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THE UNIVERSITY OF ALBERTA

THE CHEMISTRY OF ISOPRENE-$Fe(CO)_3$ ANION

by

CHRISTOPHER INGO FINCHAM

A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES AND RESEARCH

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"To my parents"

ABSTRACT

A study was undertaken to investigate the feasibility of using isoprene iron tricarbonyl as a masked form of isoprene. In this investigation it was shown that the complex could be deprotonated at -78°C by the use of the base lithium 2,2,6,6-tetramethylpiperidide in conjunction with hexamethylphosphoramide (HMPA). The resulting anion was shown to react with a variety of electrophiles to produce new iron tricarbonyl complexes. Treatment of these complexes with either ceric ammonium nitrate or trimethylamine N-oxide as oxidants, resulted in decomplexation to produce the corresponding isoprene derivatives.

Finally, it was also discovered that the isoprene iron tricarbonyl anion undergoes an isomerisation on warming from -78°C to room temperature to give, after hydrolysis, the isomeric and structurally interesting complex (methyltrimethylenemethane)iron tricarbonyl.

ACKNOWLEDGEMENTS

I would like to express my gratitude to Professor John Hooz for his guidance and supervision during the course of this work.

My thanks also go to Mess[illegible]igam and R. Swindlehurst, and Dr. A. Hogg, and their respective staffs, for the determination of the spectra.

I would also like to thank: Dr. J. Takats for his help in the identification of the methyltrimethylene-methane complex. Drs. P. McAdam, B. Mullah and S. Wnuk, for their helpful advice during the course of these experiments. Miss A. Wiseman for the typing of this manuscript.

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And finally I would like to thank my family and friends without whose support this work would not have been accomplished.

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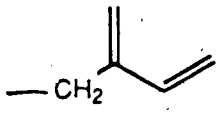
INTRODUCTION

The isoprene unit 1 is an important constituent of a number of natural products including examples such as the sesquiterpene aldehyde β-sinesal 2 (isolated from the Chinese orange, Citrus sinensus L. by Stevens and coworkers,[1] and subsequently synthesised by a number of groups[2]),



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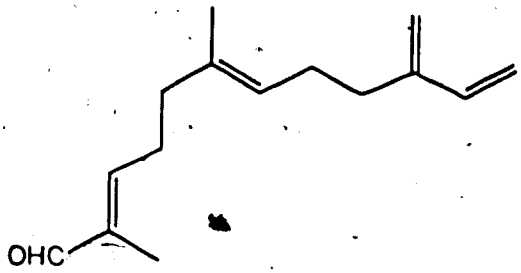
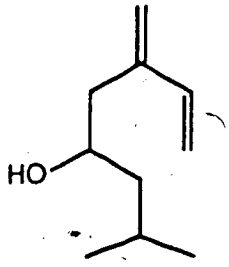


2

myrcene 3, a monoterpene found in a variety of plants,[3] and the terpene alcohols 2-methyl-6-methylene-7-octen-4-ol, 4, and 2-methyl-6-methylene-2,7-octadien-4-ol, 5, two of the major components of the sex attractant/aggregation pheromone of the male bark bettle, Ips Confusus.[4]

3



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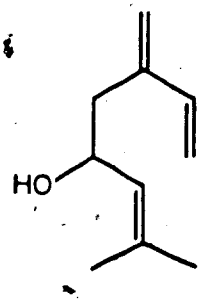
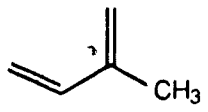


5

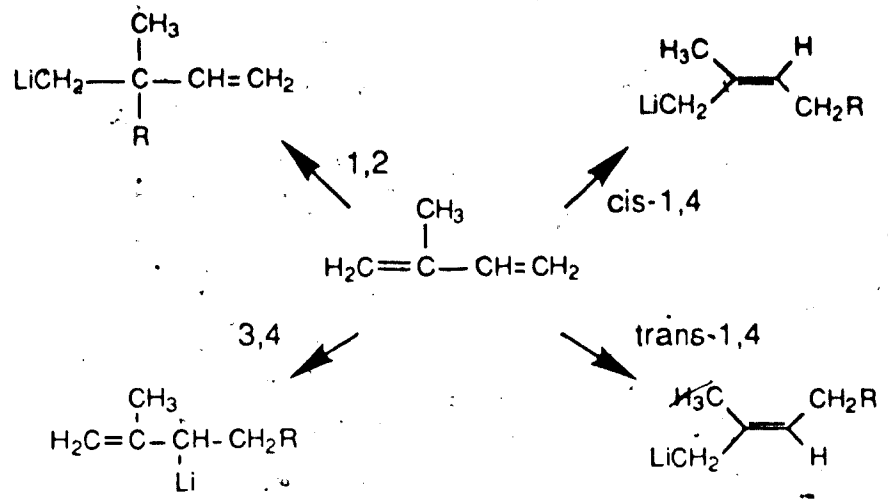
This makes the functionalisation of isoprene, 6, via deprotonation of the methyl group and reaction with electrophiles a worthwhile synthetic target.



6

The synthetic problem is made more challenging by the fact that the normal reaction of isoprene with various organolithium compounds does not result in deprotonation but rather in polymerisation, which arises from the lithium reagent acting as a nucleophile rather than a base. Nucleophilic addition to the diene produces a new organolithium species, which in turn can add to a second molecule of diene, ultimately leading to oligomerisation. The modes of addition of organolithium compounds (RLi) to isoprene are given in Scheme I.[5]

Much work has been done on these modes of addition in an attempt to find the conditions necessary to bring about repeated cis-1,4-addition since the resulting cis-polymers have properties resembling those of natural rubber. The type of addition favoured appears to depend upon the organolithium used and the solvent. For example, the



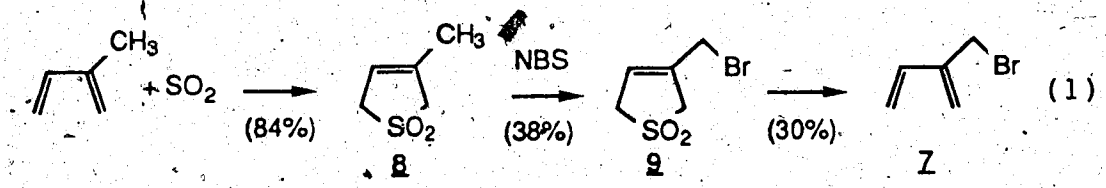
Scheme I

reaction of n-butyllithium with isoprene in cyclohexane produces mainly cis-1,4-addition, whereas changing the solvent to tetrahydrofuran results in products from the other three modes of addition, but no cis-1,4-addition.

A number of synthetic schemes have been devised to circumvent the polymerisation problems. In one approach[6] the isoprene moiety is formed in a stepwise fashion via a Wittig reaction on a suitably substituted aldehyde or ketone, whereas in others, techniques have been developed for the intact incorporation of the isoprene unit. The latter will be discussed in more detail below. Others still,[7] particularly in regard to the chiral synthesis of **4** and **5**, have used totally different approaches although

these tend not to be of general synthetic use as a method to incorporate the diene unit.

In 1968 Silverstein and coworkers[8] published details of the synthesis of the terpene alcohols 4 and 5 which they had isolated earlier.[4] Their attempts centred on the use of 2-bromomethylbutadiene 7 as an isoprene synthetic equivalent. This compound, a common synthetic equivalent in a number of papers, was first synthesised by Krug and Yen[9] via 3-methyl-2,5-dihydro-thiophene-1,1-dioxide, 8 (formed from isoprene and sulphur dioxide[10]), reaction of the latter with N-bromosuccinimide (NBS), and pyrolysis of the resulting 3-bromomethyl-2,5-dihydrothiophene-1,1-dioxide, 9, to 7 (eq. 1).

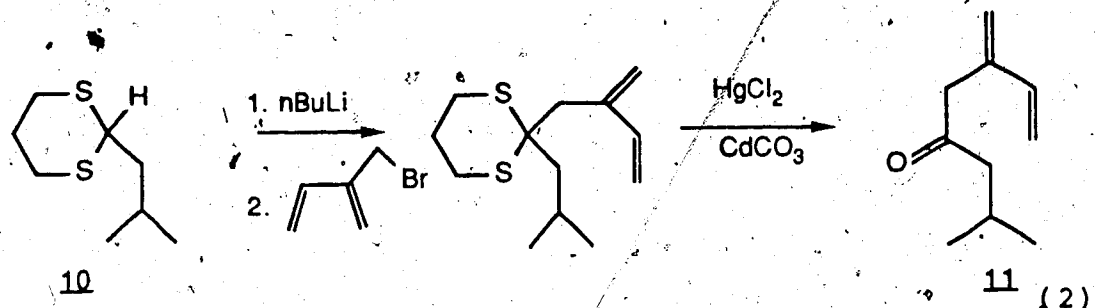


(1)

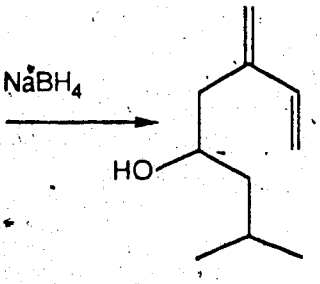
(Improved yields for the bromination (66%) and thermolysis (60-75%) have subsequently been reported.[11])

It was initially hoped that 7 could be converted into the organolithium or the Grignard reagent, and then reacted with isovaleraldehyde to produce 4. This was unsuccessful however, and no details are given of what products did result from the reaction of 7 with either

lithium or magnesium. As a result, the authors used **7** as the electrophilic component of the synthesis, and reacted it with the anion of 2-isobutyl-1,3-dithiane **10**, an activated form of isovaleraldehyde. This was followed by removal of the protecting group and reduction of the resulting ketone **11** to **4** as outlined in eq. 2.



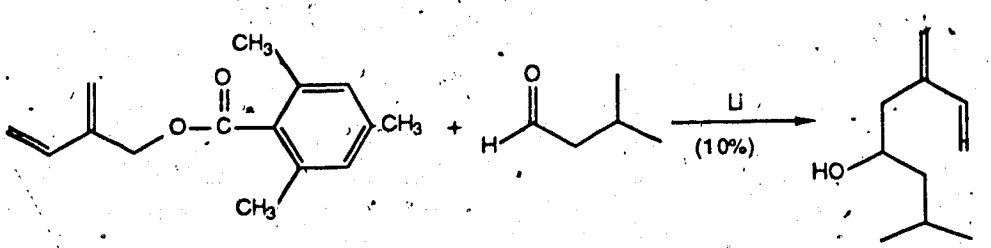
10 **11** (2)



4

The formation of an organometallic compound from **7**, and its use in the synthesis of **4**, was accomplished by Katzenellenbogen and Lenox,[12] using two procedures. The first involved the <u>in situ</u> generation of the organolithium intermediate via reaction of 2-(mesitoyloxymethyl)-1,3-butadiene **12** (prepared from the corresponding alcohol, in turn produced from **7**[13]) with lithium in tetrahydrofuran in the presence of isovaleraldehyde **13** to give **4** in 10% yield

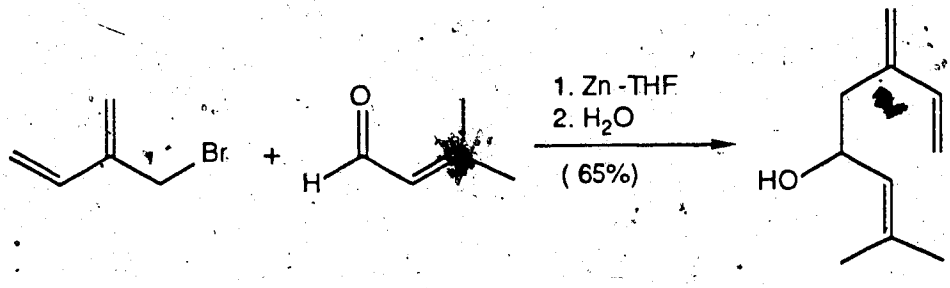
(eq. 3).



(3)

12 13 4

The second procedure used the in situ generation of an organozinc species in a "Reformatsky-type procedure", via reaction of 7 with zinc in tetrahydrofuran in the presence of 13 to produce 4 in 52% yield. This latter method was used in a collaborative study of Silverstein and Katzenellenbogen[14] to produce the other component of the pheromone, 5, via reaction of 7 with zinc and 3-methyl-2-butenal 14 (eq. 4).

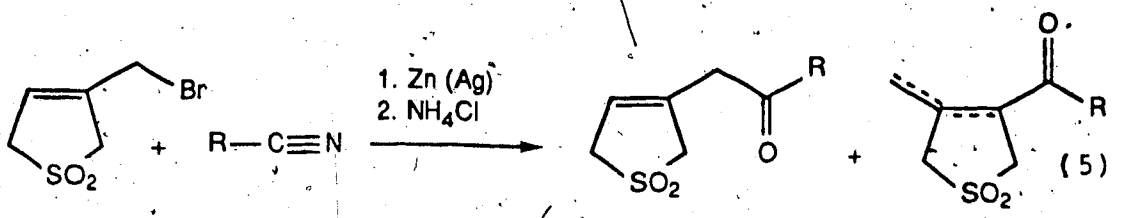


(4)

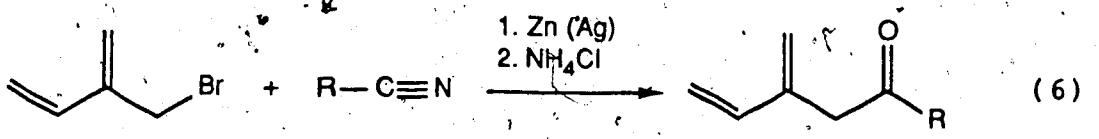
7 14 5

The reaction is also successful for the condensation of allylic bromides (of which 7 is a member) with organonitriles, and has been used by Rousseau and Drouin[15] to produce a variety of ketones containing the isoprene functionality, including the precursors to 4 and 5. The

reaction can be carried out either with the sulphur dioxide adduct of 2-bromomethyl-1,3-butadiene 9 (eq. 5), or with 2-bromomethyl-1,3-butadiene 7, itself (eq. 6), although the former results in a mixture of products, of which the desired product is only the minor component.

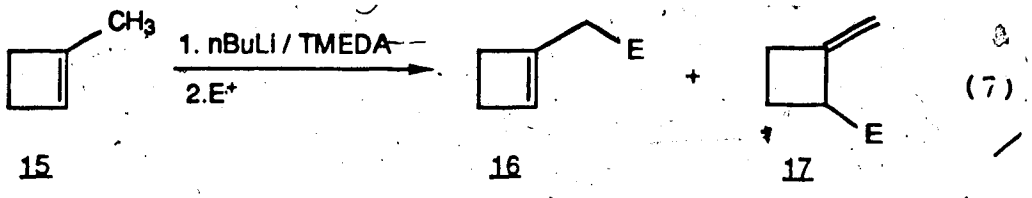


9 20-30% 80-70% (5)



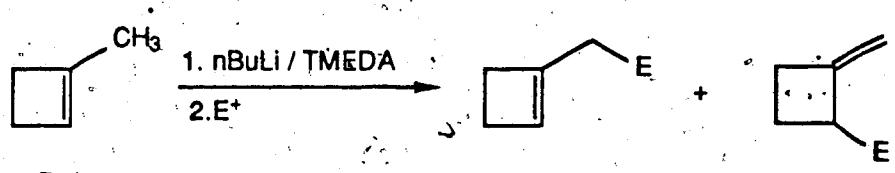
7 70-85% (6)

An alternative approach to the diene problem has been developed by Wilson and coworkers[16] utilising methylcyclobutene, 15, as an isoprene synthetic equivalent. This molecule possesses the requisite number of carbon atoms, and cyclobutenes are known to undergo thermal ring opening to give 1,3-dienes. Methylcyclobutene 15 was deprotonated using n-butyllithium/tetramethylethylenediamine (TMEDA), and then reacted with a variety of electrophiles according to eq. 7.



15 16 17 (7)

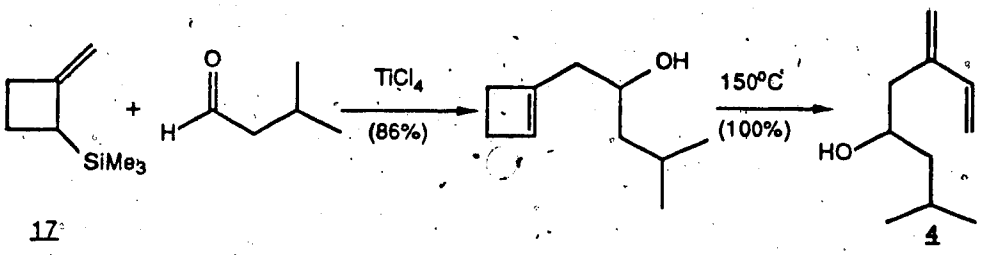
This method suffered from the drawback that two products were produced, and of these the undesired isomer **17**, tended to predominate. Although the reaction conditions (solvent and temperature) could be manipulated to favour formation of isomer **16**, the best ratio achieved was only about 2:1 and often this was done at the expense of the overall yield. A subsequent modification[17] to this procedure employed tin and silicon chemistry, in which the electrophiles were chlorotrimethylstannane and chlorotrimethylsilane, respectively (eq. 8).



15 (8)

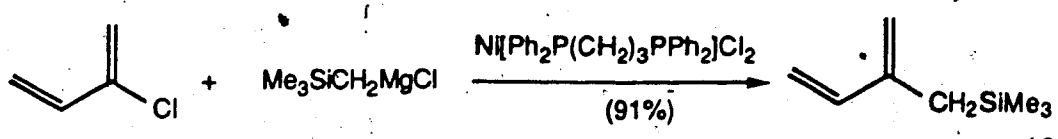
E= Me_3SnCl (85%)	80	20
E= Me_3SiCl (75%)	20	80

Compound **16** (E = $SiMe_3$) was separated from compound **17** (E = $SiMe_3$) by distillation at atmospheric pressure, and under these conditions the former underwent ring opening. In this way pure **17** (E = $SiMe_3$) was obtained, and utilised in subsequent Lewis acid ($TiCl_4$) catalysed reactions with electrophiles, including the preparation, after ring opening, of pheromone alcohol **4** (eq. 9).



17 4 (9)

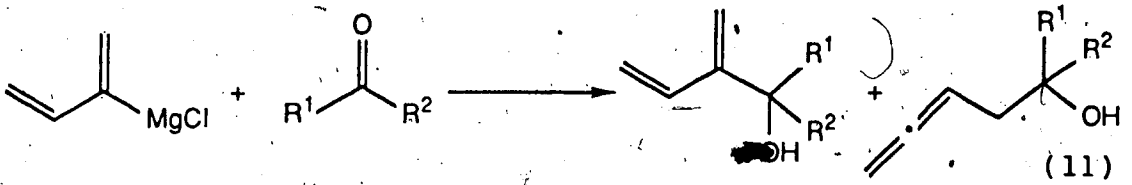
Another approach to this problem, utilising silicon chemistry, was reported by Sakurai and coworkers[18] who employed 2-trimethylsilylmethyl-1,3-butadiene **18** as an isoprene anion synthetic equivalent. This compound is formed via reaction of 2-chloro-1,3-butadiene (chloroprene) **19** with the Grignard reagent **20** formed from chloromethyltrimethylsilane under the influence of a nickel(II) catalyst (eq. 10).



19 20 18 (10)

Compound **18** was reacted with acid chlorides, acetals, and aldehydes under Lewis acid ($TiCl_4$) catalysis to produce a series of isoprenylated products, including the pheromone alcohols **4** and **5**.

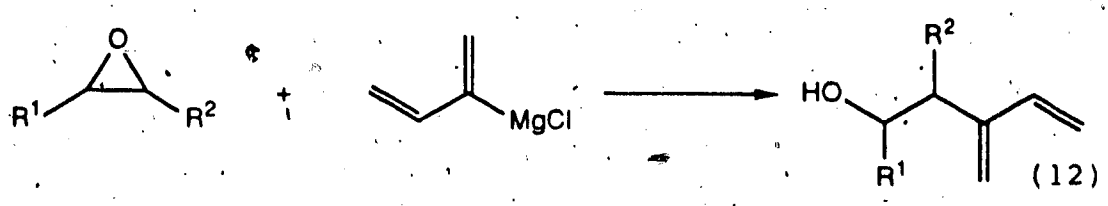
2-Chloro-1,3-butadiene **19** was also used as the starting material in another route[19] to the incorporation of the 1,3-diene moiety. From this compound was prepared the corresponding Grignard reagent, 2-(1,3-butadienyl)magnesium chloride **21**, which was reacted with a variety of ketones, aldehydes and epoxides. Reactions with the first two types of substrate resulted in a mixture of products (eq. 11), with the ratio being dependent on the steric



(11)

21 **22** **23**

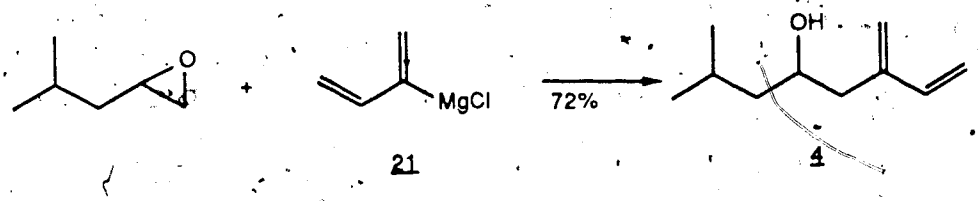
size of the substrate (e.g. if R^1 and/or R^2 are bulky groups the allene product **23** is formed, while for most aldehydes (except R^1 = Ph), the product distribution is reversed). The reaction of **21** with epoxides gave the corresponding 1,3-dienyl alcohols in moderate to good yields (eq. 12).



(12)

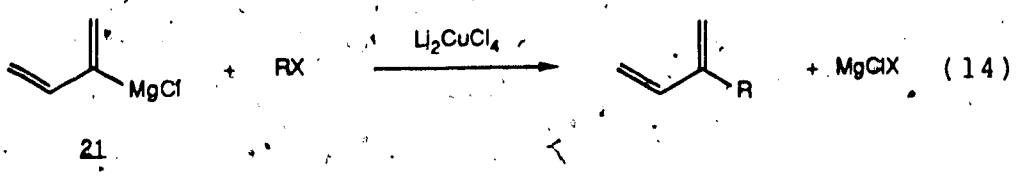
R^1=Et, R^2=H	21	77%
R^1=H, R^2=H		50%

Although 21 is a butadiene anion synthon rather than an isoprene anion synthon, by suitable choice of electrophile similar products can be obtained from 21 as were obtained via the aforementioned synthon, as evidenced by the synthesis of pheromone 4 (eq. 13).



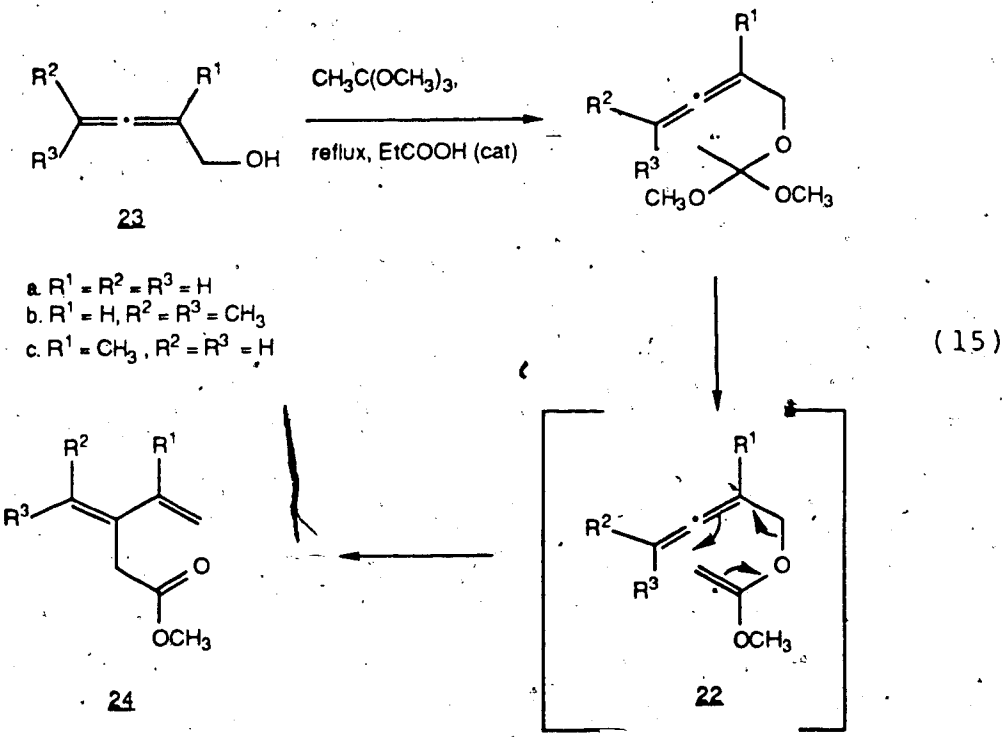
21 4 (13)

The reaction of organometallic 21 with various alkyl, aryl, and vinyl halides under the catalysis of Li_2CuCl_4 (eq. 14), has also been shown by Nunomoto and coworkers[20] to result in butadiene derivatives (i.e. isoprene derivatives) in moderate to good yields.



21 (14)

1,3-Dienyl compounds have also been prepared via the Claisen rearrangement of allenic ethers **22** produced by the reaction of allenic alcohols **23** with methyl orthoacetate as outlined in eq. 15.[21]



23

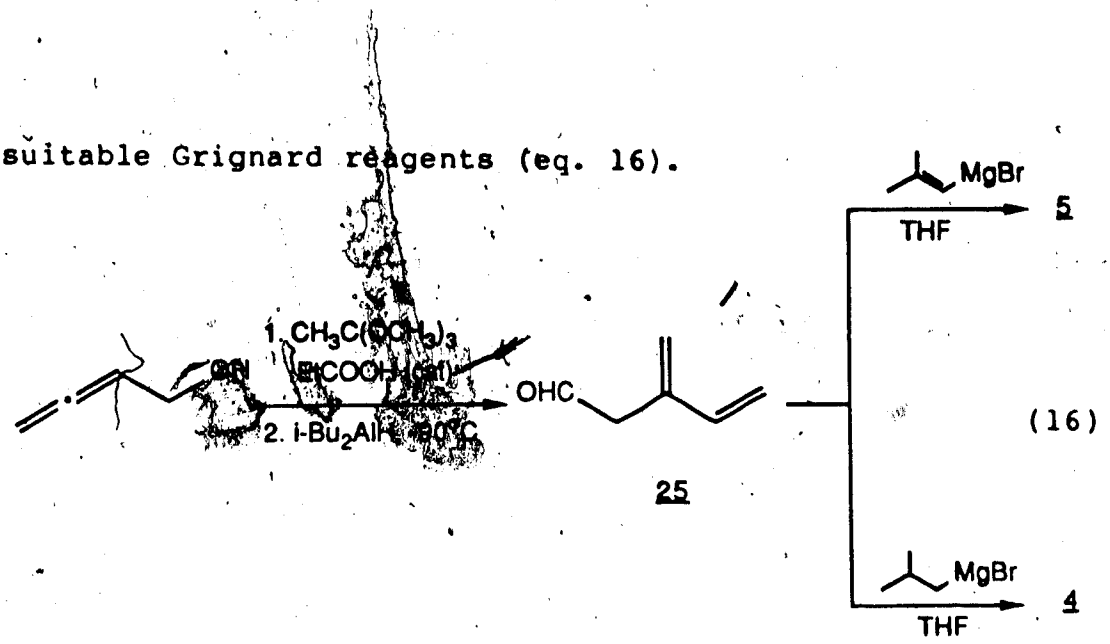
a. $R^1 = R^2 = R^3 = H$
b. $R^1 = H, R^2 = R^3 = CH_3$
c. $R^1 = CH_3, R^2 = R^3 = H$

(15)

24 22

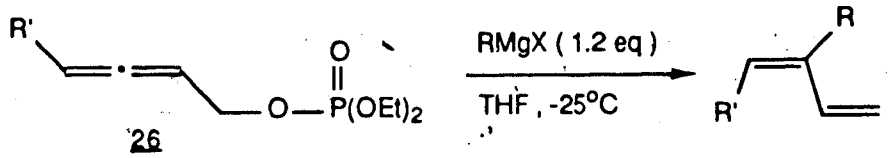
This method has been used to prepare the pheromone alcohols **4** and **5** by the reaction of aldehyde **25** (produced via diisobutylaluminum hydride reduction of **24a**) with

suitable Grignard reagents (eq. 16).



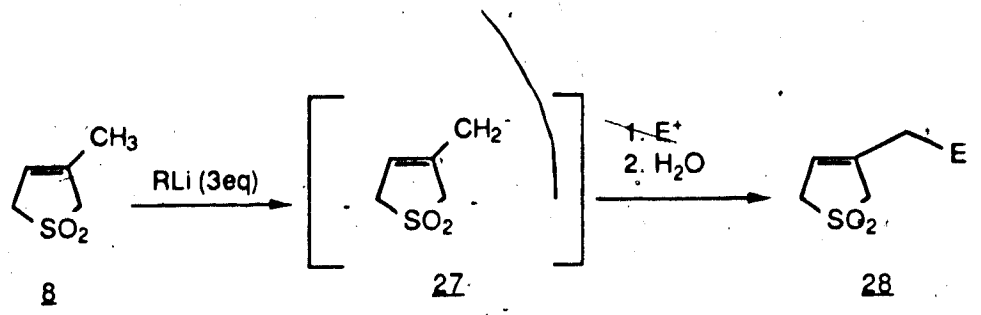
(16)

Phosphate esters of α-allenic alcohols **26** can also be used to prepare 1,3-dienes via reaction with Grignard reagents (eq. 17).[22]



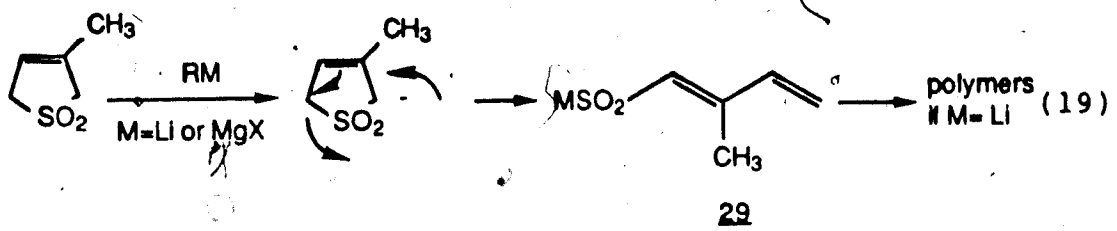
(17)

Our first approach to the "isoprene anion" problem centred upon the sulphur dioxide adduct of isoprene, 3-methyl-2,5-dihydrothiophene-1,1-dioxide, **8** (also known as 3-methyl-3-sulpholene), as a masked form of isoprene. The idea was to determine if the "trianion" **27** (or equivalent) could be formed and then reacted with various electrophiles (eq. 18).



8 27 28 (18)

It was hoped, by analogy with alkylation of the dianion of β-ketoesters,[23] that if the "trianion" was formed (trianions of polyketones have been formed[23a]), preferential attack of the electrophile should occur at the most basic position, resulting in compounds of the type indicated, **28**. The corresponding substituted isoprene derivative should then be available by thermal extrusion of sulphur dioxide. A number of organolithium bases were tried, including n-, sec- and t-butyllithium, methyllithium, and lithium tetramethylpiperidide (all with and without hexamethylphosphoramide), but none could be found to accomplish our goal. Instead, all attempts resulted in dark reaction mixtures, loss of the vinyl signal in the ^1H nmr (a phenomenon also noted by Ta-shue Chou and coworkers[24]), and polymerisation, possibly via the route indicated (eq. 19).

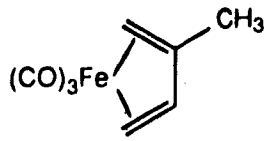


29

It should be noted that alkylation of the monoanion can be successfully achieved if the anion is generated in the presence of the alkylating agent.[24,25] Also, by use of a Grignard reagent in place of an organolithium compound, the ring opened product can be isolated as its magnesium salt **29** (M = MgBr).[26] Towards the end of this approach a paper appeared[27] in which the lifetime of the monoanion (generated using n-butyllithium/HMPA) was studied. It was reported that this anion was stable toward ring opening for up to 15 minutes when generated at -105°C, and could be successfully alkylated under these conditions. One final attempt was made to generate the "trianion" under these low temperature conditions but this again proved unsuccessful and this approach was then abandoned.

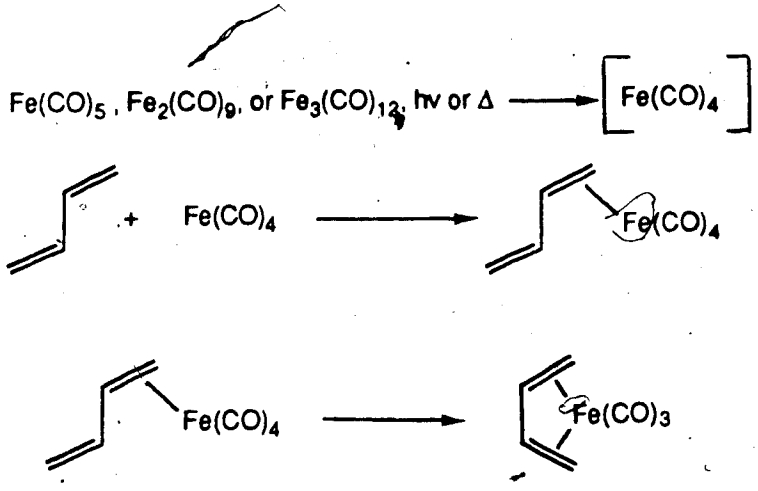
Our second approach to the problem centred on the use of tricarbonyl(2-methyl-1,3-butadiene)iron (commonly referred to as isoprene iron tricarbonyl) **30** as a masked

form of isoprene.



30

Complexes of this type are commonly formed via reaction of the diene with either iron pentacarbonyl ($Fe(CO)_5$), diiron nonacarbonyl ($Fe_2(CO)_9$) or triiron dodecacarbonyl ($Fe_3(CO)_{12}$). The reaction presumably involves the reactive intermediate species $Fe(CO)_4$ attacking one bond of the diene, with subsequent intramolecular attack by the other double bond and loss of carbon monoxide to form the η^4-coordinated species (Scheme II).

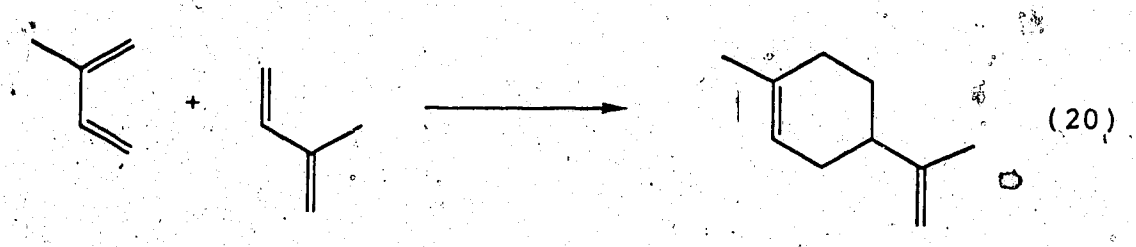


Scheme II

These compounds, while important in their own right, are of particular interest to organic chemists because of the profound effect that bonding to a metal atom has on the chemical properties of the organic segment. Some of these effects and their use in organic chemistry are described below as well as some methods that have been used to prepare these complexes.

The first acyclic diene iron tricarbonyl complex was prepared in 1930 by Reihlen and coworkers[28] via the high temperature reaction of butadiene and $Fe(CO)_5$ in a pressure vessel. $Fe(CO)_5$ is the least reactive of the common three iron carbonyl species, and a high (130-150°C) reaction temperature is necessary (presumably to generate the required $Fe(CO)_4$). Reihlen and coworkers also investigated the reaction of isoprene with $Fe(CO)_5$ under the same conditions; however, although the product obtained was given the formula $(C_5H_8)_2Fe(CO)_3$ on the basis of combustion analysis, no structure was assigned. No further work (apart from a US patent application[29]) was done in this area until 1958 when Hallam and Pauson[30] repeated Reihlen's work on butadiene, and soon after, Stone and coworkers[31] repeated the Reihlen isoprene experiment. Following Reihlen's procedure Stone and coworkers successfully prepared the complex $(C_5H_8)Fe(CO)_3$

and speculated that this was indeed the same material Reihlen had prepared in 1930 but that it was contaminated by some side product produced by Diels-Alder dimerisation of isoprene 6 (eq. 20).

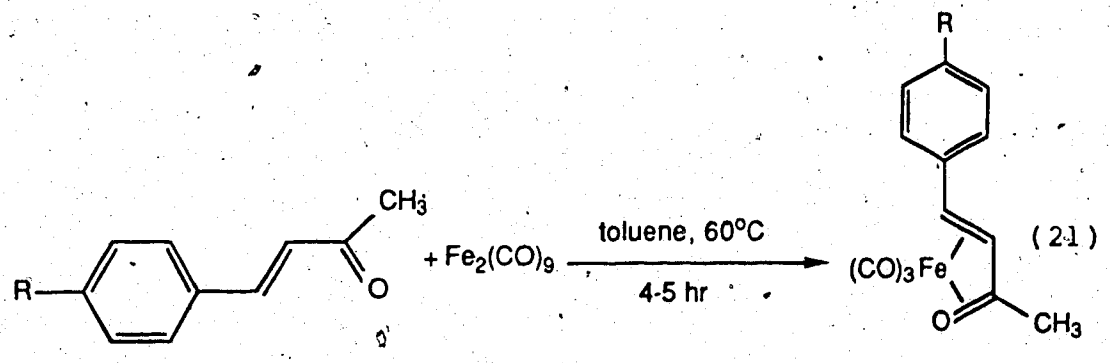


(20)

As well as repeating the thermal experiment of Reihlen, Stone and coworkers also prepared complex 30 via the photochemical reaction (sun lamp) of isoprene and $Fe(CO)_5$. Although the yield was low (1%), this technique has more recently become a rather general one since the yields have been much improved by use of more efficient ultraviolet light sources. For example, the photochemical preparation of the butadiene complex may now be accomplished in 65% yield.[32]

The high temperature (sealed tube) reaction of $Fe(CO)_5$ with dienes has also been improved by carrying out the reaction in an open system merely by refluxing the diene and $Fe(CO)_5$ in peroxide free di-n-butyl ether.[33] The open system has the advantage of allowing the carbon

monoxide produced to escape irreversibly, thus shifting the reaction equilibrium to the product side. Obviously for this method to be successful the diene itself should be thermally stable at these high temperatures; if not, then either the aforementioned photochemical reaction may be employed, or a different iron carbonyl species can be used. For example, $Fe_3(CO)_{12}$ can be used to form diene complexes under milder conditions than $Fe(CO)_5$, usually by refluxing the diene and $Fe_3(CO)_{12}$ in benzene.[31,34] Milder conditions still are needed for reactions involving $Fe_2(CO)_9$, which will produce diene complexes at ca. 50-60°C, the conditions varying from complex to complex. The latter is also the species usually employed to prepare η^4-heterodiene complexes (eq. 21).[35]



(21)

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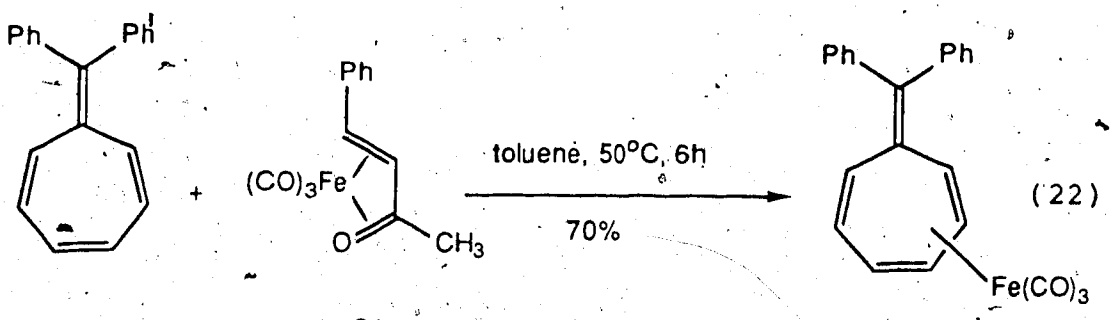
Such complexes, particularly (benzylidene acetone)iron tricarbonyl 31 (R = H) have been used as $Fe(CO)_3$ transfer reagents to produce other diene iron carbonyl complexes which could not be produced via the thermal or

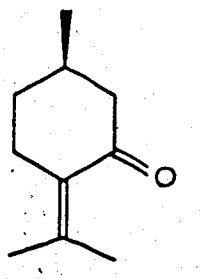
photochemical processes due to the sensitivity of the ligand to the reaction conditions, eg. the production of tricarbonyl(8,8-diphenylheptafulvene)iron **32** (eq. 22).[36]



(22)

31 **32**

Perhaps the most interesting application of heterodiene complexes lies in the preparation of optically active diene complexes, an area investigated by Birch and coworkers,[37] who prepared chiral heterodiene complexes using dienones such as (+)-pulegone, **33**, and (-)-3β-(acetyloxy)pregna-5,16-dien-20-one, **34**. These species were employed as transfer reagents to prepare chiral cyclohexadiene complexes, although only in moderate enantiomeric excess (the highest was 43% using **34**).



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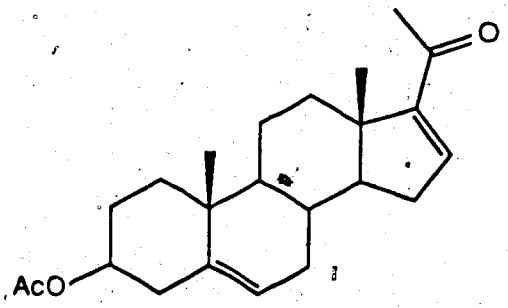
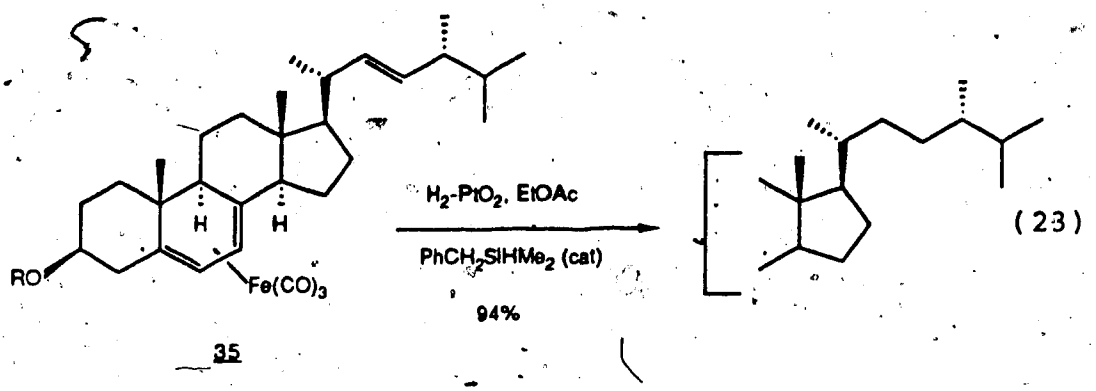


34

Another method for preparing diene iron carbonyl complexes was developed by Shvo and Hazum[38] and involves generation of the presumed $Fe(CO)_4$ intermediate by reaction of trimethylamine N-oxide (Me_3NO) with $Fe(CO)_5$ in the presence of the diene. The reaction is extremely rapid and is approximately 50% complete upon complete addition of the $Fe(CO)_5$, and is then completed by refluxing the reaction mixture for one hour.

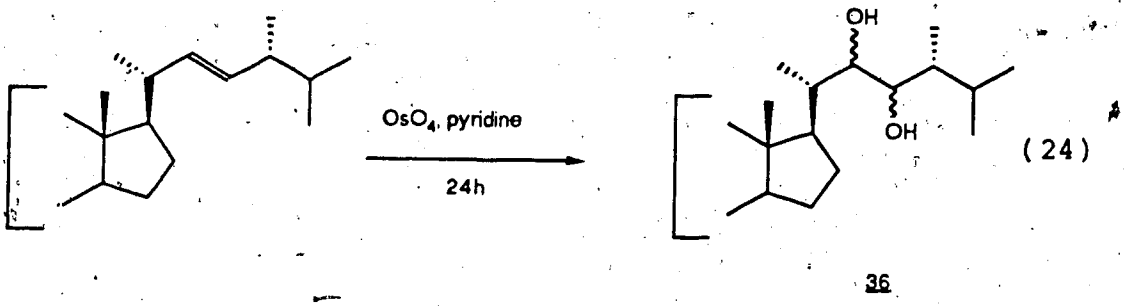
As mentioned previously, it is for their ability to modify the chemical behaviour of dienes that these complexes are of interest to organic chemists. These changes were first investigated by Hallam and Pauson[30] for the butadiene complex. They found that the complex was unaffected by hydrogen in the presence of Adam's catalyst (platinum oxide), conditions which would normally bring about the reduction of butadiene. Likewise the complex did not undergo a Diels-Alder reaction with maleic anhydride even after refluxing in benzene for 48 hours, and was only slightly affected by ozonolysis and lithium aluminum hydride reduction.

This inertness to hydrogenation was exploited by Barton and coworkers[39] as a means of protecting the diene functionality in ergosterylbenzoate 35 (R = PhCO) while the double bond in the side chain was hydrogenated (eq. 23).



35 (23)

Evans and coworkers[40] also demonstrated that in complex **35** (R = CH_3CO) the coordinated diene-$Fe(CO)_3$ unit was unaffected by treatment with osmium tetroxide, and oxidation occurred exclusively at the side chain double bond to produce diol **36** in 82% yield (eq. 24).



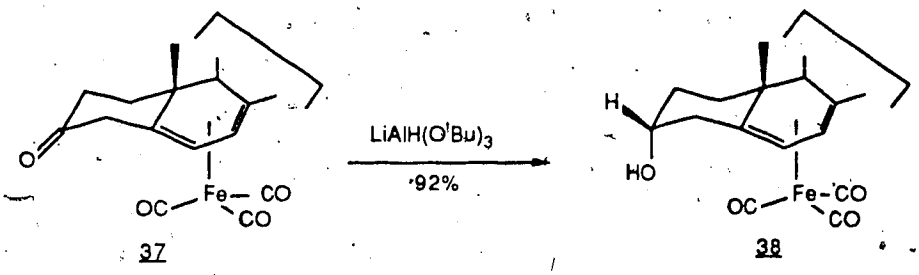
(24)

36

Similarly, reaction of **35** (R = $COCH_3$) with diborane, followed by workup with alkaline hydrogen peroxide left the diene $Fe(CO)_3$ unit intact, and produced a mixture of alcohols at the side chain double bond plus some reduction of the ester functionality.

Another interesting feature of diene-complex **35** is

that due to the crowded nature of the top (β) face of the steroid, the iron carbonyl moiety is located exclusively on the underside of the molecule and has an effect on the chemistry of the nearby 3 position. Barton and Patin[41] were thus able to bring about selective reduction of the ketone complex **37** (produced via N-chlorosuccinimide/dimethyl sulphide oxidation of **35** (R = H)), from the top face using the bulky reducing agent lithium hydridotri-t-butoxyaluminate ($LiAlH(OtBu)_3$), to give the previously unknown epimeric alcohol complex, tricarbonyl(epiergosterol)iron, **38**, in 92% yield (eq. 25).

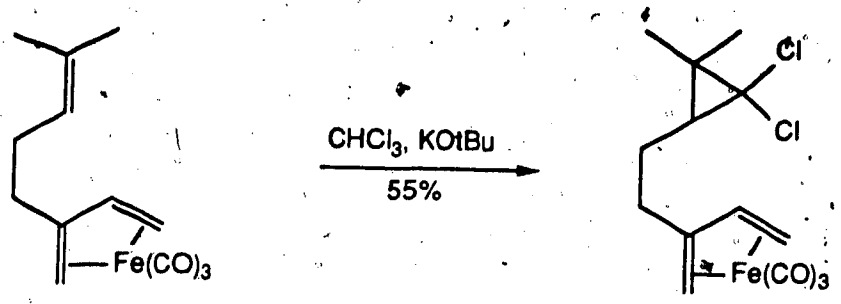


37 38 (25)

The steric bulk of the $Fe(CO)_3$ unit also has an effect on the rate of acetylation of **35** (R = H) and **38**, with the former being completely acetylated in one hour at room temperature, while the latter was unaffected.

The $Fe(CO)_3$ moiety is also unaffected by carbenes as evidenced by the formation of the cyclopropyl derivatives of myrceneirontricarbonyl **39** (eq. 26) and cyclohepta-

trieneirontricarbonyl **40** (eq. 27).[42]



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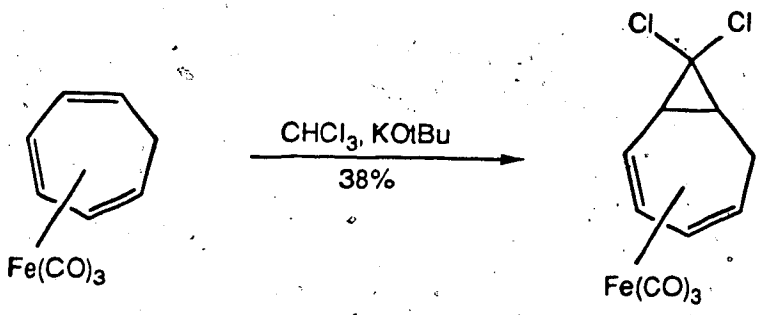
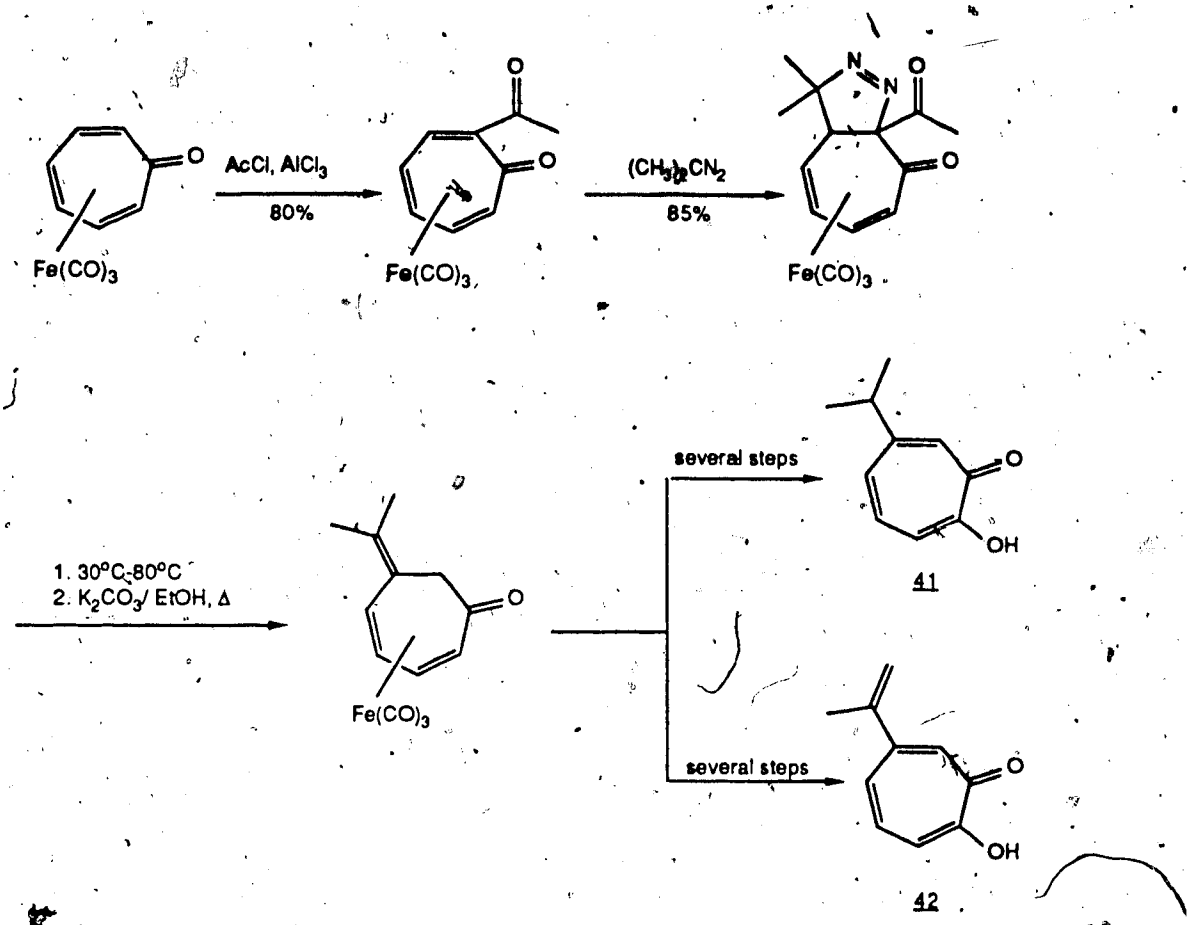


(27)

40

Complex **39** also undergoes selective acetylation at the side chain double bond under mild Friedel-Crafts conditions[43] even though acetylation of diene-$Fe(CO)_3$ complexes can be achieved fairly readily[44] (see Scheme III).

The ability to bring about acetylation of the uncomplexed double bond of a triene system has been used, in conjunction with the stability of the $Fe(CO)_3$ moiety to diazoalkanes, in the synthesis of the natural products β-Thujaplicin **41** and β-Dolabrin **42** (Scheme III).[45]

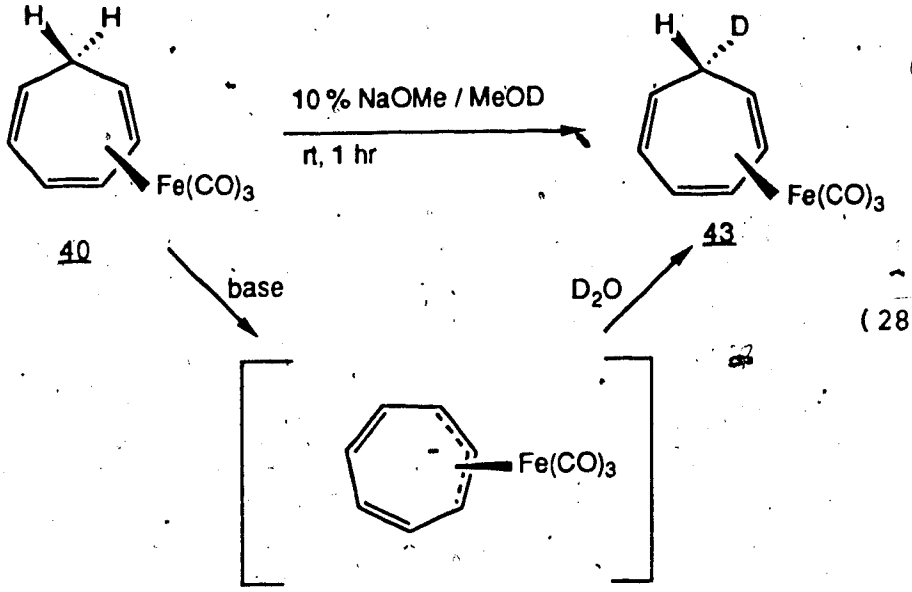


Scheme III

Complexation to a metal atom also has the ability to stabilise otherwise highly reactive or unstable species, thus allowing study and chemical modification of them (e.g. cycloheptatriene anion, butadiene and trimethylenemethane).

Although cycloheptatriene is very difficult to deprotonate (as evidenced by its high pK_a of 36[46]), the corresponding $Fe(CO)_3$ complex **40** readily undergoes deuterium exchange (10% NaOMe-MeOD, rt, 1 h) to produce

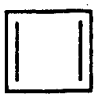
43,[47] and can be deprotonated by a variety of bases, eg. KOtBu,[42] KH[48] to produce the bright red anion **44** (eq. 28). The pK_a of complex **40** has been estimated to be 20;[42] thus, complexation brings about a 10^{16} fold increase in acidity.



40 **43** (28)

44

Similarly the highly reactive species cyclobutadiene, **45**, has been stabilised as the iron carbonyl complex, **46**



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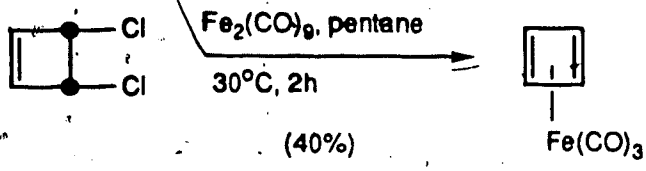
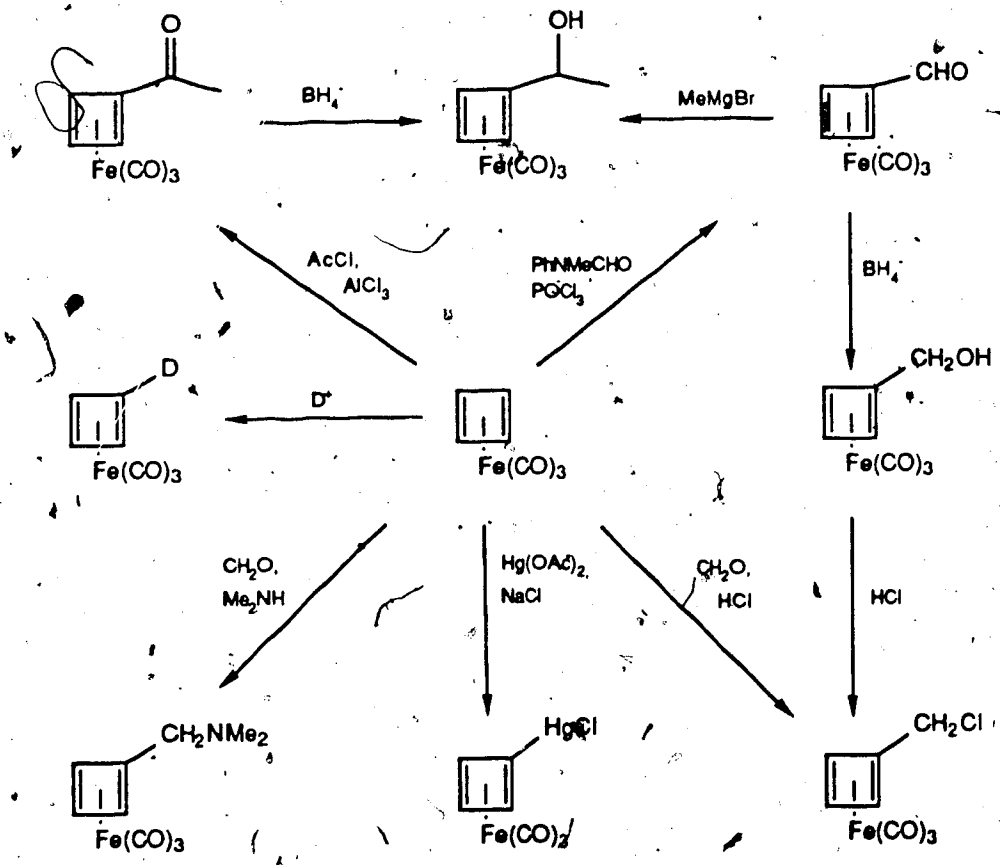
(eq. 29),[49] and numerous derivatives have been obtained



(29)

46

(Scheme IV),[50] again attesting to the stability of the $Fe(CO)_3$ moiety towards a large variety of chemical reagents and reaction conditions.

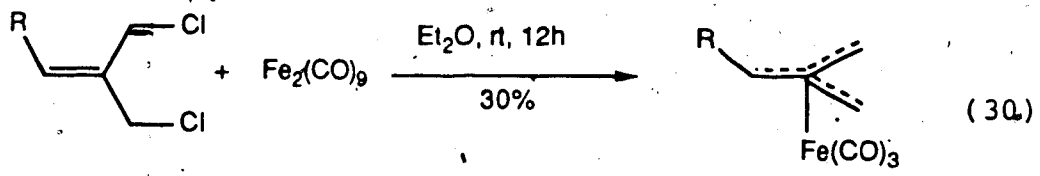


Scheme IV

Finally, the structurally interesting compound trimethylenemethane (TMM) **47** (R = H) and its derivatives

47

have also been stabilised using an iron tricarbonyl group. The parent complex **48** (R = H) was first reported in 1966[51] by the reaction of excess $Fe_2(CO)_9$ with 3-chloro-(2-chloromethyl)propene **49** (eq. 30).

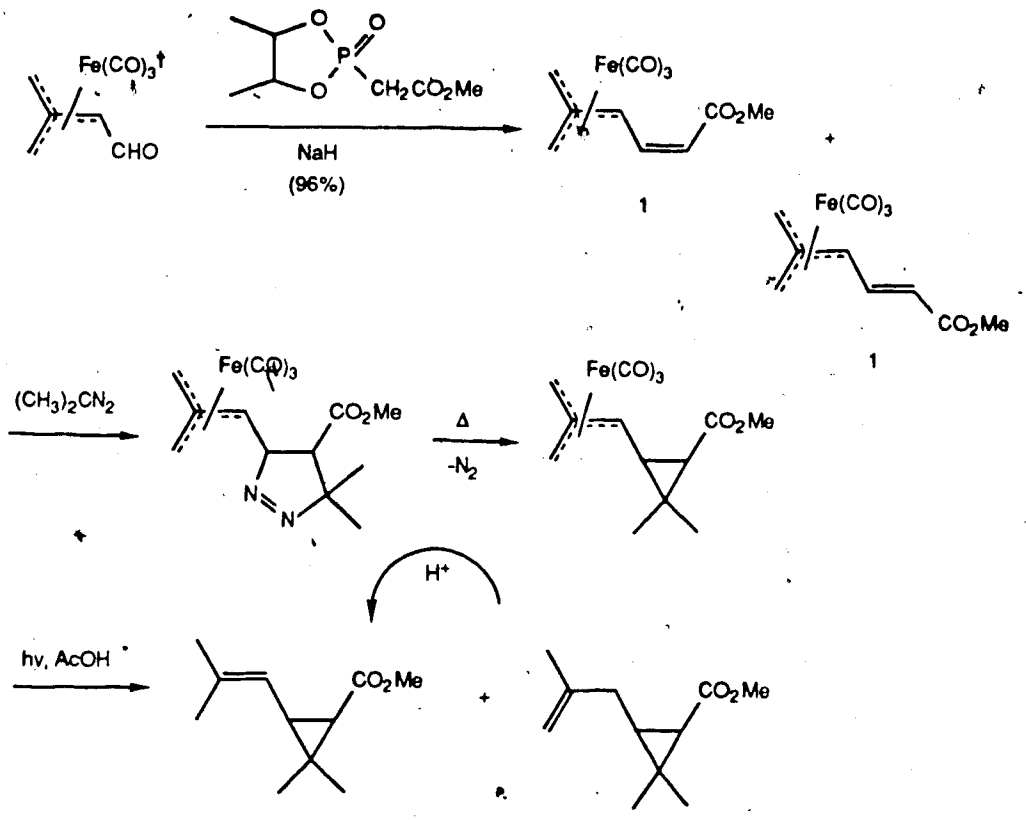


(30)

49 **48**

X-ray studies[52] of complex **48** (R = H) have shown that the organic ligand is not planar but slightly bent at the centre with the iron atom located nearest the central carbon atom and equidistant from the other three carbons. The equivalency of these three carbons, and the six protons attached to them, is demonstrated by the sharp singlet in the proton nmr spectrum. Numerous other

TMM-$Fe(CO)_3$ complexes have been reported subsequently (a review on the subject[53] lists at least 66 compounds of this type), and some have even found use as starting materials in organic syntheses, eg. preparation of chrysanthemates (Scheme V).[45]



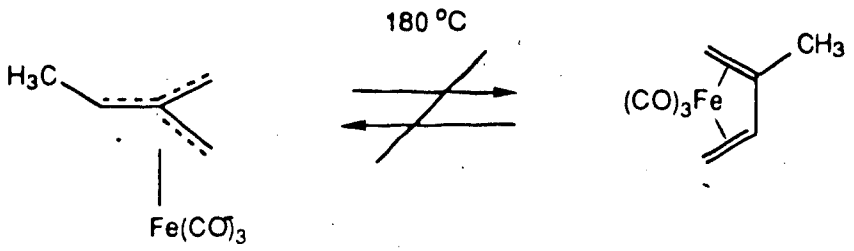
†. prepared from the corresponding alcohol complex by MnO_2 oxidation.[54]

*. reductive photodecomplextation [55]

Scheme V.

It should also be noted that methyltrimethylenemethane-$Fe(CO)_3$, **48** (R = CH_3), and isoprene-$Fe(CO)_3$, **30**, are structural isomers and have been shown to be non-

— interconvertible on heating up to 180°C[56] (eq. 31).



(31)

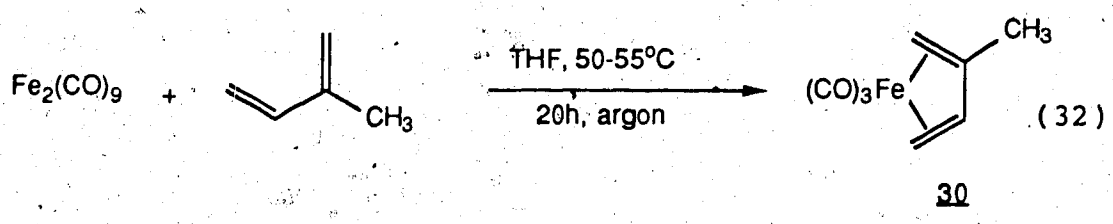
For these complexes to be of use to the organic chemist a means must be available for the removal of the iron tricarbonyl functionality without alteration of the organic ligand. Several such methods exist and these are now briefly described. Among the earliest oxidizing agents used were ferric(III) chloride and ceric ammonium nitrate (CAN),[57] and these are effective in most cases provided the free ligand is not particularly sensitive to the slightly acidic oxidation conditions. A milder technique using trimethylamine N-oxide was reported in 1974 by Shvo and Hazum,[58] and has found extensive use since then. Birch and Kelly recently reported[59] that ligand replacement (usually with phosphorous ligands) is possible using Me_3NO. Another ligand substitution/oxidation technique using alkaline hydrogen peroxide (H_2O_2/NaOH) was reported in 1979 by Davies[60] and again in 1983 by Franck-Neumann.[61] By controlling the amount of

reagent, stepwise replacement of carbon monoxide ligands by other ligands, eg. PPh_3, $P(OMe)_3$[60] can be accomplished. If a large excess of H_2O_2/NaOH is used, complete removal of the iron carbonyl moiety can be achieved and the results (75-95% yields)[61] are comparable to those using Me_3NO. A variety of other reagents have been used, eg. pyridinium chlorochromate,[62] copper(II) chloride[42], etc., although not as extensively as $FeCl_3$, CAN and Me_3NO. No particular reagent is universally applicable, and the "correct" one to use, plus the right oxidation conditions, is usually determined empirically.

During the course of this study the direct metallation of isoprene was recently reported[62a] using the specialised base combination lithium tetramethyl-piperidide-potassium tertiary butoxide. Nevertheless, this technique is intrinsically incapable of effecting chiral synthesis, which in principle is available by using transition metal complexes of (prochiral) unsymmetric dienes.

DISCUSSION

The starting complex, tricarbonyl(2-methyl-1,3-butadiene)iron, **30** (commonly called isoprene iron tricarbonyl), was prepared via the thermal reaction of nonacarbonyldiiron ($Fe_2(CO)_9$) with isoprene (eq. 32) using a modification[63] of the procedure of Dauben and Lorber.[64] Previous use of their procedure in our group



(32)

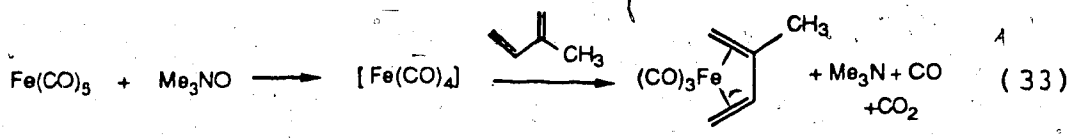
30

had produced the desired complex in only low yields (*ca.* 20-30%), and as a result, a number of changes were made.[63] Tetrahydrofuran (THF) was used in place of diethyl ether (in order to allow a higher reaction temperature), the reaction time was increased from five to *ca.* twenty hours, a large excess of isoprene was employed, and finally it was empirically determined that the yield was improved by increasing the volume of solvent used, good results being obtained with a concentration of

approximately 0.06N with respect to $Fe_2(CO)_9$. Even using this modified procedure, however, several of the early attempts resulted in only low yields (ca. 20%) and undesired side products. It was then found that the products were highly dependent upon the order in which the reagents were mixed. If $Fe_2(CO)_9$ was added to THF at room temperature, followed by isoprene, and the reaction continued in the normal manner, the desired complex **30** was produced in only low yield (ca. 20%), and the major product was an unidentified dark green oil. However, if isoprene was dissolved in THF, and the resulting solution warmed to the reaction temperature (50-55°C), followed by addition of the $Fe_2(CO)_9$, **30** was produced in good yield (59%). Cotton and Troup[65] have in fact shown that $Fe_2(CO)_9$ reacts with THF at 23°C under argon, but no products could be identified ("a...sample of diiron nonacarbonyl...stirred with 100 mL dry THF at 23°C under a flow of argon gas...acquired an orange-yellow colour immediately and then slowly (over a one hour period) changed to a deep red. IR spectra taken during this reaction sequence were very complex in the carbonyl stretching region (2100-1700 cm^{-1}) and have not been interpreted."[65])

The procedure of Shvo and Hazum[38] was also briefly investigated, in which the reactive $Fe(CO)_4$ intermediate

is produced by reaction of trimethylamine N-oxide with $Fe(CO)_5$, and the intermediate is "internally" trapped by the double bond (eq. 33). This procedure did provide the



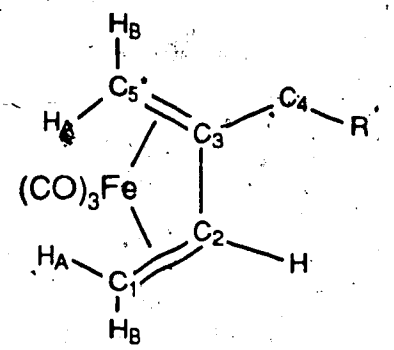
(33)

desired complex **30** in somewhat lower yield (41%) than the $Fe_2(CO)_9$ route, and also suffered from the same problems with by-products. It did, however, have the advantage of requiring a much shorter reaction time (ca. two hours) compared to the other procedure.

The base used for deprotonation of **30** was lithium 2,2,6,6-tetramethylpiperidide (LiTMP), generated by reaction of n-butyllithium with 2,2,6,6-tetramethylpiperidine (TMP) in the presence of hexamethylphosphoramide (HMPA) (10% v/v in THF).[63] The n-BuLi, TMP and HMPA were mixed in THF at -78°C for 30 minutes, after which time a solution of **30** in THF was added over a period of 30 minutes. The resulting solution was stirred for a further 30 minutes to ensure deprotonation of the complex before the electrophile was added, then the solution was allowed

to warm to room temperature overnight.

The products from these reactions were identified on the basis of their proton (^{1}H) nmr, infrared and mass spectra which, in the case of (η^4-diene)iron tricarbonyl complexes, are all particularly distinctive. The ^{1}H nmr of the complexes share the following features: (i) a broad one hydrogen triplet (J = 8 Hz) in the region δ5.1-5.4, corresponding to H2, **50**; (ii) a broad (sometimes



50

*The numbering of this carbon atom varies depending on the nature of R

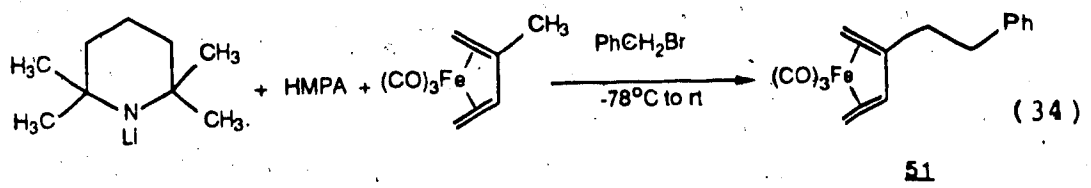
resolved) signal at *ca.* δ1.8 representing $H5_B$, in conjunction with a doublet of doublets (J = 7 and 2.3 Hz) at *ca.* δ1.7 for $H1_B$ (the "outer protons"); (iii) a similar set of signals further upfield at *ca.* δ0.3 and δ0.05 (J = 9 and 2.3 Hz) for $H5_A$ and $H1_A$ respectively (the "inner protons"). The dramatic upfield shift of the latter,

compared to their corresponding positions in the ^1H nmr spectra of the free dienes (ca. δ5.0) is believed to be caused by their proximity to the iron atom.[66]

The infrared spectra of the complexes are dominated by the signals for the carbonyl (C≡O) ligands. These appear as a sharp signal at ca. 2050 cm^{-1} and a broader signal (sometimes resolved) at ca. 1970 cm^{-1}; the former corresponds to the symmetrical stretching of the C≡O ligands and the latter to the unsymmetrical stretching. Other signals are usually much weaker but can occasionally be used for structure confirmation.

The mass spectra are also highly distinctive, with peaks resulting from the sequential loss of carbon monoxide from the molecular ion, usually ending up with a base peak corresponding to M^+-3CO. If the ionisation energy is sufficiently high the latter peak fragments further. In all the complexes the charge tends to reside on the fragment containing the iron atom due to its low ionisation potential (7.87 eV[67]), and an abundant fragment corresponding to m/e 56 (Fe) is often found in the high energy spectra.

Using the aforementioned technique the anion of **30** was generated and reacted with benzyl bromide to produce tricarbonyl(1,2,3,6-η-3-methylene-5-phenyl-1-pentene)iron **51**, in 59% yield (eq. 34).



(34)

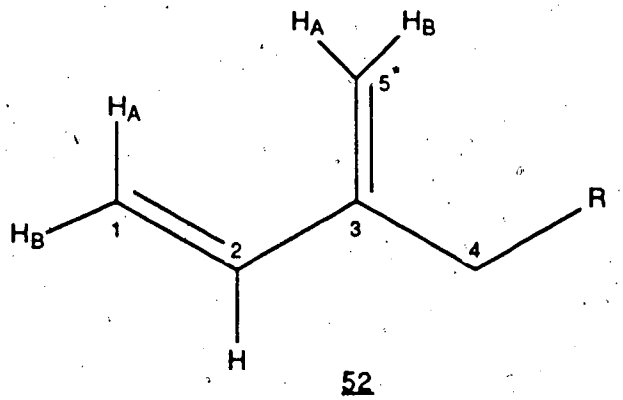
51

With this complex in hand its oxidation to the free 1,3-diene was investigated. The first oxidant studied was trimethylamine N-oxide (Me_3NO), the reagent developed by Shvo and Hazum.[58] This material is commercially available as its dihydrate (Aldrich) and although some groups have used it in this form,[68] it was felt that its activity would be increased by removing the water of crystallisation. This was achieved by refluxing the solid with dry toluene[69] in a Dean-Stark apparatus, then recrystallisation of the resulting solid from dry ethyl acetate. The oxidation technique involved heating (50-55°C) the complex in a dry aprotic solvent (both THF and toluene were used), with a large excess (14-15 equivalents of Me_3NO for several hours until TLC and/or infrared analysis (disappearance of C≡O signals) indicated that the reaction was complete. The onset of the reaction is accompanied by an evolution of gas (Me_3N and CO_2, eq. 35), and this phenomenon has been used by Birch[68]

$$(\text{diene})Fe(CO)_3 + Me_3NO \xrightarrow{\Delta} \text{diene} + Me_3N + CO_2 + \text{"iron compounds"} \qquad (35)$$

to determine reaction temperatures for oxidations using this reagent. It should however be noted that the gas evolution is not quantitative.[58]

After workup and purification (see Experimental), the free 1,3-dienes were characterised by their ^1H nmr spectra, infrared spectra, elemental analyses and/or high resolution mass spectra. The ^1H nmr of the free dienes, like those of the complexes, have a number of characteristic signals including: (i) a one hydrogen doublet of doublets (J = 18 and 11 Hz) at ca. δ6.4 corresponding to H2, **52**; (ii) a one hydrogen doublet (J = 18 Hz) at ca. δ5.3 for $H1_A$; (iii) a one hydrogen doublet (J = 11 Hz) and two broad one hydrogen singlets at ca. δ5.0 for $H1_B$, $H5_A$ and $H5_B$ respectively.



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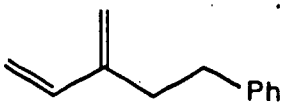
*Numbering varies depending on R.

The infrared spectra of the free dienes have two sets of signals in common: (i) absorptions at ca. 1650 and 1600 cm^{-1} resulting from C-C stretching, and (ii) absorptions at ca. 990 and 897 cm^{-1} for C-H out-of-plane deformations.[70]

Using the aforementioned technique complex **51** was oxidised to provide a pale yellow oil (55%) which was purified by flash chromatography (silica/toluene) to afford the known compound[6b,71] 3-methylene-5-phenyl-1-pentene **53** (45%).



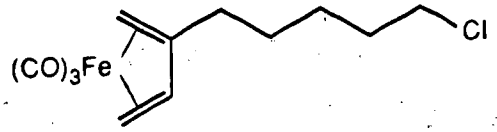
53

The oxidant ceric ammonium nitrate (CAN) was also investigated since this reagent had proven successful for several acyclic dienes in which Me_3NO had proven unsatisfactory.[72] The method employed was that of Greé and coworkers,[72] and involved reaction of the diene $Fe(CO)_3$ complex with CAN in methanol at -15°C. The addition of CAN in methanol solution (4-5 mole equivalents) to a solution of the diene-$Fe(CO)_3$ complex in methanol produces an immediate evolution of gas and the oxidations were monitored by measuring the volume of gas (in this case the reaction is quantitative producing 3 moles of carbon monoxide for each mole of diene $Fe(CO)_3$

complex[73]).

Using this procedure the oxidation of complex **51** resulted in a crude oil (87%) which was homogeneous by TLC analysis (silica/Skelly B), however purification resulted in the isolation of **53** (spectroscopically identical with that obtained previously) in only 40% yield. Washes of the chromatography column with more polar solvent systems (ether and ethyl acetate) did not result in isolation of any other identifiable products.

The anion of **30** was generated in the same manner and reacted with 1-bromo-4-chlorobutane in an attempt at differentiation between the two leaving groups. Examination of the product by mass spectroscopy indicated that the chloro complex **54** (M^+ = 298 for $C_{12}H_{15}{}^{35}ClFeO_3$ with a satellite at m/e 300 for $C_{12}H_{15}{}^{37}ClFeO_3$, ratio ca. 3:1), was the only product formed, although the yield was

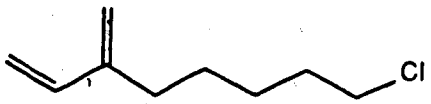


54

disappointingly low (38%). In an attempt to improve the yield, 1-bromo-4-chlorobutane was converted to 1-chloro-4-iodobutane in 82% yield via Finkelstein exchange with sodium iodide.[74] The reaction of this electrophile with

30 improved the yield of 54 to 51% along with the recovery of unreacted starting material (26%).

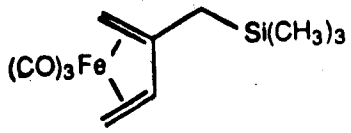
Oxidation of this complex with CAN in methanol resulted in a pale yellow oil (75%) whose infrared spectrum indicated a complete absence of carbonyl signals. The high resolution mass spectrum indicated a molecular weight of 158.0868 (calcd. for $C_9H_{15}{}^{35}Cl$: 158.0862). Purification by flash chromatography (silica/Skelly B) provided 8-chloro-3-methylene-1-octene 55 in 43% yield.



55

Reaction of the anion with chlorotrimethylsilane resulted in the isolation of an orange-brown oil (82%) but attempted purification by flash chromatography (silica/Skelly B) led to only 51% recovery of material. The main fraction was an (η^4-diene)iron tricarbonyl derivative (triplet at ca. δ5.2) and showed a strong signal at ca. δ0 for the trimethylsilyl functionality, but the expected trimethylsilyl derivative **56** could only be obtained in 2% yield.

This poor result, coupled with the low yield from the reaction with 1-bromo-4-chlorobutane, prompted a brief



56

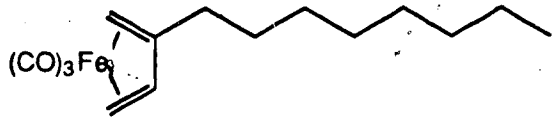
study of several reaction variables centering on the generation of LiTMP. An examination of the literature[75] indicated that the base could be prepared via reaction of TMP with either methyllithium or n-butyllithium but that the former, for some unexplained reason, gave better results. Methyllithium reacts with TMP relatively slowly at lower temperatures, and LiTMP is best prepared at room temperature.

Consequently we generated LiTMP at room temperature using methyllithium. A quantitative measure of base formation was also possible by collection of the methane evolved in a gas burette. The resulting LiTMP was then cooled to -78°C, followed by the addition of HMPA (10% v/v in ether). A solution of **30** in ether was added over a period of 30 minutes, stirred for a further 30 minutes, and then reacted with an electrophile. Using this procedure the yield of the benzylation product **51** improved to ca. 70%; the reaction with 1-bromo-4-chlorobutane produced complex **54** in an improved yield of 54% (along with recovery of starting material (21%)), and the reaction

of **30** with chlorotrimethylsilane produced complex **56** (55%) although technical difficulties were encountered in completely separating **56** from unreacted **30**.

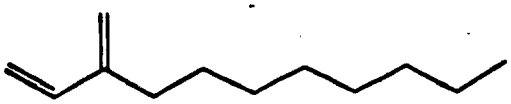
In view of these results, this method was adopted as a "standard" procedure for the generation of the anion of **30**, and the scope of the reaction was investigated using a variety of electrophiles.

The result of the reaction of **30** with 1-iodoheptane presented similar separation problems to those encountered in the chlorotrimethylsilane reaction. The crude mixture of unreacted **30**, 1-iodoheptane, and the desired product **57**, could only be partially separated by flash chromatography.



57

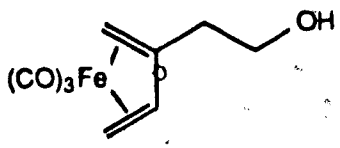
The yield of complex **57** was determined by nmr analysis to be 57%, along with 6% unreacted **30** and 7% 1-iodoheptane. Instead of further separation at this stage, the mixture was oxidised with CAN in methanol to give a crude mixture containing 1-iodoheptane and 3-methylene-1-undecene **58**. This could be separated by column chromatography (silica gel-silver nitrate, 2% ether:Skelly B), to provide **58** in 80% yield. The structure of **58** was confirmed by the following



58

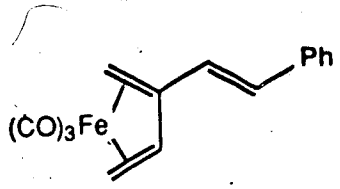
spectral data: molecular weight 166.1721 (calcd. for $C_{12}H_{22}$: 166.1722); ^1H nmr spectrum (400 MHz, $CDCl_3$) δ6.35 (d of d, J = 18 and 11 Hz, 1H, H2), 5.22 (doublet of triplets, J = 18 and 0.8 Hz, 1H, $H1_A$), 5.04 (d of d, J = 11 and 0.8 Hz, 1H, $H1_B$), 4.99 (br s, 1H), 4.98 (br s, 1H, $H12_{AB}$), 2.20 (t, J = 8 Hz, 2H, H4), 1.48 (br m, 2H), 1.30 (br m, 10H, $(CH_2)_5$), 0.90 (m, 3H, CH_3).

In 1965 Schaap and coworkers[76] reported the use of paraformaldehyde as a substitute for the experimentally difficult to handle formaldehyde, in the conversion of organolithium compounds into the corresponding primary alcohols in good yields. Consequently the reaction of the anion of **30** with this electrophile was investigated. Solid paraformaldehyde was added to the solution of anion of **30** in ether/HMPA at -78°C and the mixture then warmed to room temperature. The crude product, a yellow-orange oil (99%) was purified by flash chromatography (silica/Skelly B, followed by SkellyB:ether (1:1)) to give the desired alcohol complex **59**, albeit only in low yield (32%), along with unreacted starting material (11%).

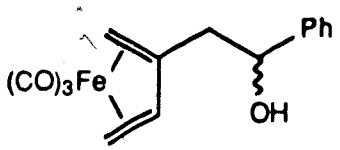


59

The reaction of the anion of **30** with benzaldehyde was also investigated. Flash chromatography (silica/toluene) of the crude product for this reaction resulted in four distinct fractions which were shown to contain, in order of elution, unreacted starting material (27%), a trace amount (4%) of a pale yellow solid identified by its ^{1}H nmr spectrum as **60** (^{1}H nmr spectrum (400 MHz, $CDCl_3$) δ8.07 (m, 2H, Ph), 7.59 (m, 1H, Ph), 7.49 (t, 2H, Ph), 5.37 (t, J = 8 Hz, 1H, H2), 4.10 (d, J = 16 Hz, 1H, H5), 3.88 (d, J = 16 Hz, 1H, H4), 1.84 (d of d, J = 2.5 and 1.5 Hz, 1H, $H6_B$), 1.78 (d of d, J = 7 and 2.5 Hz, 1H, $H1_B$), 0.48 (d of d, J = 3 and 1 Hz, 1H, $H6_A$), 0.26 (d of d, J = 9 and 2.5 Hz, 1H, $H1_A$), resulting from dehydration of **61**), recovered benzaldehyde (33%), and the desired product **61** (38%).



60

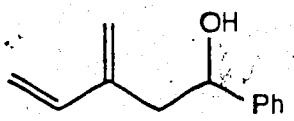


61

Although the latter appeared as a single spot in several TLC systems the ^{1}H nmr spectrum displayed two very similar sets of signals in a ratio of 3:2. The reaction was

repeated twice, and the yield of **61** could be increased to 85% using a longer reaction time (ca. 15 hours at -78°C). On each occasion however, the ratio of the aforementioned nmr signals was the same. It is believed that each set of signals belongs to one of the diasteromeric alcohols possible for **61**.

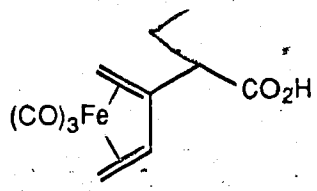
Oxidation of this complex with Me_3NO yielded an orange oil which was purified by flash chromatography to provide a 17% yield of 3-methylene-5-phenyl-penten-5-ol, **62**, with no other identifiable products. Oxidation with CAN in methanol yielded the crude alcohol **62** in 93% yield. Its identity was confirmed by high resolution mass spectroscopy 174.1043 (calcd. $C_{12}H_{14}O$: 174.1045) and by its ^1H nmr spectrum.



62

The reaction of organolithium compounds with carbon dioxide to produce carboxylic acids is often employed in synthesis. However, to obtain good yields it is important that the carbon dioxide is present in excess throughout the course of reaction (failure to do so can result in ketone and tertiary alcohol formation via further reaction of the organometallic).[77] This is normally achieved by

adding the organolithium to crushed dry ice or to a slurry of dry ice in a solvent. In this manner, addition of the anion of **30** to a slurry of dry ice in diethyl ether produced the carboxylic acid complex **63** in 89% yield after recrystallisation from ether/Skelly B (1:1).

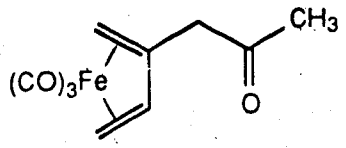


63

Oxidation of this complex with CAN resulted in a crude product which TLC analysis indicated was homogeneous. However attempted purification of this material resulted in a product which had an extremely complex ^1H nmr and mass spectra, and could not be identified. Oxidation of **63** with Me_3NO also proved unsuccessful, with the spectra (ir, nmr) of the crude reaction mixture being extremely complex.

The reaction of organolithium compounds with acid chlorides can in principle be used to produce ketones; in practise, however, the reaction must again be carefully controlled to prevent the product ketone from reacting with further organolithium to form the tertiary alcohol. The normal procedure was modified and the anion was gradually added via double ended needle, to an excess (3 mole equivalents) of acetyl chloride in ether at -78°C.

The resulting mixture was kept at -78°C and monitored by TLC, then quenched with water when all the starting material had been consumed (2 hours). This produced ketone **64** in 40% yield.

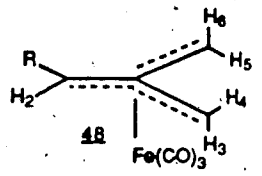


64

Attempts at oxidation of **64** with either Me_3NO or CAN were unsuccessful and produced products with complex, uninterpretable ^1H nmr and mass spectra.

Other less reactive electrophiles such as ethyl benzoate, benzonitrile and N,N-dimethylacetamide were also explored as substrates but in no case was there produced any identifiable ketone complexes (as evidenced by the lack of a signal at *ca.* 1720 cm^{-1} in the infrared spectra of the crude products). However, a new compound was isolated in moderate yield (53%) during the reaction of **30** with N,N-dimethylacetamide. The infrared spectrum, while showing no signal at *ca.* 1720 cm^{-1} did display signals at 2055 and 1980 cm^{-1} typical of an iron carbonyl complex. The ^1H nmr spectrum was unlike that of any of the isoprene complexes prepared previously, and showed the following signals: δ3.01 (doublet of quartets, J = 6.9 and 2.2 Hz, 1H), 2.48 (d, J = 4.4 Hz, 1H), 2.09 (d, J = 2.2 Hz, 1H), 1.69 (s, 1H), 1.55 (d, J = 4.4 Hz, 1H) and 1.28 (d, J =

6.9 Hz, 3H). The ^{13}C nmr spectrum showed signals for five different carbon atoms at δ104, 73, 52, 50 and 15, suggesting that the compound was possibly a structural isomer of **30**. The spectrum also showed three distinct signals for the carbonyl ligands at δ212.0, 211.5 and 211.0, indicating a fairly high barrier to rotation of the carbonyl ligands. The latter is not the case for typical (η^4-diene)$Fe(CO)_3$ complexes where only one carbonyl is seen.[78] On this basis a trimethylenemethane structure **48** (R = CH_3) was assigned. Compounds of this type are known

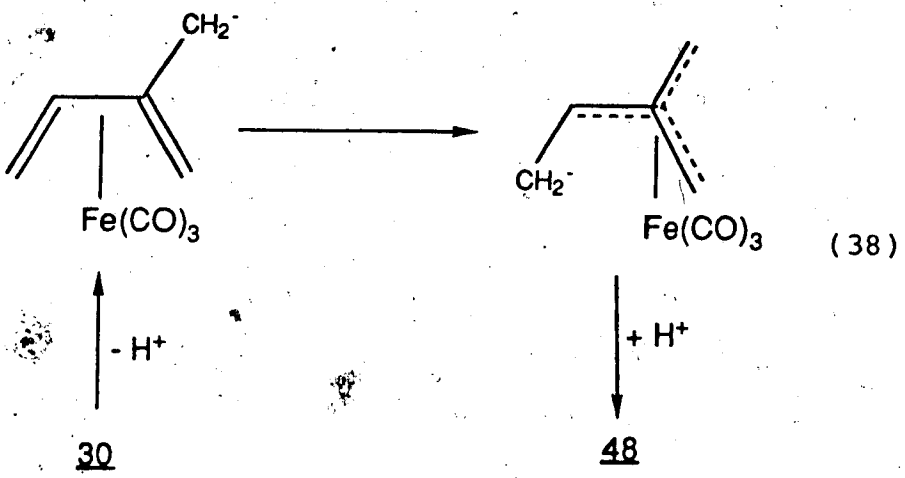


48

to be sufficiently "rigid" to display three carbonyl signals in their ^{13}C spectra,[79] and to have distinctive, simple ^1H nmr spectra due to the limited amount of coupling between the protons, long range W coupling being the only type seen.[80] Confirmation of the structure was provided by a comparison with the reported ^1H nmr, and infrared spectra:[80] δ3.14 (H2), 2.51 (H6), 2.11 (H5), 1.77 (H4), 1.63 (H3), $J_{H_2H_5}$ = 2.5 Hz and $J_{H_3H_6}$ = 4.5 Hz; infrared 2058 and 1979 cm^{-1} (C≡O).

The possible thermal isomerisation of (isoprene)iron tricarbonyl to (methyltrimethylenemethane)iron tricarbonyl, and vice versa, was investigated by Aumann

and coworkers,[56] and was found not to occur even upon heating to 180°C (eq. 31). However, formation of the isoprene $Fe(CO)_3$ (or $MTMMFe(CO)_3$) anion (or cation) may in principle allow the "isomerisation" to occur via a formal redistribution of electrons, and a slight "migration" of the iron atom from a position under the central carbon atom in **48** to a position midway between carbon atoms 2 and 3 in compound **30** (eq. 38).



(38)

30 **48**

In order to investigate the relationship between the anion of complex **48** and that of complex **30**, the latter was generated in the usual manner, and the resulting solution divided into two aliquots. One portion was quenched with water at -78°C, whereas the other was warmed to room temperature prior to being quenched. During the warm-up period the latter underwent a series of colour changes from lemon-yellow (-78°C) through orange-yellow, finally attaining a deep orange-red colour at room temperature. The product from the "-78°C quench" was **30** (55%) and that

from the "room temperature quench" was **48** (32%), although nmr integration showed the latter contained ca. 8% of **30**.

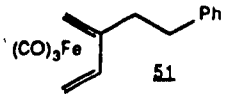
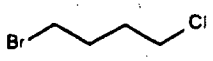
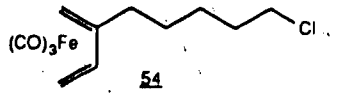
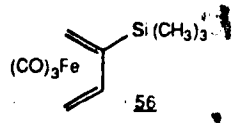
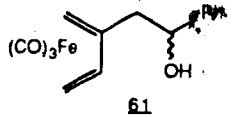
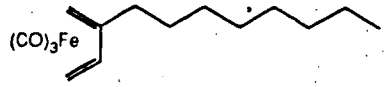
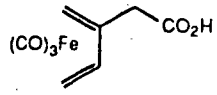
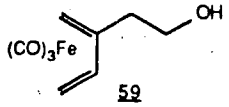
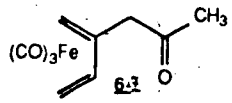
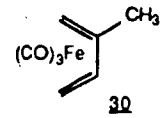
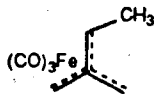
An attempt was made to determine if an alkylated derivative of **48** could be obtained by generating the anion of **30**, warming it to room temperature, then treating it with methyl iodide. However none of the desired (ethyltrimethylenemethane)iron tricarbonyl could be detected - the only identifiable product, after reduced pressure distillation of the crude mixture, was a small amount (<10%) of (methyltrimethylenemethane)iron tricarbonyl.

The results described above for the reaction of **30** with electrophiles are summarised in Table 1 and the results of the oxidation of the (η^4-diene)iron tricarbonyl complexes are summarised in Table 2.

In conclusion it has been shown that formation of the isoprene iron tricarbonyl complex allows deprotonation of the isoprene moiety to occur without polymerisation, and that the anion of the complex reacts with a variety of electrophiles to produce new iron tricarbonyl complexes in moderate to good yields. From these complexes the free dienes can be obtained via oxidative decomplexation using either ceric ammonium nitrate or trimethylamine N-oxide. Although the yields for the latter are low at present, it is hoped that future experimentation will provide these

TABLE 1.

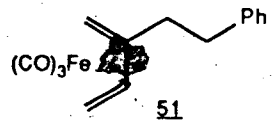
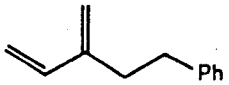
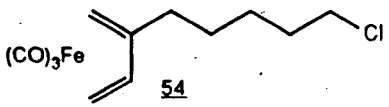
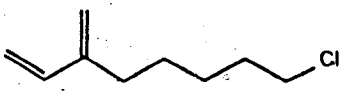
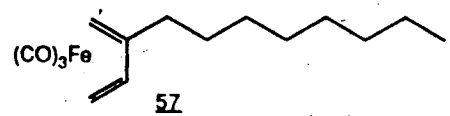
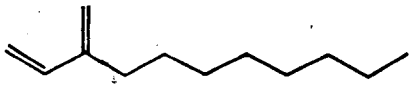
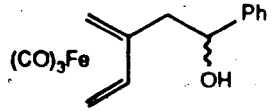
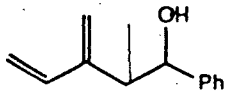
REACTION OF (ISOPRENE) IRON TRICARBONYL WITH ELECTROPHILES

ELECTROPHILE	PRODUCT	YIELD [a]
$PhCH_2Br$	 51	70% [b]
	 54	54%
$(CH_3)_3SiCl$	 56	55% [b]
PhCHO	 61	85% [c]
	 57	57% [b]
CO_2	 63	89%
$(CH_2O)_n$	 59	32%
CH_3COCl	 64	40%
H_2O -78 °C	 30	55%
H_2O room temp.	 48	29% [b]
$CH_3CON(CH_3)_2$	48	53%

a. Isolated yield.
b. Yield from NMR integration.
c. 3:2 mixture of isomers.

TABLE 2.

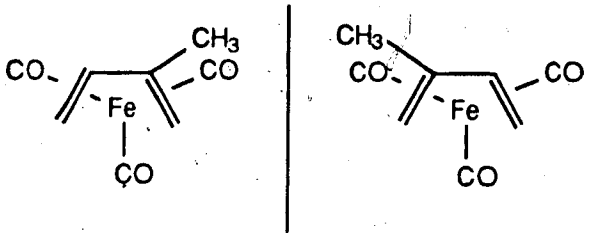
OXIDATION OF IRON TRICARBONYL COMPLEXES.

COMPLEX	OXIDANT	PRODUCT	YIELD (%)[a]
 51	Me_3NO	 53	45
	CAN		40
 54	CAN	 55	43
 57	CAN	 58	80
 61	CAN	 62	93[b]
	Me_3NO		17

a. Isolated yield.
b. Crude yield.

dienes in better yields. If this can be achieved, this method, because of its brevity and easy availability of starting materials, offers a valuable alternative to current multistep procedures.

This method also has the advantage of providing the possibility of chiral synthesis. For example, complexation of an unsymmetrical (prochiral) diene to a transition metal produces a racemic mixture of two enantiomers. While no simple means exists for the resolution of such a racemic mixture, some prior work of Birch[37] has indicated that chiral **30** might be available



30

(via chirality transfer) using the reaction of suitable optically active heterodiene-$Fe(CO)_3$ complexes with isoprene, eg.

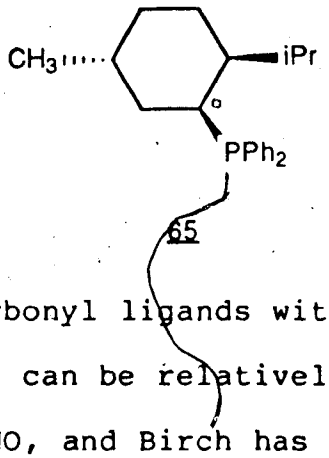
Prochiral Diene + (Chiral Auxiliary)$Fe(CO)_3$ →

(Chiral Diene)$Fe(CO)_3$ + Auxiliary

The interesting possibility exists, then, that

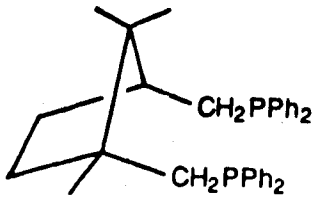
deprotonation of chiral **30** and subsequent alkylation could produce optically active isoprenoids by chiral induction.

The design of this method also offers other possibilities to be explored. For example, Howell and Thomas[81] have reported that substitution of one of the carbon monoxide ligands in a (1,3-cyclohexadiene)iron tricarbonyl complex with the chiral monodentate phosphorous ligand neomenthyldiphenyl phosphine **65**, led to modest chiral induction.

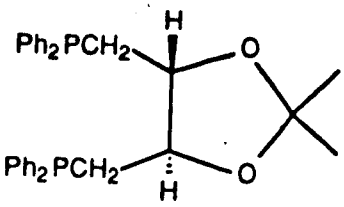


65

Substitution of carbonyl ligands with monodentate phosphorous ligands can be relatively easily achieved by treatment with Me_3NO, and Birch has reported[59] the replacement of a carbon monoxide ligand with **65** in 68% yield using this procedure. An alternative would be to use the technique of Davies[60] (H_2O_2/NaOH) to replace two of the carbonyl ligands with one of the many chiral bidentate phosphorous ligands developed in the field of chiral catalytic hydrogenation (eg. (+) CAMPHOS = (+) - (1R,3S) - 1,2,2-trimethyl-1,3-bis(diphenylphosphinomethyl)cyclopentane, **66** and (-)-DIOP = (-)-2,3-O-iso

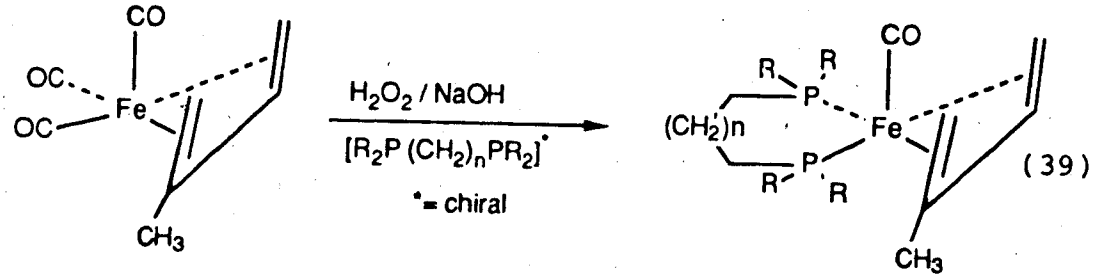


66



67

propylidene-2,3-dihydroxy-1,4-bis(diphenylphosphino)butane **67**). By judicious choice of reagent, it may be possible to prepare suitable optically active derivatives for study (eq. 39) of chiral induction.



30 (39)

Finally, it has also been shown that the anion of the isoprene complex undergoes an interconversion on warming from -78°C to room temperature to produce, after hydrolysis, the isomeric complex (methyltrimethylene-methane)iron tricarbonyl. It remains for future work to investigate this phenomenon in greater detail in achiral, and possibly chiral form. The consequences of such a study would be of interest, both from the standpoint of structure and synthesis.

EXPERIMENTAL

General Procedures

Nuclear magnetic resonance (nmr) spectra were run on a Bruker WP-80, Bruker WH-200, Bruker AM-300 or Bruker WH-400 spectrometers (as indicated), using deuterated chloroform ($CDCl_3$), and tetramethylsilane (TMS) as an internal standard. Chemical shifts are reported as δ values in parts per million downfield from TMS, followed by the multiplicity, coupling constant(s) in Hertz, number of hydrogens and signal assignment. The following abbreviations are used in the text: s = singlet, d = doublet, t = triplet, d of d = doublet of doublets, m = multiplet, br = broad. H_A and H_B refer, respectively, to the inner and outer protons of the diene unit as outlined in the Discussion (see page 35), and typified by structure 50.

Infrared spectra (ir) were recorded on a Nicolet 7199 FTIR in the manner indicated. The following abbreviations are used: s = strong, m = medium, w = weak, sh = shoulder.

Low resolution mass spectra of the iron complexes were recorded on an AEI MS12, and exact mass measurements on a Kratos MS50 spectrometer. Both are reported as m/e (relative intensity).

Analyses were performed by the Microanalytical Laboratory of this department.

Melting points were measured on a Reichert hot stage apparatus and are uncorrected.

Commercial methyllithium in diethyl ether (Aldrich), was standardised by titration using diphenylacetic acid as an indicator.[82]

All reactions involving the tricarbonyl(diene)iron complexes were performed under argon, using standard techniques.[83] Glassware was oven-dried at 140°C for several hours. All solvents were distilled under argon (purified further by passage through a tower of reduced BASF catalyst R3-11 and a drying tower filled with Drierite and sodium hydroxide pellets); diethyl ether and tetrahydrofuran were refluxed over potassium, benzophenone added and distilled; toluene was refluxed over sodium, benzophenone added, and distilled; ethyl acetate and Skellysolve B were distilled from calcium hydride, 2,2,6,6-tetramethylpiperidine (TMP) was dried over sodium hydroxide pellets, filtered, and distilled at atmospheric pressure,[75] then stored in the dark under argon. Trimethylamine oxide dihydrate (Aldrich) was dehydrated by the azeotropic removal of water with toluene,[69] followed by recrystallisation, under argon, from ethyl acetate. All electrophiles were purified by standard procedures.[84]

Flash chromatography was performed using MN Silica Gel 60 minus 200 mesh ASTM (Macherey Nagel and Co.), according to the technique of Still.[85] Thin layer chromatography (TLC) was carried out using precoated Merck Silica Gel 60 F254 plates. Compounds were visualised by ultraviolet light and/or iodine vapour. Alumina (II) refers to aluminum oxide Brockmann activity (II) manufactured by British Drug House (BDH).

Preparation of Tricarbonyl(2-methyl-1,3-butadiene)iron

Method A:[63] A 2 litre 3-neck round bottom flask was oven-dried, capped with rubber septa and cooled under a stream of argon. One litre of freshly distilled tetrahydrofuran (THF) was transferred to the flask via double ended needle from a solvent still, followed by 70 mL (700 mmol) of freshly distilled isoprene. The flask was then fitted with a water condenser topped by a dry-ice condenser, a magnetic stirring bar and a thermometer. The solution was slowly warmed, with stirring, to 50-55° (internal temperature), the remaining rubber septum was replaced with a Schlenk tube containing 23.79 g (65.3 mmol) diiron nonocarbonyl which was added to the solution. The mixture was kept at this temperature for 20 hours, then cooled to room temperature, filtered, and evaporated <u>in vacuo</u> using a rotary evaporator (water

aspirator). The resulting dark orange-brown oil was chromatographed over alumina (II), eluting with Skellysolve B, then distilled to yield tricarbonyl(2-methyl-1,3-butadiene)iron **30** (8.01 g, 59%), as an orange oil bp 63-65°C (6.5 torr);[86] ^{1}H nmr (400 MHz, $CDCl_3$) δ5.28 (t, J = 8 Hz, 1H, H3), 2.18 (s, 3H, CH_3), 1.84 (t, J = 1.5 Hz, 1H, $H1_B$), 1.65 (d of d, J = 7 and 2.4 Hz, 1H, $H4_B$), 0.32 (d of d, J = 1.5 and 0.8 Hz, 1H, $H1_A$), 0.05 (d of d, J = 9 and 2.4 Hz, 1H, $H4_A$); ^{13}C nmr (75.5 MHz, $CDCl_3$) δ211.59, 103.11, 84.77, 43.90, 38.05, 22.90; IR (liquid film) 2058 (s), 1990 (s), 1966 (s) cm^{-1}; MS, m/e (relative intensity) 208(43) M^+ (for $C_8H_8FeO_3^+$), 180(36), 152(24), 124(100), 108(5), 96(14), 84(21), 82(20), 56(65).

Method B: A slurry of 6.22 g (82.8 mmol) anhydrous trimethylamine oxide in 60 mL dry THF was placed in a 100 mL 3-neck R.B. flask equipped with an addition funnel, magnetic stirring bar, septum inlet, and a dry-ice condenser with an outlet to a mineral oil bubbler. The mixture was cooled to -78°C and 2.1 mL (21 mmol) freshly distilled isoprene were added via syringe, followed by the dropwise addition, with stirring, of 5.4 mL (41 mmol) iron pentacarbonyl. When the addition was complete the cooling bath was removed and the solution was warmed to room temperature over a period of 1 h, followed by one hour of reflux. The solution was cooled to room temperature,

filtered, [redacted]ncentrated on a rotary evaporator. The resulting [redacted]as purified by flash chromatography (silica/Skelly B), to yield tricarbonyl(2-methyl-1,3-butadiene)iron **30** (1.75 g, 41%) with spectral properties identical to those of the material obtained via method A.

General Procedure for the Generation of the Anion of Tricarbonyl(2-methyl-1,3-butadiene)iron, and its Reaction with Benzyl Bromide:

Into an oven-dried 100 mL 3-neck R.B. flask, equipped with an addition funnel, septum inlet, low temperature thermometer, and magnetic stirring bar, was placed dry diethyl ether (36 mL). To this was added 2,2,6,6-tetramethylpiperidine (0.58 mL, 3.4 mmol), followed by a solution of methyllithium in diethyl ether (2.4 mL, 3.6 mmol). The solution was stirred at room temperature until methane evolution ceased (approximately 10 min), and then cooled to -60°C (internal temperature), at which point hexamethylphosphoramide (HMPA) (4 mL, 10% v/v with respect to ether), was added. To this solution of lithium 2,2,6,6-tetramethylpiperidide/HMPA was introduced tricarbonyl(2-methyl-1,3-butadiene)iron (628 mg, 3.02 mmol) in ether (5 mL) over a period of 30 min, and the funnel rinsed with ether (5 mL). This solution was stirred for a further 30 min after which time benzyl

bromide (0.57 mL, 4.8 mmol) was added. The reaction mixture was stirred for one hour at -78°C, then allowed to warm to room temperature overnight. This solution was poured into brine, the ether layer was separated, and the aqueous layer extracted with Skelly B (4×50 mL). The combined ether-Skelly B extract was successively washed with water (25 mL), then brine (25 mL), dried over anhydrous magnesium sulphate, and concentrated on a rotary evaporator (water aspirator; bath temperature approximately 30°C). The resulting oil was purified by flash chromatography (silica/Skelly B) to give, in order of elution, recovered starting material (62 mg; 10%), and product (707 mg, 79%). The nmr spectrum of the latter showed it to be 11% benzyl bromide and 89% desired product. The overall yield of tricarbonyl(1,2,3,6-η-3-methylene-6-phenyl-1-pentene)iron 51 was 70%. ^{1}H nmr (200 MHz, $CDCl_3$) δ7.28 (m, 5H, Ph-), 5.20 (t, J = 8 Hz, 1H, H2), 3.00 (m, 2H, $-CH_2-$), 2.65 (m, 2H, $-CH_2-$), 1.80 (brs, 1H, $H6_B$), 1.68 (d of d, J = 7 and 2.5 Hz, 1H, $H1_B$), 0.29 (d, J = 1.5 Hz, 1H, $H6_A$), 0.10 (d of d, J = 9 and 2.5 Hz, 1H, $H1_A$); IR (liquid film) 2046 (s), 1963 (s) cm^{-1}; MS m/e (relative intensity) 298(3), M^+ (for $C_{15}H_{14}FeO_3^+$), 270(3), 242(4), 214(100), 158(2), 91(18), 65(3), 56(14), 39(2).

Reaction of Complex 30 with 1-Bromo-4-chlorobutane:

The anion of the starting complex was generated as described above from 2,2,6,6-TMP (0.81 mL, 4.8 mmol), methyllithium (3.2 mL, 4.8 mmol), and 30 (652 mg, 3.1 mmol). 1-Bromo-4-chlorobutane (0.64 mL, 5.6 mmol) was introduced via syringe, and the solution stirred for 35 min at -60°C (internal temperature), then warmed to room temperature and stirred for a further 45 min. The reaction was quenched with brine, the layers separated, and the aqueous layer extracted with Skelly B. The combined ether-Skelly B extract was successively washed with water, then brine, dried over magnesium sulphate and concentrated on a rotary evaporator. The resulting crude oil was purified by flash chromatography (silica/Skelly B), to yield starting material (136 mg, 21%), and tricarbonyl(1,2,3,9-η-8-chloro-3-methylene-1-octene)iron 54 (497 mg, 54%). ^{1}H nmr (400 MHz, $CDCl_3$) δ5.24 (t, J = 8 Hz, 1H, H2), 3.54 (t, J = 6.5 Hz, 2H, $-CH_2-$), 2.38 (m, 1H, H4), 2.25 (m, 1H, H4), 1.68 (m, 8H, H5,6,7,1_B, 9_B), [expansion: 1.78 (t, J = 2 Hz, $H9_B$), 1.65 (d of d, J = 7 and 2 Hz, 1H, $H1_B$)], 0.26 (d, J = 2 Hz, 1H, $H9_A$), 0.05 (d of d, J = 9 and 2 Hz, 1H, $H1_A$); IR ($CDCl_3$ cast) 2047 (s), 1985 (s) cm^{-1}; MS m/e (relative intensity) 300(7) M^+ (for $C_{12}H_{15}{}^{37}ClFeO_3{}^+$), 298(20) M^+ (for $C_{12}H_{15}{}^{35}ClFeO_3{}^+$), 270(12), 242(43), 214(100), 178(100), 158(27), 124(81), 91(51), 56(95).

Reaction of Complex 30 with Chlorotrimethylsilane:

Metallation of the parent complex was accomplished in the manner previously described using 2,2,6,6-TMP (0.64 mL, 3.8 mmol), methyllithium (2.5 mL, 3.8 mmol), and complex **30** (658 mg, 3.2 mmol). A solution of chlorotrimethylsilane (0.60 mL, 4.8 mmol) in ether (5 mL) was added and the resulting solution stirred for 45 min at -78°C and then allowed to warm to room temperature overnight. Workup of the reaction was as described in the general procedure. The yellow oil obtained was partially purified by fractional bulb-to-bulb distillation (using two receiver bulbs). This technique yielded two fractions; A, 178 mg bp 58-62°C (11 torr) and B, 337 mg bp 68-75°C (11 torr). Fraction A (by nmr) was 10% starting material, 90% tricarbonyl(1,2,3,5-η-3-methylene-4-trimethylsilylbutene)-iron **56**; fraction B was pure **56**. The overall yield of **56** was 55%. ^{1}H nmr (200 MHz, $CDCl_3$) δ5.14 (t, J = 8 Hz, 1H, H2), 2.02 (d, J = 13 Hz, 1H, H4), 1.75 (t, J = 2 Hz, 1H, $H5_B$), 1.65 (d of d, J = 7 and 2 Hz, 1H, $H1_B$), 1.63 (d, J = 13 Hz, 1H, H4), 0.29 (d of d, J = 2 and 1 Hz, 1H, $H5_A$), 0.13 (s, 9H, $(CH_3)_3Si$), 0.06 (d of d, J = 9 and 2 Hz, 1H, $H1_A$); IR ($CDCl_3$ cast) 2017 (s), 1965 (s) cm^{-1}; MS m/e (relative intensity) 280(14) M^+ (for $C_{11}H_{16}FeSiO_3^+$), 252(13), 224(35), 196(100), 73(16), 56(12).

Reaction of Complex 30 with Benzaldehyde:

The anion was generated from complex **30** (549 mg, 2.64 mmol), 2,2,6,6-TMP (0.55 mL, 3.3 mmol), and methyllithium (2.1 mL, 3.3 mmol) in the manner previously described. Benzaldehyde (0.40 mL, 4.0 mmol) was added via syringe at -78°C. The solution was stirred at this temperature for one hour and then warmed to room temperature and quenched with brine. The reaction mixture was then worked up in the usual manner using ether for the extraction of the aqueous phase in place of Skelly B. The residue remaining, after concentration (rotary evaporator) was purified by flash chromatography (silica/toluene) to yield, in order of elution, unreacted starting material (150 mg, 27%), tricarbonyl(1,2,3,6-η-3-methylene-5-phenyl-1,4-pentadiene)iron, **60** (29 mg, 4%), a pale yellow solid (mp 98-100°C). ^{1}H nmr (400 MHz, $CDCl_3$) δ8.01 (m, 2H), 7.59 (m, 1H) and 7.49 (t, 2H, Ph), 5.37 (t, J = 8 Hz, 1H, H2), 4.10 (d, J = 16 Hz, 1H, H5), 3.88 (d, J = 16 Hz, 1H, H4), 1.84 (d of d, J = 2.5 and 1.5 Hz, 1H, $H6_B$), 1.78 (d of d, J = 7 and 2.5 Hz, 1H, $H1_B$), 0.48 (d of d, J = 3 and 1 Hz, 1H, $H6_A$), 0.26 (d of d, J = 9 and 2.5 Hz, 1H, $H1_A$); benzaldehyde (140 mg, 33%), and impure alcohol. The latter was purified by flash chromatography (silica/40% ether:Skelly B[illegible]e tricarbonyl(1,2,3,6-η-methylene-

5-phenyl-1-penten-5-ol)iron, **61** (318 mg, 38%) as a mixture of isomers (approximately 3:2). ^{1}H nmr (400 MHz, $CDCl_3$) δ7.31 (m, 5H, Ph), 5.31 (t, J = 8 Hz, 0.3H, H2), 5.14 (t, J = 8 Hz, 0.7H, H2), 4.97 and 4.92 (doublet of multiplets, 1H, H5), 2.84 (m, 1H, H4), 2.67 (m, 0.6H, H4) and 2.46 (m, 0.4H, H4), 2.21 (d, J = 3 Hz, 1H, OH), 1.78 (t, J = 2 Hz, 0.6H, $H6_B$), 1.74 and 1.71 (t + d of d, J = 2 Hz and J = 7 and 2.5 Hz, 0.8H, $H6_B$ + $H1_B$), 1.61 (d of d, J = 7 and 2.5 Hz, 0.6H, $H1_B$), 0.28 (d, J = 2 Hz, 0.6H, $H6_A$), 0.24 (d, J = 2 Hz, 0.4H, $H6_A$), 0.12 (d of d, J = 9 and 2.5 Hz, 0.4H, HI_A), 0.05 (d of d, J = 9 and 2.5 Hz, 0.6H, $H1_A$); ^{1}H nmr (D_2O exchange) δ4.97 (d of d, J = 14 and 11 Hz), 4.92 (d of d, J = 17.5 and 8.5 Hz); ^{13}C nmr (100 MHz, acetone-d_6) δ212.82 (C≡O), 145.73 (C_1', Ph), 128.94, 128.90, 127.96, 127.02, 126.86 ($C_{2'-6'}$, Ph), 104.79 (C^3), 87.82, 87.00 (C^2), 75.76, 75.54 (C^5), 49.27, 48.78, 45.50, 39.28 (C' and C^6), 30.37, 30.19 (C^4); IR (film) 334 (broad, w), 2049 (s), 1963 (s), 1040 (w), 755 (w), 701 (w) cm^{-1}; MS m/e (relative intensity) 314(0.2) M^+ (for $C_{15}H_{14}FeO_4$), 286(9), 258(4), 230(100), 212(34), 56(23).

The reaction was repeated with the solution of the anion and benzaldehyde being kept at -78°C overnight, and then warmed to room temperature and [illegible] in the previous manner. This yielded, after [illegible] tography, unreacted starting material (72 mg, [illegible]) and **61** (1.10 g,

85%) in the same ratio of isomers (by nmr) as previously observed.

Reaction of Complex 30 with 1-Iodoheptane:

1-Iodoheptane (0.45 mL, 2.8 mmol) was added via syringe to a solution of the anion of **30** (573 mg, 2.75 mmol) in ether/HMPA (40 mL). The resulting solution was stirred for one hour at -78°C, then warmed to room temperature and left overnight. The reaction mixture was poured into ice-cold water (150 mL), followed by workup in the usual manner (extraction with Skelly B). The resulting crude oil was purified by flash chromatography (silica/Skelly B) into two fractions. The first (386 mg), was shown by nmr to be a mixture of 1-iodoheptane (7%) and product (93%), and the second (177 mg) was 71% product, 21% starting complex and 8% 1-iodoheptane. The overall yield (by nmr integration) of tricarbonyl(1,2,3,12-η-3-methylene-1-undecene)iron, **57**, was (483 mg, 57%). ^{1}H nmr (400 MHz, $CDCl_3$) δ5.22 (t, J = 8 Hz, 1H, H2), 2.30 (m, 2H, H4), 1.80 (t, J = 2.5 Hz, 1H, $H12_B$), 1.66 (d of d, J = 7 and 2.5 Hz, 1H, $H1_B$), 1.42 and 1.32 (m, 12H, H5→10), 0.90 (t, J = 7.5 Hz, 1H, H11), 0.28 (d, J = 1.5 Hz, 1H, $H12_A$), 0.06 (d of d, J = 9 and 2.5 Hz, 1H, $H1_A$); IR (neat film) 2949 (sh), 2928 (m), 2847 (m), 2049 (s), 1970 (s) cm^{-1}; MS m/e (relative intensity) 306(15) M^+ (for $C_{15}H_{22}FeO_3$),

278(8), 250(7), 222(96), 166(10), 152(49), 138(21), 124(75), 56(100).

Reaction of Complex 30 with Carbon Dioxide:

The anion of complex **30** (866 mg, 4.16 mmol) was generated as before using 54 mL of ether and 6 mL of HMPA as the solvent system. After stirring for 30 min at -78°C the solution was added via double ended needle to a slurry of dry ice/ether in a 1 L R.B. flask, fitted with a rubber septum and a large gauge needle outlet to a mineral oil bubbler. The mixture was allowed to warm to room temperature overnight with the carbon dioxide escaping through the bubbler. Ice cold water (100 mL) was added, followed by the addition of concentrated hydrochloric acid (0.34 mL, 4.2 mmol) in water (1 mL). The mixture was then acidified with 1N HCl until the aqueous layer had a pH of 2 (this is best performed with the mixture in a separatory funnel). The ether layer was separated, and the aqueous fraction extracted further with ether (3×75 mL). The combined ether extract was successively washed with water (50 mL), brine (50 mL), and dried over $MgSO_4$. The ether was removed on a rotary evaporator to give a crude pale yellow solid. This was recrystallised from ether-Skelly B (1:1) to yield tricarbonyl(3,6,4,5-η-3-methylene-4-pentenoic acid)iron **63** (929 mg, 89%) as a pale yellow

solid, mp 75-77°C. ^{1}H nmr (400 MHz, $CDCl_3$) δ9.47 (very broad s, 1H, $CO_2\underline{H}$), 5.42 (t, J = 8 Hz, 1H, H4), 3.48 (d, J = 16 Hz, 1H, H2), 3.27 (d, J = 16 Hz, 1H, H2), 1.85 (brs, 1H, $H6_B$), 1.77 (d of d, J = 7 and 2.5 Hz, 1H, $H5_B$), 0.39 (brd, J = 2.3 Hz, 1H, $H6_A$), 0.19 (d of d, J = 9 and 2.5 Hz, 1H, $H5_A$); IR ($CHCl_3$ cast) 3239-2525 (s), 2062 (s), 1989-1953 (s), 1710 (s), 1412 (m) cm^{-1}; MS m/e (relative intensity) 244(14) M^+-CO (M^+ for $C_9H_8FeO_5$ absent), 196(54), 168(84), 140(100), 122(37), 84(32), 56(98), 28(98).

Reaction of Complex 30 with Paraformaldehyde:

A solution of the anion of **30** (603 mg, 2.90 mmol) was formed as before. Solid paraformaldehyde (0.44 g, 14.7 mmol) was added and the mixture stirred for 2 hours at -78°C, then warmed to room temperature and left stirring overnight. The reaction mixture was poured into saturated ammonium chloride (200 mL) then worked up in the usual manner (extraction with ether). The crude orange-yellow oil was purified by flash chromatography, eluting with Skelly B followed by ether-Skelly B (1:1). This resulted in recovered starting material (65 mg, 11%), and tricarbonyl(1,2,3,6-η-3-methylene-1-penten-5-ol)iron **59** (220 mg, 32%). ^{1}H nmr (400 MHz, $CDCl_3$) δ5.38 (t, J = 8 Hz, 1H, H2), 3.96 (m, 2H, CH_2), 2.68 (m, 1H, H4), 2.48 (m, 1H,

H4), 1.83 (t, J = 2 Hz, 1H, $H6_B$), 1.72 (d of d, J = 7 and 2.5 Hz, 1H, $H1_B$), 1.65 (brs, 1H, OH), 0.31 (d, J = 2 Hz, 1H, $H6_A$), 0.11 (d of d, J = 9 and 2.5 Hz, 1H, $H1_A$); IR (neat film) 3340 (m, broad), 2055 (s), 1971 (s), 1050 (m), 1030 (sh) cm^{-1}; MS m/e (relative intensity) 238 (2) M^+ (for $C_9H_{10}FeO_4$), 210(7), 182(13), 154(43), 136(7), 126(30), 108(57), 84(38), 56(100).

Reaction of Complex 30 with Acetyl Chloride:

A solution of the anion of complex **30** (827 mg, 3.98 mmol) in ether/HMPA (54 mL:6 mL) was added via double ended needle to a solution of acetyl chloride (0.85 mL, 12 mmol) in ether (5 mL), in a 100 mL R.B. flask fitted with a rubber septum, at -78°C. The reaction was monitored by TLC (silica/toluene) which indicated complete consumption of **30** in 2 hours; then the reaction mixture was poured into brine (250 mL). The layers were separated, and the aqueous layer extracted with ether (3×50 mL). The combined ether extract was washed with saturated sodium hydrogen carbonate (50 mL), then water (50 mL), and dried over $MgSO_4$. The solvent was removed on a rotary evaporator and the crude oil purified by flash chromatography (silica/SkellyB:ether (2:1)) to yield tricarbonyl(4,7,5,6-η-4-methylene-5-hexen-2-one)iron **64** (395 mg, 40%). ^1H nmr (400 MHz, $CDCl_3$) δ5.33 (t, J = 8

Hz, 1H, H5), 3.56 (d, J = 16 Hz, 1H, H3), 3.28 (d, J = 16 Hz, 1H, H3), 2.31 (s, 3H, CH_3), 1.78 (t, J = 2 Hz, 1H, $H7_B$), 1.77 (d of d, J = 7 and 2.5 Hz, 1H, $H6_B$), 0.41 (d, J = 2 Hz, 1H, $H7_A$), 0.22 (d of d, J = 9 and 2.5 Hz, 1H, $H6_A$); IR (CH_2Cl_2 cast) 2050 (s), 1967 (s), 1720 (m) cm^{-1}; MS m/e (relative intensity) 250(13) M^+ (for $C_{10}H_{20}FeO_4$), 222(12), 194(34), 166(100), 56(46).

Reaction of Complex 30 with N,N-Dimethylacetamide:

The anion of complex 30 (543 mg, 2.6 mmol) was prepared as before. N,N-Dimethylacetamide (0.36 mL, 3.9 mmol) was added at -78°C, the mixture warmed to room temperature overnight, then cooled to 0°C and quenched with saturated ammonium chloride (25 mL). The layers were separated and the aqueous layer extracted with Skelly B (3×50 mL). The combined organic extract was successively washed with 1N hydrochloric acid (50 mL), water (50 mL), saturated sodium hydrogen carbonate (50 mL), water (50 mL), and brine (50 mL), then dried over magnesium sulphate, and concentrated (rotary evaporator) to yield tricarbonyl(methyltrimethylenemethane)iron 48 (342 mg, 53%). Yellow oil, bp ca. 50°C (8 torr). ^1H nmr (300 MHz, $CDCl_3$) δ3.01 (d of quartets, J = 6.9 and 2.2 Hz, 1H), 2.48 (d, J = 4.4 Hz, 1H), 2.09 (d, J = 2.2 Hz, 1H), 1.69 (s, 1H), 1.55 (d, J = 4.4 Hz, 1H), 1.28 (d, J = 6.9 Hz, 3H);

^{13}C nmr (75.5 Hz, $CDCl_3$) δ212.08, 211.54, 211.01, 103.99, 73.26, 52.33, 50.28, 15.03; IR (neat film) 2055 (s), 1980 (s) cm^{-1}; MS m/e (relative intensity) 204 (4) M^+ (for $C_8H_8FeO_3$), 180(0.6), 152(2.3), 124(100), 56(67).

Oxidation of Tricarbonyl(diene)iron Complexes (General Procedures), as Illustrated by the Reaction of Complex 51:

Two oxidants were used, trimethylamine oxide and ceric ammonium nitrate.

Method A (Trimethylamine oxide): Complex 51 (398 mg, 1.34 mmol) was dissolved in THF (30 mL), anhydrous trimethylamine oxide (1.052 g, 14.00 mmol) was added, and the slurry heated to 50-55°C (internal temperature). The mixture was kept at this temperature, under argon, for 15 h, then cooled to room temperature and filtered. The filtrate was successively washed with water, then brine, dried over magnesium sulphate, and concentrated (rotary evaporator). The crude oil was purified by flash chromatography (silica/toluene), to yield 3-methylene-5-phenyl-1-pentene **53** (95 mg, 45%). ^1H nmr (400 MHz, $CDCl_3$) δ7.29 (m, 2H), 7.21 (m, 3H), 6.40 (d of d, J = 18 and 11 Hz, 1H), 5.28 (d, J = 18 Hz, 1H), 5.04 (m, J = 11 Hz, 3H), 2.81 (m, 2H), 2.52 (m, 2H); IR ($CHCl_3$ cast) 3080-3020 (m), 2980-2860 (m), 1600 (m), 1500 (m), 990 (m), 897 (s), 745 (m) 698 (s) ~~cm^{-1}; MS~~ m/e (relative intensity) 158(30),

91(100), 65(12); calcd. for $C_{12}H_{14}$ 158.1096, found 158.1098. Anal. Calcd for $C_{12}H_{14}$: C, 91.08; H, 8.92. Found: C, 91.07; H, 8.98.

Method B (Ceric Ammonium Nitrate (CAN)): Complex 51 (674 mg, 2.26 mmol) was dissolved in methanol (10 mL), the solution cooled to -16°C, using a 1-octanol/dry-ice cooling bath, and a solution of CAN (2.5 g, 4.6 mmol) in methanol (20 mL) added dropwise with stirring. The progress of the oxidation was monitored by collecting the carbon monoxide evolved in a gas burette. When the addition was complete 73% of the theoretical volume was liberated and so a further two mole equivalents of CAN/MeOH were added. The final volume collected was 95% of the theoretical amount. The cooling bath was removed and the solution warmed to room temperature. The methanol was removed (rotary evaporator) and the residue taken up in water (100 mL). This aqueous solution was extracted with ether (3×50 mL), and the ether washed with brine (50 mL), dried over magnesium sulphate and concentrated (rotary evaporator). The crude oil (310 mg, 87%) was purified by flash chromatography (silica/Skelly B) to yield 53 (143 mg, 40%) identical to that obtained via method A.

Oxidation of Complex 54:

Complex 54 (403 mg, 1.35 mmol) was oxidised using CAN/MeOH as described above. The yield after workup was 160 mg (75%). This pale yellow oil was purified via flash chromatography (silica/Skelly B) to give 8-chloro-3-methylene-1-octene 55 (92 mg, 43%). ^{1}H nmr (400 MHz, $CDCl_3$) δ6.37 (d of d, J = 18 and 11 Hz, 1H), 5.22 (d of d, J = 18 and 0.5 Hz, 1H), 5.06 (d, J = 11 Hz, 1H), 5.03 (brs, 1H), 4.99 (brs, 1H), 3.54 (t, J = 7 Hz, 2H), 2.22 (brt, J = 7 Hz, 2H), 1.80 (quintet, J = 7 Hz, 2H), 1.50 (m, 5H); IR ($CDCl_3$ cast) 2932 (m), 2860 (w), 1633 (s), 855 (m) cm^{-1}; MS m/e (relative intensity) 160(4), 158(8), 109(5). 95(14), 81(16), 68(100), 67(34), 53(14); calcd. for $C_9H_{15}{}^{37}Cl$ 160.0833, found 160.0840, calcd. for $C_9H_{15}{}^{35}Cl$ 158.0862, found 158.0868.

Oxidation of Complex 57:

Complex 57 (175 mg, 0.59 mmol) was oxidised with CAN in the usual manner. The crude oil was purified by column chromatography using silica gel/silver nitrate and eluting with 2% ether:Skelly B. This yielded 3-methylene-1-undecene 58 (78 mg, 80%) as a colourless oil. ^{1}H nmr (400 MHz, $CDCl_3$) δ6.35 (d of d, J = 18 and 11 Hz, 1H), 5.22 (doublet of triplets, J = 18 and 0.5-0.8 Hz, 1H), 5.04 (d of d, J = 11 and 0.8 Hz, 1H), 4.99 (brs, 1H), 4.98 (brs,

1H), 2.20 (t, J = 8 Hz, 2H), 1.48 (brm, 2H), 1.30 (brm, 10H), 0.90 (m, 3H); IR (neat film) 2960 (sh), 2927 (s), 2855 (s), 1640 (m), 1600 (m), 990 (m), 905 (sh), 890 (m) cm^{-1}; MS m/e (relative intensity) 166(46), 139(7), 123(7), 109(21), 98(12), 95(42), 81(60), 68(100), 67(47), 57(43), 53(14); calcd. for $C_{12}H_{22}$ 166.1722, found 166.1721.

Oxidation of Complex 61:

A solution of complex 61 (370 mg, 1.18 mmol) in THF (5 mL) was added to a cooled (-16°C) slurry of CAN (2.64 g, 4.81 mmol) in THF (20 mL). After the addition was complete the cooling bath was removed and the mixture warmed to room temperature. The reaction mixture was filtered, the solid washed with THF and the washings added to the filtrate. The solvent was removed (rotary evaporator), brine (50 mL) was added, and the solution extracted with ether (3×25 mL). The combined extracts were successively washed with water (2×5 mL), then brine (2×5 mL), dried with sodium sulphate and concentrated (rotary evaporator). The crude oil was purified by flash chromatography (silica/40% ether:Skelly B) to yield 3-methylene-5-phenyl-1-penten-5-ol, 62 (68 mg, 33%). ^1H nmr (400 MHz, $CDCl_3$) δ7.32 and 7.24 (d of multiplets, 5H), 6.39 (d of d, J = 18 and 11 Hz, 1H), 5.31 (d, J = 18 Hz, 1H), 5.15 (m, 1H), 5.12 (d, 1H), 5.08 (brs, 1H), 4.82 (d of d, J = 9 and 4 Hz, 1H), 2.70 (d of d, J = 14 and 4 Hz,

1H), 2.56 (d of d, J = 14 and 9 Hz, 1H), 2.06 (brs, 1H); IR ($CHCl_3$ cast) 3420 (m, br), 3080 (m), 306 (sh), 3020 (m), 3000 (sh), 2920 (m), 1630 (w), 1590 (m), 1490 (m), 1450 (m), 1055 (m), 990 (m), 901 (s), 757 (m), 700 (s) cm^{-1}; MS m/e (relative intensity)174(0.8), 156(1.1), 107(100); calcd. for $C_{12}H_{14}O$ 174.1045, found 174.1039. Anal. Calcd for $C_{12}H_{14}$: C, 82.72; H, 8.10. Found: C, 81.95; H, 7.99.

Oxidation of Complex 61 with Trimethylamine Oxide:

Complex **61** (1.10 g, 3.50 mmol) and trimethylamine oxide (3.9 g, 53 mmol) in THF (100 mL) were stirred at room temperature for one hour and then heated to 54°C where it was kept for 3 hours (until gas evolution ceased). The slurry was cooled to room temperature, filtered, and the THF removed (rotary evaporator). The residue was taken up in water (100 mL) and extracted with ether (3×30 mL). The combined ether extracts were successively washed with water (10 mL), then brine (10 mL), dried over magnesium sulphate and concentrated (rotary evaporator) to yield an orange oil (505 mg, 83%). This oil was purified by flash chromatography (silica/30% ether:Skelly B) to yield **62** (105 mg, 17%), identical with that obtained via CAN oxidation.

Reaction of the Isoprene Anion Complex with Water at -78°C and Room Temperature:

The anion complex of **30** was generated by the addition of **30** (64 mg, 3.11 mmol) in 10 mL ether, to a solution of LiTMP (4.4 mL, 4.7 mmol) in 54 mL ether and 6 mL HMPA at -78°C as before. A portion (38 mL) of this solution was removed via double ended needle into a septum-capped measuring cylinder and allowed to warm to room temperature over a period of 2 hours during which time the colour changed from lemon yellow (-78°C) to orange-yellow, to orange-red (room temperature). After reaching room temperature the aliquot was quenched with water (100 mL) and the layers separated. The aqueous layer was extracted with Skelly B (3×50 mL), the combined organic extract washed with 0.1N HCl (25 mL) to remove any TMP, then washed successively with water (25 mL), sodium hydrogen carbonate (25 mL) and brine (25 mL), dried over anhydrous magnesium sulphate and concentrated on a rotary evaporator to yield an orange oil (234 mg, 72%). TLC (silica/Skelly B) showed this to be two spots plus some baseline material and this oil was purified by flash chromatography (silica/Skelly B) to yield **48** (104 mg, 32%) as a yellow oil. ^{1}H nmr showed the presence of **30** (8%) in this oil.

The remaining 37 mL of the anion solution at -78°C was quenched with water (100 mL), then warmed to room

temperature and worked up in the same manner as the first aliquot, to yield an orange oil (220 mg, 69%). This oil was purified by flash chromatography (silica/Skelly B) to yield **30** (176 mg, 55%) as a yellow oil.

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Uptake, elimination and tainting involving benzothiophene and dibenzothiophene by rainbow trout (*Salmo gairdneri*)

BY

Edel M. Dromey

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THE UNIVERSITY OF ALBERTA FACULTY OF GRADUATE STUDIES AND RESEARCH

The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research for acceptance, a thesis entitled Uptake, Elimination and Tainting Involving Benzothiophene and Dibenzothiophene by Rainbow Trout (*Salmo gairdneri*), submitted by Edel Mary Dromey in partial fulfilment of the requirements for the degree of MASTER OF SCIENCE, in Environmental Science.

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Supervisor

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Date: Oct 21/87

Abstract

Aquatic species in the nearshore environment may be exposed to petroleum derived pollutants. Certain of these pollutants may be bio-accumulated by the organisms, and can often impart an offensive odour to the fish flesh. Rainbow trout (*Salmo gairdneri*) were exposed to benzothiophene and dibenzothiophene, to determine if they could readily accumulate and depurate these chemicals; and to look for evidence of metabolism. Muscle, liver, and bile samples were extracted in the laboratory and analysed for parent compound by gas chromatography, using both flame ionization and flame photometric detectors. Extracts were further derivitized in the search for metabolites. In addition rainbow trout were exposed to varying concentrations of benzothiophene and a sensory evaluation was performed on the resulting flesh. Sensory experiments were focused on benzothiophene as it was suspected of having a low odour threshold in fish. Exposed flesh was compared with fortified flesh, to ascertain if there was a difference between the two.

The results indicate that benzothiophene and dibenzothiophene are readily bioconcentrated by rainbow trout, with the greatest accumulation being found in the liver. Exposure/depuration experiments demonstrated that after 65h depuration, significant amounts of both chemicals could still be found in the tissues examined. Although no metabolites of either compound were found with the methods employed, further research is needed in order to confirm or deny the lack of metabolites.

Experiments with the sensory panel confirmed that: (i) benzothiophene had a very low detectable threshold for exposed fish and (ii) panelists had a preference for exposed over fortified flesh. Fish exposed to benzothiophene were found to be significantly more tainted than those fortified with the chemical. The median odour

threshold value was found to be 0.01 mg/kg. This was the lowest concentration presented to the panelists, therefore it is suspected that the actual odour threshold could be lower.

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1. Introduction and Problem Statement

Oil discharged to rivers can have deleterious effects upon aquatic organisms. Of particular concern is that portion of oil which is soluble in water. The concentration and composition of hydrocarbons in the aqueous phase is different from the parent oil as more soluble components, such as aromatics, are concentrated there. This fraction can achieve intimate contact with fish and other pelagic organisms and it is not removed by gravity oil separation devices (Birkholz, *et al.*, 1987).

In January, 1982, a fire and series of equipment failures at the Suncor oil sands plant, north of Ft. McMurray, Alberta resulted in a spill of coker distillate fractions (thermally cracked bitumen, which has not been completely hydrotreated to remove sulphur and nitrogen compounds) to the plant's wastewater pond. Over the subsequent 10 week period, plant records reported the release of more than 50 tons of oil and grease into the Athabasca River under winter ice cover.

Subsequent analysis on fish (walleye) taken from the Athabasca River, by Wellington Environmental (1983, cited in Hrudey and Nelson, 1986) indicated the presence of aliphatics, polycyclic aromatic hydrocarbons (PAH) and polycyclic aromatic sulphur hydrocarbons (PASH) in the fish flesh. These contaminants can pose environmental problems at very low concentrations. Bioaccumulation can lead to much higher concentrations of some contaminants in fish tissue than found in water (Eastmond *et al.*, 1984). Many of these contaminants cause taste and odour problems in water at extremely low concentrations (Zoeteman *et al.*, 1971).

Birkholz *et al.* (1987) characterized the spilled oil from the Suncor plant. Alkylated benzenes, alkylated benzothiophenes and alkylated dibenzothiophenes were identified and

confirmed. Studies have shown that the PASH may be the most persistent of the polycyclic aromatic hydrocarbons in the environment (Lee *et al.*, 1972) and they have been found to bioconcentrate in fish and shellfish (Ogata *et al.*, 1977; Ogata and Miyake, 1980; and Ogata and Fujisawa, 1983).

Additional work by Wellington Environmental matched specific compounds found in the tissue with those identified in the Suncor waste oil. Although PAH and PASH were contained in the tainted fish from the Athabasca River, direct correspondence between identified compounds and those primarily responsible for the sensory taint was not established. There is very little known about which contaminants and what quantities are necessary to impart an offensive odour/taste to fish, or indeed to what extent they bioconcentrate. The PASH have been labelled as a possible major source of tainting in fish (Connell and Miller, 1981) but there is no experimental evidence to prove this. Consequently, there is a need to explore the nature of these contaminants which arise from the petroleum industry.

This particular research was designed to determine:

* if certain PASH, namely benzothiophene and dibenzothiophene readily accumulate in fish tissue.
* if fish can readily depurate these compounds after exposure.
* at what level benzothiophene imparts an offensive odour (taint) to the fish.
* if there is any sensory difference between fish flesh from fish exposed to benzothiophene in water and those spiked with benzothiophene after processing.
* if there is any evidence of metabolism for either of these chemicals.

2. Literature Review

2.1 Oil Tainting Problem

2.1.1 Introduction

Tainting of commercial species of fish crustaceans, oysters and muscles by petroleum products has been extensively reported in the literature (Fetterolf, 1964; Connell, 1974; Connell and Miller, 1980; Motohiro, 1983; and Hrudey and Nelson, 1986). Most of the tainting in the flesh of fish is due to the activities of the petroleum and chemical industries (Nitta, 1970, cited in Motohiro, 1974). Connell and Miller (1981) report that the taint which is associated with petroleum in aquatic species is due to an extremely complex mixture of substances associated with petroleum oil. They suggest that if fish ingest petroleum products, there is a possibility that tainting will occur. In addition, low levels of dissolved petroleum hydrocarbons in the water soluble fraction can be absorbed into muscle tissue of aquatic organisms and impart a taint to them. Thus tainting can occur through adsorption of dissolved components from the water through the gills (Lee, *et al.*, 1972, 1976; Varanasi, *et al.*, 1979; Roubal, *et al.*, 1977 and Sanborn and Malins, 1977) and adsorption of the dissolved component through the skin (Varanasi, *et al.*, 1978).

Although there are numerous references on the occurrence of tainting due to petroleum and petroleum products, there is little reported on the specific causative compounds. Stansby (1978) reports this area of research as being most neglected and recommends further research. Motohiro (1983) corroborates this view, suggesting that additional research is required on the behavior *in vivo* and the biochemical reaction between petroleum compounds and living tissues *in vitro* , with reference to tainting of fish flesh.

Connell and Miller (1981) report high sensitivity of the olfactory system to sulphur compounds. They suggest that such minor components of a petroleum substance may be major contributors to its aroma. Hrudey and Nelson (1986) reported that tainted fish from the Athabasca River contained both PAH and PASH, but the causative compound(s) primarily responsible for the taint was not identified. While there are few reports in the literature on specific PAH imparting an offensive odour to fish, there are even less (if any) on PASH. It is for this reason that PASH are specifically dealt with in this report.

2.2 Sources of PASH in the Environment:

2.2.1 PASH in petroleum and petroleum products:

Crude petroleum and most refined petroleum products are extremely complex mixtures of thousands of organic compounds (Neff, 1979). Hydrocarbons are most abundant, usually representing more than 75% of the weight of the oil (Jones *et al.*, 1977). The remainder is made up primarily of various sulphur, nitrogen and oxygen containing organic compounds. PASH include such compounds as benzothiophene and dibenzothiophene. Unfortunately, PASH comprises an incredibly complex mixture of individual specific compounds. For example Nishioka *et. al* (1986) presented structures for 62 different sulphur heterocycles (presented in Figure 1) which could occur in petroleum. This list did not include any of the many alkyl substituted components which occur. If the latter, which are known to exist in petroleum, were considered, the total number of possible PASH compounds becomes very large.

The composition of petroleum products is dependent upon the raw source (crude oil, coal, shale oil, etc.) the degree of chemical modification (such as cracking) and the physical separation method employed. Coleman *et al.* (1973, 1984) studied the

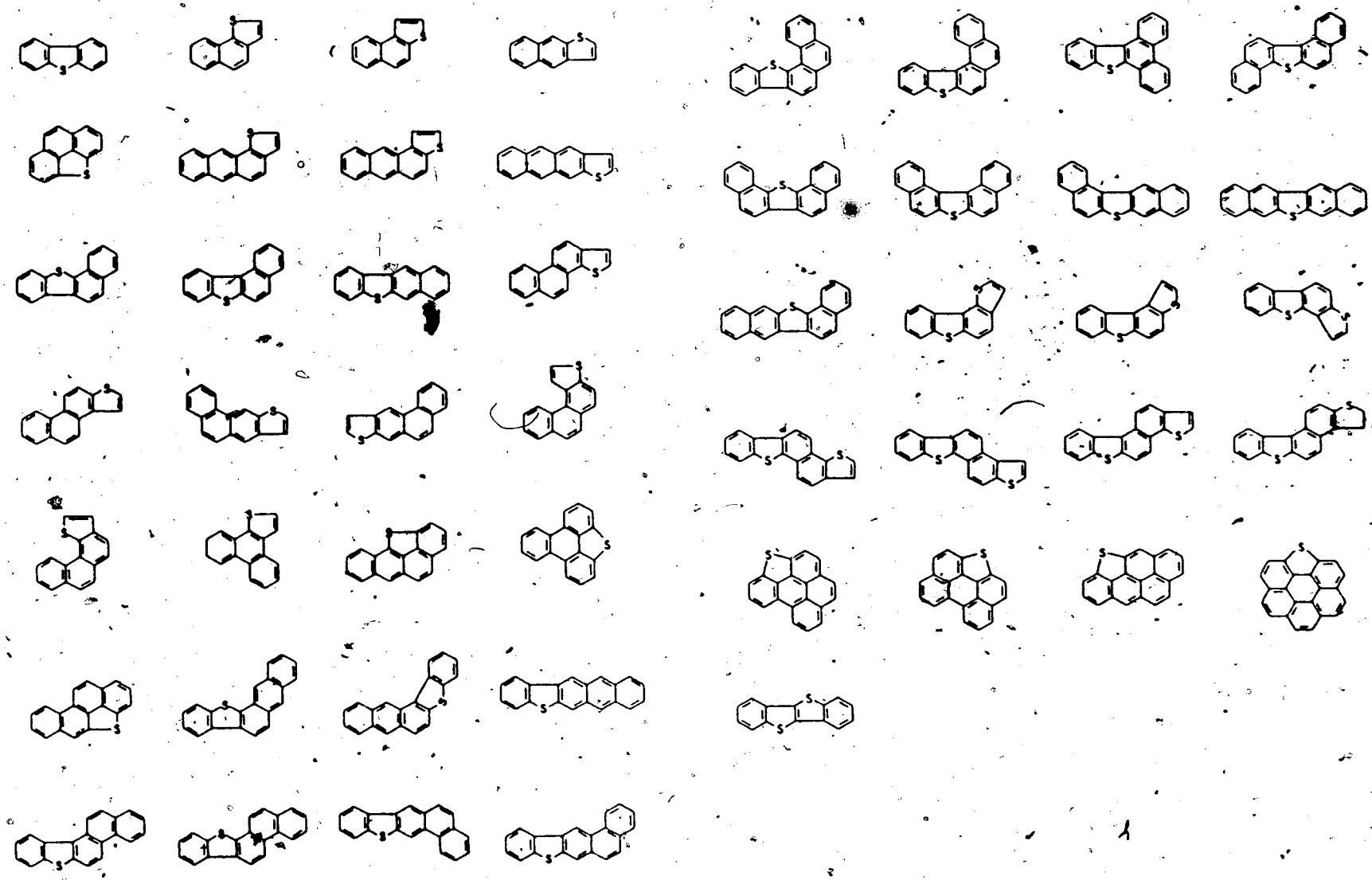


Figure 1. Example of possible sulphur heterocycle structures.
(Adapted from Nishioka, et al., 1986).

composition of the water soluble fractions (WSF) of certain petroleum products namely: gasoline, kerosene and No. 2 Fuel oil. The WSF was chosen in order to answer the question as to which components are likely to enter the drinking water supplies. It was shown that for all three fuels, aromatics comprised 50% or less of the weight of the product when analyzed directly. However, in each case aromatics comprised >93% by weight of the water-soluble fraction. Of the total aromatic content, PASH were found to represent between 3 and 15% by weight.

2.2.2 PASH in coal:

The organic matter in coal consists largely of a macromolecular material of complex and variable composition. A large number of complex PAH and heteroaromatic compounds are formed when coal is pyrolyzed (Eaton *et al.*, 1978; and Poston & Purdy, 1986). However, it is difficult to relate the products formed in this way to the original structure of coal. According to Neff (1979), 70 to 75% of the carbon present in bituminous coal is aromatic. PASH may comprise from 0.2 to 12% of the total aromatic carbon content.

2.2.3 PASH in bitumen:

Selucky *et al.* (1977) performed a detailed analysis on Athabasca bitumen. The bitumen analysed was separated into two groups: saturates and unsaturates. The latter was separated into mono-, di- and polyunsaturates. The polyaromatics (which contain the PASH series) were found to contain 7.07% by weigh sulphur compounds. The mono- and di- aromatics were found to contain 0.26 and 2.94% sulphur content by weight. Mass

spectral analysis of the polyaromatic fraction suggested that about 12% of the total sulphur content was of the benzothiophene series.

2.2.4 PASH in bitumen (oil sands) wastewater:

Hrudey & Nelson (1986) found the data base on characterization of the oil sands wastewaters to be limited. Wastewater characterization indicated a variety of alkylated aromatics and heterocyclic compounds with substantial quantities of sulphur compounds. Birkholz *et al.* (1987) characterized the water soluble components of an oil sands wastewater obtained from the Suncor plant in Ft. McMurray. Aromatic compounds predominated with alkylated dibenzothiophenes being positively identified and confirmed. The results suggest that C_1-C_3 dibenzothiophenes are likely present in the water soluble fraction.

Strosher & Peak (1976) found that tailings pond dyke drainage from the Great Canadian Oil Sands (now Suncor) plant contained 100 to 120 mg/L of total organic carbon. The total organic carbon contained 69% extractable carbon, which was mainly oxygenated compounds (92%). These oxygenated compounds were found to contain 5.3% sulphur compounds. Within the upgrading plant wastewater, Strosher & Peake found an average of 36 mg/L of total organic carbon. This total organic carbon was found to contain 42% extractable carbon. The extractable carbon was found to contain 17% organic sulphur compounds. Among compounds tentatively identified in the upgrading plant wastewater were benzothiophenes and dibenzothiophenes.

At a later stage, Strosher and Peake (1979) found the upgrading plant wastewater contained 47% extractable carbon for an average of 36 mg/L of organic carbon. The extractable carbon was found to contain 24% organic sulphur compounds which were

mainly substituted benzothiophenes. Although this number differs from the 1976 findings, Hrudey & Nelson (1986) are not surprised at the scale of variation because of the large portion of organic matter which cannot be accounted for.

2.3 Research into PASH Related Tainting Problems

There have been no papers in the literature dealing with the uptake and subsequent taintability of specific PASH in fish species. Certain authors have demonstrated that fish bioaccumulate PASH (Eastmond *et al.*, 1984; and Ogata and Fujisawa, 1983) but they have made no connection between uptake and subsequent tainting.

Ogata *et al.* (1977) demonstrated that eels reared in crude oil accumulate organic sulphur compounds. Ogata and Miyake (1978, 1980) characterized these sulphur compounds as being alkylated benzothiophenes. Ogata and Fujisawa (1983) found accumulation of alkylated dibenzothiophenes (C_1 to C_3) to tissue concentrations of 6 and 9 ppb in oyster and mussel respectively. These correspond to bioconcentration factors from 1845 to 28500.

Motohiro (1983) cites Yoshida and Uezuieu (1961) and Kaneda and Yasumoto (1974) as reporting sulphur compounds to be found in tainted fish. Wellington (1983) reported PASH ranging from 30 to 1500 ppb in tainted Walleye from the Athabasca River.

Connell and Miller (1981) suggest sulphur compounds may be a major contributor to the aroma of petroleum. GESAMP (cited in Connell and Miller, 1981) report dibenzothiophenes as being one major tainting component, in addition to several other classes of compounds.

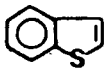
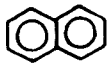
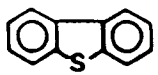
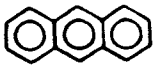
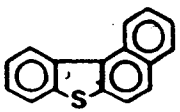
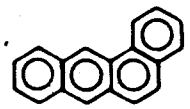
Paasivirta *et al.* (1981) [redacted]kkonen (1982) measured a range of parts per million concentrations of alkyl dibenzothiophenes in fish and muscle from the Baltic. They found a good correlation between total oil residues and the alkyl dibenzothiophenes.

Vassilaros *et al.* (1982) analyzed catfish in the vicinity of a coking plant outfall for PAH and PASH. Parts per million concentrations of specific compounds were found from each class. The authors presented the argument that PASH would be of greater environmental significance than PAH, owing to their higher water solubilities. Structures, solubilities and octanol/water partition coefficients of the specific compounds are presented in Table 1.

Eastmond *et al.* (1984) compared a series of PASH and their sterically and structurally similar PAH for bioconcentration, elimination and toxicity in *Daphnia magna*. Benzothiophene was compared to naphthalene, dibenzothiophene with phenanthrene and benzo(b)naptha [2,1-d]thiophene with chrysene. Their results are summarized in Table 2. These suggest a greater degree of bioconcentration for the PASH than for the PAH. There was no obvious trend for observed elimination differences between PASH and PAH. The PASH were generally more toxic than their analogous PAH.

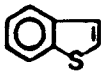
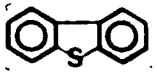
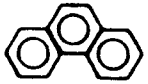
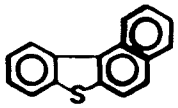
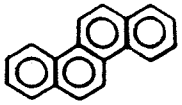
Vassilaros *et al.* (1982) and Vandermeulen (1981) cited in Eastmond *et al.* (1984), indicated that PASH associated with oil spills may be depurated more slowly than their PAH analogues and are more resistant to microbial degradation. There is no evidence in the literature of uptake and elimination of PASH by fish as related to tainting problems.

Table I Comparison of octanol/water partition coefficient and water solubility for Selected PASH and PAH[a]

PASH	Log P	Water Solubility	PAH	Log P	Water Solubility
thiophene 	1.6	1430.	benzene 	1.6	1515.
benzothiophene 	3.1	113.	naphthalene 	3.4	30.
dibenzothiophene 	4.4	1.7	anthracence 	4.4	0.04
benzo[*b*]naphtho-[1,2-*d*]thiophene 	5.6	0.06	benz[*a*]-anthracene 	5.6	0.01

[a]after Vassilaros et al.(1982)

Table . Summary of peak bioconcentration factors and elimination half lives for PASH and PAH in *Daphnia magna*.[a]

PASH	Peak BCF	Elimination half-life (h)	PAH	Peak BCF	Elimination half-life (h)
benzo[*b*]thiophene 	750	27	naphthalene 	50	b
dibenzothiophene 	600	12	phenanthrene 	600	b
benzo[*b*]naphtho-[2,1-*d*]thiophene 	8000	23	chrysene 	5500	18

[a]after Eastmond et al. (1984) [b]elimination not first order

2.4 Pathways of PASH in the Aquatic Environment

PASH may reach the aquatic environment by various routes including: combustion or spillage of fossil fuels; industrial and domestic wastewater discharges; runoff from land; and atmospheric fallout. Crude oil and distillate products entering the aquatic environment each year from material and anthropogenic sources was estimated by the National Academy of Sciences (1973, cited by Neff, 1979) to be approximately 6.1 million metric tons per year. Neff, assuming a mean concentration of 2.8% PAH in crude oil, estimated that 1.7 million tons of PAH are entering aquatic systems each year from all sources. Of course, PASH would be some fraction of that estimate.

Very little is known about the amounts of PASH entering the aquatic environment each year. Although estimates have been done for PAH, they cannot be reliably transferred to PASH.

Metabolism of PASH:

2.5.1 Introduction:

A detailed knowledge of the metabolism of PAH in aquatic species is essential to assessing the consequences of petroleum pollution. The mixed function oxygenase (MFO) system is an enzyme system which responds readily to the presence of foreign chemicals (Malins, 1977; Varanasi, 1979). There are numerous studies in the literature detailing the activation of the MFO's by PAHs, but there have been no studies on the metabolism of PASH.

Owing to a lack of such research , PAH analogues must be reviewed in order to obtain an indication of the possible metabolic pathways of the corresponding PASH. The limitations of such a comparison must be stressed. PASH do not necessarily follow the same pathways as PAH. For the purpose of this review, naphthalene has been chosen as the PAH analogue to benzothiophene.

2.5.2 Overview: enzyme systems governing the metabolism of aromatic hydrocarbons:

MFO enzymes, which are NADPH-dependent, catalyze the introduction of oxygen into the aromatic nuclei (Malins, 1979). This enzyme system is variously known as cytochrome P450 dependent MFO, oxygenases, aryl hydrocarbon hydroxylases (AHH) or drug metabolizing system. The MFO system is located in the liver and sometimes in other organs such as the spleen (Neff, 1979). Within the cell, it is localized primarily in association with the endoplasmic reticulum (Andersson and Forlin, 1985) and consists of several components, including a nicotinamide adenine dinucleotide phosphate (NADPH) cytochrome reductase, a phospholipid and the heme protein called cytochrome P448 or P450. These components function as an electron transport system when combined with NADPH as a source of reducing power, molecular oxygen and an appropriate substrate such as PAH (Neff, 1979). Neff reports that such a system catalyzes the incorporation of one atom of molecular oxygen into water and one atom into the substrate. The general reaction involved is as follows:

$$RH + NADPH + O_2 + H^+ \longrightarrow NADP^+ + H_2O + ROH$$

The oxygenases are believed to account for the formation of virtually all the primary metabolic products of aromatic hydrocarbon degradation (Von Hofe & Pufer, 1986). The presence of this xenobiotic biotransformation enzyme system in fish is now well established (Lech, 1973, Lech and Bend, 1980, Andersson and Forlin, 1985 and Oikari and Kunnamo-Ojalo, 1987). Recently, electrophilic intermediates (such as the epoxide group) in the formation of the hydroxy derivatives, have been viewed with interest (Andersson and Forlin, 1985; and Oikari and Kunnamo-Ojalo, 1987). Reactions involving the conjugation of epoxides and phenols with water soluble substances give rise to mercapturic acid derivatives, glucuronides, sulphates and glycosides in liver and kidney (Lech and Bend, 1980 and Andersson & Forlin, 1985).

Collier *et al.* (1978) and Bend *et al.* (1979) have shown that metabolites of several aromatic hydrocarbons excreted into the bile are hydroxylated and glucuronidated products, whereas sulphates are present in very low amounts, or absent. These reports indicate that foreign compounds such as BaP and naphthalene in fish are metabolized via sequential enzyme systems in the liver and that conjugated xenobiotics are the endproducts of xenobiotic biotransformation.

2.5.3 Metabolism of naphthalene

Studies with naphthalene may be of some help in trying to identify metabolites of PASH, as they may follow the same or similar pathways of metabolic degradation. However further research is required, specifically in the area of developing new analytical techniques for identifying PASH metabolites. Lee et. al (1972) studied the uptake and metabolism of 14C naphthalene in three species of marine fish. They found that the compound was metabolized in the liver and subsequently deposited in the bile: a major storage site of hydrocarbon metabolites. In all tissues the major compound after 48h of exposure was found to be 1,2-dihydro-1,2-dihydroxynaphthalene.

Malins *et. al* (1979) exposed *S.gairdneri* to radiolabeled naphthalene. Isolation of metabolites from the liver resulted in the identification of two non-conjugates (1-napthol and 1,2-dihydro-1,2-dihydroxynaphthalene) and three conjugates (1 napthyl glucuronic acid, 1-napthyl sulphate and 1-napthyl glucoside). Major components of the metabolite fractions were 1,2-dihydro-1,2-dihydroxynaphthalene and glucuronic acid derivitives.

Krahn *et. al* (1980) force fed rainbow trout gelatin capsules containing naphthalene. Metabolites were determined using HPLC/UVF. As such, no attempts were made to identify 1,2-dihydro-1,2-dihydroxynaphthalene since it does not fluorece strongly. The bile was examined and one non conjugate (1-napthol) and two conjugated metabolites (1-napthyl glucuronide and 1-napthyl sulphate) were identified.

Varanasi *et al.* (1978, 1979, 1982a and 1982b); and Varanasi and Gmur (1980, 1981) carried out numerous experiments on the metabolism of naphthalene in aquatic species. Varanasi *et al.* (1979) force fed juvenile starry flounder and rock sole naphthalene dissolved in salmon oil. They reported high concentrations of naphthalene and its metabolic products 24h after the force feeding commenced in starry flounder, with maximum concentrations being reached at 48h. Significantly greater amounts of naphthalene and its metabolites were found in rock sole.

In the bile of both fish, glucuronic acid conjugates were the major metabolites. Small amounts of mercapturic acid derivatives were present also. Non-conjugated metabolites, such as 1 and 2-naphthols and 1,2-dihydro-1,2 dihydroxynaphthalene constituted less than 10% of the total extracted metabolites. In the livers of both fish, 1,2-dihydro, 1,2-dihydroxynaphthalene was the major metabolite (38.7% for rock sole and 41.5% for starry flounder) at 24h after feeding. Considerable proportions (31.9%) of conjugates (eg. glucuronides, mercapturic acids, glucosides/sulphates) were also found in the liver of rock sole, whereas metabolites in starry flounder were mainly non-conjugated

(76.7%). However, with time, there was an increase of conjugated metabolites found in the liver. Varanasi & Gmur (1981) found that glucuronides were the major metabolites of naphthalene in liver of English sole. On hydrolysing these glucuronides the authors revealed that a large proportion (70%) was the 1,2-dihydro-1,2-dihydroxynaphthalene. This was the major metabolite found in the liver of rock sole in the preceeding study, by Varanasi *et al.* (1979).

Varanasi *et al.* (1978) studied the uptake and release of naphthalene and its metabolites through the skin of fish. The skin is a primary site of contact with environmental pollutants and had been largely overlooked in studies concerning the fate of petroleum in aquatic organisms. Rainbow trout were exposed to naphthalene through: force feeding, via injection; and water immersion studies. The authors found that regardless of mode of exposure, the skin accumulated significant concentrations of naphthalene and its metabolites. The results also indicated that, similar to the liver, the skin has the ability to preferentially retain metabolites.

3. Materials and Methods

3.1 Materials:

Benzothiophene and dibenzothiophene were obtained from Fisher Scientific Company. Purity was confirmed to be 98% using GC/FID and GC/MS. Celite, florisil, sodium bicarbonate (solid), acetic anhydride (97%), anhydrous sodium sulfate, phosphoric acid (85%), ammonium hydroxide (30%) and pesticide grade solvents (chloroform, methylene chloride, methanol and hexane) were also obtained from Fisher Scientific. The anhydrous sodium sulphate and celite were purified by soxhlet extraction using methylene chloride for 12 hours. Following extraction, the solvent was evaporated in a vacuum fume hood and the materials then stored in a convection oven maintained at 130 °C, until required.

Ammonium phosphate buffer (pH 6.5) was prepared by adding 1.0 mL of phosphoric acid to 1 L organic free water and titrating to pH 6.5 by dropwise addition of ammonium hydroxide (30%). Potassium phosphate buffer (pH 7.0) was prepared by titrating 50 mL of 0.1 M potassium dihydrogen orthophosphate (obtained from Fisher Scientific) with 0.1 M sodium hydroxide (Fisher grade) to pH 7.0. Acetate buffer (0.2M) was prepared titrating 50 mL of 0.2 M acetic acid with 0.2 M sodium acetate to pH 5.0. Acetate buffer (2.0 M) was prepared by titrating 50 mL of 2.0 M acetic acid with 2.0 M sodium acetate to pH 5.0.

a-Napthyl-b-D-glucuronic acid and glucuronidase (containing sulphatase activity) were obtained from Sigma chemical company. The b-glucuronidase solution (Sigma, G.8132) was prepared by dissolving 10-12 mg of the enzyme in 500 mL of 0.2 M acetate buffer. 100 mL of this solution gave approximately 2000 units of enzymatic activity.

Organic free water was obtained by passing distilled water through a trace organic removal cartridge (Millipore Corp.).

Rainbow trout were obtained from the Alberta Environmental Centre, Vegreville, Alberta. They averaged 150-300 g in weight and 200-300mm in length. The fish were held in a 1400 L fibreglass tank, at an average temperature of 11 °C. The tank was aerated with the aid of three aquarium size air diffuser stones. While in the holding tank, the trout were fed a diet of pellet food. A diurnal cycle of 8 h light and 16 h dark was observed throughout the experiments.

3.2 Equipment:

Gas chromatography was performed using a Varian (model 3500) GC and a Hewlett-Packard (model 5880) GC. The Varian 3500 instrument was equipped with a flame photometric detector, a model 1077 Grob type split/splitless injector (operated in the splitless mode) and a model 600 data system. The column used was a 30 m X 0.32 mm (i.d.) fused silica, wall-coated DB-5, obtained from J&W Scientific Inc.. The carrier gas was helium and the temperature increased from 40 to 280 °C at 10 °C/min., beginning 1 min. after injection. The oven temp was maintained at 280 °C for 10 min. and the detector temperature maintained at 300 °C.

The H-P model 5880 was equipped with a model 1883B Grob type split/splitless injector system (operated in the splitless mode), flame ionization detector, model 7672A autosampler and level four data processing capability. It contained a 30 m X 0.32 mm i.d. fused silica, wall-coated, DB-1301 capillary column, obtained from J&W Scientific Inc.. The carrier gas was helium and the temperature increased from 40 to 280 °C at 10 °C/min., beginning 1 min. after injection. The oven temperature was maintained at 280 °C for 20

min. and the injector and detector temperature were maintained at 270 °C and 300 °C respectively.

Substances were confirmed using a HP model 5970 quadrupole mass spectrometer interfaced with a HP model 5980 GC. This GC was equipped with a model 1883B Grob type split/splitless injector (operated in the splitless mode) and a 12.5 m X 0.2 mm (i.d.) fused silica, wall-coated, HP-1 capillary column. The carrier gas was helium and the temperature increased from 40 to 300 °C at 10 °C/min. beginning 1 min. after injection. The oven temperature was maintained at 300°C for 8 min. and the injector maintained at 250 °C. The data system was a HP model 5997, with data acquisition beginning 2 min. after injection. Mass spectral scans were obtained every 1.36 seconds.

The HPLC (High Performance Liquid Chromatograph) Waters Chromatography Division system consisted of the following units: two model 6000A pumps, a Waters automated gradient controller, a model 680 solvent programmer, a model 710B autosampler, a model 450 variable wavelength detector and a Hewlett-Packard model 3388A integrator for recording and integrating chromatograms. Separations were performed using a 15 cm X 4.6 mm i.d. Supelcosil LC-PAH column, frit size 5 µm (Supelco Inc.). Analysis was performed in the isocratic mode using a solution of acetonitrile (70%) and ammonium phosphate buffer, pH 6.5 (30%). The flow rate was maintained at 3.0 mL/min. and absorbance was set at 233 nm.

The soxhlet extraction apparatus consisted of a soxhlet extractor, Friedrich condensor and a 300 mL round bottomed flask obtained from Kontes Scientific Glassware Inc. Extraction thimbles (45 mm X 130 mm frit, porosity 40 µ) were obtained from John Scientific Inc. The six soxhlet extractors were mounted in an electrothermal extraction

assembly (Fisher Scientific) which consisted of electrothermal heaters and the necessary support apparatus to mount the soxhlet apparatus.

The nitrogen evaporator used was a Meyer Organomation model III. The water bath employed with this evaporator unit was maintained at 50°C. The electric shaker used was a Burnett, model 75 wrist action shaker. The polytron was obtained from Brinkman instruments, a Kinematica type PT10-35. The centrifuge employed was a model CL obtained from International Equipment Co (I.E.C.).

3.3 Fish Exposure Facilities and Procedures:

3.3.1 Initial Studies:

Fish exposure methodologies were initially assessed in the Department of Zoology, University of Alberta. The response of rainbow trout to various different parameters was quantified.

Fish of similar size in the same aquaria were compatible. However, when a large and a small fish were placed together, aggressive behavior was observed. The smaller fish was unable to protect itself and incurred substantial superficial injuries as a result. Thus in addition to allowing 1 L of water for every 5- 7 g of fish weight, similar sized fish were always placed together in the same aquarium, to reduce this stress associated with the aggressive behavior.

In these preliminary studies oxygen levels in non-aerated tanks were carefully monitored and the response of rainbow trout noted. Fish became visibly stressed around 4.7 mg/L dissolved oxygen and below 4.0 mg/L death was imminent. Dissolved oxygen levels, therefore, were never allowed to drop below 4.0 mg/L at any time during

the exposure experiments. Although this level was reached on two occasions only, the water was re-aerated to bring D.O. levels back up to 7.0 mg/L or better. A water temperature of 12 °C was maintained in the exposure tanks and appeared to be an adequate temperature for the trout as well.

Little was reported in the literature regarding the toxicity of PASH to fish. Therefore, LC_{50}'s for benzothiophene and dibenzothiophene were determined and estimated as 8.0 and 5 mg/L respectively. The experimental method for determining the LC50's consisted of exposing a number of fish (6 fish with 2 per tank) to varying concentrations of the chemical, ranging from 0.01 mg/L to 10 mg/L. The LC50 was defined as that concentration where >50% responded (died) in a specified time period (8 h). From these experiments a concentration of 1 mg/L was chosen for exposure and exposure/depuration studies for both the benzothiophene and dibenzothiophene. This choice was based on the LC50 findings: 1 mg/L would not likely kill the trout for the exposure period contemplated.

Trial experiments were also run to determine the extent of loss of the chemicals from the exposure tank due to factors such as volatilization and adsorption to the liner bags. In order to obtain the desired concentration in water, it was necessary to add the chemicals dissolved in water soluble solvents; methanol for benzothiophene and acetone for dibenzothiophene.

Logistical problems, such as how to add the chemicals to the water, were also worked out at this time. When added directly with no solvent and no mixing, very low concentrations of chemicals were found in the water. However, with the aid of a hand-held rotary mixer and the carrier solvents mentioned above, the desired concentrations could be obtained during the initial phases of the experiments at least.

Overall the initial experiments proved valuable in the development of the exposure methodology. As a result, the actual exposure/depuration studies were run smoothly and in the minimum of time.

3.3.2 Main Holding Tank

A 1400 L circular fibre glass tank was used to maintain fish stocks for experimental purposes (Figure 2). Water within the tank was maintained through a combination of recirculation (with filtration and cooling) and fresh water input. Organic- and chlorine-free water was provided by running available tap water through two 1.2 m long X 10 cm diameter granular activated carbon columns. This treated water was allowed to flow into the holding tank at an average rate of 1.5 L/min. An overflow was provided at the opposite end of the tank to allow drainage.

The tank water was continually cleaned and cooled through a recirculation system (Figure 2). Water was pumped from the bottom of the holding tank through a sediment trap (for large particle removal) followed by a fine mesh cloth filter (25 mm polyester) for a more thorough cleanup. The water was finally passed through a refrigeration unit (Coardley Broadview Model R2-10) and cooled to a temperature of 8-9 ^{o}C prior to re-entry (along with the fresh water) into the holding tank.

Water temperature, dissolved oxygen and pH were monitored continuously using a Yellow Springs Instrument(Y.S.I.), model 310 Dissolved oxygen/temperature meter and an Fisher model 805 MP pH/MV meter. Filtered, freshwater, was checked biweekly for free chlorine using the DPD (N,N-Diethyl-p-phenyl-enediamine) colourmetric method (

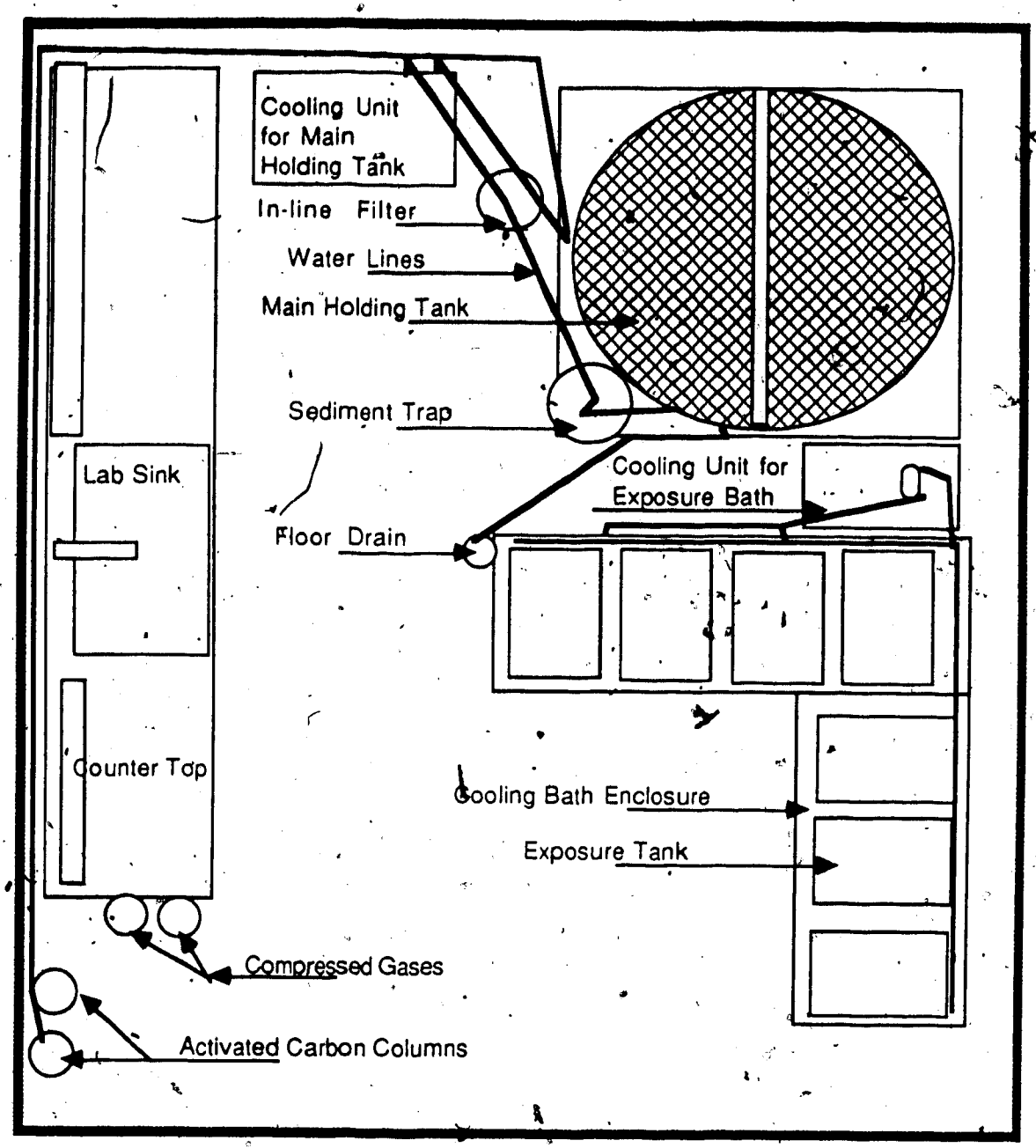


Figure 2. Schematic of fish holding facilities.

APHA, 1986). In addition, the filtration unit was removed and thoroughly cleaned once a week.

Fish were fed a diet of large pellet food approximately 50 g/day/tank. The top of the holding tank was covered with a screen to prevent fish from jumping out. A diurnal periodicity of 8 h light/16 h dark was maintained through artificial lighting.

3.3.3 Exposure Aquaria

The exposure set-up consisted of seven glass aquaria (68 L capacity). These aquaria were placed within a cooling bath unit. The cooling water circulated around the bottom 8 cm of each aquarium (Figure 2) maintaining the temperature of the aquarium water between 11-13 ^{o}C. The cooling water itself was kept at 6-8 ^{o}C by circulation through a refrigeration unit. The aquaria were lined with polyethylene plastic liner bags of 6 mil. thickness. This prevented adsorption of the exposure chemicals onto the silicone joints and glass of the aquarium. Fresh liners were used for each experiment.

Static 8 h exposures were conducted in the aquaria, filled with 50 L of carbon filtered water. The exposure compounds of interest were benzothiophene and dibenzothiophene. Fish ranged from 200 to 350 g in weight and 150 to 300 mm in length.

3.3.4 Exposure Procedure

Prior to an exposure, two fish were placed in each aquarium for a minimum period of 48 h acclimatization (to the aquarium environment). Aeration was provided with diffuser stones and the fish were fed pellets. Feeding was terminated 24 h prior to exposure. Separate aquaria were prepared for the exposures. Fresh water within the

exposure aquaria was aerated with pure oxygen for a period about 10 to 15 minutes (raising D.O. to ≥16 mg/L). Prior to the commencement of the exposure aeration was terminated and the required amount of chemical was then mixed into the aquarium water using a rotary lab.-mixer. The fish (2) were then transferred from their acclimatization aquaria into designated exposure aquaria to begin the exposure. The aquaria were not aerated during the experiment unless the D.O. level fell below 3.7 mg/L (point of visible stress). Water samples were taken at equal intervals from each aquarium during an exposure and stored in teflon sealed vials at 4 °C until analysis could be performed.

3.3.5 Exposure/Depuration Studies:

An exposure concentration of 1.0 mg/L (+/- 0.25 mg/L) was used for each of benzothiophene and dibenzothiophene for a period of 8-9 hrs (refer to Figures 11 to 16). Physical and chemical properties of both benzothiophene and dibenzothiophene are presented in Table 2a. For the purpose of exposure/depuration experiments, fish were removed from the exposure tanks after 8 h and placed in "depuration" aquaria filled with fresh water, for a period of 65 h.

At the end of each experiment, the fish were sacrificed by a blow to the base of the skull. Wet weight and lengths were measured immediately. Each fish was then fillétted and all organs individually weighed; muscle, head, viscera, skin and bones and liver. Bile was also extracted (using a 10cc syringe fitted with a 16 gauge, 15 mm needle) from the gall bladder and frozen in micro-centrifuge vials (-80 °C) until required for analysis.

Table 2a. Physical and chemical properties of benzothiophene and dibenzothiophene.

Compound	Melting Point (°C)	Boiling Point (°C)	Solubility in water @ 20 (°C)	Molecular Weight
Benzothiophene	32	221	113 mg/L	134
Dibenzothiophene	99-100	332	17 mg/L	184

3.3.6 Water Analysis

Since benzothiophene and dibenzothiophene had low solubilities in water it was necessary to dissolve them in ethanol and acetone (pesticide grade) respectively. An equal volume of either ethanol or acetone was similarly added to the control tank. Water samples were taken from the aquaria at regular intervals throughout the experiment.

Analysis for dibenzothiophene and benzothiophene in water for trout exposure was performed by high pressure reverse phase liquid chromatography (HPLC) Analysis was performed isocratically using acetonitrile (70%)/ammonium phosphate buffer (pH 6.5, 30%). Volume Injection volume was maintained at 15 µL. Quantitation was performed by the external standard method with benzothiophene and dibenzothiophene stock solutions as the external standard. Area counts obtained for the water samples were compared to the external standard.

3.4 Flesh Samples:

3.4.1 Preparation

Fish muscle was prepared according to the method of Benville and Tindle (1970). Whole frozen fillets were diced into pieces using a knife and mallet. These were then placed in a Waring blender along with 1.5 times as much dry ice. The mixture was then pulverized to a fine powder. The pulverized mixture was then placed in a glass beaker, labelled and sealed with aluminum. It was stored at -80 °C until required (8 h minimum storage, to allow the dry ice to sublime).

For the soxhlet extraction procedure, 20 g of the thawed powder was vigorously mixed with 80 g of anhydrous sodium sulphate and then packed into a glass thimble (see also Stalling *et al.*, 1972). The thimble contained approximately 2 cm of extracted celite at the bottom. The mixture was extracted with 280 mL methylene chloride for a minimum of 5 h. Following soxhlet extraction, the extract was placed in a refrigerator at -4 ^{o}C, until required. All samples were clearly labelled according to a pre-arranged coding system.

3.4.2 Gel Permeation Columns:

Clean-up of the fish extract followed the method of Hessleberg and Johnson (1972) using gel permeation chromatography (GPC), followed by a florisil partitioning step. Stalling et al. (1972) found GPC to be an effective and reproducible technique for removing lipids from fish extracts. Rainbow trout has a high fat content of about 7-10% of body weight, therefore GPC was considered a suitable technique for removal of the fat. Mulder and Buytynhuys (1970) reviewed applications of gel-permeation to organic compounds and reported separations of a wide range of molecules using bio-beads and various organic solvents; benzothiophene and dibenzothiophene both fall within this molecular range. Both these chemicals, having molecular weights between 130 and 200 are readily separated from lipids, which have molecular weights between 600 and 1500.

The chromatographic column (Figure 3) was prepared by inserting a small amount of glass wool in the bottom of a 750 mm X 19 mm i.d. glass column and packing with bio-beads, SX-3, swollen with elution solvent (Methylene Chloride:Hexane, 1:1). The column was packed by tapping with a glass rod. Tightness of packing and sample texture determined the solvent flow rate during extraction (Hesselberg and Johnson, 1972). The column was topped with 2.5 cm of sea sand for removal of larger lipid particles. A

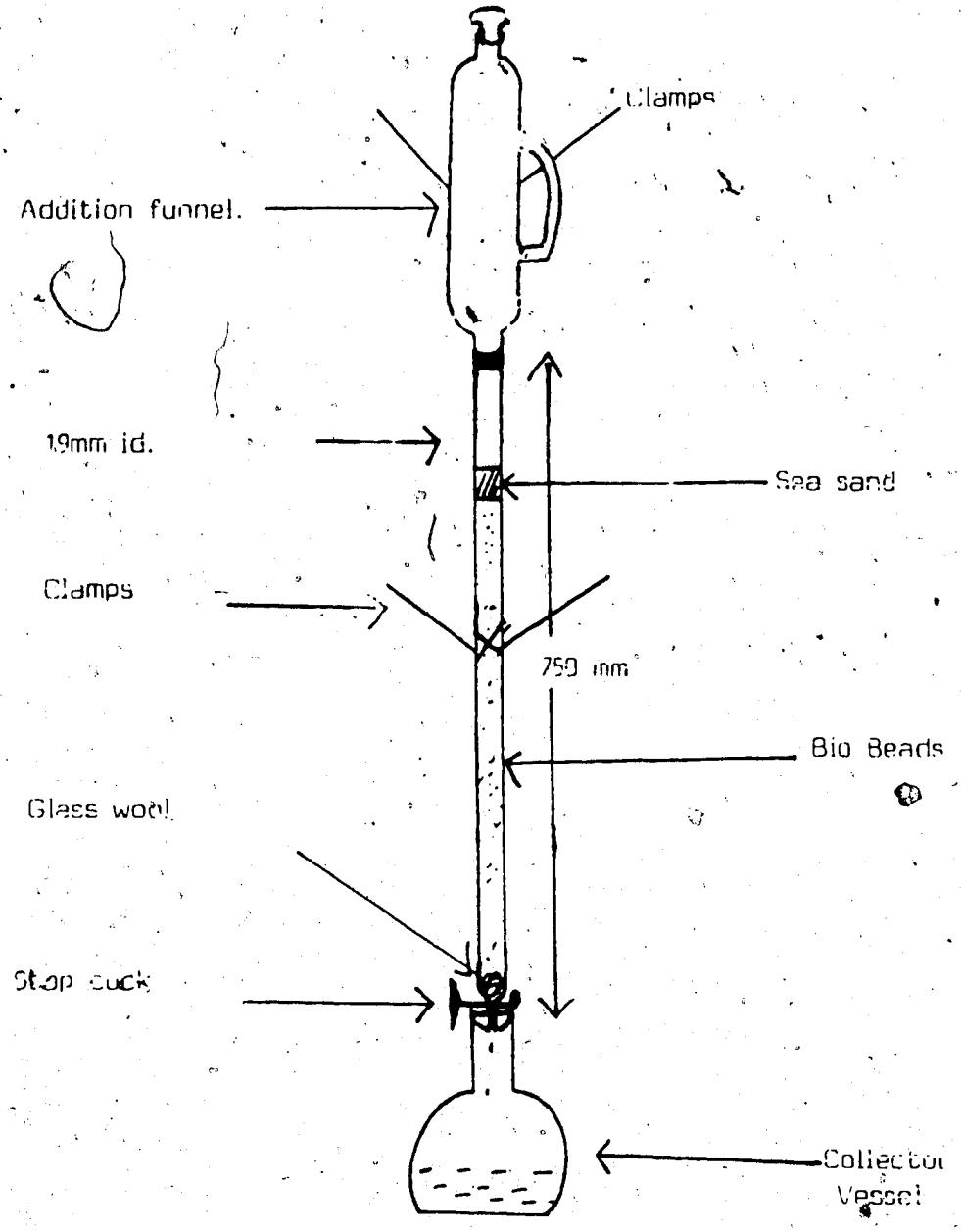


Figure 3. Gel permeation column.

graphical representation of the gel permeation chromatographic column is presented in Figure 3.

3.4.3 Florisil Columns:

Florisil columns (Figure 4) were prepared by activating 10 g (accurately weighed) of 60-80 mesh florisil (Fisher) for 4 h at 650 °C. The florisil was then deactivated by the addition of 5% or 10% deionized water (i.e. 5% water/95% florisil). When not in use, the florisil was stored in scintillation vials and sealed with teflon tape. During an extraction, a small amount of glass wool was placed in the bottom of a 400 mm X 19 mm i.d. glass column and then wet packed with deactivated florisil using hexane as solvent. All glassware was rinsed with hexane prior to use. The florisil was packed with the aid of a glass rod so as to ensure the absence of air. A 2.5 cm layer of sodium sulphate was placed on top of the column so as to remove any water. The hexane level was dropped to within 2 mm of the sodium sulphate layer. The column was then ready for the extract to be cleaned.

3.4.4 Tissue Clean-up:

The tissue extract (280 mL from the soxhlet procedure) was then placed into a 300 mL Kuderna-Danish (K.D.) evaporative concentrator, (refer to Figure 5) and concentrated down to 4 mL. The 300 mL apparatus was then rinsed 3 times with 2 mL of of DCM, for a total of 10 mL of solvent and extract in the Mills tube. This 10 mL was then quantitatively transferred to the head of the GPC column (previously flushed with 100 mL of 50:50 DCM:Hexane) and allowed to run through the column until the extract was within 1-2 mm of the sea sand. The Mills tube was replaced on the K.D. apparatus and the walls rinsed with 5 X 2 mL of DCM. This 10 mL was again run through the GPC column to

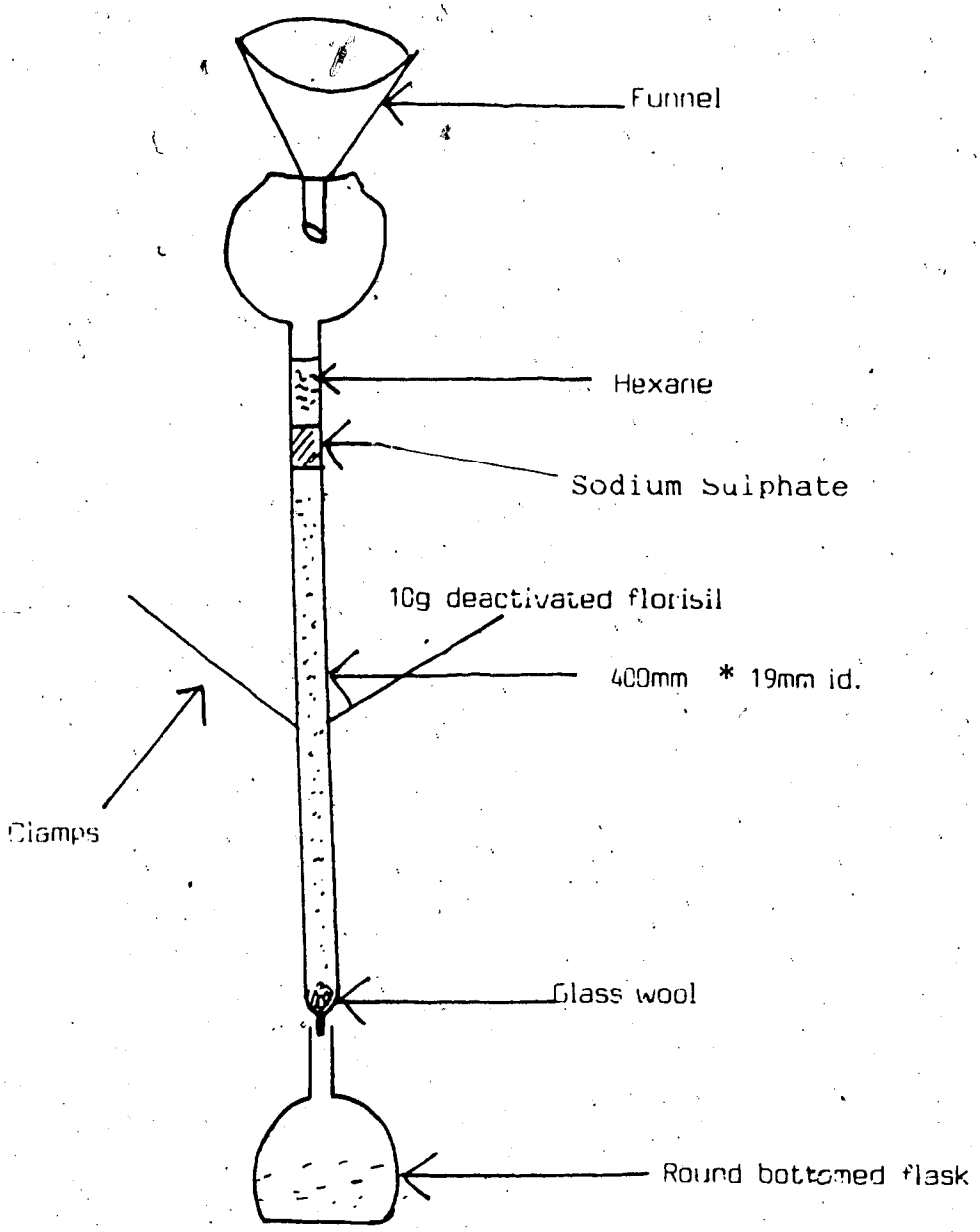


Figure 4. Florisil column.

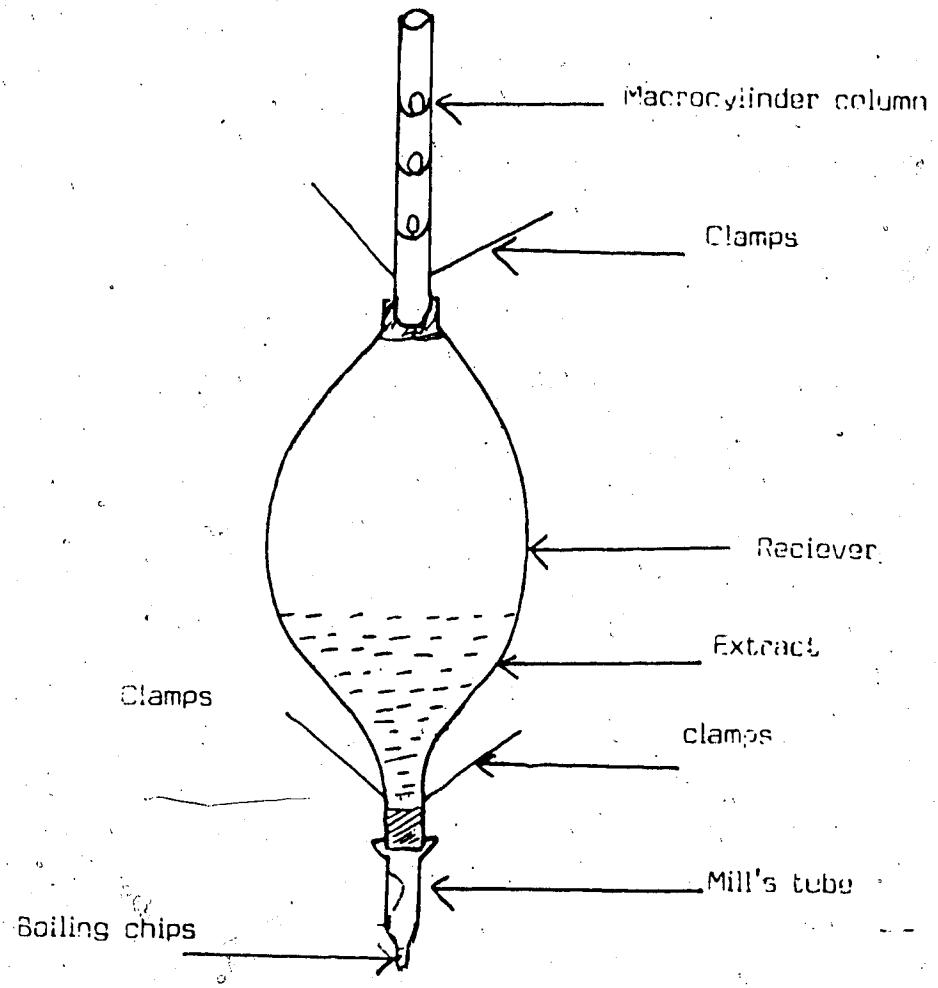


Figure 5. Kuderna-Danish apparatus.

with 1-2 mm of the sand. A 250 mL addition funnel was then placed on top of the column (see Figure 3) and filled with 230 mL of 50:50 DCM:Hexane for elution. The first 80 mL collected was discarded as waste and the next 80 mL retained as sample in a 125 mL round bottom flask.

The 80 mL of eluate retained was then concentrated down to about 4 mL using clean K-D. glassware. The glass wall of the concentrating flask was rinsed with 3X2 mL of DCM, for a total of 10 mL in the Mills Tube. This 10 mL was then quantitatively transferred to the florisil column and eluted to within 2 mm of the sodium sulphate (this step was repeated again with a 3X2 mL rinsing of the mills tube). The florisil column was then eluted with 40 mL of hexane and drained to within 2 mm of the sodium sulphate . All of the extract was collected and transferred to a 125 mL K.D. apparatus.

The eluate was concentrated down to 2 mL and the apparatus rinsed with 3X2 mL hexane. This 8 mL was then transferred to a 15 mL centrifuge tube, in addition to 2X2 mL of hexane rinse from the Mills tube. This final extract was concentrated to 1 mL with the aid of a Model III Nitrogen Evaporator.

The tissue extract was analysed for benzothiophene and dibenzothiophene by GC/FID and GC/FPD using a HP model 5880 and Varian model 3500 GC respectively.

3.5 Liver Sample Preparation:

All liver samples were analyzed for benzothiophene and dibenzothiophene using a modification of the method of Varanasi *et al.* (1982a). Potassium phosphate buffer (pH 7.0) was used instead of saline. Frozen liver (2-4 g) was thawed and transferred to a 50 mL centrifuge tube with 4 mL of potassium phosphate buffer (pH 7.0). The mixture was

homogenized with a polytron (Brinkman instruments) for approximately 1 min.. The polytron probe was then washed with 5X2 mL methanol into the centrifuge tube. Chloroform (5 mL) was then added and the mixture vortexed for 1 min. This step was repeated followed by the addition of 5 mL of organic-free water. The mixture was vortexed for 1 min. and then centrifuged at 2000 rpm for 5 min. (Centrifuge Model C.L. (I.C.S.). After centrifuging, there was three distinct layers: an upper water layer, middle solids layer and a lower chloroform/methanol layer. The lower chloroform/methanol layer was removed and dried through a 10 g sodium sulphate column and collected in a 50 mL centrifuge tube. The aqueous/solids layer was then extracted with 10 mL chloroform and vortexed for 1 min. The lower chloroform layer was removed and dried through the sodium sulphate column. The extract was then concentrated to 1 mL on the N-evaporator as the final step in preparation for analysis by GC. The aqueous portion was saved for further analysis.

3.5.1 Liver Metabolites

The aqueous-methanol phase of the liver extraction (with protein removed by centrifugation) was evaporated to 6 mL with the nitrogen evaporator and made up to 0.2M in acetate, buffered at pH 5.0. It was incubated with 2000 units of b-glucuronidase (containing sulphatase activity) for 4h at 37°C following the procedure of Varanasi *et al.* (1982a).

Following hydrolysis, the solution was extracted with 10 mL methanol and vortexed for 1 min. This was followed by 2X5 mL chloroform with vortexing and 5 mL organic free water. The lower chloroform-methanol layer was dried through a 10 g sodium sulphate column and collected in a clean 50 mL centrifuge tube. A further extraction with 10 mL chloroform was performed, the lower layer dried through the sodium sulphate

column and the resulting 35 mL of extract concentrated to 1.0 mL with the aid of a N-evap. It was then analysed on a Varian model 3500 GC, with FPD detector.

3.6 Bile Analysis:

3.6.1 Enzymatic hydrolysis of bile

Bile samples were analysed for conjugated metabolites by the following method: an aliquot of bile (100 mL) was placed into a 15 mL centrifuge tube and dissolved in 0.5 mL of a 0.2M acetate buffer. This mixture was incubated together with 2000 units of b-glucuronidase (dissolved in 0.1 mL of the acetate buffer) for 2 h at 37°C, in a shaking incubator.

After enzyme treatment, the solution was extracted with chloroform-methanol according to the following procedure:

-2.5 mL of methanol and 1.25 mL of chloroform were added to the solution and vortexed for 1 minute.

-an additional 1.25 mL of chloroform was added to the vessel and vortexed for 1 min., followed by 1.25 mL of organic free water with vortexing.

-two layers are formed; the bottom phase being chloroform. This lower layer was removed and dried by passage through a 1 g sodium sulphate column and collected in a 5 mL calibrated centrifuge tube.

-the remaining aqueous phase (upper layer) was further extracted with an additional 2 mL of chloroform, vortexed and the lower layer dried through the sodium sulphate column as before.

-the combined extracts were concentrated down to 1.0 mL on the Nitrogen-evaporator and the extract analysed on the Varian model 3500 GC, with FPD.

-parent compounds found in extracts were quantified using acenapthene as an internal standard.

3.6.2 Acetylation of bile

Enzymatically hydrolyzed and extracted bile samples were acetylated according to the procedure of Hargesheimer *et. al* (1981). The bile (100 mL) was placed in a 25 mL centrifuge tube, along with 4 mL demineralized water and vortex for 1 min. Solid sodium bicarbonate was slowly added until there was an excess of sodium bicarbonate and the evolution of carbon dioxide ceased. This solution was then extracted with 3X2 mL of chloroform and the chloroform layer dried through a sodium sulphate column. The resulting extract was concentrated to 1 mL on the nitrogen evaporator. The sample was analysed on the HP model 5880 GC/FID and the Varian model 3500 GC/FPD.

3.6.3 Caustic hydrolysis of bile

Caustic hydrolysis of bile was performed in the search for first pass metabolites. Aliquots of bile (100 mL) were placed in a 15 mL centrifuge tube along with 1 mL of 6N sodium hydroxide. This mixture was vortexed for 1 min. and then extracted with chloroform and methanol according to the procedure outlined in pp. . The final extract was analyzed using GC/FPD.

3.7 Tainting Potential of Benzothiophene:

3.7.1 Apparatus

All test were performed in an odour-free room located in the Department of Food Science , in the University of Alberta. This sensory testing laboratory was equipped with individual booths and red lights to disguise possible color differences between samples as outlined in ASTM (1986). A constant temperature of 21°C and a relative humidity of approximately 50% was maintained. The panel booth design followed the guidelines as set out in ASTM (1986) as did the plan of the sensory testing laboratory. Panelists were given tap-water and dilute lemon solution as rinsing material and unsalted soda crackers (as outlined by Iredale and York, 1976) were provided to allow the judges to clear the previous sample from their palates before continuing . Glassware was cleaned with hot soapy water and rinsed several times with fresh water. Samples were kept warm in a glass 2L beaker filled with water placed on top of a hot plate. Napkins and miniature garbage bags were supplied to each panelist. Equipment used in testing was stored in the odour-free room and used only for these tests as outlined by Alexander *et al.*, 1982. All samples were prepared in a sample preparation area connected to the sensory lab by a cupboard door pass-through.

3.7.2 Ethics:

An ethics committee was set up in the University of Alberta to review the research projects of three graduate students (including this project) using humans as sensory "guinea pigs". Prior to any sensory analysis, statements on the conduct of the tests were examined and approved by this committee. The statements assured that:

- the individual panelists were thoroughly instructed on techniques they were to employ.
- the panelists were supervised to preclude consumption of the fish. Any deviations from this policy by panelists were to be documented in writing.
- there were procedures to abort the test if a panelist complained of any discomfort.
- there were procedures to ensure prompt medical action should the latter occur.

3.7.3 Panelists:

Judges chosen for the study were picked from an original group of 27 people on their ability to be consistent organoleptically and to identify an "off-flavor" in fish. The panelists ranged in ages from 20 to 39 and the majority were graduate and undergraduate students at the University of Alberta. Each selected judge signed a waiver and release form prior to the sensory analysis of experimental fish. A copy of this release and waiver is supplied in Appendix 1. In addition, all panelists completed a personal medical information document, to ensure the researchers that no medical complications would interfere with the sensory analysis. A copy of this form is supplied in Figure 6. Each panelist was thoroughly instructed on the techniques employed and was supervised during the actual procedure.

3.7.4 Screening and training tests

All panelists were carefully screened and trained prior to the actual sensory evaluations as outlined by Jellinek, 1985. The work involved in screening and training the

PERSONAL HISTORY

Name: _____

Address: _____

_____

Telephone: _____

Age: _____ Sex: _____

Do you have any history of cardiac or respiratory ailments? (Yes or No)

_____

If yes, please provide details below:

_____

_____

_____

Do you have any allergies? (Yes or No) _____

If yes, please provide details below:

_____

_____

_____

Were you ever a habitual smoker of cigarettes? (Yes or No) _____

If yes, when, approximately, did you quit? _____

Figure 6. Personal medical history form.

panelists was shared by two other graduate students. The screening process consisted of two phases: the first phase measured both the ability of each panelist to distinguish the four basic tastes of sweet, sour, bitter and salty (Jellinek, 1985 and Piggott, 1984) (Table 3), and to distinguish and describe familiar odours (Table 4) as outlined by Amerine, *et al.* (1963). The second phase consisted of a series of tests in which two actual fish samples were compared and the panelist was asked to determine which sample was more "tainted". This was a forced-choice procedure, disallowing a "no-difference" response. The tainted sample was fortified with naphthalene. In the second phase of panel sessions, each judge tasted seven pairs of samples (Figure 7), each pair evaluated consisted of one sample of the "untainted" fish and one of the "tainted" fish. The samples were all coded according to the randomized design . Questionaires for the screening tests are supplied in Appendix 2. The 11 selected panelists (out of an original group of 27) were chosen because of their ability to consistently discriminate the different tastes and odours, as well as their high scoring of correct responses. The selected panelists were then required to go through training sessions to familiarize them with the particular testing procedure and the medium being investigated.

3.7.5 Questionaire for benzothiophene:

The procedure used was a modification of the quantitative descriptive analysis of Stone *et al.* (1974) and Lockhart *et al.* (1986). Each panelist was presented with eight pairs of coded samples. Panelists were asked to record their response to each sample by placing a check mark inside a "rating box" which corresponded to the intensity of the off-flavor in the sample. (refer to sample questionaire, Figure 8). These responses were later converted to numerical scores using a template. Each sample was evaluated 3 times by each panelist. Mean scores of fortified and exposed fish are given in Table 14 in section 4.6.2.

Table 3. List of suggested compounds for taste standards.

Sweet	*Sour*	*Salt*	*Bitter*
glucose	acetic acid	NaCl	Caffeine
Sucrose	Citric acid	NH_4Cl	Nicotine
Almonds	Malic acid	$MgCl_2$	Quinine
Lactose			

Table 4. List of suggested standards for odors. The list has been compiled from Jellinek, 1985 and Piggott, 1984.

Odor	*Standard*
Bitter	Quinine
Earthy	Geosmin
Chlorinous	Na-Hypochlorite, Bleach
Onion	Isopropylmercaptan
Medicinal	Iodine
Model paint	Methyl-isobutylketone
Cloves/spicy	Cloves
Soapy	Soap
Grassy/rotten nuts	Hexanal
Moth balls	Naphthalene
Sweet	Vanilla
Sweet	Almonds
Sweet	Nonal
Model glue	Toluene
Fishy	Tuna
Oily	Oil sands effluent

SCREENING TEST FOR FISH TAINTING

NAME_____ DATE_____

Seven pairs of coded samples are provided. Each of these foil packets contains a sample of fish. Some samples have been artificially "tainted" with varying concentrations of a common household substance (moth balls). Other samples are untainted.

To evaluate the samples, tear off the end of the foil packet and open it as much as possible. Sniff each pair of samples separately and in the order indicated. Circle the sample within each pair which is most tainted. After you have completed your evaluation, close each packet by folding over the open end.

Rinse your mouth with the lemon water provided and take a bi[illegible] cracker after smelling each sample pair. Wait approximately 15 se[illegible] between sample pairs.

Pair	Samples	
1	321	332
2	621	448
3	554	416
4	481	197
5	871	763
6	223	323
7	787	576

Figure 7. Questionaire for phase 2 of screening process.

FISH FLESH SENSORY EVALUATION RUN #2

NAME _____ DATE _____

Eight pairs of coded fish samples are provided. In each pair, one of the samples contains fish that has been exposed to benzothiophene only. The other sample contains fish that has either been exposed to dechlorinated tap water or spiked with benzothiophene. In order to evaluate the samples, tear off the end of the aluminum packet and open it as much as possible. Sniff each pair of samples individually and in the order indicated. If a taste cannot be detected by sniffing alone, you are requested to taste the sample by placing it in your mouth for approximately 20 seconds. Please expectorate it after this time. You are requested not to swallow the sample. Circle the sample within each pair which is most tainted. Evaluate the degree of taint of this sample by ticking within the most appropriate category (see example below). Rinse your mouth with the lemon water provided, and take a bite of cracker after evaluating each pair of samples. Wait approximately 15 seconds between pairs.

Pair No.	Samples		Degree of Taint
Example	770	(888)	barely detectable · detectable · highly detectable
	832	610	barely detectable · detectable · highly detectable
2	808	671	barely detectable · detectable · highly detectable
3	444	498	barely detectable · detectable · highly detectable
4	855	826	barely detectable · detectable · highly detectable
5	233	307	barely detectable · detectable · highly detectable
6	901	808	barely detectable · detectable · highly detectable
7	308	777	barely detectable · detectable · highly detectable
8	221	229	barely detectable · detectable · highly detectable

Figure 8. Questionaire for benzothiophene.

3.7.6 Preparation of fish: fortified samples

Frozen flesh was thawed in cold running water (while still in vacuum sealed bags) and ground in a Waring blender. The minced flesh was fortified with the required amount of chemical dissolved in ethanol (95% ethanol, 5% water; Fisher grade). The desired amount was calculated on a mg/kg basis and stock solutions were prepared. For example, from a 1000 ppm stock solution of benzothiophene in ethanol, 5X200 µL aliquots were added to 1000 g of fish flesh to obtain a 1 ppm sample. Each 200 µL was added separately and then blended in the stainless steel Waring blender to obtain as homogeneous a sample as possible. An equal amount of ethanol was added to the control samples as was used above for dissolving the benzothiophene.

3.7.7 Preparation of flesh: exposed samples

In the case of exposed flesh, fish were exposed to varying concentrations in aquaria (Section 3.3.4). The concentrations used were 0.01, 0.03, 0.08, 0.5 and 1 ppm. The fish were exposed under static conditions in a non-aerated tank for 8 h. They were then killed by a blow to the base of the skull, eviscerated, washed in distilled water and the fillets vacuum sealed and frozen at -20 °C until required. Water samples were not taken during these experiments as we were only interested in flesh concentration. Analysis of the flesh by GC/FPD indicated that these samples needed to be diluted with control fish flesh to give an actual concentration of 0.03, 0.08, 0.5 and 1.0 ppm. Exposed flesh was diluted with control flesh by mixing both together in the Waring blender, until the required concentration was obtained (as determined by GC/FID analyses of the samples). This procedure was necessary for all but the 0.01 ppm concentration, as samples of exposed fish were available which contained a level of 0.01 ppm. Finally, an equal amount of ethanol was added to the exposed fish as to the fortified fish, in order to nullify any

discrepancies caused by the ethanol. All prepared flesh was re-analyzed by G.C./F.I.D. prior to sensory evaluation to ensure the correct concentration.

3.7.8 Sample Preparation

Individual samples for sensory analysis were prepared according to the procedure of Iredale and York (1976) and Lockhart, *et al.* (1986). For each sample, a 15 g portion was wrapped in aluminum foil and the aluminum coded with a wax pencil for identification during sensory analysis. Each pair of samples was individually vacuum sealed, labelled and frozen. Eight pairs were placed in one larger bag and frozen until required.

For presentation, the wrapped, coded samples (8 pairs) were heated in water at 60 ^{o}C for 10 minutes and served in a beaker of water on a warming plate. The panelists were instructed to open each sachet containing one pair and to compare the two samples. A copy of the questionaire is provided in Figure 8.

4. Results

4.1 Quality Control:

Portions of tissue (muscle and liver) were obtained from control fish and analyzed before and after fortification with benzothiophene and dibenzothiophene. Recovery of benzothiophene and dibenzothiophene from fortified tissue (liver and muscle) is summarized in Tables 5 and 6. From these results it is apparent that acceptable levels of both benzothiophene and dibenzothiophene were obtained from fortified fish tissue. The chromatograms of the liver samples indicated a large co-elution of biogenic material (Figure 9); however, the level of this interference was not so high as to warrant further sample cleanup.

The efficiency of the enzymatic hydrolysis was tested by hydrolyzing replicate aqueous solution of α-Napthyl-β-D-glucuronic acid (200 βg/mL) and extracting and analyzing the resulting solution for 1-napthol by GC/FID. The hydrolysis was found to be greater than 95% effective. The efficiency of the GPC columns was tested by eluting the columns with 1 mL of standard solutions of benzothiophene and dibenzothiophene (100 μg/mL) and analyzing the eluates for percent recovery. Figure 10 depicts the chromatograms obtained from the standard as compared to the quality control. Recovery efficiency was found to be 98%.

Analysis of tissue (muscle and liver) as well as bile, was performed on both GC/FPD and GC/FID. The former was used primarily to identify sulphur compounds whereas the latter was used to quantify amounts because, the linear response of the GC/FID, being independent of the shape of the peak , is more favorable to use in quantifying amounts. The FPD when used in the sulphur mode is inherently non-linear

Table 5: Recovery of benzothiophene from fortified fish tissue for the purpose of quality control.

Tissue	*Replicate Analysis*	*Fortification Level (µg/g)*	*Mean Recovery benzo (%)*
Muscle	3	1.0	97
Liver	2	4.5	90

Table 6: Recovery of dibenzothiophene from fortified fish tissue for the purpose of quality control.

Tissue	*Replicate Analysis*	*Fortification Level (µg/g)*	*Mean Recovery benzo (%)*
Muscle	3	1.0	97
Liver	2	4.0	93

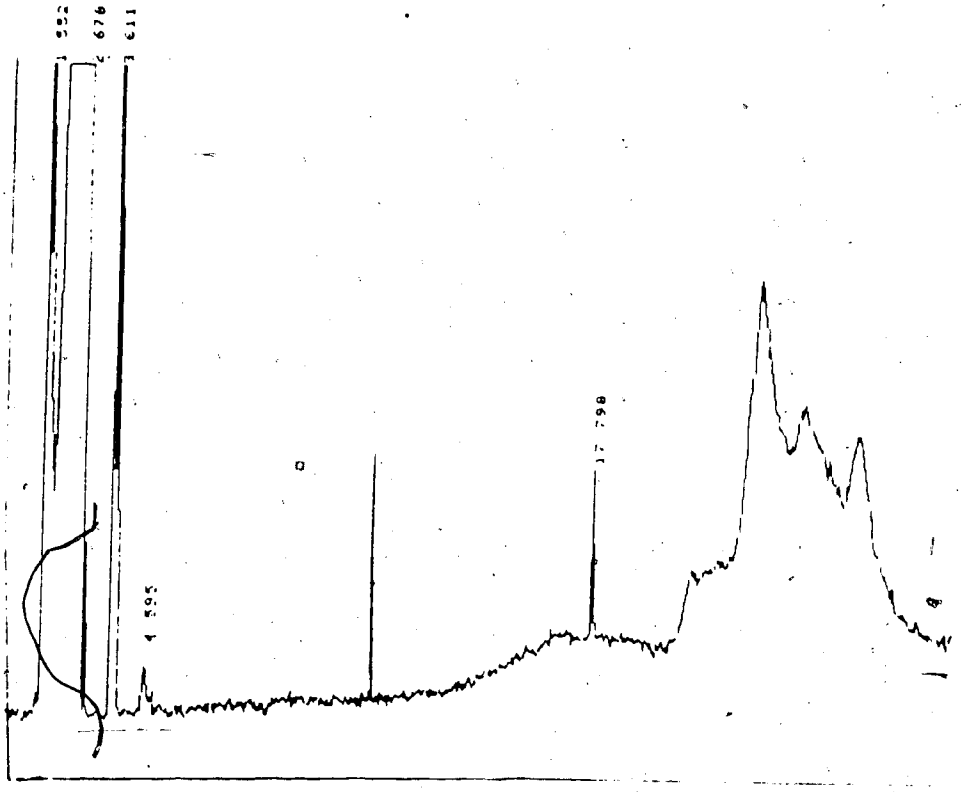


Figure 9. Chromatogram of fortified liver.

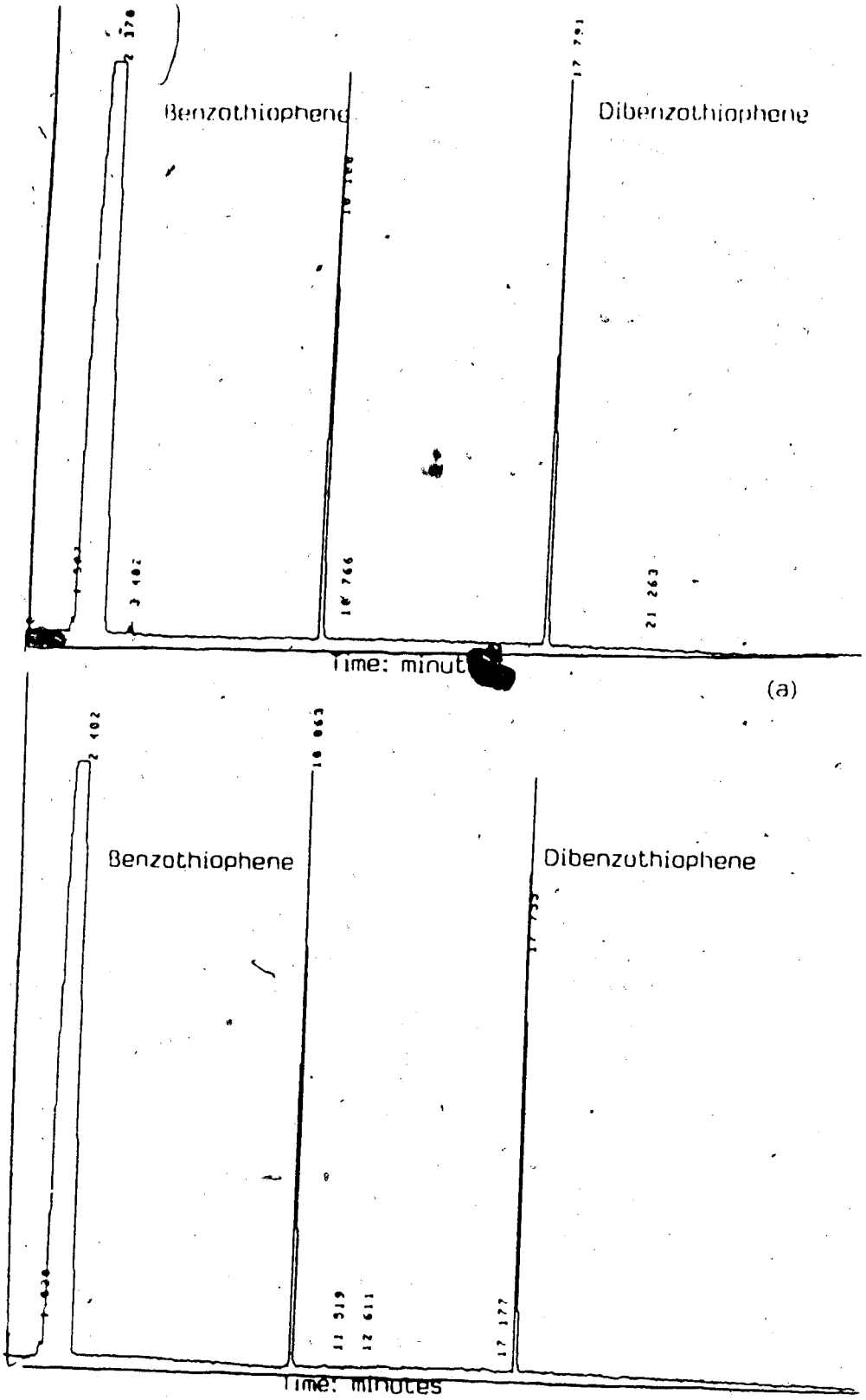


Figure 10. Gc/fpd chromatograms of (a) quality control eluate from GPC and (b) standard of comparison

(Attar *et al.*, 1977; Bowmann and Benoza, 1968) as a result both the maximum height and the area will be a function of the shape of the peak resulting in large errors.

4.2 Benzothiophene and Dibenzothiophene in Water:

The average concentrations of benzothiophene and dibenzothiophene in water, as determined by HPLC, were 0.7 and 0.52 mg/L respectively. Water samples were taken at regular intervals during the exposures. There was a significant drop in concentration from the start to the finish of the experiment in both cases (by as much as 50% in the case of some tanks, see Figures 1.1 to 16). This drop in concentration is results from the volatility and low solubility of both compounds. Water solubilities for benzothiophene and dibenzothiophene are 113 and 1.7 mg/L respectively. Additional losses of the chemical may have occurred through adsorption of the chemicals onto the polyethylene liners of the aquaria. The most dramatic decrease occurs in the first three hours; thereafter the concentration levels off somewhat.

4.3 Benzothiophene:

4.3.1 Accumulation and elimination studies

Table 7 illustrates the tissue distribution of fish exposed to benzothiophene. Table 8 illustrates the tissue distribution of exposed fish followed by 65 h depuration. Fish exposed to benzothiophene in water readily accumulated the chemical, but they can depurate this substance to a large extent after 65 h in fresh water. Mean bioconcentration factors were determined to be 37, 45 and 29 for the muscle, liver and bile respectively. These were calculated by dividing the mean tissue concentration by the mean exposure concentration.

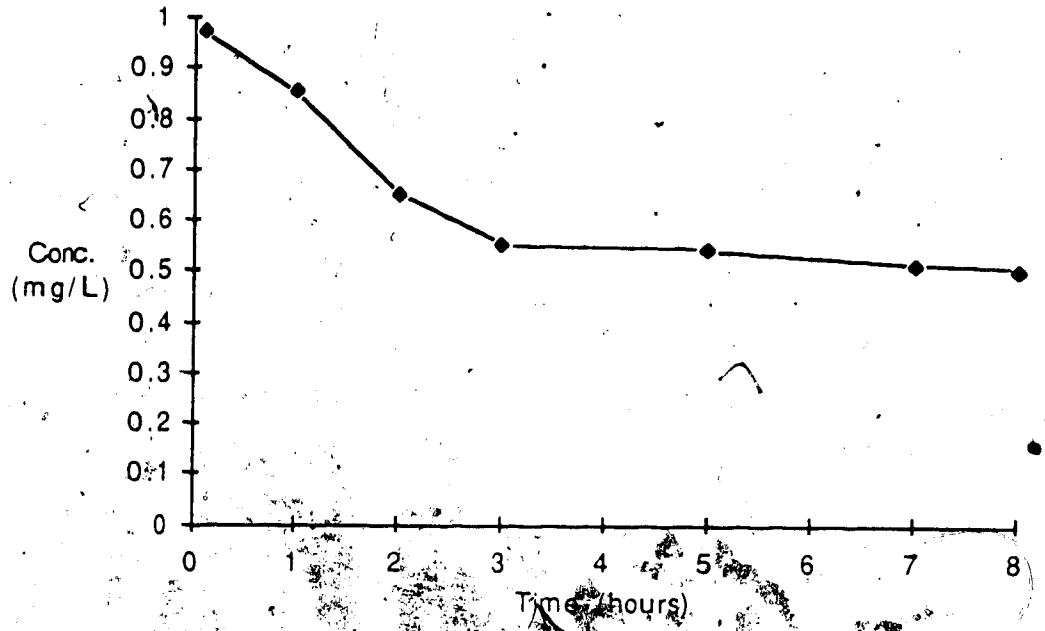


Figure 11. Benzothiophene exposure concentration in Tank #1, with two fish present.

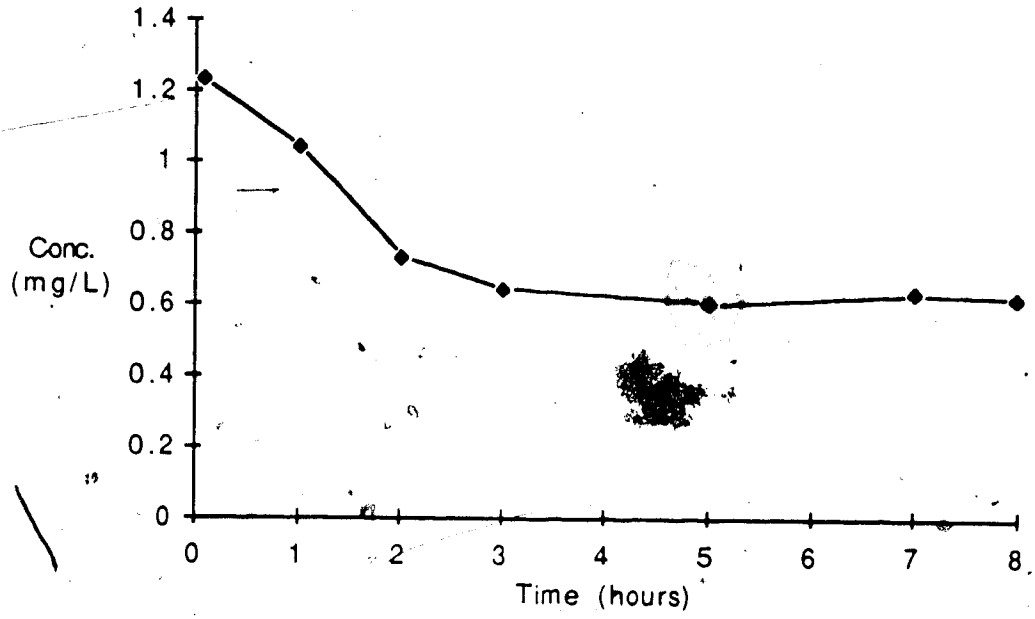


Figure 12. Benzothiophene exposure concentration in Tank #2, with two fish present.

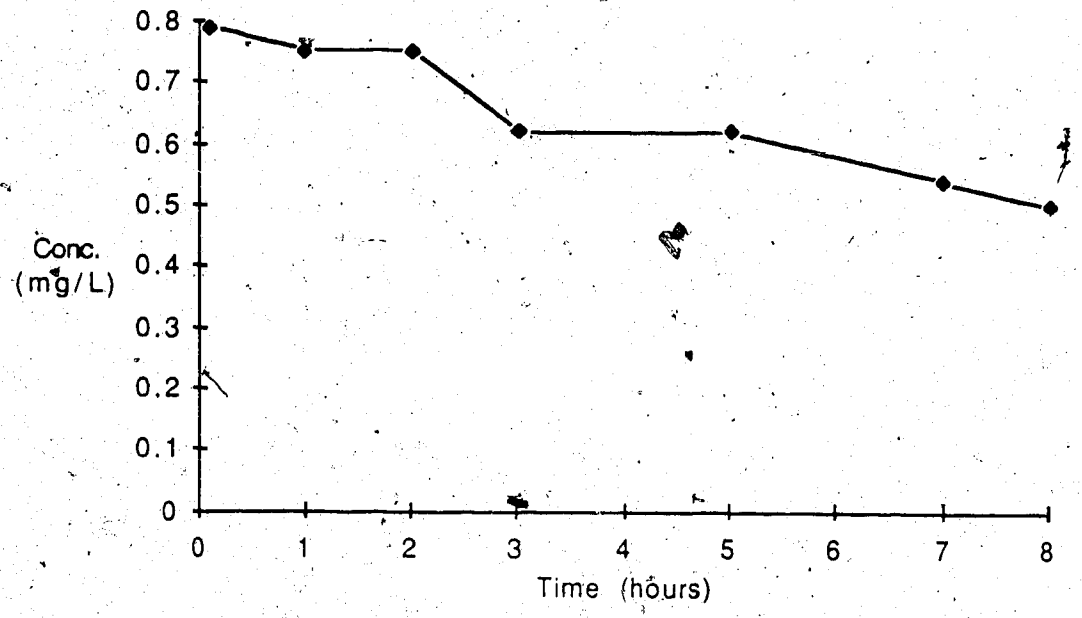


Figure 13. Benzothiophene exposure concentration in Tank #3, with two fish present.

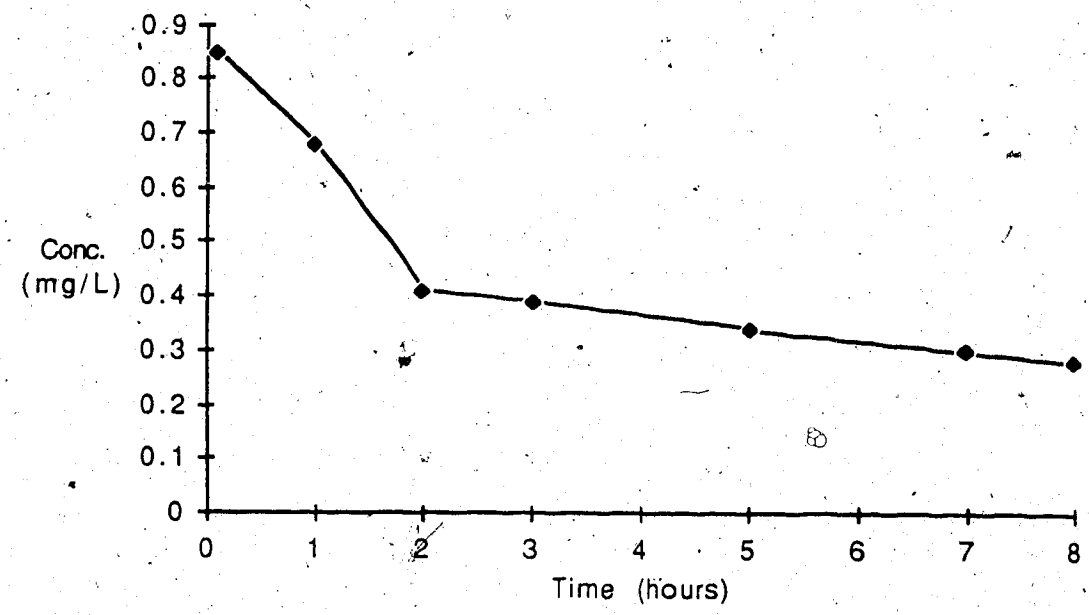


Figure 14. Dibenzothiophene exposure concentration in Tank #1, with two fish present.

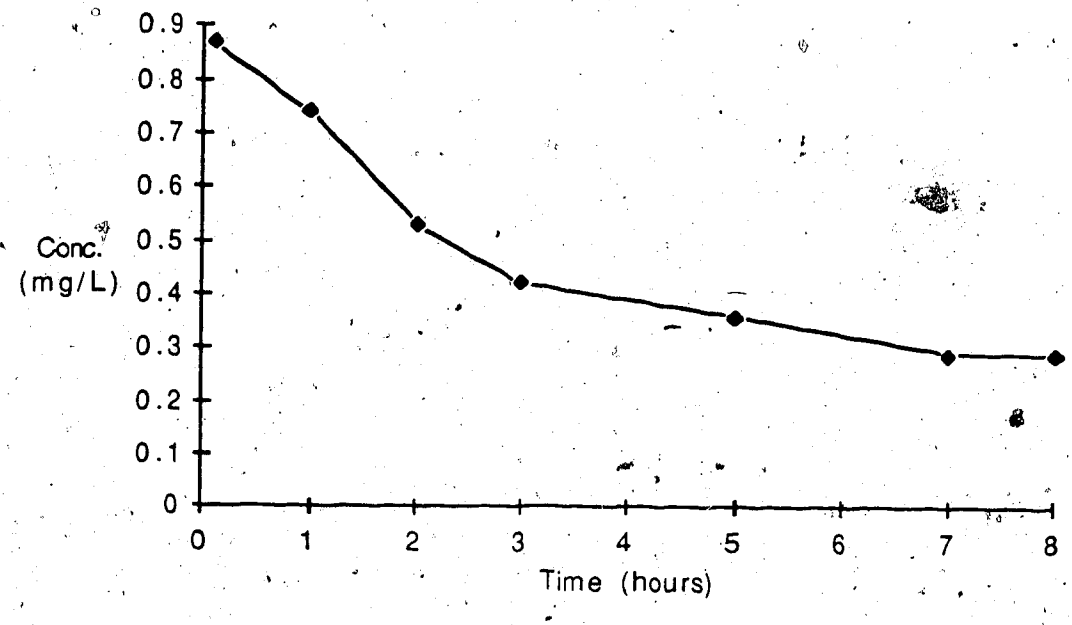


Figure 15. Dibenzothiophene exposure concentration in Tank #2, with two fish present.

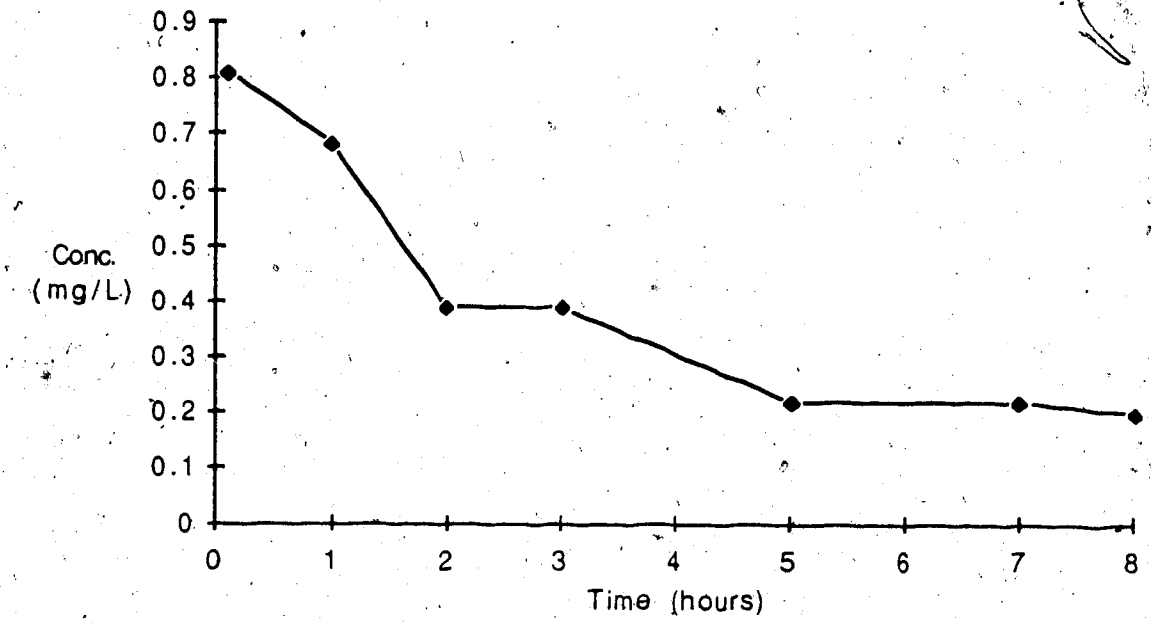


Figure 16. Dibenzothiophene exposure concentration in Tank #3, with two fish present.

Table 7. Distribution of benzothiophene in exposed fish (no depuration).

Replicate #	1	2	3
Exposure time (h)	8	8	8
Mean Exposure concentration (mg/L)	0.67	0.81	0.55
Conc. Range (mg/L)	0.99-0.50	1.23-0.64	0.78-0.54
Length of fish (mm)	272	242	271
Weight of fish (g)	254	163	261
Muscle weight (g)	139	89	133
Muscle conc. (μg/g)	18.7	30.6	26.3
Liver weight (g)	2.9	2.4	4.0
Liver conc. (μg/g)*	24.3	36.2	31.0
Bile conc. (μg/g)**	5.4+	10.3	3.9

* The quantities of benzothiophene found in the liver were from direct chloroform methanol extraction. Treatment of the liver sample with B-glucuronidase indicated an absence of the parent compound.

** The represents the concentration found in the bile after enzymatic hydrolysis followed by chloroform methanol extraction.

+ This particular extract evaporated to dryness in the vial before analysis: thus the residue was diluted with 50 mL of chloroform prior to analysis. This value, therefore represents a conservative estimate of the actual concentration.

Table 8. Distribution of benzothiophene in fish with 65h depuration.

Replicate #	1	2	3
Exposure time (h)	8	8	8
Mean Exposure concentration (mg/L)	0.67	0.81	0.55
Conc. Range (mg/L)	0.99-0.50	1.23-0.64	0.78-0.54
Length of fish (mm)	256	232	290
Weight of fish (g)	217	171	300
Muscle weight (g)	103	89	121
Muscle conc. (μg/g)	2.25	1.6	4.9
Liver weight (g)	3.0	2.6	4.5
Liver conc. (μg/g)*	2.5	1.7	1.3
Bile conc. (μg/g)**	0	0	0

* This value represents that concentration found in the bile after enzymatic hydrolysis followed by chloroform methanol extraction.

** This value represents that concentration found in the liver after direct extraction (no enzyme treatment).

Evidently the liver bioconcentrates benzothiophene to the greatest extent. One liver chromatogram obtained from a fish exposed to the hydrocarbon, is presented in Figure 17. The concentration in the liver dropped from 31.1 µg/g to 1.8 µg/g following depuration and that of the muscle dropped from an average of 25.2 µg/g to 2.8 µg/g, There was no evidence of the parent compound in the bile after depuration, whereas levels immediately after exposure averaged 6.5 µg/g. Chromatograms of bile and flesh from fish exposed to benzothiophene (in addition to controls) are presented in Figures 18 and 19. The parent compound is eluted from the column at 10.11 mins. Parent compound observed in livers and bile of these fish, were positively identified using GC/FID in conjunction with GC/MS. The total ion chromatogram of one fish sample is given in Figure 20. The spectrum appearing at 8.18 min. was identified as the parent compound, because the observed spectrum and retention time matched that observed for reference material. The retention time on the GC/MS differs from that of the GC/FID because the columns in both are dissimilar; temperature programming is also different in both these GCs.

Despite depuration, detectable levels of the chemical were present in the muscle and liver. Mean concentrations in both were determined to be 2.9 and 1.8 µg/g respectively. From these results, it would appear that benzothiophene is eliminated to a large extent (89-100%) from muscle, liver and bile.

4.3.2 Metabolism studies : benzothiophene:

Liver and bile from fish exposed to benzothiophene were analyzed for evidence of metabolism. Both tissues were enzymatically hydrolyzed using glucuronidase solution containing sulphatase activity. Following hydrolysis, a chloroform-methanol extraction was performed (refer to methods section). However, analysis of the extracts revealed parent compound only with little evidence of conjugated metabolites. Direct acetylation and

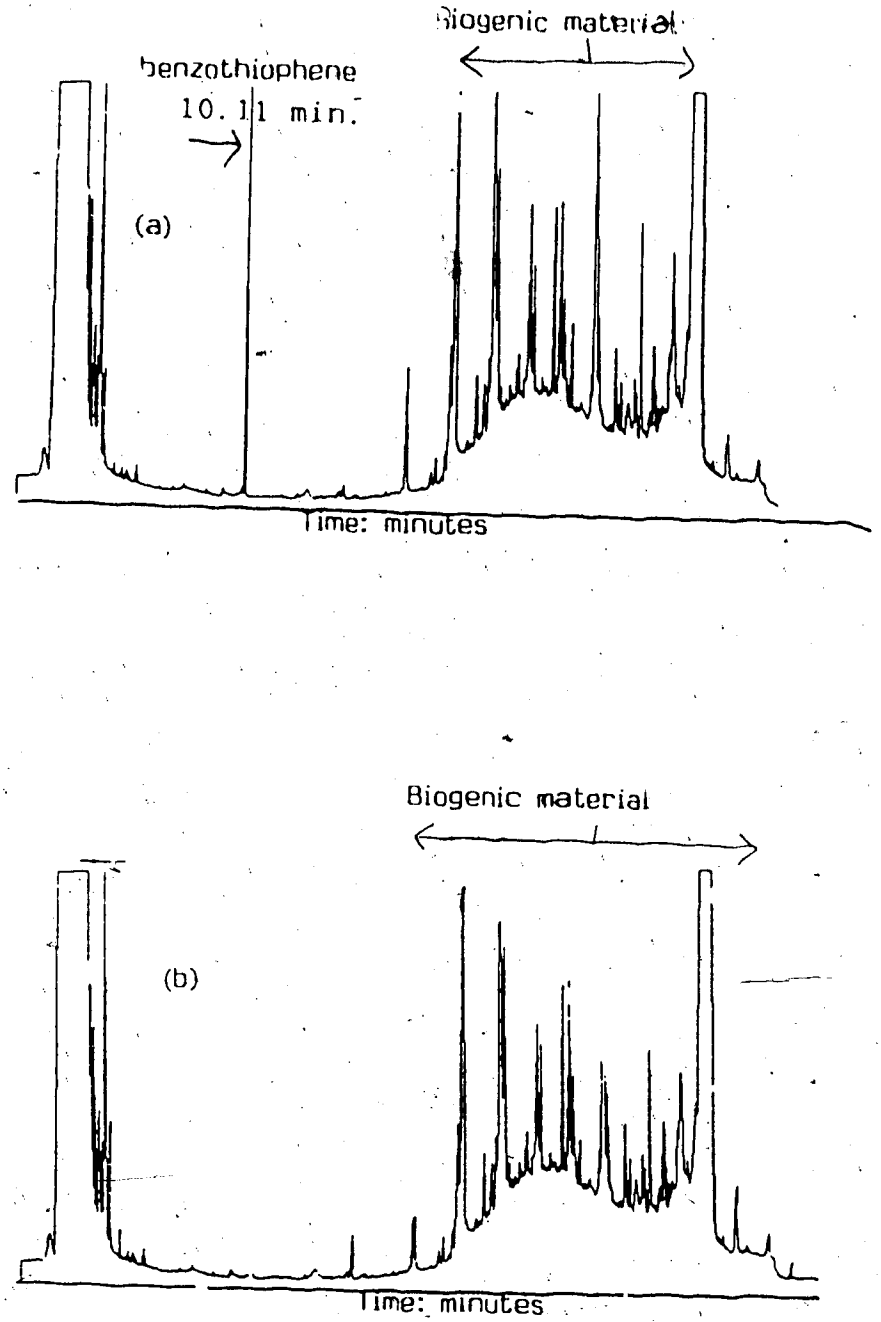


Figure 17. GC/FPD chromatograms from liver of fish (a) exposed to benzothiophene (b) control fish.

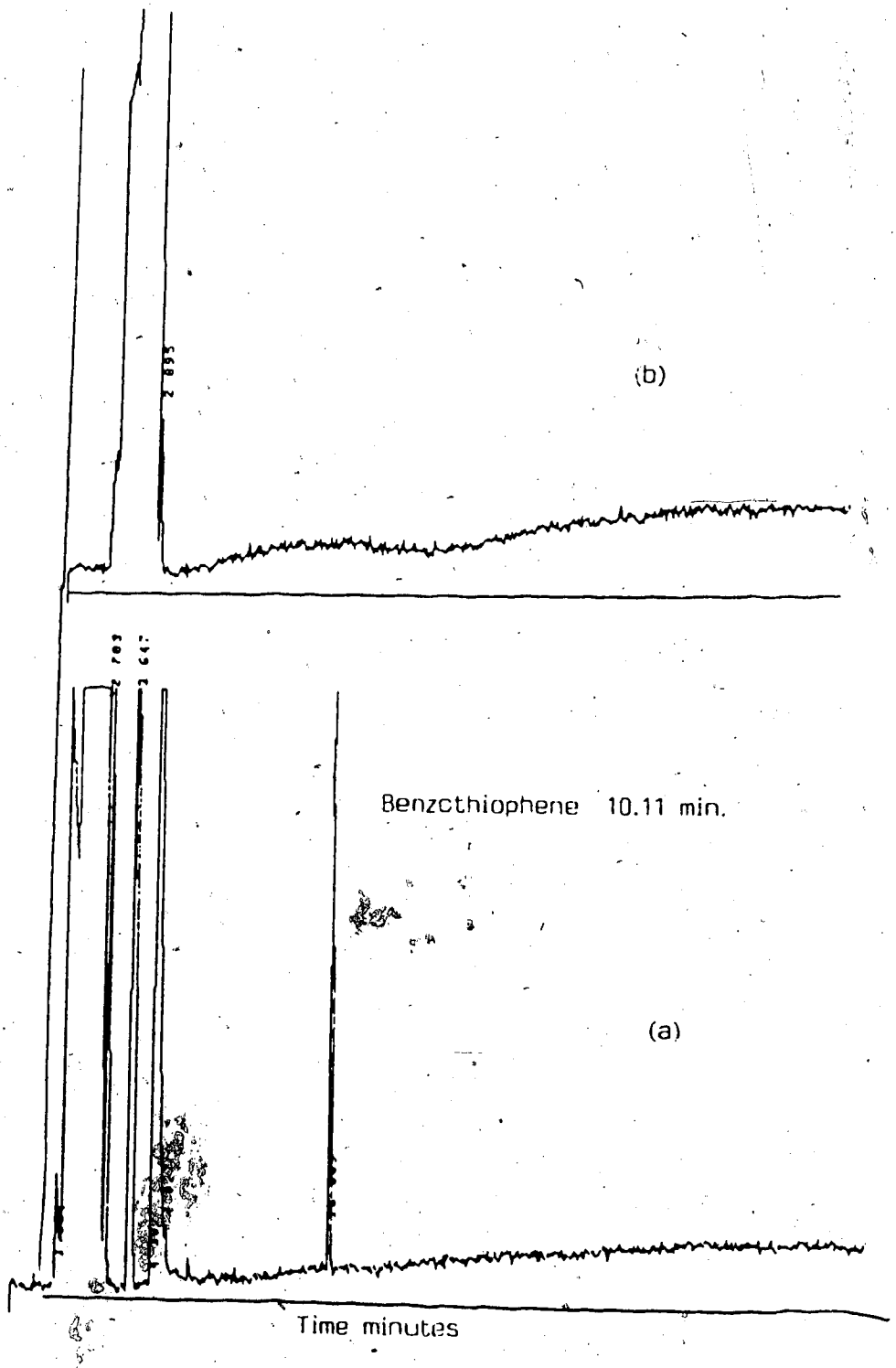


Figure 18. Chromatogram of bile from; (a) fish exposed to benzothiophene, and (b) control fish.

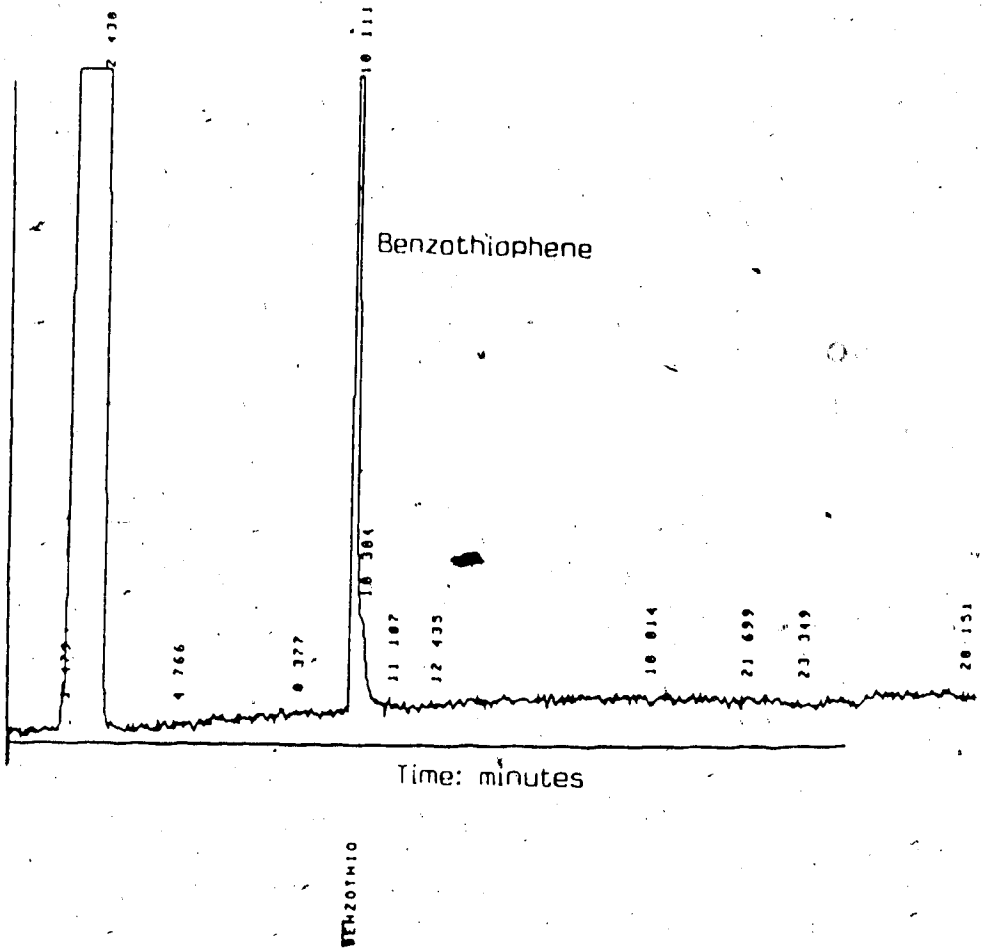


Figure 19. Chromatogram of flesh from fish exposed to benzothiophene

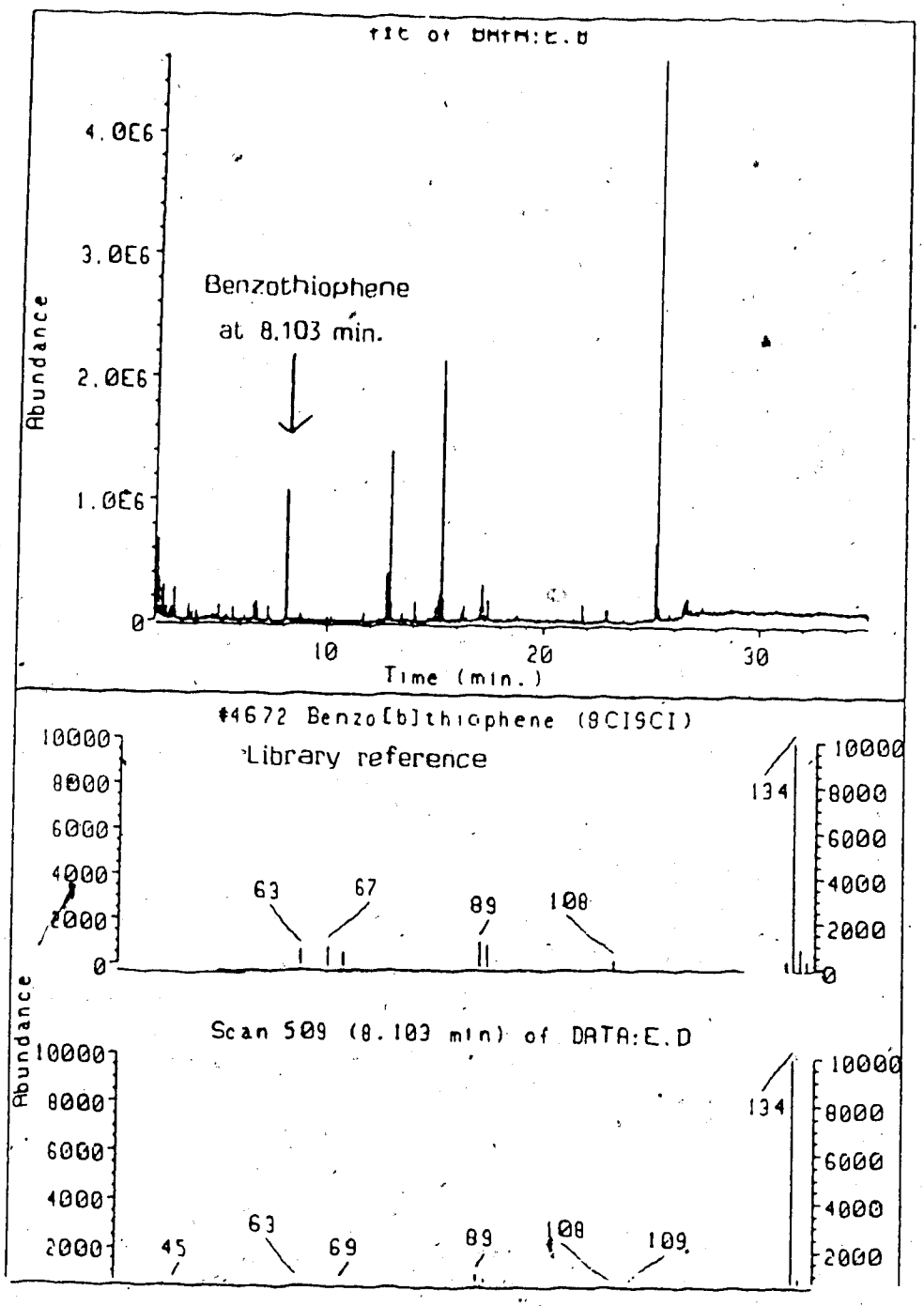


Figure 20. Total ion chromatogram of flesh exposed to benzothiophene Scan of peak at 8.103 min. matches this compound with that of reference benzothiophene.

caustic hydrolysis was also performed on bile samples in search of metabolites, but none were found. One possible pathway for the mobilization of benzothiophene is represented in Figure 21. However, no glucuronide conjugates could be detected by analysis.

4.4 Dibenzothiophene

4.4.1 Accumulation and elimination studies.

Table 9 illustrates the tissue distribution observed for fish exposed to dibenzothiophene. Table 10 illustrates the tissue distribution of exposed fish followed by 65 h depuration. Table 11 shows that rainbow trout can readily accumulate dibenzothiophene in muscle and liver. Mean bioconcentration factors were 43 and 32 for muscle and liver respectively. Mean concentrations in the muscle and liver were 23 and 18.5 mg/g respectively. Chromatograms of liver and flesh from fish exposed to dibenzothiophene are presented in Figures 22 and 23. They are compared to control samples. As can be seen from these figures, the parent compound is eluted from the column at 17.798 mins. The parent hydrocarbon was positively identified using G.C./MS in conjunction with GC/FID. Standards of benzothiophene were used to positively identify the chemical in GC/FID, whereas a comparison with library references was used on the mass spectrometer. An example of one total ion chromatogram is presented in Figure 24. The spectrum appearing at 15.403 min. was correlated with the parent compound, as it matched library reference material.

Both an enzymatic hydrolysis and a caustic hydrolysis were performed on the bile in search of parent compound. There was no evidence of the parent compound in the bile. This would lead one to suspect that the chemical had possibly been metabolized in the liver prior to deposition in the bile.

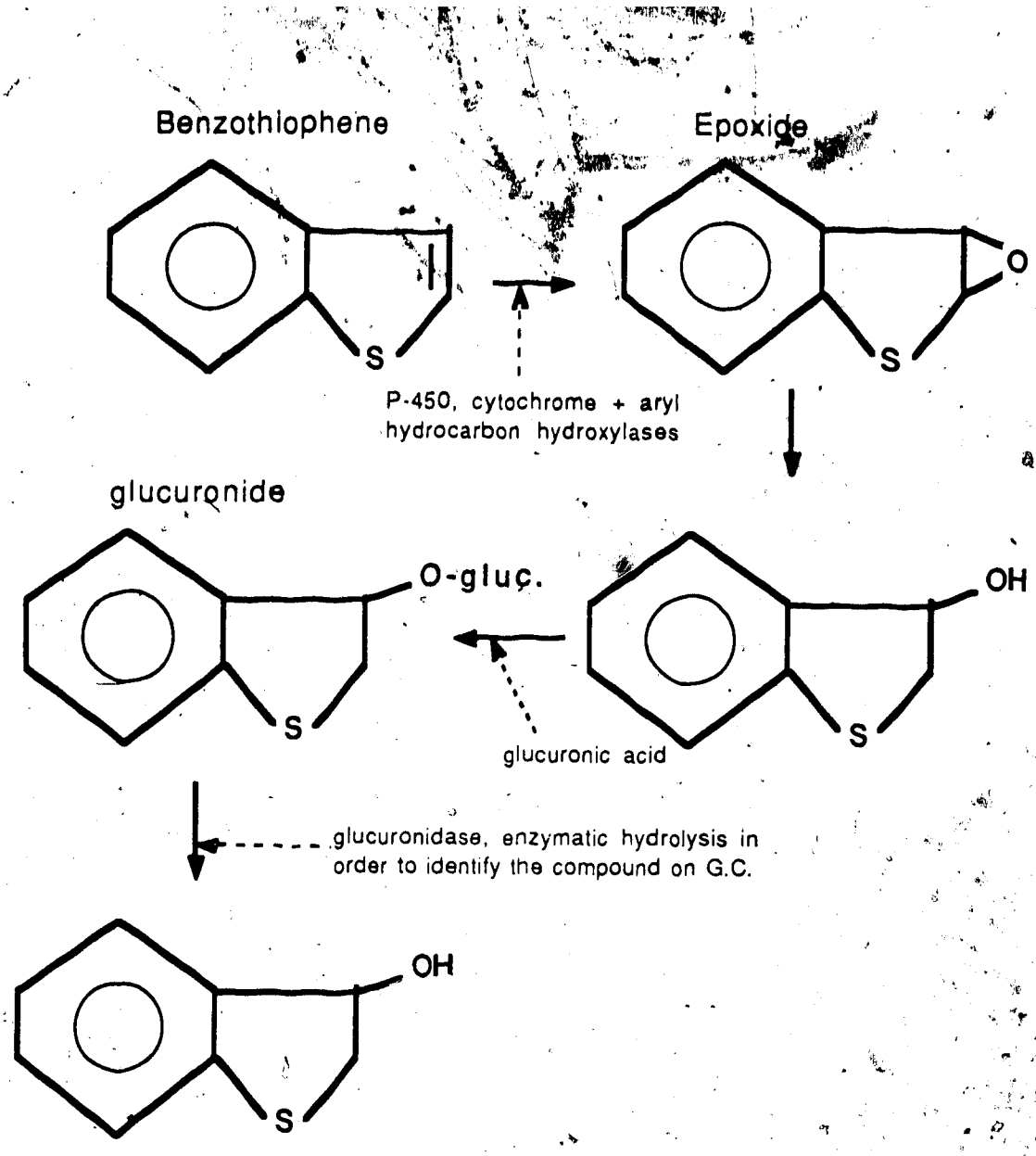


Figure 21. Graphical representation of suspected metabolic pathway of benzothiophene. In this scheme benzothiophene is converted to the epoxide via the cytochrome P450 enzyme system and converted to the monohydric alcohol followed by an additional reaction of the molecule: glucuronic acid is incorporated into the molecule. This compound is then enzymatically hydrolysed in the laboratory with glucuronidase in order to identify the alcohol by GC-FID. However, no standard was available in the laboratory for comparison and this compound was not identified.

Table 9. Distribution of dibenzothiophene in exposed fish (no depuration).

Replicate #	1	2	3
Exposure time (h)	8	8	8
Mean Exposure concentration (mg/L)	0.53	0.56	0.49
Conc. Range (mg/L)	0.86-0.42	0.86-0..27	0.81-0.49
Length of fish (mm)	236	195	227
Weight of fish (g)	162	102	143
Muscle weight (g)	81	45	73
Muscle conc. (µg/g)	26.6	24.0	18.2
Liver weight (g)	3.1	2.5	1.6
Liver conc. (µg/g)*	13.8	18.9	23.0
Bile conc. (µg/g)**	0	0	0

* This concentration represent that found in the liver after direct extraction (no enzyme treatment).

** This concentration represents that found in the bile after enzymatic hydrolysis followed by chloroform methanol extraction.

Table 10. Distribution of dibenzothiophene in exposed fish with 65h depuration.

Replicate #	1	2	3
Exposure time (h)	8	8	8
Mean Exposure concentration (mg/L)	0.53	0.56	0.49
Conc. Range (mg/L)	0.86-0.42	0.86-0..27	0.81-0.49
Length of fish (mm)	257	254	278
Weight of fish (g)	232	201	256
Muscle weight (g)	72	144	37
Muscle conc. (µg/g)	10.5	11.2	8.8
Liver weight (g)	5.0	5.4	1.3
Liver conc. (µg/g)*	16.8	12.3	13.7
Bile conc. (µg/g)**	0	0	0

* This concentration represent that found in the liver after direct extraction (no enzyme treatment). Enzymatic-hydrolysis was performed on the liver sample, but indicated an absence of parent compound.

** This concentration represents that found in the bile after enzymatic hydrolysis followed by chloroform methanol extraction.

Table 11. Bioconcentration factors for benzothiophene and dibenzothiophene in muscle, liver and bile of exposed fish. These were calculated by dividing the mean water concentration by the mean tissue and bile concentrations.

Compound		Bio-Concentration Factors		
		Muscle	*Liver*	*Bile*
Benzothiophene	1	28	36	8
	2	38	45	13
	3	48	56	7
Dibenzothiophene	1	50	26	-
	2	43	34	-
	3	37	47	-

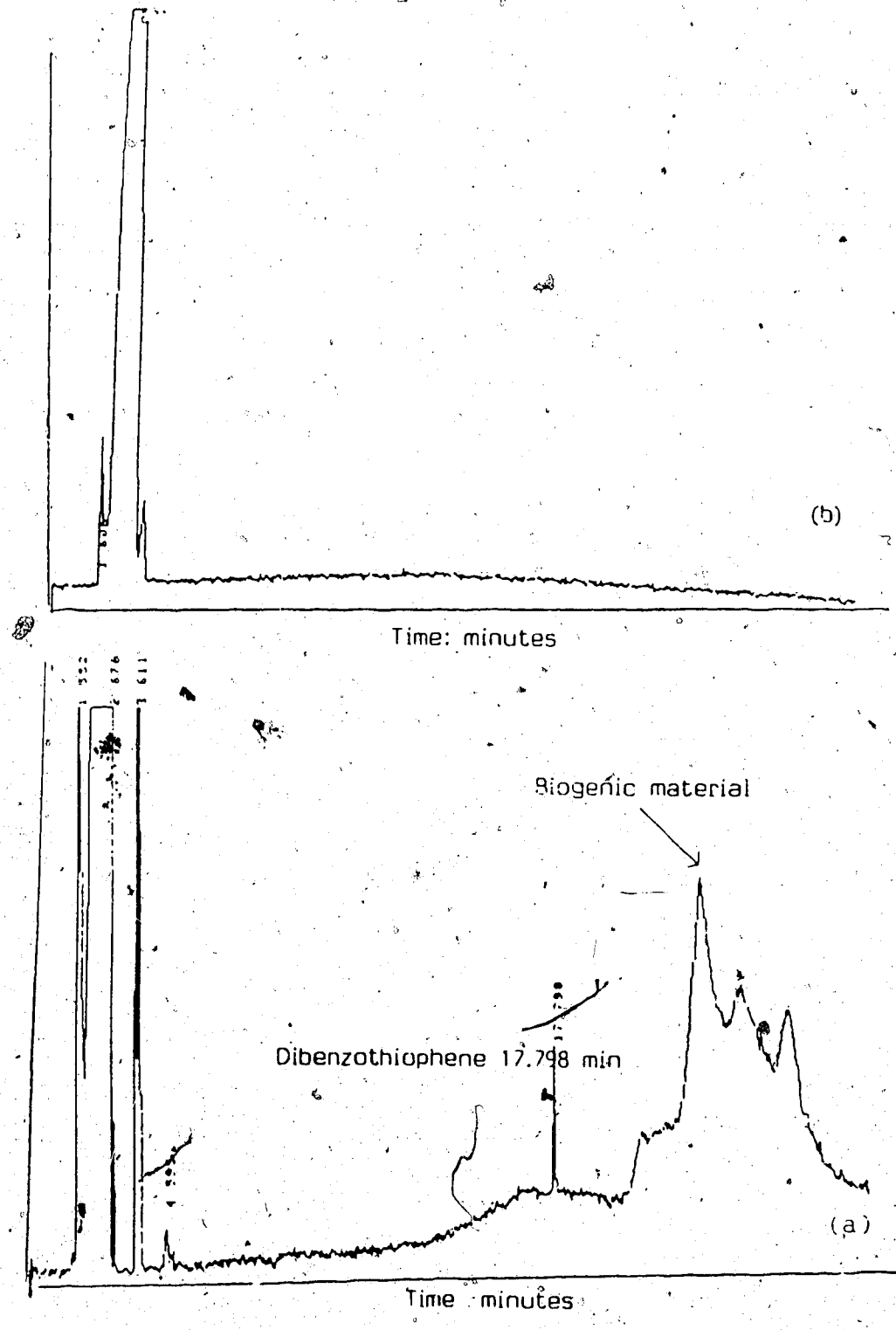


Figure 22. Chromatogram of liver from: (a) fish exposed to dibenzothiophene, and (b) control fish.

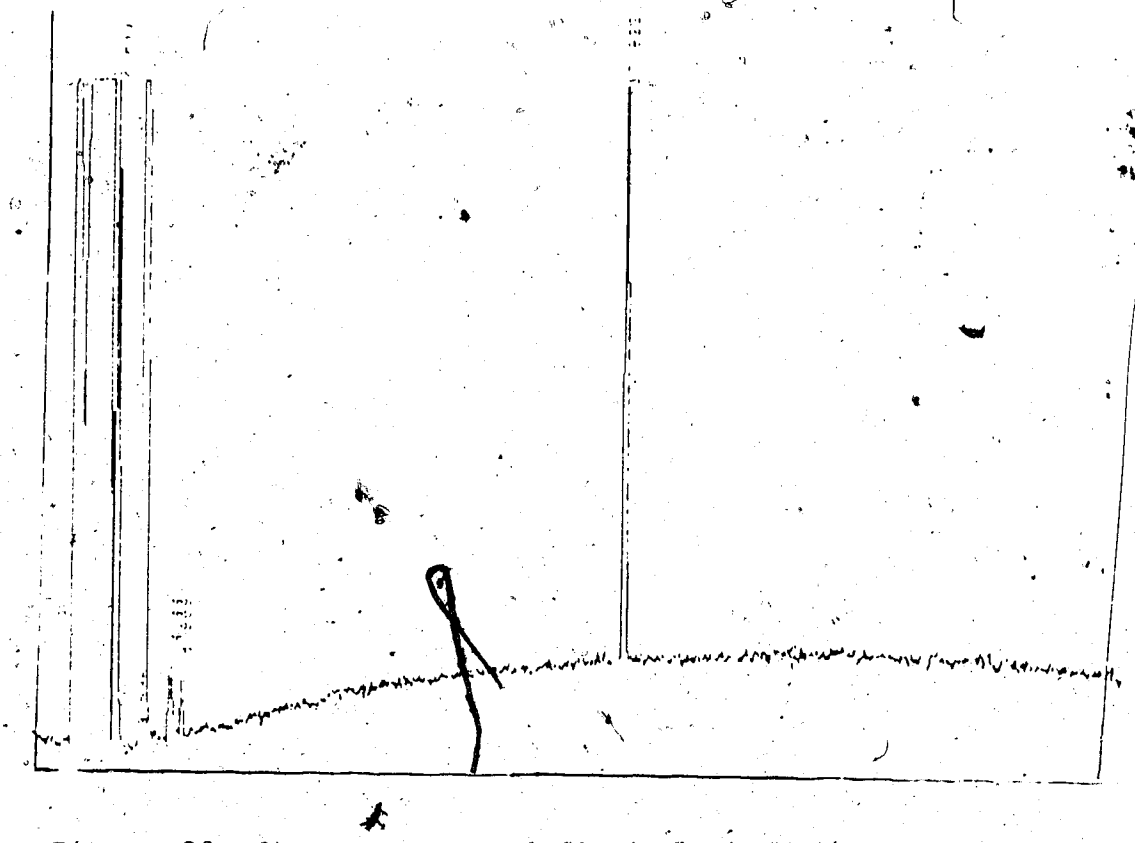


Figure 23. Chromatogram of flesh from fish exposed to dibenzothiophene

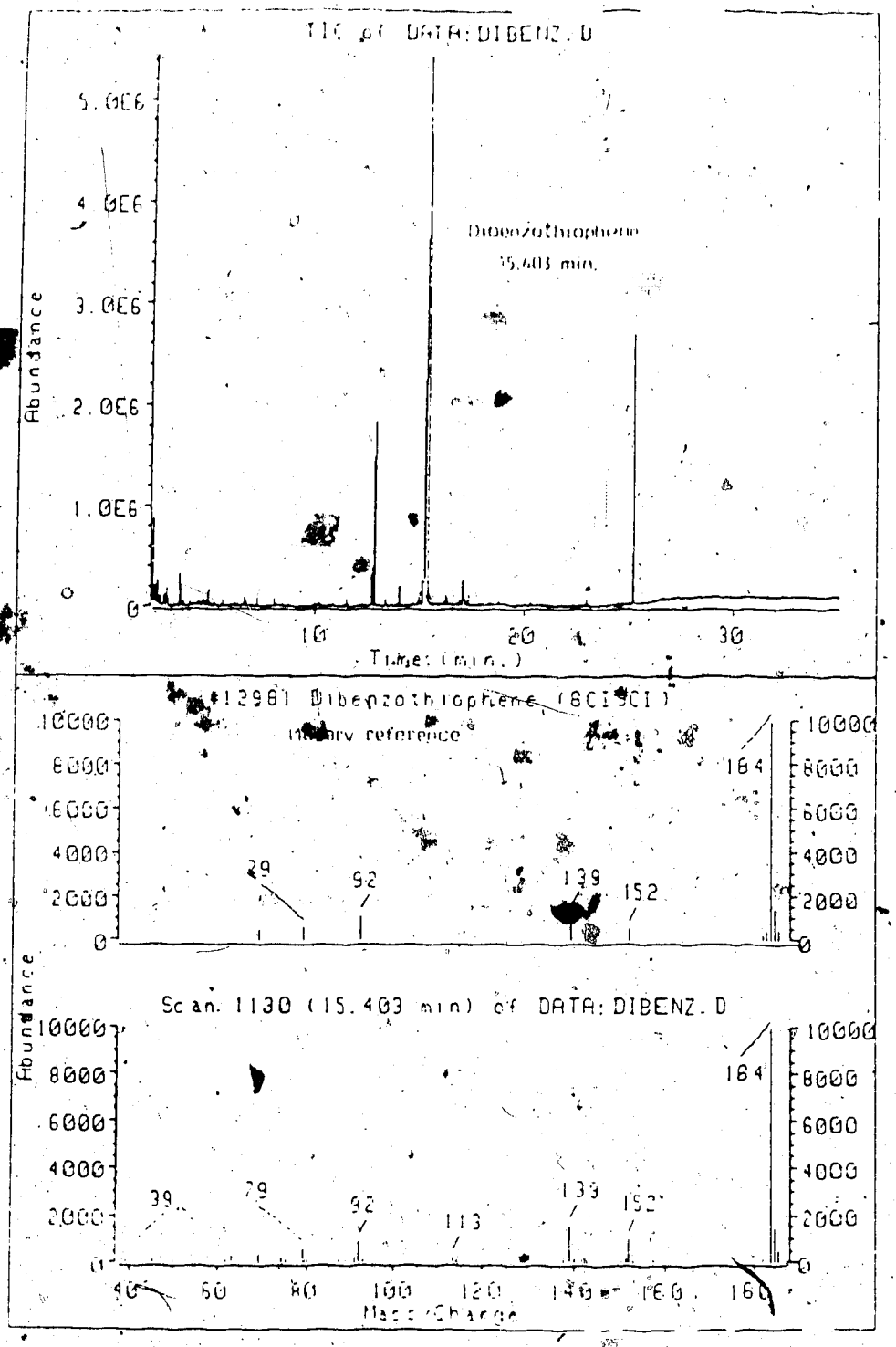


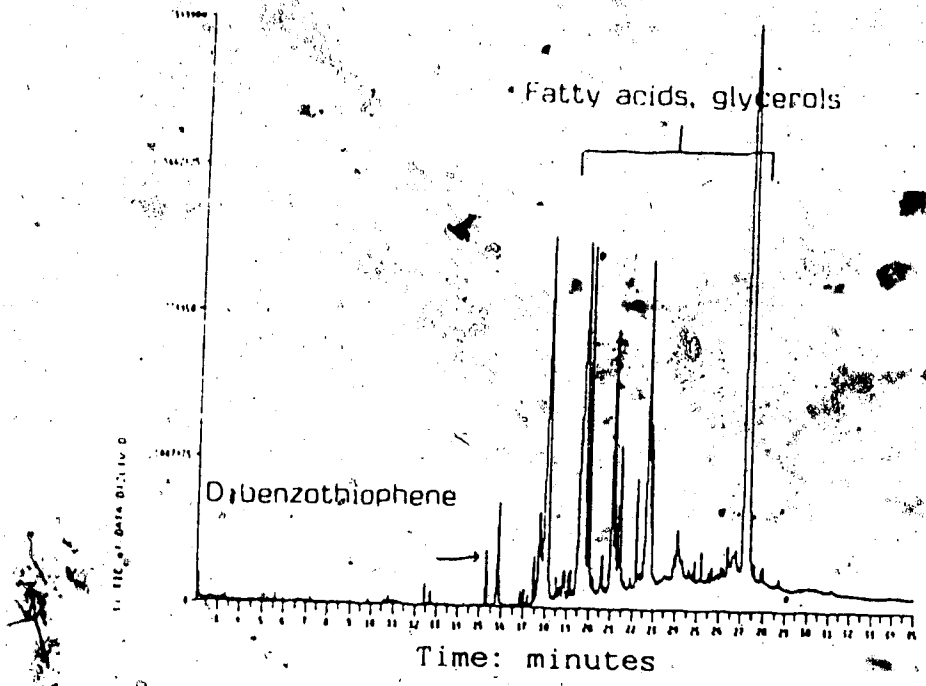
Figure 24. Total ion chromatogram of dibenzothiophene fish flesh.

Although rainbow trout can readily accumulate dibenzothiophene in muscle and liver with an average of 23 and 18.5 µg/g respectively, they are slow to depurate the same compound. After 65h of depuration the fish contained an average of 10.1 and 14.3 µg/g in muscle and liver tissue respectively. These levels of chemical are quite high, considering the fish were in fresh water for 65 h. Such high levels of the compound following depuration, indicate the fish had some difficulty mobilizing dibenzothiophene. Dibenzothiophene appears to be more persistent than benzothiophene in the tissues of sal

4.4.2 Metabolism studies; dibenzothiophene:

Liver and bile samples from fish exposed to dibenzothiophene were examined for the presence of metabolites. Neither primary nor secondary metabolites were found. However, in the case of one depurated liver, there were a number of unidentified peaks which appeared on the GC/FPD and GC/FID chromatograms, approximately 4 minutes after the parent compound peak (Figure 25). This particular extract was then run on GC/MS, in order to try and identify these peaks. However, the relevant spectra did not match any reference library compounds. The extract was then run (with the help of the staff chemist), on a high resolution mass spectrometer (VG Model 7070) and the spectra were determined to be fatty acids and glycerols. There was no evidence of metabolites.

On analysis of bile samples, neither parent compound nor metabolites were found. While the fish were in fresh water for depuration studies, they were fed (and observed to eat) pellet food. We can assume therefore, that digestion occurred, resulting in production and flow of bile. As such, it is surprising that no detectable levels of dibenzothiophene



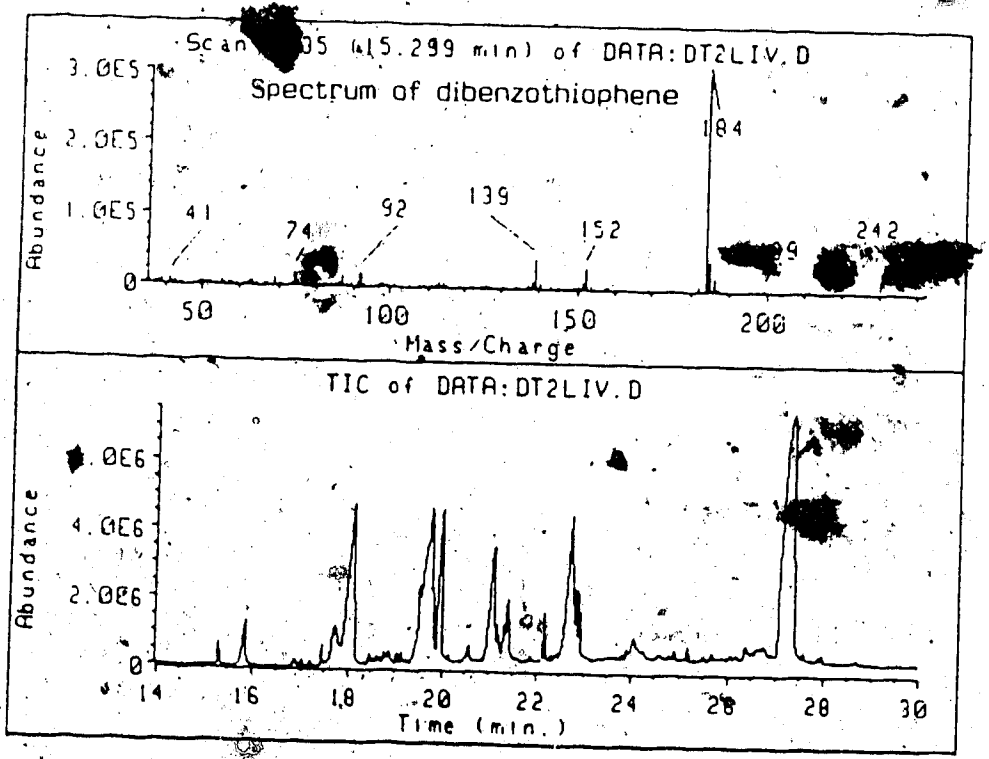


Figure 25 Depurated dibenzothiophene liver. . G.C./M.S. chromatogram of extract with identification of parent compound.

were found in the bile, given that the related compound, benzothiophene, was found in ample quantities in the bile of similarly exposed fish.

Dibenzothiophene may be metabolized, but no evidence of this was found. In comparison to benzothiophene, dibenzothiophene is accumulated to a similar extent in both liver and muscle tissue, but is eliminated more slowly. Following depuration in both cases, the muscle contained an average of 2.9 µg/g in the case of benzothiophene and 10.2 µg/g in the case of dibenzothiophene. It appears that fish can eliminate benzothiophene more rapidly than they can dibenzothiophene. This may result from dibenzothiophene having a higher octanol-water partition coefficient (more lipophilic) than benzothiophene. However this is unlikely an explanation for the "all or nothing" results obtained. The reason is more likely to be an analytical problem in finding the metabolites.

4.5 Bioconcentration of benzothiophene and dibenzothiophene.

Bioconcentration factors (BCF) for benzothiophene and dibenzothiophene in fish tissues are reported in Table 11. Both chemicals are bioconcentrated to a similar extent in the muscle and liver of rainbow trout. However there was no evidence of dibenzothiophene being bioconcentrated in the bile, whereas benzothiophene exhibited a mean BCF of 9. One possible explanation for this is that the fish may have had more difficulty in mobilizing dibenzothiophene in the liver and subsequently depositing it in the bile, than they did with benzothiophene. Alternatively, analytical techniques for the recovery of PASH from fish tissues may be lacking and require further development.

The results reported in Table 12 were used to determine additional BCFs of benzothiophene. BCFs in this flesh ranged from 10 to 500. With such a scatter of

Table 12. Tissue concentrations for fish exposed to benzothiophene.

*Exposure Concentration (mg/L)**	*Flesh Concentration (mg/Kg)* **
0.01	0.01
0.03	3
0.08	47
0.5	62
1.0	84

* This value is based on the amount of chemical added to the water.

** The 15 samples from each concentration were pooled together by means of a Waring blender. Analyses were performed on these pooled samples using GC/FID.

Table 12a. Exposed flesh as prepared for sensory evaluation panel.

Flesh Concentration (mg/Kg) *
0.01
0.043
0.092
0.54
1.04

* The exposed flesh was mixed with control flesh in order to obtain the desired concentration.

Table 12b. Tissue concentration for fish fortified with benzothiophene. This flesh was fortified for the purpose of sensory evaluation only.

Target Fortification Level (mg/Kg)	*Flesh Concentration (mg/Kg)* *
0.01	0.021
0.03	0.034
0.08	0.086
0.5	0.61
1.0	1.08

BCFs, it is difficult to predict water levels that will produce related concentrations of the chemical in fish.

4.6 Sensory Evaluation:

4.6.1 Taste threshold:

Individual taste and odour thresholds were determined by panelists who made a judgement as to the least detectable concentration of benzothiophene that they could recognize correctly (Jellinek, 1985). Correct responses are taken to be those, above which panelists do not mistakenly identify the controls as having a taint. There were three controls interspersed among the eight pairs.

The sensory threshold determination was carried out with rigorous environmental and procedural controls as outlined in ASTM (1979). The median taste/odour threshold was determined as the lowest concentration where >50% of the population detected a correct response and was found to be 0.01 ppm. The geometric mean odour thresholds were calculated for each panelist. Table 13 gives the individual, individual mean and group mean threshold values. There was no distinction made between taste and odour thresholds, as it has been demonstrated that these two senses are inter-related. The combination of the two senses was used for the sensory evaluations.

For each replicate evaluation, bar charts were construeted depicting the % response of exposed or fortified versus controls for each of the three pairs where a control was included. These charts demonstrate that [illegible] spiked over controls at all values, with the percentage increasing for higher concentrations. The odour

Table 13: Individual, individual mean and group mean thresholds (all calculated means are geometric).

Panelist	*Replication Analysis*			*Mean Individual Threshold (Geometric Mean) (ppm)*
	1	*2*	*3*	
	Individual Thresholds (ppm)			
1	0.01	0.03	1.0	0.067
2	0.01	0.01	0.01	0.01
3	0.01	0.08	0.01	0.02
4	0.01	0.01	0.01	0.01
5	0.01	0.01	0.01	0.01
6	0.01	0.08	0.08	0.04
7	0.5	0.03	0.5	0.20
8	0.01	0.01	0.01	0.01
9	0.01	0.01	0.01	0.01
10	0.03	0.5	0.01	0.05
11	0.03	0.01	0.08	0.03
Geometric Group mean	0.02	0.03	0.03	0.04*
				0.02**

* Total Group Mean Threshold

** Recalculated group mean

threshold was thus determined to be 0.01 mg/kg. These charts are depicted in Figures 26 to 28.

The individual mean thresholds ranged from 0.01 mg/kg to 0.20 mg/kg (geometric means, refer to Table 13). Note the large differences between these values for individual panelists. There are three judges in particular who deviate considerably from the rest of the group. On checking the raw data it was noticed that these particular panelists repeatedly identified the controls as having an "off-flavour" and were inconsistent from session to session. Thresholds obtained from these panelists in each of the three replicates were plotted (Figures 29 and 30) and compared with their geometric means. Each of the three exhibited at least one replication greater than four times or less than one quarter times (>4X or <1/4X) his/her geometric mean. According to Rosen *et. al* (1962) and Hamilton *et. al* (1978) a given panelist should distinguish concentrations which differ by a factor of two (2X or >1/2X); it was our decision to use twice their factor of two as a safety measure. This is sufficient criterion for rejecting panelists. Consequently the results of the particular panelists in question were ignored.

A new group mean threshold was calculated based on the remaining 8 panelists. This new group mean threshold was determined to be 0.02 mg/kg as opposed to the old value of 0.04 mg/kg. The geometric mean threshold value of 0.02 mg/kg is more in keeping with the median threshold value of 0.01 mg/kg. Since 0.01 mg/kg is the lowest concentration presented to the panelists, we can safely assume that the actual threshold concentration for benzothiophene is 0.01 mg/kg or less.