were to be found in the extracts. However, GC/MS data of both the diterpene as well as the fatty acid methyl ester fractions contained only small amounts of diterpene acids of the abietane skeleton. This is contrary to what other researchers have found (Swan, 1973; Westfelt, 1966; Ekman, 1979). It is possible that the tree was cut down too early in the year and that the *sap* had not risen; also that it would have been easier to extract these compounds from an older tree. The following data shows the extraction of the bark (only) of the same tree.

4.1. Extraction and hydrolysis of Douglas Fir bark

The extraction of bark from Douglas Fir and hydrolysis of that extract are summarized in Table 4.1.

Table 4.1. Extraction and hydrolysis of Douglas Fir bark.



4.2. Esterification and collection of resin acids from Douglas Fir bark

Esterification and isolation of the methyl esters of the resin acids from Douglas Fir are summarized in Table 4.2. The acidic water left from the above extractions contained a significant precipitate; it was re-extracted with diethyl ether. This fraction, as well as the esters and diterpene acids listed in Table 4.2 was methylated with diazomethane to assure complete esterification.

ESTERIFICATION WITH DIAZOMETHANE:
GENERATION of 700 mg CH_2N_2 in 55 ml diethyl ether from Diazald™:

1) 5 g KOH dissolved in 10 ml 95% ethanol + 8 ml water in ice bath
2) dropwise addition of 5.0 g Diazald™ dissolved in 45 ml diethyl ether followed by a 10 ml ether rinse.

EXTRACTED FRACTIONS METHYLATED WITH DIAZOMETHANE (CH_2N_2:acids: 5:1)	WT. RECOVERED SAMPLE
1) Douglas fir bark, sonicated in methanol, diterpene acid esterified with TSA/methanol.	11.9 mg
2) Douglas Fir bark, sonicated in methanol, free acids only (diterpene acids)	11.9 mg
3) Douglas Fir bark—acidic H_2O extract	2.6 mg

From GC/MS data later obtained, it was observed that 1) and 3) showed diterpene acids, especially fraction 3).

4.3. GC-MS Ion chromatogram and mass spectrum for resin acids

The esters were analyzed on a Hewlett-Packard Model 5880 gas chromatograph-mass spectrometer under the following conditions:

COLUMN:	007-methyl silicone, I.D. 0.32 mm, length: 10 m
MASS RANGE:	10→450
TEMP.PROGRAMMING:	from 50°C→170°C at 30°C/min.
	from 170°C→275°C at 5°C/min.

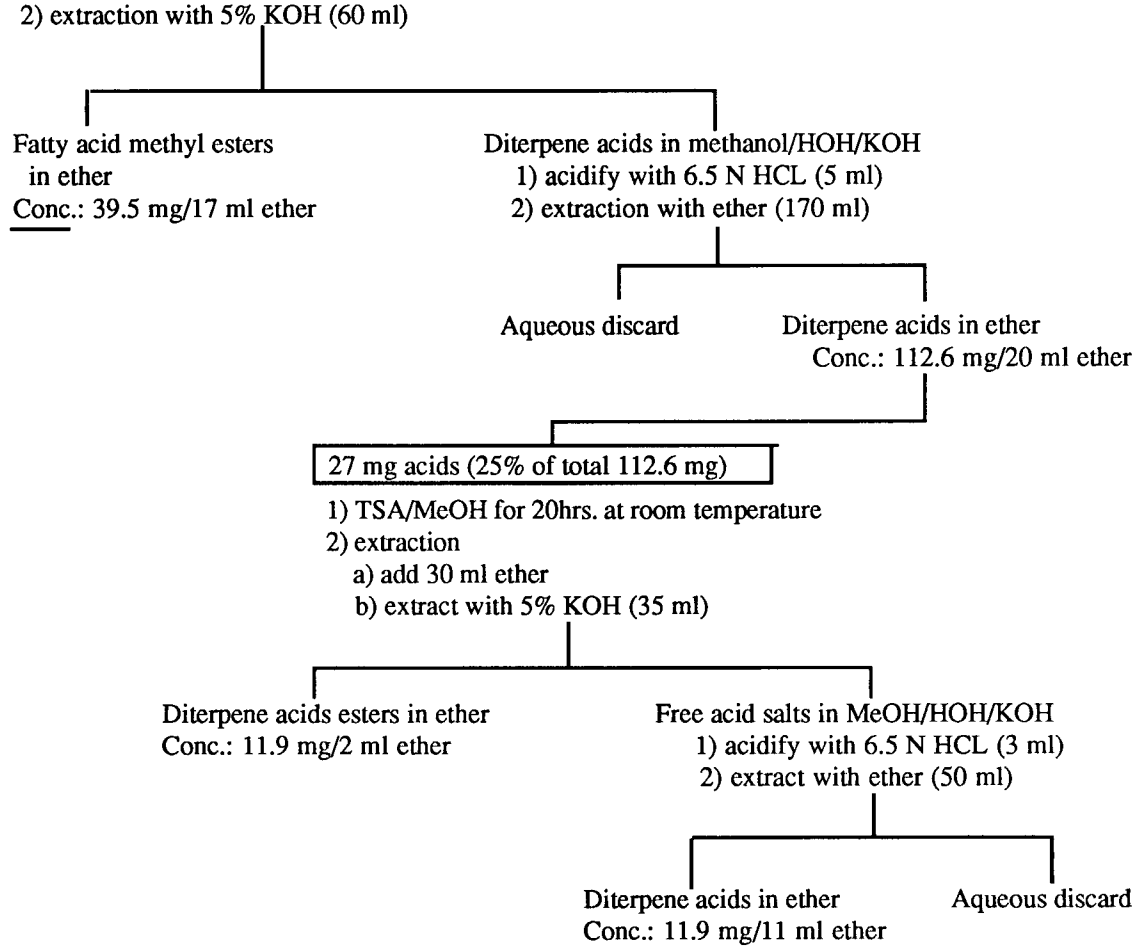
The mass spectrum of dehydroabietic acid methyl ester and a total ion chromatogram of the resin acids are shown in Figure 4.1.

Table 4.2. Esterification and isolation of resin acids.

For esterification:

240 mg (subfraction of 1220 mg FREE ACIDS) in 10 ml ether

1) TSA/MeOH (4 hrs.) - 4% TSA (by weight)
2) extraction with 5% KOH (60 ml)



SUMMARY:

	NEUTRALS	FREE ACIDS
1) DOUGLAS FIR BARK sonicated in ether	1215 mg	21 mg
2) DOUGLAS FIR BARK sonicated in methanol	725 mg	1220 mg

Of the original 240 mg used in the first stage of esterification, 152.1 mg of acids and esters were recovered. Of the recovered compounds, 39.5 mg were fatty acid methyl esters (26.0%) and 112.6 mg were diterpene acids (74.0%). In the second stage of esterification, only 27.9 mg were diterpene methyl esters (44%) and 11.9 mg were diterpene acids (44%).

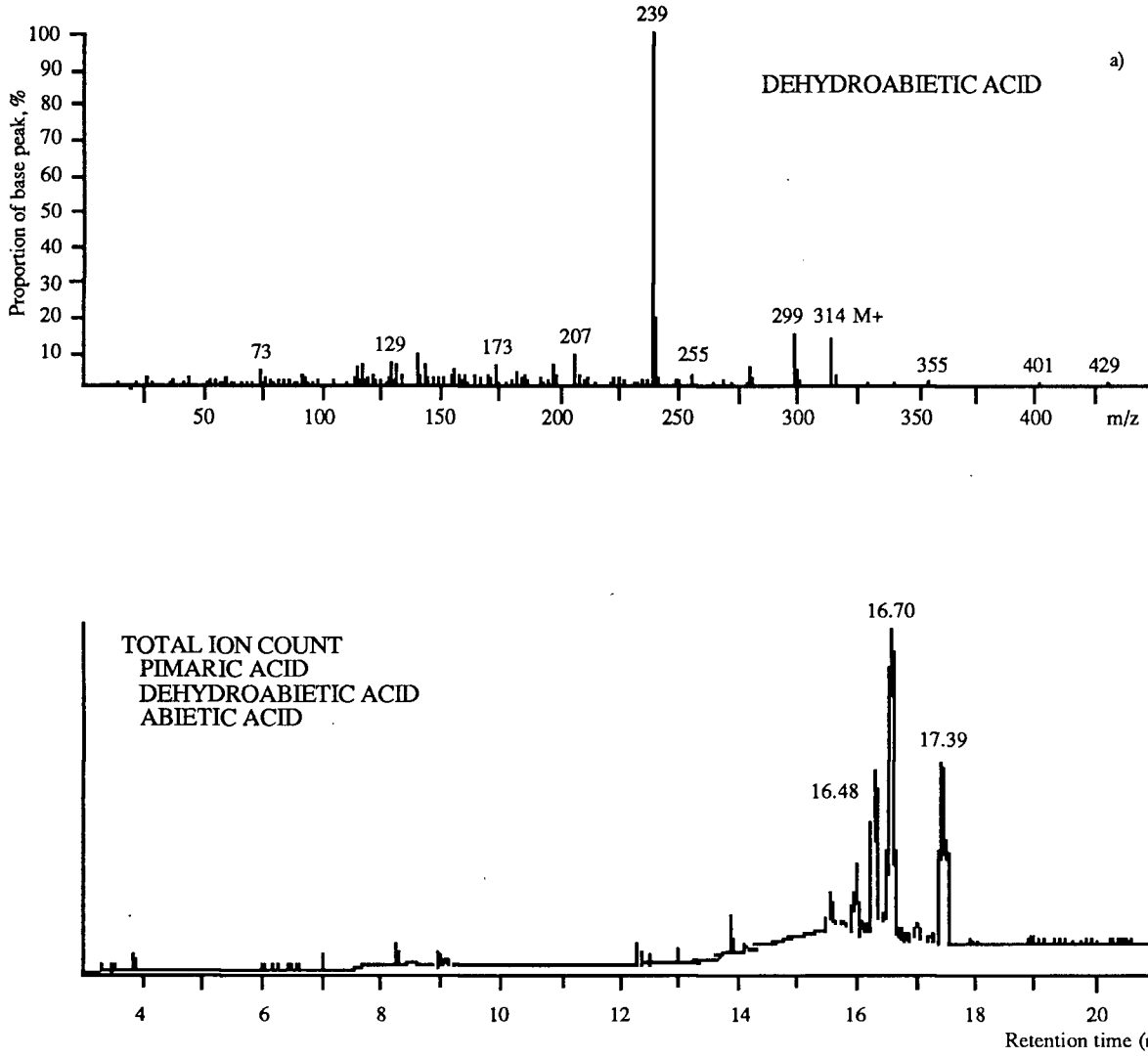


Fig 4.1. a) Mass spectrum of dehydroabietic acid methyl ester: base peak: 239;M^+ 314 ($C_{21}H_{30}O_2$); M-15: 299; b) total ion chromatogram of resin acids

5. SCIMS ANALYSIS OF EXTRACTED AND SEPARATED COMPOUNDS.

For this analysis, a VG Isogas PRISM™ triple collector mass spectrometer and a C,H,N analyzer (Carlo Erba Model 1106) were used. The C,H,N analyzer was directly connected to the mass spectrometer through a narrow stainless steel tubing interface. The organic compounds were combusted in the analyzer and the evolved CO_2 (H_2O, etc.) was carried through a water trap at –92°C, where the water and other impurities were removed by freezing. Subsequently, the CO_2 was collected in a liquid N_2 trap at –196°C. Both traps were attached to the mass spectrometer.

5.1. Analytical procedures for $\delta^{13}C$ analysis

When $\delta^{13}C$ analyses have been performed on organic material, it is common to combust the samples in quartz or borosilicate tubes with copper oxide at a temperature of 900°C or 550°C, depending upon preference and the type of silicate used (Sofer, 1980).

The samples used in this study were quite small and were all contained in a solvent; instead of trying to transfer them in a liquid state to a quartz tube, then evaporate off the solvent through heating without affecting the sample, it was decided to develop a method using the C,H,N analyzer connected to the mass spectrometer in order to do the stable carbon isotope analysis.

The C,H,N, analyzer is a simplified chromatograph; it is designed to combust organic material, producing CO_2, N_2 and H_2O. The combustion column contained chromium oxide and cobaltous-cobaltic oxide with silver. The reduction column contained reduced copper. The gases were separated on a chromatographic column before they, in this case, go through a stainless steel tube attached to the inlet system of the mass spectrometer. Here, the water is frozen out first, then CO_2 and N_2 are carried into a second trap cooled with liquid nitrogen to –196°C. The CO_2 gas freezes out, but N_2 and helium (carrier gas) are bled through another valve into the atmosphere.

The PRISM™ used in this work is the latest version of a stable isotope mass spectrometer developed by VG Isogas, Ltd., England. Its design of electromagnet, length of flight tube, reduced volume of pipework in the preparation side of the instrument and microinlet designed to minimize memory effects affecting reference or sample gases makes it very sensitive. Measurements of natural abundances of stable isotopes in organic matter and/or carbonate in the range of 20 μg of sample are obtainable. The mass spectrometer is fully automated and is run from an IBM PS2/50™ series microcomputer with factory designed software written in Pascal.

The Carlo Erba model 1106 C,H,N analyzer is able to combust samples in the range of 100 μg carbon (Howarth, 1977) which makes this instrument the limiting factor with regard to size of sample.

The problems to be resolved were how to transfer liquid samples into tin foil containers for the C,H,N analyzer, elimination of the solvent and finding the lower limit with respect to sample size. In order to examine and solve these problems, a commercial standard, 98% pure retene, was used. Through mainly simple trial and error it was found:

a) adding solvent (hexane) to retene to remove it through evaporation under reduced pressure with a water aspirator for 24 hours did not change the isotopic signature for the compound. Solvent was either completely removed or its isotopic signature was so close to the value of retene that it did not make any difference. It turned out that all compounds analyzed were reasonably close in isotopic ratios. Had the range been much larger, it would have been necessary to find another standard of different $\delta^{13}C$ to ascertain whether hexane still did not have any effects on these values;

b) tin foil containers were run as "blanks" and showed a content of 2–3 μg, with a $\delta^{13}C$ value of –21.4‰ which would shift the sample values by approximately +0.3‰; and

c) the lower limit of sample size is about 45–50 μg of retene (see Table 5.1).

Table 5.1. $\delta^{13}C$ analysis of commercial retene in a *dry* and *wet* state.

	WEIGHT	$\delta^{13}C$ VALUE w.r.t. PDB	INTERNAL PRECISION
dry:			
Retene	103 µg	–25.248	0.004‰
Retene	43 µg	–25.168	0.004‰
Retene	30 µg	–24.531	0.002‰
Retene	30 µg	–24.739	0.009‰
Retene	52 µg	–25.360	0.002‰
wet (hexane):			
Retene	35 µg	–24.909	0.006‰
Retene	35 µg	–25.001	0.005‰
Retene	35 µg	–24.893	0.005‰
Retene	35 µg	–25.002	0.005‰
Retene	35 µg	–24.958	0.005‰

One serious problem was observed with the analysis of very small samples. Water from the combustion was carried through the water trap due to:

1) entrainment of the small number of water vapour molecules by the helium carrier gas;

2) nucleation problems;

3) the combination of 1) and 2); and

4) entrainment and carriage of frozen water (with the aid of He) into the liquid N_2 trap for CO_2.

If water is carried into the analytical system of the mass spectrometer, it causes instability in the measurement process and lowers the reproducibility of the results. This problem was partly eliminated by extending the 'pump-out' times between samples and by running standards, before and after each sample. This was done to extend further the 'pump-out' times of the analyzer of the mass spectrometer and to see that the isotopic value for the laboratory standard (acetanilide; –27.3‰) was still obtainable.

5.2. $\delta^{13}C$ values for diterpene acids, diterpene hydrocarbons, perylene, and 6-methyl hexadecane.

It can be seen from the values in Table 5.2 that for simonellite, tetrahydroretene and perylene, the reproducibility was not very good. Improper combustion could have been the problem; complete combustion varies for the compounds, even when surplus oxygen is used.

Table 5.2. $\delta^{13}C_{PDB}$ values for separated compounds*

	AVERAGE VALUE	# REPLICATES
Abietane† and Pimarane esters	–29.5‰ ± 0.3§	2
Dehydroabietane	–22.7‰ ± 0.4	2
Dehydroabietin	–23.9‰ ± 0.1	2
Simonellite	–23.5‰ ± 0.9	3
Tetrahydroretene	–23.6‰ ± 0.6	3
Retene	–24.5‰ ± 0.3	3
Perylene	–26.0‰ ± 0.7	3
6-methyl hexadecane	–25.1‰ ± 0.1	2

* The standard used for the carbon isotopic analyses is the Peedee Belemnite (calcite) that has been assigned a $\delta^{13}C$ value of 0.0. Sample isotopic ratios are calculated in terms of $\delta^{13}C$:

$$\delta^{13}C = \frac{R_{sample} - R_{standard}}{R_{standard}} \times 10^3, \text{ where } R = {}^{13}C/{}^{12}C.$$

† It was established that the extra carbon added during the esterification did not have an isotopic ratio that would cause a shift in the value for the acids.

§ Impurities (Sect. 5.1) would add +0.3‰ to each value

6. DISCUSSION

It was only in 1939 that the molecular formula of one of the reduced resin hydrocarbons, fichtelite, was established, yet it was isolated as early as 1841 (Burgstahler and Marx, 1969). Burgstahler and Marx established the stereochemistry of fichtelite and showed its origin to be a degraded product of abietic-type resin acids and that the remaining methyl group at C-4 was of β-configuration.

Softwoods (conifers) from which the resin acids were extracted are, in general, rich in resin acids; however, some spruces will show a low content. Hardwoods (birch, alder) do not contain resin acids (Swan, 1973). Softwoods and hardwoods both belong to the C_3-plants and their biosynthetic pathways are considered to be the more primitive way of incorporating CO_2 from the atmosphere during photosynthesis.

6.1. Biosynthetic considerations in the formation of secondary metabolites: resin acids

The group of diterpenes that was investigated in this work were the resin acids. Primary and secondary metabolites are quoted to be, respectively:

> "derjenigen Atomkomplexe, an welche das Leben geknüpft ist, hingegen diejenigen, welche nicht in jeder entwicklungsfähigen Zelle gefunden werden, als sekundäre" (Luckner, 1984).

In very general terms, primary metabolism is necessary for survival, whereas the extent of secondary metabolism depends upon the organism.

The function of secondary metabolites is not always known. Some more functionalized diterpenes of the labdane skeleton, grindelic acids, are produced by species of *Grindelia* as protection against insects (Timmermann *et al.*, 1985). Figure 1.2 shows the biosynthetic pathway to abietic acid as it has been stated by many investigators (Mann, 1987; Haslam, 1985; Luckner, 1984). In order to explain the $^{13}C/^{12}C$ ratio of these particular compounds, it is necessary to refer to earlier work from the 1960's and 1970's. The now classic paper in this respect by Park and Epstein (1961) discussed the kinetic effect associated with isotopic fractionation. Why is it that plant material is so much *lighter* than the carbon of atmospheric CO_2 which is used in the photosynthesis? Park and Epstein suggested that isotopic selection at the acetate and/or pyruvate stage was a likely mechanism.

From a series of papers on aerobic microbial alterations by *Acetobacter suboxydans*, one can conclude that:

1) the natural abundance of $^{13}C/^{12}C$ in acetate from apple cider results in C-carboxyl which is 8.4‰ heavier than the C-methyl (Rinaldi *et al.*, 1974a);
2) in the oxidation of synthetic ethanol, bacteria preferentially select a lighter fraction to oxidize to an acetic acid which is 4.4‰ lighter than the precursor alcohol and leaves unreacted residual ethanol which is 1.7‰ heavier than the precursor. The difference between acid product and residual precursor alcohol is 6.1‰ (Rinaldi *et al.*, 1974b); and
3) where oxidized and reduced products are synthesized from the same precursor pool by auto-oxidation-reduction, as is the case in the synthesis of acetoin (3-hydroxy-butanone-2), the more oxidized groups will be enriched in ^{13}C and the more reduced will be depleted: C=O (–18.5‰) > –HCOH (–29.1‰) > CH_3 (–34.9‰) (Meinschein *et al.*, (1974).

Where a common precursor pool is used for biosynthesis, the lighter fraction is selected for reaction; heavier unreacted compounds accumulate and the residual pool gets progressively heavier; more reduced compounds (CH_4 in methanogenesis) or more oxidized compounds (acetic acid in *Acetobacter* oxidation of synthetic ethanol) are lighter relative to the residual pool of unreacted precursors (Meinschein *et al.*, 1974; Rinaldi *et al.*, 1974b).

Later work by Monson and Hayes (1980; 1982a; 1982b) and Vogler and Hayes (1980) determined the natural abundances of ^{13}C at specific positions within bacterial fatty acids. The carboxyl position in acetyl-CoA is depleted in carbon-13 and the methyl is enriched; it is this intermediate which is used in the biosynthesis of lipids (see Figure 1.2). In fatty acids, the carboxyl group is also *light* in contrast with the ^{13}C-enriched carboxyl found in acetate as a byproduct of ethanol oxidation by *Acetobacter suboxydans* (Rinaldi *et al.*, (1974a, 1974b). From this it follows that there is an isotopic order, an intramolecular order, of concentrations of carbon isotopes in biologically produced compounds. Resin acids (like other lipids) are depleted overall in ^{13}C, as is evident from the stable isotope analysis of the acids and their structurally related hydrocarbons.

6.2. Degradation of the diterpene acids

The organic compounds found in the Powell Lake sediments were microbially degraded. The isolation of traces of 7-keto dehydroabietic acid and various branched and hydroxylated fatty acids indicate microbial activity (Ekman, 1979; Matsumoto *et al.*, 1985; Summons, 1987; Goossens *et al.*, 1986; Barnes and Barnes, 1978). The reducing

environment and the cyclic structures of the diterpenes are most likely to be the reasons that these compounds have been retained and have not been degraded to CO_2 or CH_4, respectively (Barnes and Barnes, 1983).

The diterpene acids are derived from the condensation of repeating C_2-units and the abietene skeleton (Fig. 6.1) will have alternating *heavier* and *lighter* carbons depending upon whether they were derived from the methyl or carboxyl carbon of acetyl-CoA (Fig. 1.2).

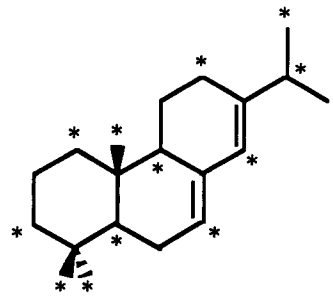
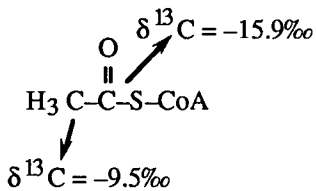


Fig. 6-1. Abietene skeleton. * = heavier carbon.

Some of the diterpenoids isolated from Powell Lake retained their original abietene skeleton, while others had lost carbons at C-4 or C-10, either as methyl or carboxyl groups. Groups at both C-4 and C-10 are *heavier* since they all were derived from the methyl of acetyl-CoA. According to estimates by Monson and Hayes (1982a) and DeNiro and Epstein (1977), if *Escherichia coli* is grown on a glucose substrate with $^{13}C_{PDB}$ of –9.96‰, the lipids (overall) will have a value of –16.84, fatty acids –12.85, and neutrals –25.38. So, lipids are approximately 7‰ *lighter* than the precursor substrate. Further experiments showed specifically that in acetyl-CoA, this fractionation has already occurred, apparently at a branch point in the enzymatic decarboxylation of pyruvate by pyruvate decarboxylase; this yields the following compound where the carbonyl is 6.4‰ lighter than the methyl groups.



The average value for fatty acids (–12.85‰) which arise from multiple condensations of C_2 units derived from acetyl-CoA is very similar to the average value for acetyl-CoA (–12.7‰) shown above (Monson and Hayes, 1982a).

6.3. Results of $\delta^{13}C$ analysis of diterpenes

It was the intention, through stable isotope analysis, to establish degradation pathways; i.e., to confirm which methyl groups were primarily used for microbial growth and the sequence of degradation steps. Table 5.2 shows the values obtained for the diterpenes, perylene, and 6-methyl hexadecane.

While mass spectral evidence indicates the presence of abietic and dehydroabietic acids in the sediments of both the West and South Basins of Powell Lake, the quantities were too small to isolate. The dehydroabietic acid used in this study was isolated from a Douglas Fir, *Pseudotsuga menziesii*; it is about –6‰ lighter than the diterpenoid hydrocarbons isolated from the sediments. These degraded products represent about 200 years of sedimentation and reflect an isotopic value averaged over that time period. A number of factors could contribute to the lighter acid isolated from the Douglas Fir:

1) juvenile effects: increase in $^{13}C/^{12}C$ ratio by +2‰ (Freyer and Belacy, 1983);
2) light intensity: max –4.5‰ change; conditions of low intensity light favor an increased isotopic fractionation with the formation of compounds depleted in ^{13}C (Smith *et al.*, 1976);
3) canopy effects: possible depletion in ^{13}C (Wilson and Grinsted, 1977; O'Leary, 1981);
4) anthropogenic effects: decrease in $^{13}C/^{12}C$ ratio from 1850 to present of –2‰ due to the combustion of fossil fuels (Freyer and Belacy, 1983);
5) CO_2 origin: max. change of ±9‰ (Smith *et al.*, 1976); and
6) winter versus summer: depletion in winter, –2 to –5‰; lower temperatures favour an increased isotope fractionation effect (Wilson and Grinsted, 1977; Francey and Farquhar, 1982).

Differences in temperature, light intensity and the source of CO_2 may all have contributed to the lighter values measured for dehydroabietic acid (–29.5‰); based on the discussion in Chapter 1, one might have expected a value closer to –26.0‰ for the diterpenoids.

Dehydroabietane (C_{20}) has been reported in conifers as a natural product (Rogers *et al.*, 1979). The corresponding C_{20}-acid is –6.8‰ lighter than dehydroabietane. If dehydroabietane is the original hydrocarbon from which the acids are biologically synthesized in conifers (Hafizoglu, 1983; Lorbeer and Zelman, 1988), one could expect the formation of a lighter acid fraction and the accumulation of a heavier residual precursor hydrocarbon pool which is subsequently contributed to the lake sediments.

If dehydroabietane accumulating in the sediments is the precursor of the other diterpenoid hydrocarbons, simple progressive loss of carbons at C-4 and C-10 with the formation of lighter C_{19} and C_{18} hydrocarbons would nearly account for the observed depletion in ^{13}C (Table 6.1). The C_{19} and C_{18} diterpenes are –0.3‰ to –0.7‰ lighter relative to C_{20} than is predicted through loss of heavy carbons, suggesting a slight isotope effect. On the assump-

tion that the lighter fraction of the precursor is selected for alteration, a heavier residual fraction of dehydroabietane will accumulate and the product (oxidized—more aromatic) will be lighter. If dehydroabietane is the precursor in

Table 6.1. Estimate of $\delta^{13}C$ values for diterpenes with progressive loss of carbon at C-4 and C-10.

diterpenes

heavy C/total C	C_{20}	C_{19}	C_{18}
	12/20	11/19	10/18
% heavy C	60%	57.895%	55.55%
relative to C_{20}		2.105%	4.45%
		lighter	lighter

relative to C_{20} precursor with isotope signature similar to the value found for dehydroabietane

	DH-abietane[a]	DH-abietin[b] simonellite	TH-retene[c]	Retene
observed $\delta^{13}C$	–22.7	–23.5 to –23.9	–23.6	–24.5
		–0.8 to –1.2 lighter	–0.9 to	–1.8 lighter
predict from loss of heavy C	$C_{20}\rightarrow$	$C_{19}\rightarrow$	C_{18}	
	-22.7	0.4778 lighter	1.01015 lighter	
		–23.18	–23.71	
	C_{20} Precursor	DH-abietin[b] simonellite	TH-retene[c]	Retene
observed		–23.5 to –23.9	–23.6	–24.5
–predicted		–(–23.18)	–23.71	–23.71
observed Δ		–0.32 to –0.72 lighter	0.11 heavier	–0.79 lighter

[a]DH-abietane = dehydroabietane; [b]DH-abietin=dehydroabietin; [c]TH-retene=tetrahydroretene;

both the tree and in the sediments, one would predict the presence of a heavy fraction accumulating in the sediments. Only comparatively small amounts of this hydrocarbon were extracted. Luckner (1984) gives the half life of diterpenes in pine trees as: a) cortex—170 days; b) needles—46 days. It is therefore probable that part of the residual dehydroabietane is degraded and used in the synthesis of other tree metabolites. The C_{20} dehydroabietane may be the

precursor of simonellite, which retains the C-4 methyl, whereas the origin of diterpenes which have lost the C-4 methyl is more likely to have taken place via decarboxylation of the resin acids; however, in view of the isotope values for these compounds, a precursor acid is needed with an isotope signature similar to dehydroabietane (Table 6.1).

Resin acids comprise a large fraction of the resin diterpenes in soft woods (Zinkel and Engler, 1977; Zinkel *et al.*, 1985). Only small amounts were found in West and South Basins which suggests that aerobic conditions, which initiate alteration of diterpenoid acids, must lead predominantly to products other than those with the abietane skeleton. The principal hydrocarbons found in Powell Lake (Barnes and Barnes, 1983) are summarized in Figure 6.2.

The hydrocarbon concentrations from South Basin sediments range from 200-fold (retene) to 2-fold (dehydro-abietane) the values for the same hydrocarbons isolated from below the redox boundary in West Basin. West Basin has an oxic water column as well as a few centimetres of oxic sediments, whereas South Basin has a partly anoxic water column and anoxic sediments. The combination of a shorter oxic water column and totally anoxic sediments in South Basin contribute to the very high concentrations of hydrocarbons that are found there.

If dehydroabietane arises from an abiotic reduction of the C-4 carboxyl in dehydroabietic acid in the sediments due to low Eh associated with sulphide production in West Basin and methanogenesis in South Basin, one would expect similar ^{13}C signatures for the acid as precursor and the hydrocarbon as end product. If it were due to microbial degradation, one would expect a lighter rather than a heavier hydrocarbon as the end product. Because the amount of dehydroabietane increases with reducing conditions and depth in West Basin, it must represent a mixed pool of products from the original conifer and processes in the sediments. There are a number of references to decarboxylation and the formation of hydrocarbons from diterpenoid acids (Kieslich, 1976), but there is no evidence to date for the microbial reduction of a carboxyl group to a methyl group with the formation of dehydroabietane (Berry *et al.*, 1987); however, methanogens utilize H_2 and CO_2 formed by other microbial systems in a mixed culture to reduce the CO_2 to methane (Novelli *et al.* , 1988). In the laboratory benzoate is reduced, undergoing ring fission to form n-heptanoic acid and finally short-chain acids and H_2 under anaerobic conditions (Evans, 1977; Berry *et al.*, 1987). These reductive processes could explain both the saturated analogue of dehydroabietin (fichtelite) in South Basin and the reduction of dehydroabietic acid to dehydroabietane in the sediment. However, dehydroabietane must be stable in a reducing environment, because there was no evidence for the occurrence of the saturated analogue, abietane.

In sediments the majority of products represent alterations by a mixed microbial community. The effects are more complex and more difficult to predict than in the case where the products represent the effect of branching biosynthesis within the same cellular environment. If the diterpenoid acids, which are the dominant components of the softwood resins, are substantially altered by aerobic microbial oxidation, the lighter fractions may undergo ring fission and subsequent oxidation to provide energy and release CO_2 and water. The acids and hydrocarbons which do survive and accumulate represent a residual pool of diterpenoids which are significantly heavier than the original

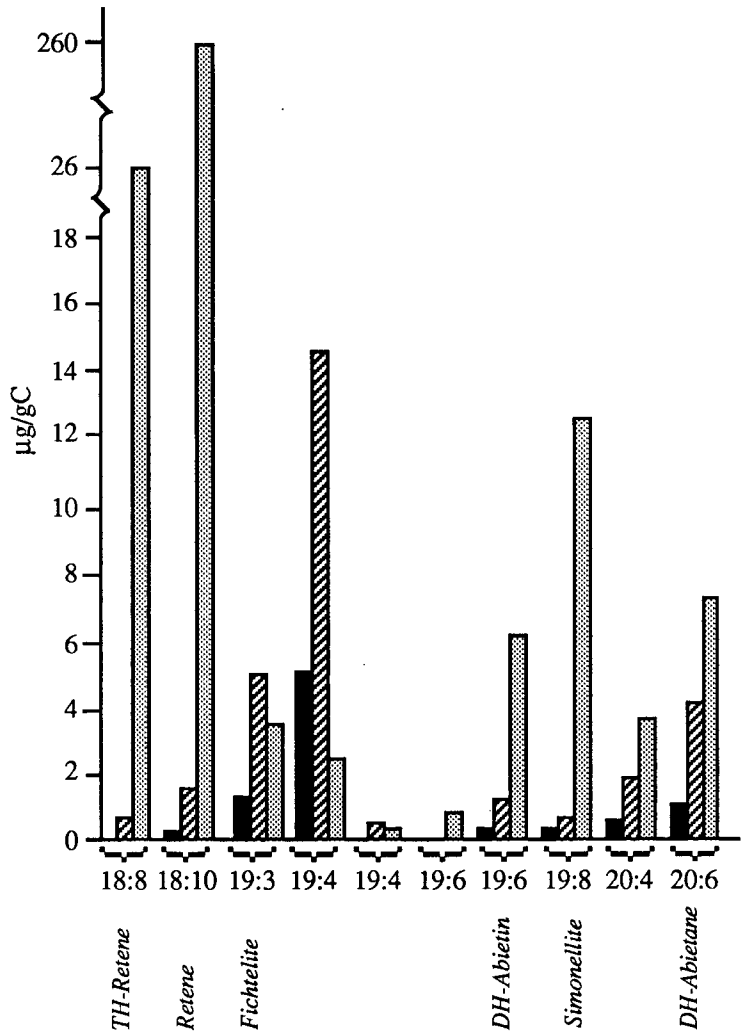


Fig. 6.2. Distribution of diterpenes isolated from the sediments of Powell Lake expresed in µg g^{-1} organic carbon. Solid bar represents West Basin sediments above the redox boundary; hatched bar, West Basin sediments below the redox boundary; and stippled bar, anoxic sediments from South Basin (adapted from Barnes and Barnes, 1983).

diterpenoid acid contributed by conifers. Fig. 6.3 shows the possible relationships between the precursor and the degraded hydrocarbons. At this time it is not possible to determine the relative proportions of sources contributing

to the pool of dehydroabietane or the proportion of the resin acid(s) that are decarboxylated, totally degraded in the aerobic environment, or reduced to a C_{20} hydrocarbon.

6.4. Results of $\delta^{13}C$ analysis of perylene

Various origins have been suggested for perylene. Perylene is ubiquitous and Allport and Bu'lock (1958; 1960) suggested it is a fungal pigment, a perylene-quinone. Assante *et al.* (1977) examined another fungal species and found similar quinones. Insects have been quoted as possible sources (Bowie *et al.*, 1966). Perylene has been found in both terrestrial and marine environments (Hites *et al.*, 1980; Kawamura and Ishiwatari, 1985; Kawamura *et al.*, 1987; Simoneit, 1986; Prahl and Carpenter, 1983; Baker and Louda, 1982; Louda and Baker, 1984).

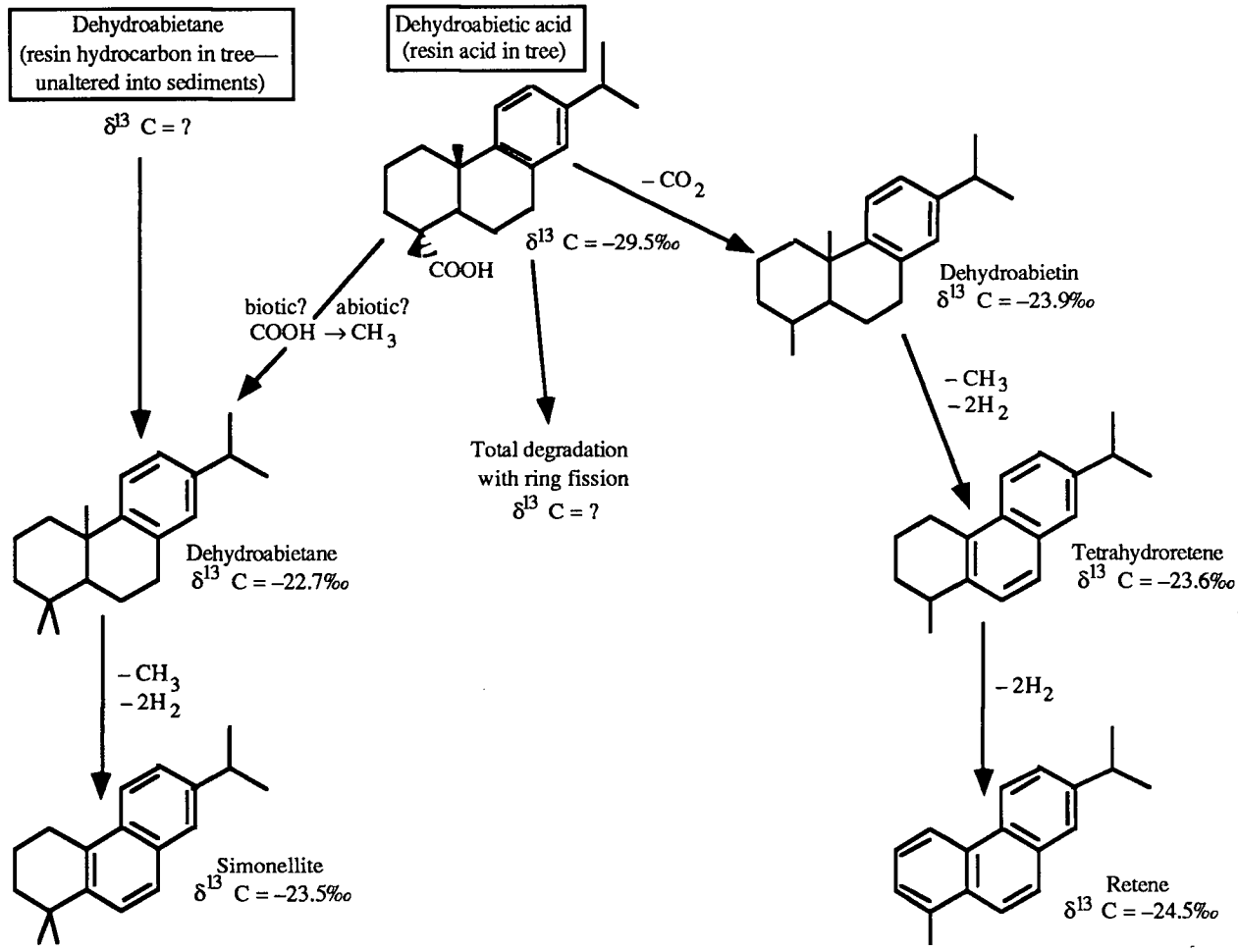


Fig. 6.3. Possible relationship between diterpene precursors and end products.

In Powell Lake sediments, the origin of perylene is most likely terrigenous, and its $\delta^{13}C$ is –26.0‰, which is what could be expected for terrigenous C_3-plant material. This does not exclude other origins for perylene, but, in the reducing environment of South Basin, comparable to anoxic river sediments from Rhode Island, U.S.A. (Hites *et al.*, 1980), the most likely origin is terrigenous. The light carbon signature suggests the origin is probably degraded terrestrial plant material which was used by fungi to produce pigments, either perylene-quinones or cercosporins (Okubo *et al.*, 1975). If perylene is an anthropogenic product, it should have an even lighter isotopic signature, less than –28‰ (Farran *et al.*, 1987).

6.5. Results of $\delta^{13}C$ analysis of 6-methyl hexadecane

The most prominent producers of branched-chain alkanes are the cyanobacteria (Bird and Lynch, 1974). The 7- and 8-methyl heptadecanes (Han *et al.*, 1968)are more common, but 6- and 7-methyl hexadecanes are found as well (Gelpi *et al.*, 1970). They can be synthesized from a fatty acid (cis-vaccenic acid); a methyl group is added across the double bond, followed by reduction and decarboxylation (Bird and Lynch, 1974). The role of these hydrocarbons in the microorganisms is not well known. In fungal species, similar hydrocarbons were found either on spore walls, where they may create a hydrophobic surface, or were located in the cell wall (Bird and Lynch, 1974).

In Chapter 1, it was stated that $\delta^{13}C$ could range from –16 to –21‰ or be as light as –32‰. The value found was –25.1 ±0.1‰. Estep and Vigg (1985) stated the following whole cell values for cyanobacteria in freshwater lakes:

a freshwater, eutrophic lake	–26 to –30‰
a saline, terminal lake	–17‰
a pluvial lake	–24‰

The observed value of –25.1‰ is very close to the quoted value for an eutrophic lake It does, most likely, reflect the source of CO_2 used by this prokaryote and phototroph to generate its own material. More ^{13}C-enriched values are characteristic of a marine environment, which should give a much heavier isotopic value since it is the dissolved CO_2 in the water that the bacterium uses and the isotopic value of the CO_2 would be different, reflecting the different source. More ^{13}C-depleted values would be expected in a freshwater environment.

6.6. Conclusions

Through stable carbon isotopic analysis, it was confirmed that diterpenes of the abietane skeleton are of C_3-plant origin. The degraded diterpene hydrocarbons resulting from microbial degradation of diterpene acids were heavier than the original precursor acids, but lighter than the possible hydrocarbon precursor (dehydroabietane). It was, however, not possible to show the exact degradation pathway for these hydrocarbons. The isotope values did, however, show that:

1) there is a family relationship, based on similar isotopic signatures, for the diterpenes in the sediments and the diterpenoids of higher plants with the abietane skeleton; the probable precursor would be functionalized at C-4 and have one or more double bonds in the *B* or *C* rings;
2) they are microbially degraded, and show slight isotopic effects;
3) they are reduced or oxidized without the breaking of C–C bonds, but instead through the addition of H_2 across double bonds or dehydroxylation; and
4) the mode of degradation and origin of precursor will determine the isotopic values of degraded organic compounds.

The $\delta^{13}C$ value for perylene in the South Basin sediments is consistent both with a fungal origin and the reduction of perylene-quinone or cercosporin pigments in an anoxic environment.

The $\delta^{13}C$ value of the branched alkane, 6-methyl hexadecane, which originates from cyanobacteria, indicates that the source material, CO_2 or HCO_3^-, is lighter than values quoted for most marine cyanobacterial mat communities and is consistent with a freshwater lake environment.

The procedures and results in this study have shown that it is possible to measure $\delta^{13}C$ for individual compounds, working with amounts as small as 50 µg. The measurements obtained can give information as to the origin of the compounds; the variation in signatures is source dependent (algal, fungal, higher plant) and can vary as much as 6‰. Environmental conditions such as freshwater versus marine and oxic versus anoxic will determine the final isotopic values.

For diterpenes of the abietane skeleton, aromatization and defunctionalization can take place rapidly and very early in diagenesis, prior to burial and thermal stress. This will limit their use as biomarkers for assessing thermal maturation of sediments. The use of isotopic signatures of individual compounds may be better applied to solving environmental pollution problems. Oil spills could be traced to their source through the analysis of single compounds; the same applies to effluents from various industrial facilities. In addition isotopic signatures for individual compounds could be used for oil-source rock correlation.

This study has broken new ground in the identification of precursor-product relationships for individual compounds. Future work is needed to reproduce the trends that are suggested here for higher plant precursors and their degradation products. This work should be extended to oxic freshwater and marine environments.

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