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For my parents

STUDIES OF THE SYNTHESIS, ENVIRONMENTAL OCCURRENCE AND TOXICITY OF UNRESOLVED COMPLEX MIXTURES (UCMs) OF HYDROCARBONS

by

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Emma Jane Wraige

ABSTRACT

The occurrence of unresolved complex mixtures of hydrocarbons (UCMs) in the aliphatic fraction of marine sediments and organisms from areas impacted by petroleum hydrocarbons is well documented and widely accepted as an indication of fossil fuel contamination. In contrast, the presence of an aromatic UCM is often ignored and environmental concentrations of aromatic UCM hydrocarbons in marine biota and sediments are rarely reported. The aims of this study were to establish the quantitative significance of aromatic UCMs in environmental samples and to assess the toxicological significance of both aliphatic and aromatic UCMs.

A reproducible method was developed and validated for the analysis and quantification of petroleum hydrocarbons in mussel (*Mytilus edulis*) tissue. Emphasis was placed upon development of a method which minimized losses of more volatile, lower molecular weight, toxicologically significant hydrocarbons, without compromising recovery of higher molecular weight compounds which are useful for source identification in environmental monitoring schemes. Analysis of mussels from a small number of U.K. coastal locations indicated that aromatic hydrocarbon UCMs may form a significant proportion (*ca* 20 %) of the total hydrocarbon body burden of mussels from areas contaminated with petroleum hydrocarbons. Aromatic UCM hydrocarbons were not observed in mussels from relatively uncontaminated areas but concentrations of 430 $\mu g\ g^{-1}$ (dry wt tissue) were measured in mussels from heavily impacted areas. Aliphatic UCM concentrations ranged from 7 - 3445 $\mu g\ g^{-1}$ (dry wt tissue).

For the purposes of toxicological studies, a low molecular weight model aliphatic UCM hydrocarbon, 4-propyloctane (4-PO) was synthesised. Two low molecular weight model aromatic hydrocarbons 7-cyclohexyltetralin and 7-cyclohexyl-1-propyltetralin were also synthesised using a modification of the Haworth synthesis. All three target compounds and synthetic intermediates were characterised by NMR, MS and IR.

Exposure of *M. edulis* to 4-PO caused a significant reduction in mussel ciliary feeding activity indicating that 4-PO was indeed toxic as measured by this bioassay. The demonstrable narcotic activity of 4-PO is presumably related to the greater aqueous solubility of branched hydrocarbons compared with similar straight chain hydrocarbons. Further experiments investigating the effect of 4-PO over exposure periods up to 120h provided a unique and detailed insight into the relationship between concentration of toxicant in the gills of *M. edulis* and observed feeding rate. The established method of mussel feeding rate determination was modified in light of this relationship to produce an improved rapid and reproducible screening technique.

Both of the model aromatic UCM hydrocarbons were also found to be toxic to mussel ciliary feeding activity. This appears to be the first report of investigations into the toxicity of the aromatic UCM and suggests that previous studies have ignored an environmental burden of toxicological significance.

Estimates of the tissue effective concentration (TEC_{50}) for the model UCM hydrocarbons gave comparable values with those reported for the effect of other narcotic hydrocarbons upon mussel feeding rate, providing support for the theory that non-specific narcosis occurs at a relatively constant tissue concentration of toxicant.

The demonstrated narcotic activity of each of the three model UCM hydrocarbons has extended the molecular weight range of narcotic hydrocarbons studied to date. The results presented herein suggest that a small proportion of low molecular weight aliphatic UCMs and perhaps a greater proportion of aromatic UCMs are of toxicological significance.

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AUTHORS DECLARATION

At no time during the registration for the degree of Doctor of Philosophy has the author been registered for any other University award.

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Wraige, E.J., P.Donkin and S.J.Rowland The Effects of Unresolved Complex Mixtures of Hydrocarbons upon the Mussel *Mytilus edulis*' A low molecular weight model UCM hydrocarbon; 4-propyloctane (4-PO). Plymouth Marine Laboratory, March 1994

Wraige, E.J., P.Donkin and S.J.Rowland The Effects of Unresolved Complex Mixtures of Hydrocarbons upon the Mussel *Mytilus edulis*' A low molecular weight model UCM hydrocarbon; 4-propyloctane (4-PO). SETAC-UK annual meeting. University of Sheffield, September 1994 [Awarded runner up in Student poster competition]

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Signed .....

Date.....

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LIST OF COMMON ABBREVIATIONS

BCF	bioconcentration factor
CBR	critical body residue
BB	body burden
BSTFA	*N,O*-bis[trimethylsilyl]trifluoracetamide
CI-MS	chemical ionisation- mass spectrometry
DCM	dichloromethane
DEPT	Distortionless enhancement by polarisation transfer
EI-MS	electron impact-mass spectrometry
EPA	Environmental Protection Agency
FI-MS	field ionisation-mass spectrometry
FT-ICR	fourier-transform ion cyclotron mass spectrometry
GC	gas chromatography
GC-MS	gas chromatography-mass spectrometry
GPC	gel permeation chromatography
HAc	glacial acetic acid
HPLC	high performance liquid chromatography
IR	infra-red spectroscopy
K_{OW}	octanol-water partition co-efficient
K.D.	Kuderna Danish apparatus
LC_{50}	concentration of toxicant which results in 50 % mortality
MS	mass spectrometry
MeOH	methanol
MW	molecular weight
NMR	nuclear magnetic spectroscopy
PAH	polycyclic aromatic hydrocarbon
QSAR	quantitative structure-activity relationship
RSD	relative standard deviation
SFG	scope for growth
SRC	Syracuse Research Corporation
SRM	standard reference materials
TEC_{50}	tissue concentration to reduce a toxicant induced response by 50%
TEL	total extractable lipid
TJP	Tia Juana Pesado
TMS	trimethylsilyl
TOE	total organic extract
UCM	unresolved complex mixture
UCM_{ox}	unresolved complex mixture of oxidised hydrocarbons
UV	ultra-violet spectroscopy
4-PO	4-propyloctane
7-CHT	7-cyclohexyltetralin
7-C-1-PT	7-cyclohexyl-1-propyltetralin

CHAPTER ONE

Introduction

1.1 Unresolved complex mixtures (UCMs) of hydrocarbons

Petroleum is made up of a complex mixture of hydrocarbons and minor amounts of other compounds. Despite advances in analytical techniques, a substantial proportion of the hydrocarbons of both crude oil and some refined petroleum products remains unresolved and hence unidentified, even by such high resolution methods as gas chromatography (GC). When examined by these methods, the hydrocarbons of many crude oils can be shown to contain chromatographically unresolved 'humps' (Figure 1.1a). These 'humps' have been termed unresolved complex mixtures (UCMs; *e.g.* Thompson and Eglinton, 1978).

Hydrocarbon UCMs vary in carbon number range and appearance. Analysis of an unweathered, undegraded crude oil by gas chromatography usually produces a chromatogram dominated by a well resolved distribution of *n*-alkanes, isoprenoids, alkylbenzenes, naphthalenes and phenanthrenes with little evidence of an UCM (Figure 1.1b). However, weathering processes (*e.g.* microbial degradation) result in loss of the resolved compounds and hence a relative increase in the UCM, which is considered to be relatively inert to microbial degradation (Gough, 1989). Consequently, the gas chromatograms of biodegraded crude oils are often dominated by an UCM (Figure 1.1a). Hydrocarbon UCMs are also a common feature of a number of refined petroleum products, in particular the middle and heavy distillate fractions. The UCM becomes enriched in these fractions as a result of post distillation refining processes. For example, hydrocarbon base stocks of lubricating oils consist almost entirely of UCMs (Figure 1.1c), with very few resolved components. The latter are removed by solvent extraction (unsaturated and aromatic compounds) or by 'dewaxing' (*n*-alkanes; Wauquier, 1995).

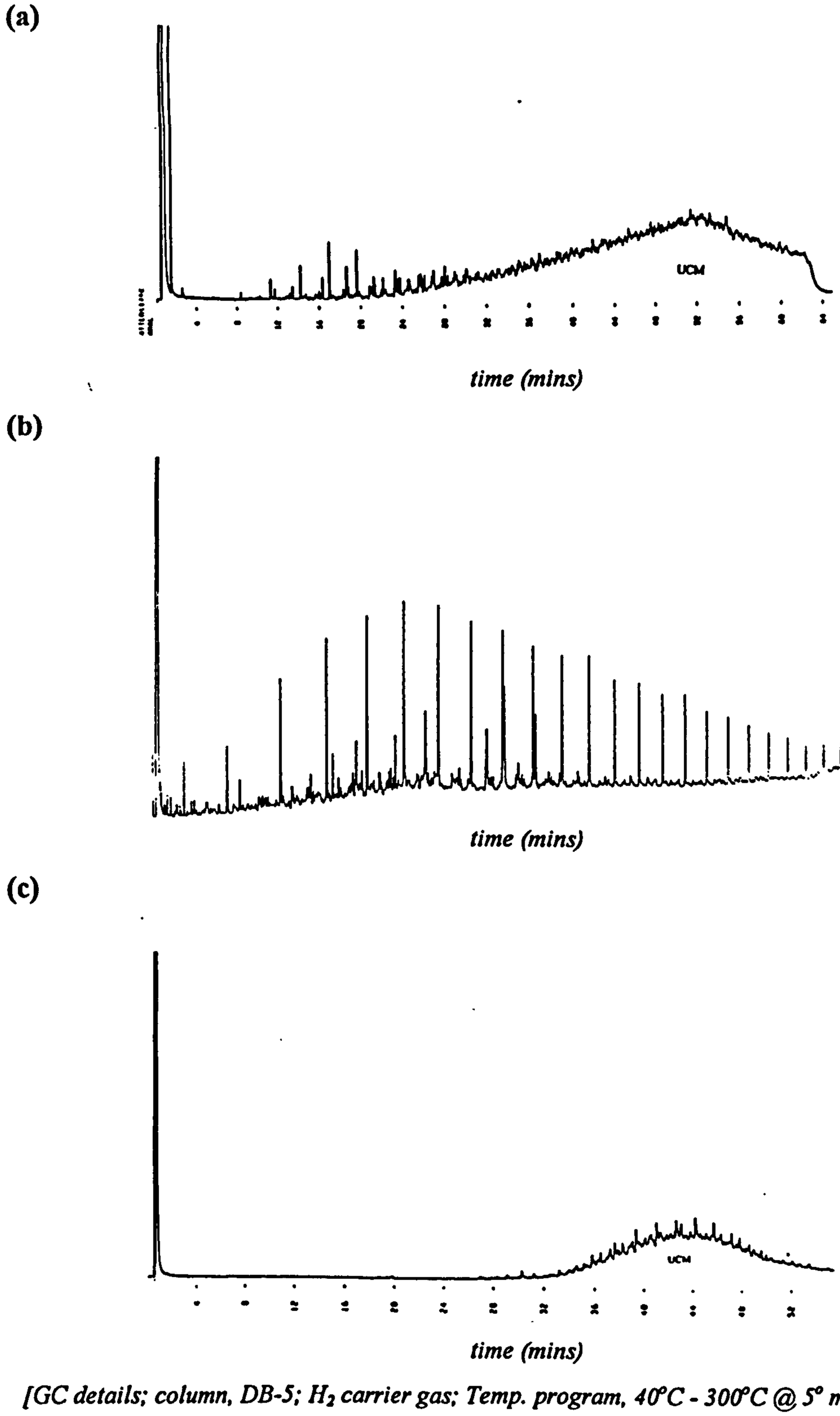


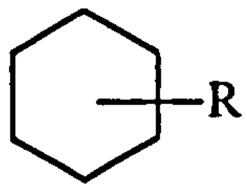
Figure 1.1 Gas chromatograms of the aliphatic fraction of (a) a biodegraded crude oil (Tia Juana Pesado); (b) fresh crude oil (Forties) and (c) lubricating oil (Silkolene 150 lubricating base oil)

1.2 Compositional studies of hydrocarbon UCMs

It is only since the 1950s that the compositions of hydrocarbon UCMs have been investigated in any detail. Although the exact structural composition of any one hydrocarbon UCM has not been elucidated, a number of studies have attempted to identify the structural components of hydrocarbon UCMs and some have proposed model structures (*e.g.* Rossini *et al.*, 1953; Gough and Rowland, 1990,1991; Killops and Al-Juboori, 1990; Revill, 1992; Thomas, 1995; Revill *et al.*, 1997; Thomas *et al.*, 1997). These studies have recently been comprehensively reviewed (Revill, 1992; Thomas, 1995) and therefore the present discussion is but a brief summary.

Lubricating oil hydrocarbons are mainly unresolved by GC and, consequently, have become the most widely studied of UCMs. Early studies (*e.g.* Rossini *et al.*, 1953) estimated the 'average' molecular composition of heavy petroleum fuels by determination of the elemental composition and physical properties of the lubricant fraction of Ponca City crude oil. By comparison of the data with that of authentic hydrocarbons, alkylcycloalkanes (Figure 1.2, I) were proposed as the major hydrocarbon type in the aliphatic fraction, whilst naphthenoaromatic compounds (Figure 1.2, II) were identified in the aromatic fraction. This was later confirmed by mass spectrometry (MS) (Clerc *et al.*, 1955). By combining mass spectral data with nuclear magnetic resonance (NMR) spectroscopy and infra-red (IR) analyses, Hood *et al.* (1959) proposed acyclic and monocyclic structures (Figure 1.2, III-VI respectively) for the aliphatic fractions of a lubricating oil.

Since elemental analysis and spectroscopic studies provided only limited compositional information, a number of recent studies (Gough, 1989; Gough and Rowland, 1990; Killops and Al-Juboori, 1990; Revill, 1992; Thomas, 1995; Revill *et al.* 1997; Thomas *et*



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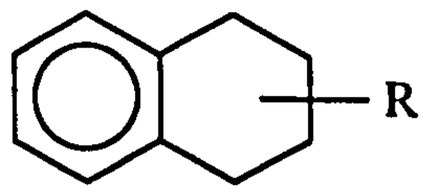

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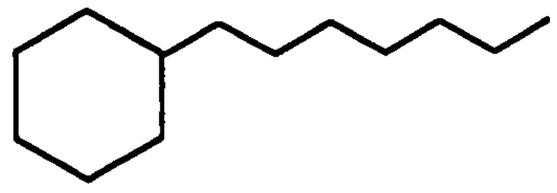

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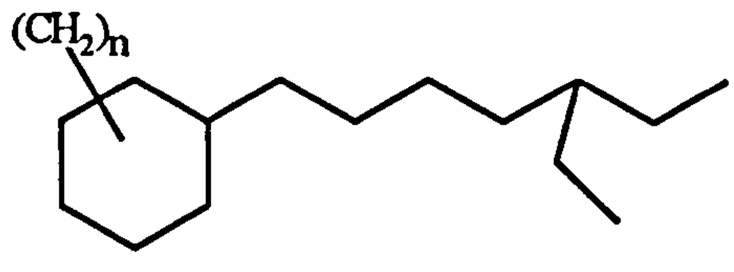

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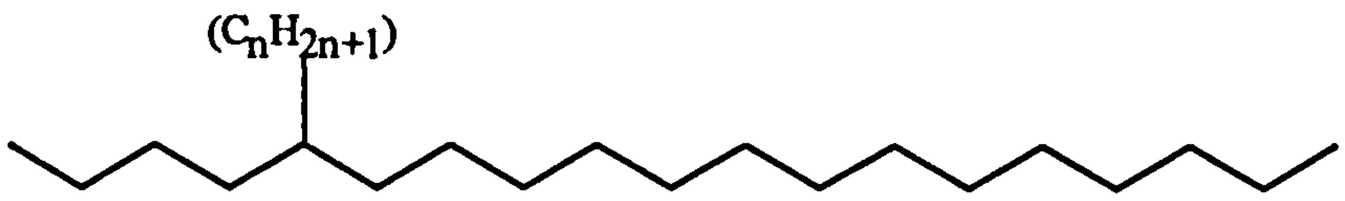
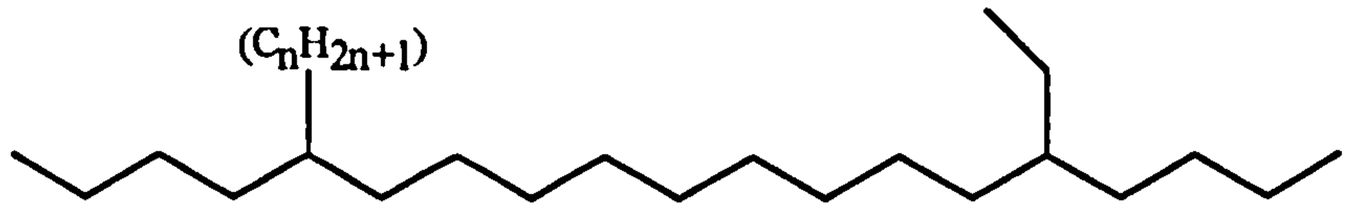


(V)

(n = 2 or 3)



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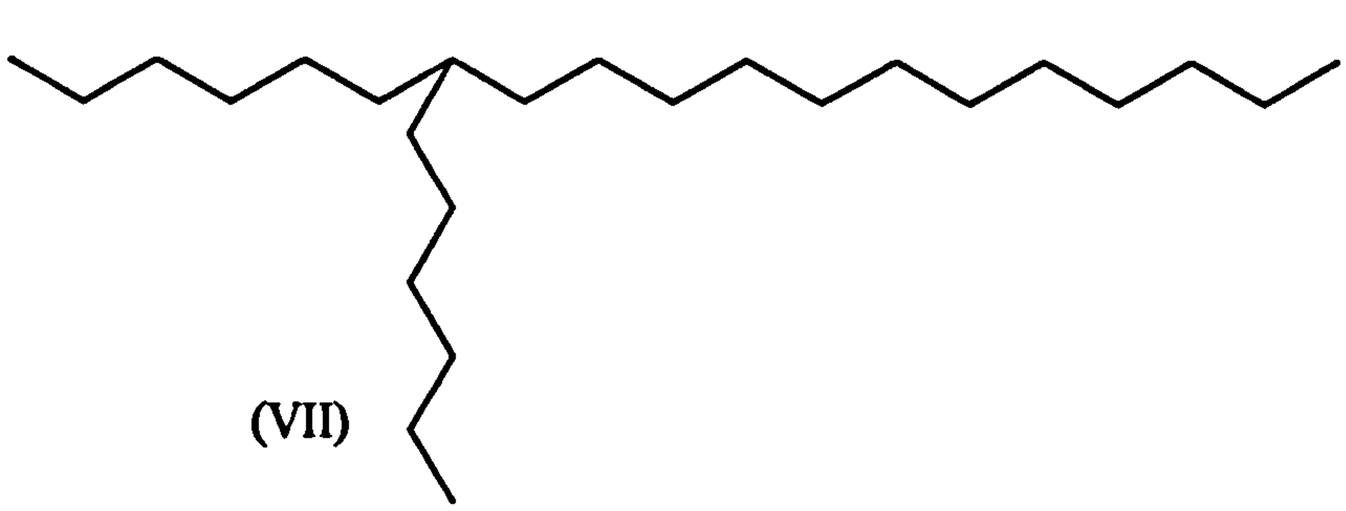

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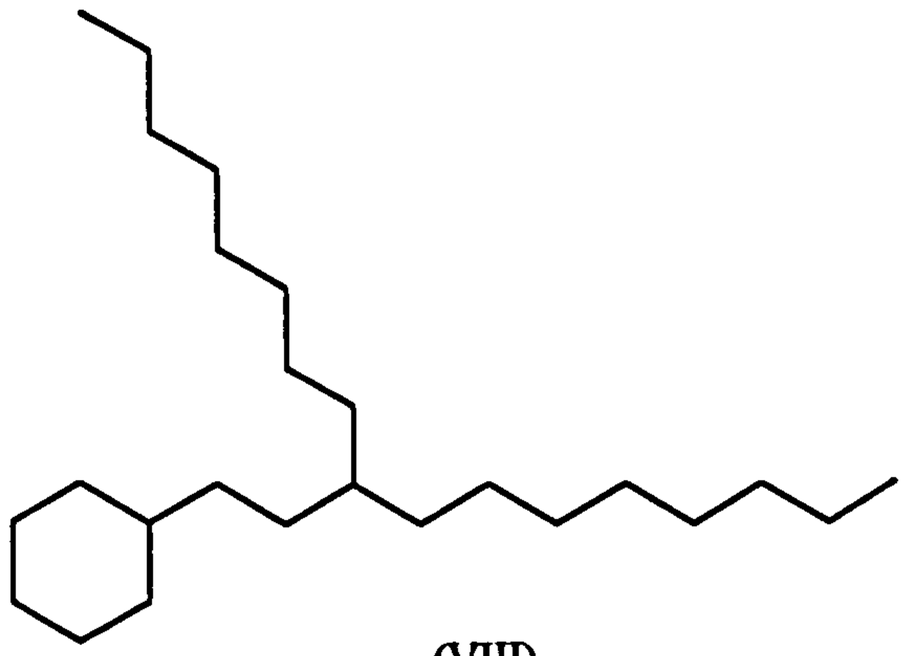


(VIII)

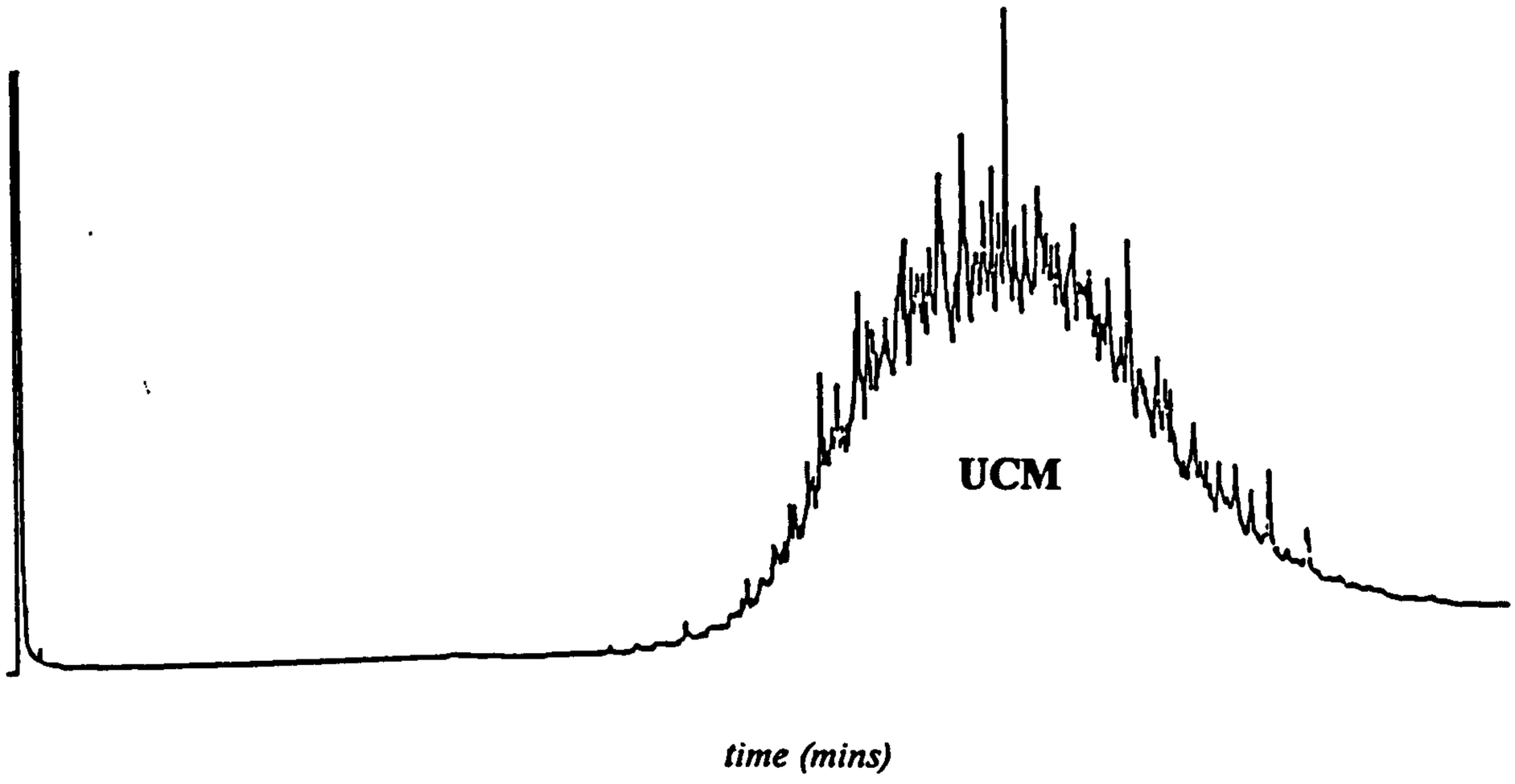
Figure 1.2 Proposed model aliphatic UCM compounds (Hood *et al.*, 1959; Gough and Rowland, 1990, 1991)

al. 1997) have used oxidative degradation techniques in conjunction with bulk analytical techniques to characterise hydrocarbon UCMs isolated from lubricating oils and biodegraded crude oils.

Gough and Rowland (1990) oxidised aliphatic UCM hydrocarbons isolated from a biodegraded crude oil and the hydrocarbon base stocks of two lubricating oils with a chromium trioxide/glacial acetic acid (CrO_3/HAc) mixture. The oxidation products contained a greater proportion (*ca* 20 %) of resolved functionalised components than the unoxidised aliphatic UCM, as illustrated in Figure 1.3. The oxidation products were examined by electron impact (EI-MS) and chemical ionisation (CI-MS) mass spectrometry and the principal oxidation products identified as a homologous series of straight chain mono-carboxylic acids, together with smaller amounts of α,ω-dicarboxylic acids, γ-lactones and ketones (Gough and Rowland, 1990). The relatively simple structures of the oxidation products and the absence of polymethyl and cyclic carboxylic acids was somewhat surprising, considering that most previous workers had assumed aliphatic UCMs to be mainly composed of highly branched and/or cyclic hydrocarbons. However, it should be remembered that whilst the aliphatic UCM hydrocarbons were almost completely oxidised, about 80 % of the oxidation products were still unresolved and unidentified.

The mechanism of the CrO_3/HAc oxidation (reviewed by Gough, 1989) involves attack by CrO_3 (probably as the acid chromate) of branched hydrocarbons at the most substituted carbon position, for example a tertiary carbon, to produce the corresponding tertiary alcohol thus;

(a)



(b)

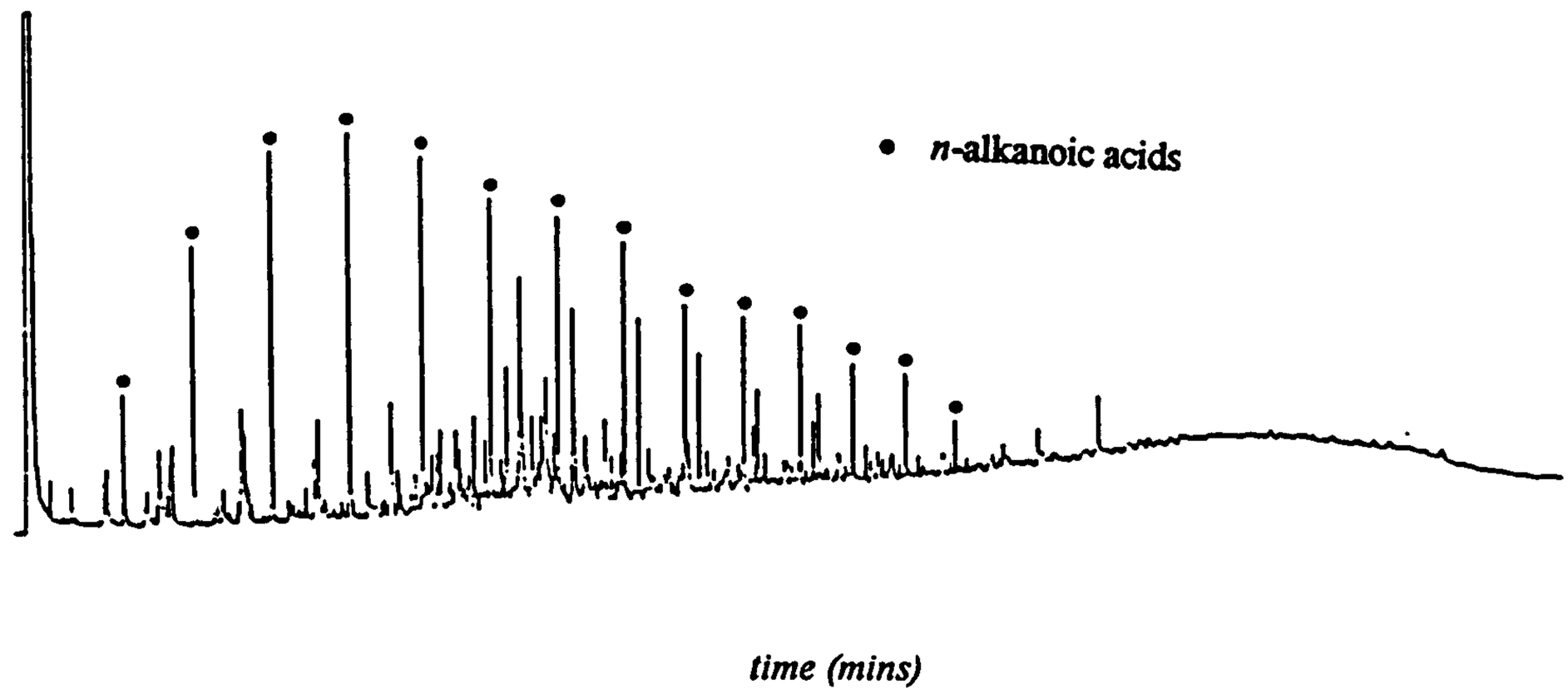
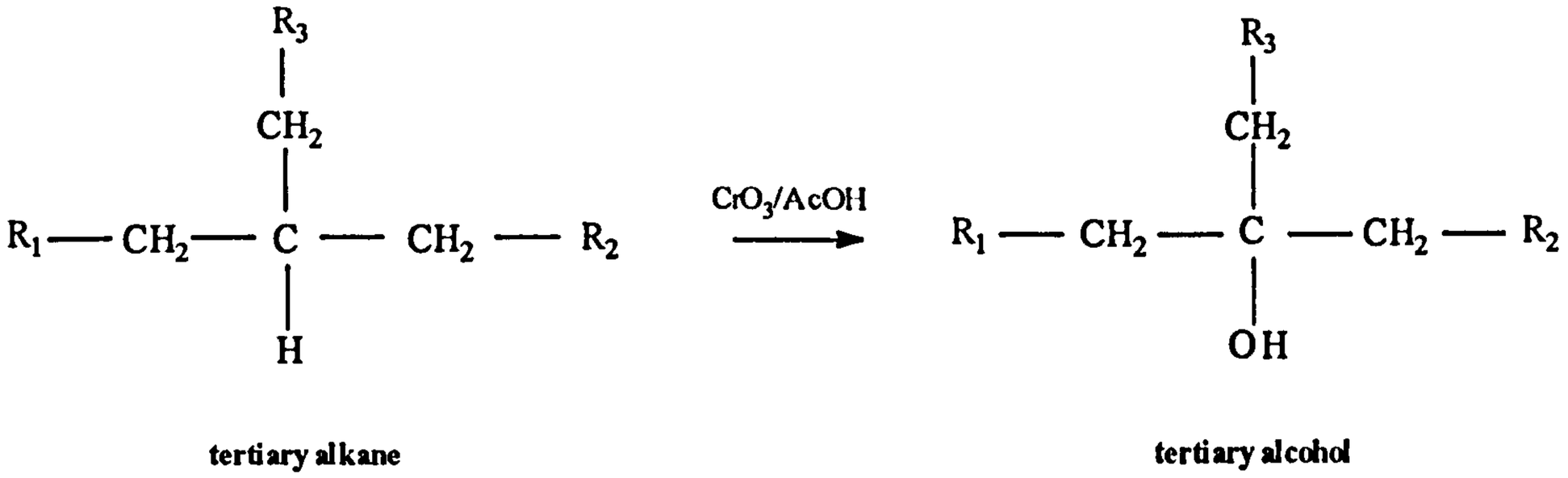


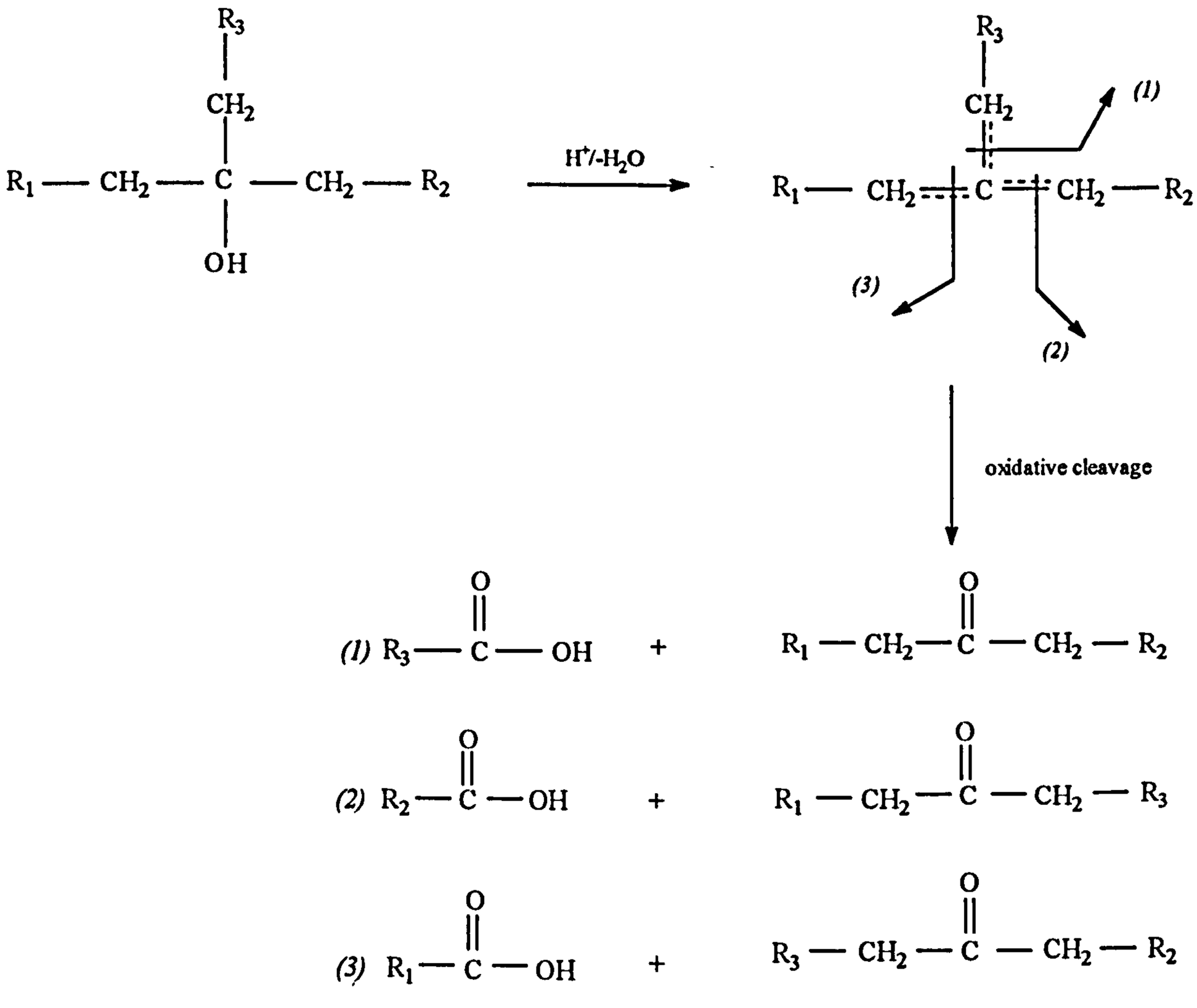
Figure 1.3 Gas chromatograms of (a) the aliphatic UCM before oxidation and (b) the aliphatic UCM oxidation products (Gough, 1989)

[GC details; column, DB-5; H_2 carrier gas; Temp. program, 50°C - 300°C @ 5° min^{-1} hold 20 min]



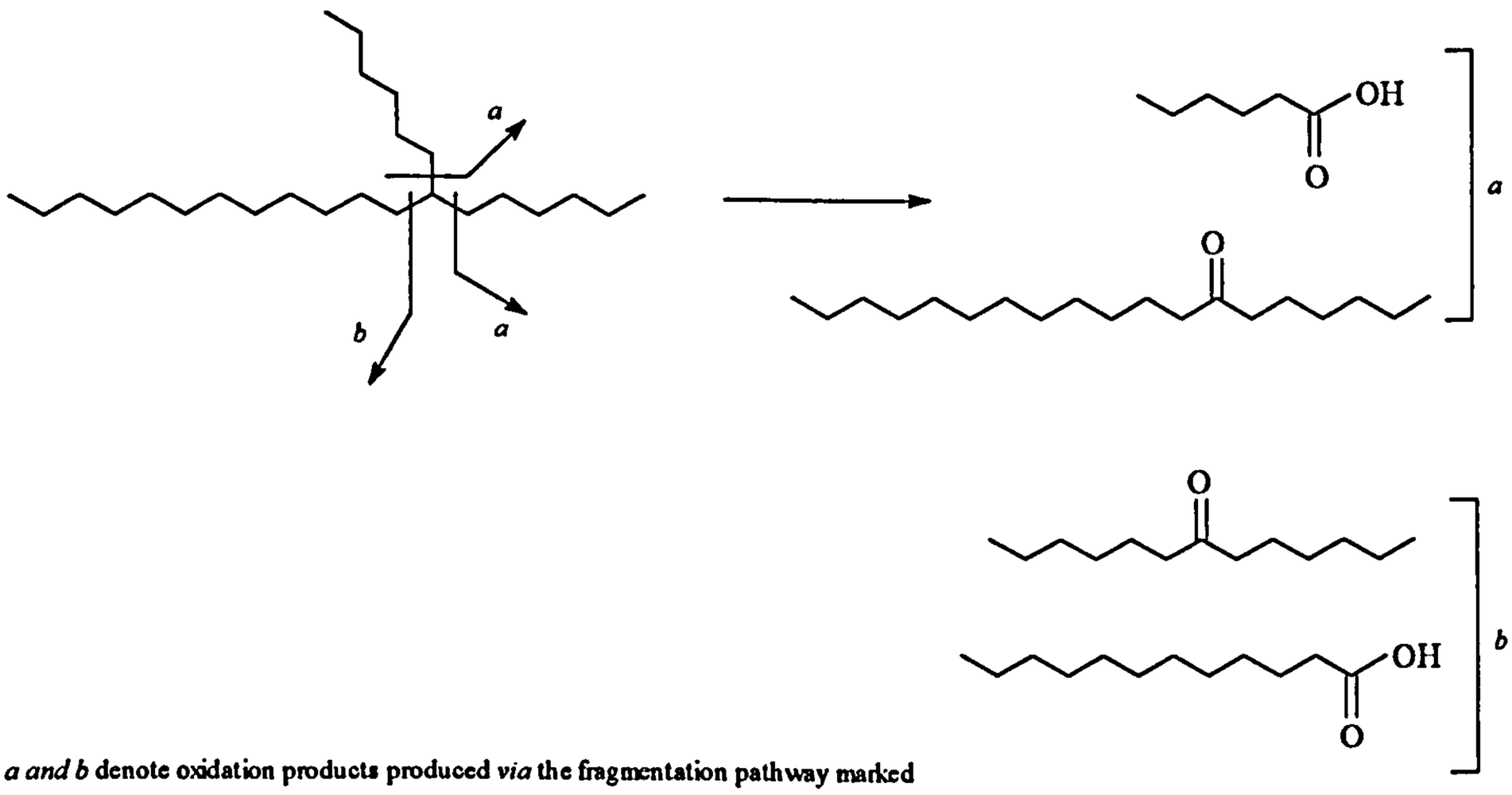
tertiary alkane **tertiary alcohol**

In acidic conditions the alcohol undergoes acid-catalysed dehydration to form a mixture of alkenes. The resulting alkenes are then subjected to further oxidation, resulting in formation of a number of products, usually acids and ketones, depending upon the degree of alkyl substitution (Gough, 1989).



oxidation products

From a consideration of the mechanism of oxidation and the UCM oxidation products, Gough and Rowland (1990,1991) proposed a number of aliphatic UCM compounds. Since straight chain monocarboxylic acids were identified as the principal resolved oxidation products, Gough and Rowland (1990, 1991) proposed that these resulted from oxidation at the tertiary centre of relatively simple monoalkyl branched acylic (or possibly monocyclic) alkanes (*e.g.* Figure 1.2, VII and VIII). Further evidence to support the theory that some aliphatic UCMs are comprised, at least in part, of relatively simple monoalkyl cyclic and acyclic compounds was provided by oxidation of a number of the proposed model UCM compounds. Gough (1989) synthesised, amongst other compounds, a C_{25} 'T-branched alkane, 7-*n*-hexylnonadecane (Figure 1.2, VII). Oxidation with CrO_3/HAc yielded dodecanoic acid, nonadecan-7-one and tridecanone, each of which is a predicted product from oxidative cleavage adjacent to the tertiary centre (Figure 1.4), thereby confirming that the UCM was comprised, in part, of simple monoalkyl compounds such as 7-*n*-hexylnonadecane.



a and b denote oxidation products produced *via* the fragmentation pathway marked

Figure 1.4 Predicted oxidation products of 7-*n*-hexylnonadecane oxidised with CrO_3/HAc

Gough (1989) postulated that the lack of cyclic moieties in the UCM oxidation products of naphthenic oils may be attributable to oxidation at ring-alkyl chain junctions, resulting in ring opening. Oxidation of a synthetic model cyclic UCM component (9-(1-cyclohexylethyl)-heptadecane (Figure 1.2, VIII) yielded only the predicted *n*-octanoic acid as the major product, suggesting that ring opening did occur (Gough, 1989).

Since UCMs are known to be relatively resistant to biodegradation (hence their abundance in biodegraded crude oils), Gough *et al.* (1992) compared the rate and extent of biodegradation of their synthesised model compounds (*e.g.* Figure 1.2, VII and VIII) relative to that of an UCM isolated from a lubricating oil. The degree of resistance to biodegradation of these structurally simple model compounds was greater than straight chain and monomethyl branched compounds, and importantly, was comparable with the rate of degradation of the UCM, suggesting that such compounds are indeed reasonable models for some UCM components.

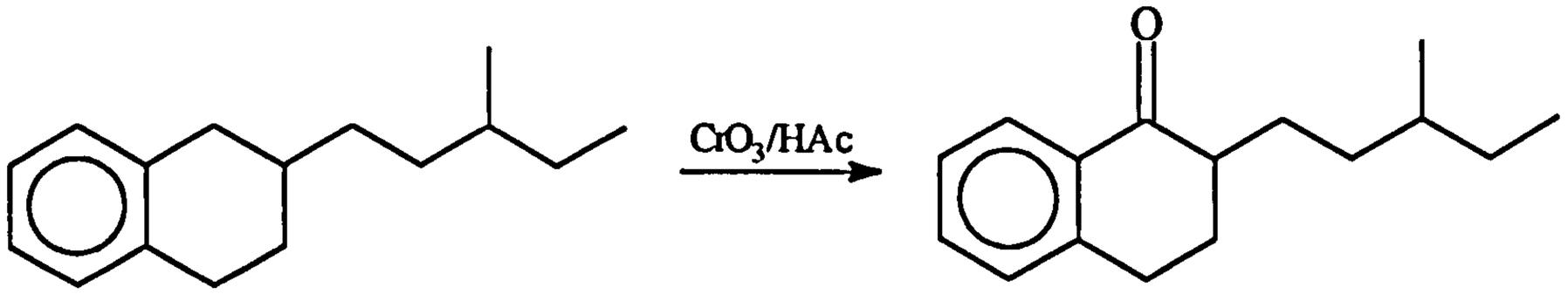
Killops and Al-Juboori (1990) used a combination of ^{1}H and ^{13}C NMR, FT-IR, UV, elemental analysis, EI-MS, CI-MS and chromic acid oxidation to characterise the total hydrocarbon fraction of a heavily biodegraded crude oil. These authors proposed that in this oil, in agreement with characterisation studies of other oils, the UCM was principally acyclic in nature but with cycloalkanes also making a significant contribution to the UCM. Similar precursor compounds to those postulated by Gough and Rowland (1990,1991) were suggested as model UCM components.

In an attempt to reduce the structural diversity of an UCM prior to oxidation studies, Thomas (1995) fractionally distilled an UCM isolated from a lubricating oil hydrocarbon base stock into smaller UCMs. Only a slight increase in GC resolution of the distillate

fractions was observed compared with the undistilled oil, and oxidative degradation (CrO_3/HAc) of each of the distillate fractions yielded similar distributions of resolved oxidation products to those obtained from the unfractionated UCM. This led Thomas (1995) to propose that the aliphatic UCM of this oil was a fairly homogenous mixture of branched monoalkyl acyclic and monocyclic alkanes. This author concluded that characterisation of the remaining unresolved oxidation products was required before any further conclusions could be drawn.

Most hydrocarbon UCMs have been shown to be highly aliphatic in nature, and even the aromatic hydrocarbons are thought to be present mainly as alkyl substituted benzenes and naphthenoaromatic compounds (Rossini *et al.*, 1953; Gough, 1989; Killops and Al-Juboori, 1990; Revill *et al.*, 1997; Thomas *et al.*, 1997). A few model aromatic UCM structures have been proposed in recent years from use of similar spectroscopic and degradative techniques to those applied to the characterisation of aliphatic UCMs (Gough, 1989; Revill *et al.*, 1997; Thomas *et al.*, 1997).

Gough (1989) oxidised the aromatic UCM isolated from a lubricating oil with CrO_3/HAc. In contrast to the oxidation of branched alkanes, which are attacked preferentially at tertiary carbons, the oxidation of alkylaromatics with CrO_3/HAc occurs almost exclusively at the benzylic position thus;

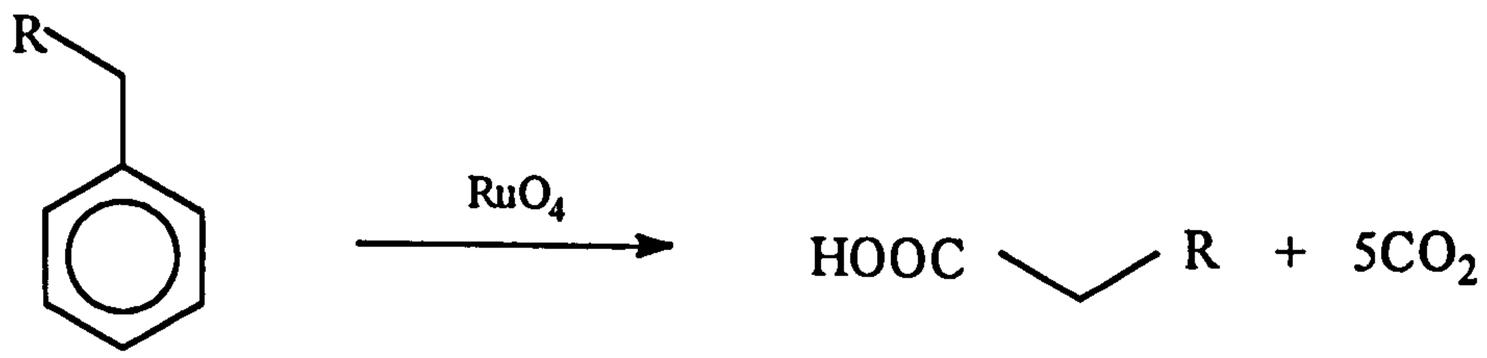


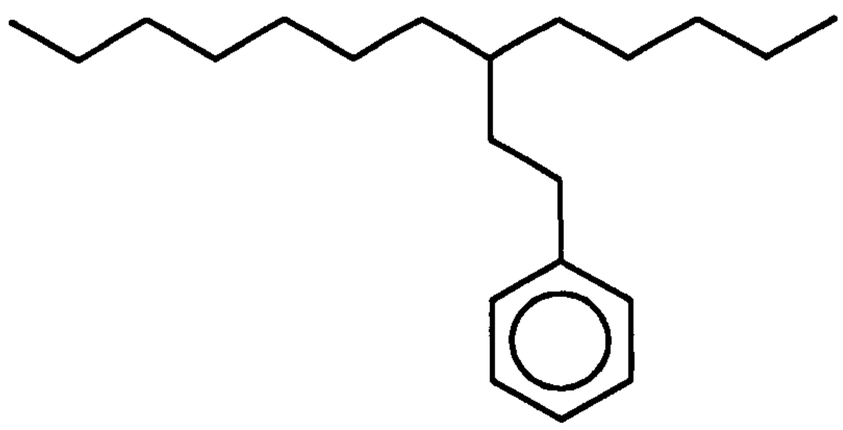
The principal aromatic oxidation products of the oxidised aromatic UCM were identified as unsubstituted or methyl substituted benzoic acids or monoaromatic ketones. In addition, *n*-monocarboxylic acids were identified as the principal aliphatic resolved

oxidation products. Combining these data with those from instrumental analyses such as EI-MS, gel permeation chromatography (GPC) and field ionisation mass spectrometry (FI-MS), Gough (1989) proposed alkyl substituted monoaromatic structures as one constituent of the aromatic UCM (Fig, 1.5, (I)).

Revill *et al.* (1997) investigated the aromatic fraction of a biodegraded crude oil (Tia Juana Pesado, TJP) from which most of the resolved components had been removed by natural bacterial action. These authors conducted a number of preliminary studies using non-oxidative techniques followed by oxidation with CrO_3/HAc or ruthenium tetroxide (RuO_4).

The highly aliphatic nature of the aromatic UCM of TJP was highlighted by IR spectroscopy, with absorptions due to CH_2 and CH_3 dominating the spectrum, whilst aromatic absorptions were weak (Revill *et al.*, 1997). The authors proposed that *ortho*-disubstituted benzenoid structures similar to tetralin may be of importance, as indicated by the presence of a strong absorption at *ca* 730 cm^{-1}. Chromium oxide (CrO_3/HAc) oxidation of the aromatic UCM of TJP (Revill *et al.*, 1997) produced similar results to those of Gough (1989) for the aromatic UCM of a lubricating oil. Ruthenium tetroxide is known to attack substituted aromatic hydrocarbons at the *ipso*-carbons of the benzene ring, preserving any alkyl substituents as aliphatic carboxylic acids and oxidising the aromatic ring to carbon dioxide, *e.g.* ;





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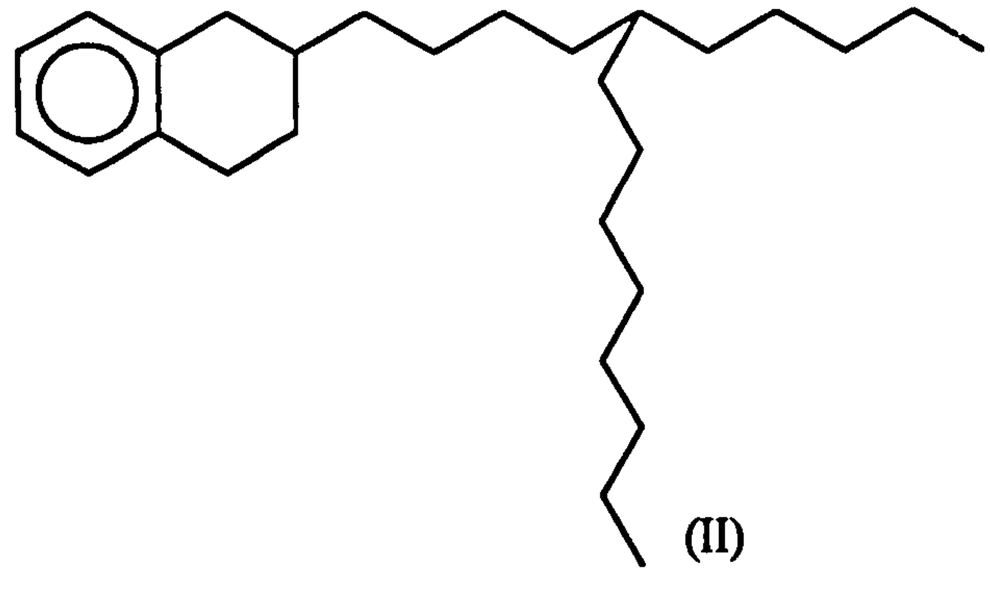

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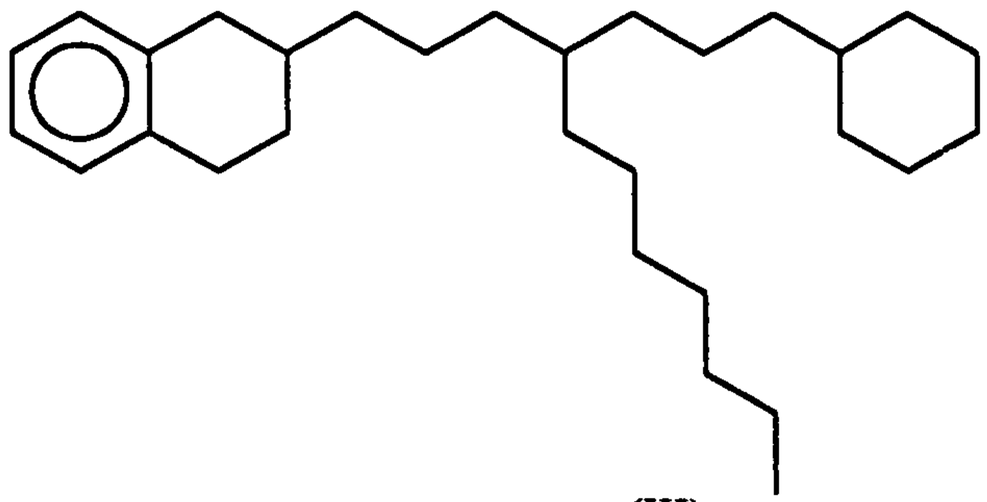

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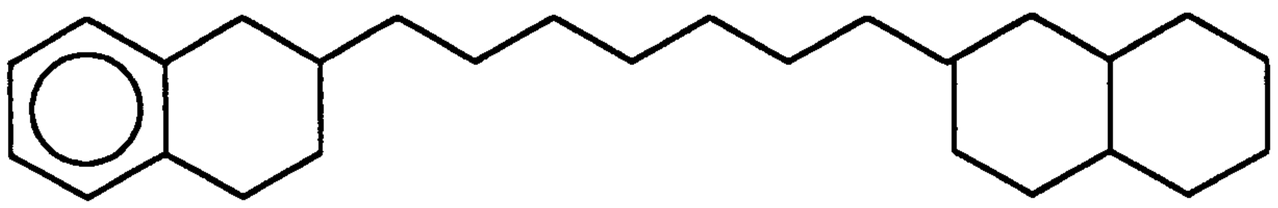

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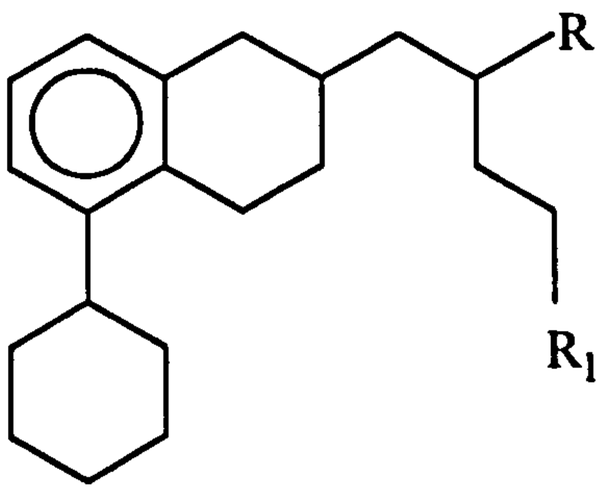
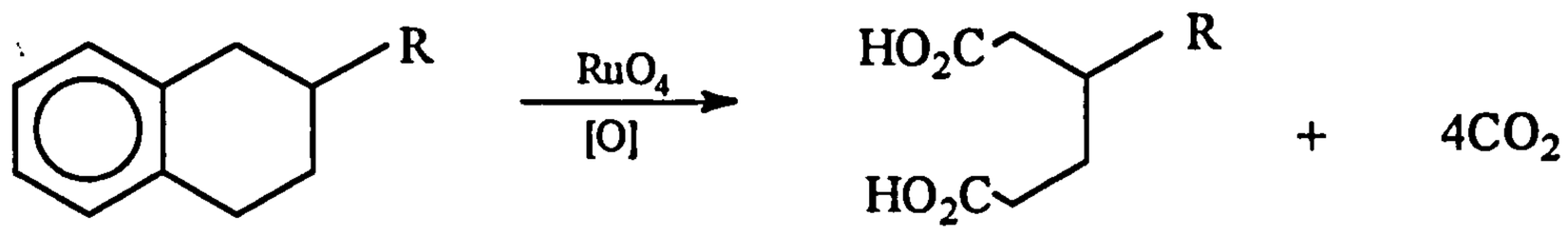


(V)

Figure 1.5 Proposed model aromatic UCM structures (Gough, 1989; Revill, 1992; Thomas, 1995; Revill *et al.*, 1997; Thomas *et al.*, 1997)

Revill *et al.* (1997) noted the presence of a significant UCM in the RuO_4 oxidation products and attributed this to the presence of branched alkyl substituents present as acids. Fractionation of the oxidation products and subsequent analysis of the acidic fraction by EI-MS and ^1H NMR suggested the presence of dicarboxylic acids within the RuO_4 oxidised UCM (Revill *et al.*, 1997). Such compounds would be produced from oxidation at the *ipso*-carbons of tetralin type compounds, as illustrated below;



providing evidence for the presence of naphthenoaromatic compounds within the UCM, rather than alkylated benzenes. Mass spectrometry of the oxidised UCM also provided strong evidence for the presence of alicyclic moieties. Combining the spectroscopic and chemical oxidation data, Revill *et al.* (1997) proposed that naphthenoaromatic compounds such as those illustrated in Figure 1.5 (II, III and IV) are important constituents of the aromatic UCM of TJP crude oil.

Thomas *et al.* (1997) applied RuO_4 oxidation, with an attempt to mass balance the oxidation products, to a study of a number of authentic aromatic compounds, to a suite of aromatic UCM distillates and to a number of unresolved aromatic refinery fractions. Although all the steps of the mechanism of RuO_4 oxidation are not fully understood, as mentioned previously, alkyl benzenes are known to be attacked preferentially at the *ipso*-carbon of the aromatic ring, and the alkyl substituents are preserved as aliphatic carboxylic acids. The aromatic ring is oxidised to carbon dioxide and the degree of aromaticity can be inferred from amount of carbon dioxide evolved. These authors coined the term '*retro*-structural analysis' for the process of reconstructing the precursor molecule from analysis of the oxidised products. A summary of the reconstruction of

precursor molecules by *retro*-structural analysis is presented in Fig 1.6. Using this approach, Thomas *et al.* (1997) concluded that alkyl hydroaromatic groups and/or bridging groups were prevalent within the UCMs studied. This is also consistent with some of the structures proposed by Revill *et al.* (1997; Figure 1.5 herein). In addition, Thomas *et al.* (1997) also proposed that biphenyls and alkylphenylcyclohexanes were important structural moieties.

A possibly significant advance in the characterisation of oil hydrocarbon UCMs was made by Thomas (1995) who used Fourier transform-ion cyclotron resonance spectroscopy (FT-ICR) to examine the total oxidation products, including those unresolved by GC (the so called UCM_{ox}). Quantitatively, the UCM_{ox} comprises the major part (75% - 90%) of the oxidation products from oxidative degradation of most hydrocarbon UCMs (Thomas, 1995). Analysis of the total oxidation products of a number of aromatic refinery fractions led Thomas (1995) to identify, in addition to the GC resolved *n*-mono and dicarboxylic acids, numerous series of cycloalkyl carboxylic acids from the abundant molecular ions created by negative ion CI FT-ICR. The very high resolving power of the mass spectral technique (Hsu *et al.*, 1994) allowed accurate masses to be calculated and effectively replaced the poor GC resolution with high mass spectral resolution. Interpreting the GC, FT-ICR and mass balance data, using the *retro*-structural analysis approach, Thomas (1995) postulated a general model aromatic UCM structure (Figure 1.5, V).

Whilst the components of any one particular UCM cannot be unequivocally characterised, the work described above represents the current state of knowledge concerning the general molecular composition of various hydrocarbon UCMs isolated from a number of crude oils and lubricating oils. For the purposes of the present study, the model compounds proposed by Gough and Rowland (1990, 1991), Revill *et al.*

(1997) and Thomas *et al.* (1997; Figures 1.2 and 1.5 herein) are accepted as the best currently available data and provide, at the least, some indication of hydrocarbon UCM components on which to base the analytical and toxicological studies discussed in this thesis.

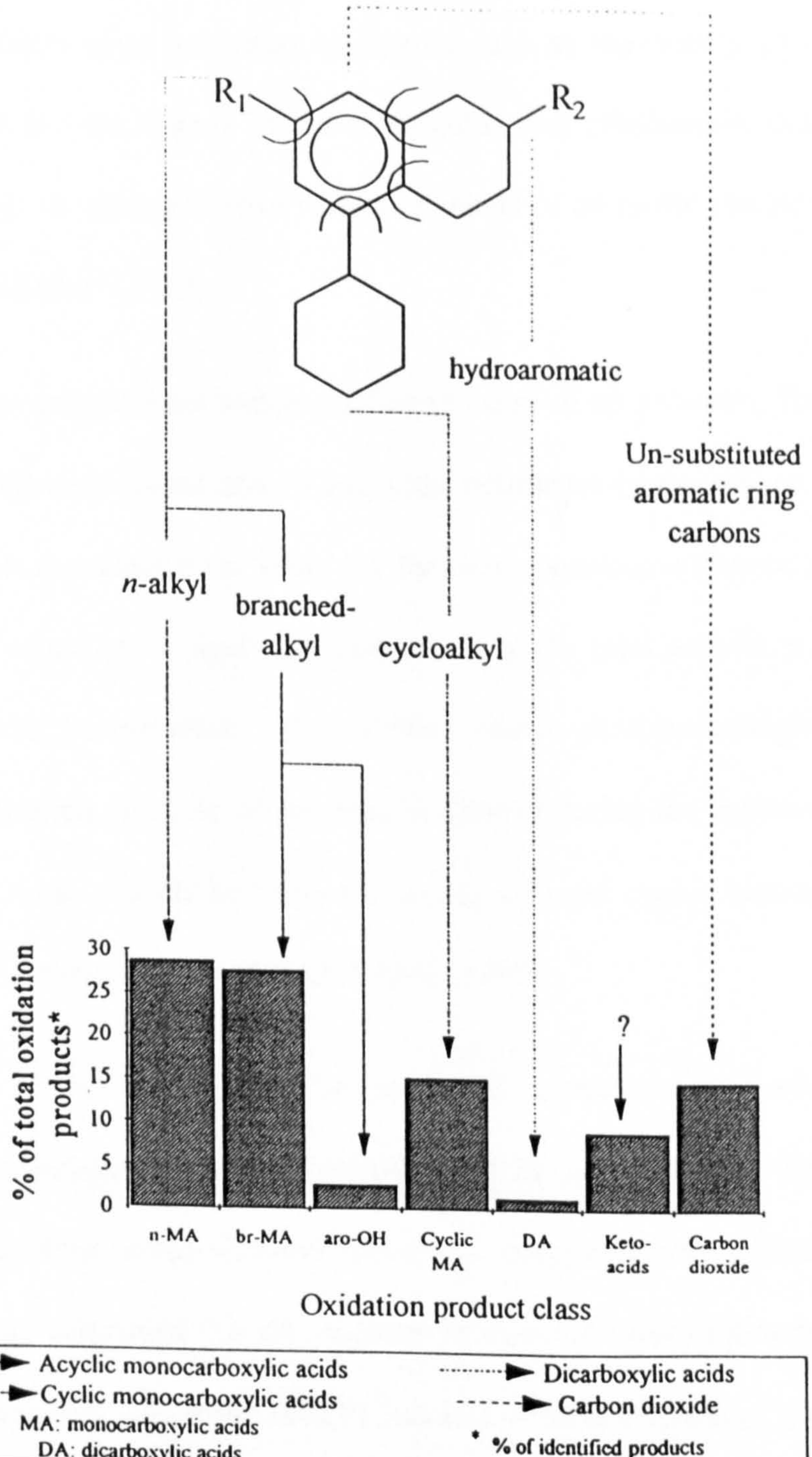


Figure 1.6 *Retro*-structural analysis of the UCM of a mono-aromatic hydrocarbon fraction isolated from a heavy cycle oil by HPLC (method IP 391/90; Thomas, 1995)

1.3 Sources and environmental occurrences of UCM hydrocarbons in the marine environment

The annual contributions of the principal inputs of petroleum to the oceans are summarised in Table 1.1 (Clark, 1992). The relative importance of each input varies in different geographical areas according to factors such as the density of shipping and offshore activities and the degree of industrialisation and urbanisation in coastal areas (GESAMP, 1993). A comprehensive review of inputs of oil to the marine environment is provided by GESAMP (1993).

Tanker disasters are the most widely publicised cause of oil pollution. However, such incidents contribute only about 5% of the total petroleum hydrocarbons entering the oceans annually. It is evident from Table 1.1 that less conspicuous chronic sources such as urban run-off contribute a significant proportion of the total petroleum hydrocarbon inputs to the marine environment. In particular, engine oil is increasingly becoming a contaminant of concern, because of the large volumes entering the aquatic environment through sewers, urban run-off and direct dumping of used engine lubricating oil into municipal drains (Latimer *et al.*, 1990; GESAMP, 1993).

Latimer *et al.* (1990) investigated the sources of petroleum hydrocarbons in urban run-off and demonstrated that the hydrocarbons at four different land use sites (commercial, residential, motorway and industrial) originated mainly from used engine oil. These authors concluded that the majority of engine oil had originated from either oil drops from vehicles within the driving lanes/road surfaces/parking areas, or direct dumping of waste engine oil into storm drains (Latimer *et al.*, 1990). Likewise, Bomboi and Hernandez (1991) identified engine lubricating oils as the primary source of hydrocarbons in urban run-off. UCM concentrations ranging from 450 - 1926 $\mu g\ l^{-1}$

were reported in the hydrocarbon fractions extracted from urban run-off samples, contributing 80 % or more of the total aliphatic hydrocarbon burden and demonstrating that lube oils are sometimes a significant source of UCM hydrocarbons to the marine environment.

Source		Total
Transportation		
Tanker operations	0.158	
Tanker accidents	0.121	
Bilge and fuel oil	0.252	
Dry docking	0.004	
Non-tanker accidents	0.020	
		0.555
Fixed installations		
Coastal refineries	0.10	
Offshore production	0.05	
Marine terminals	0.03	
		0.180
Other sources		
Municipal wastes	0.70	
Industrial waste	0.20	
Urban run-off	0.12	
River run-off	0.04	
Atmospheric fall-out	0.30	
Ocean dumping	0.02	
		1.380
Natural inputs		0.250
Total		2.365

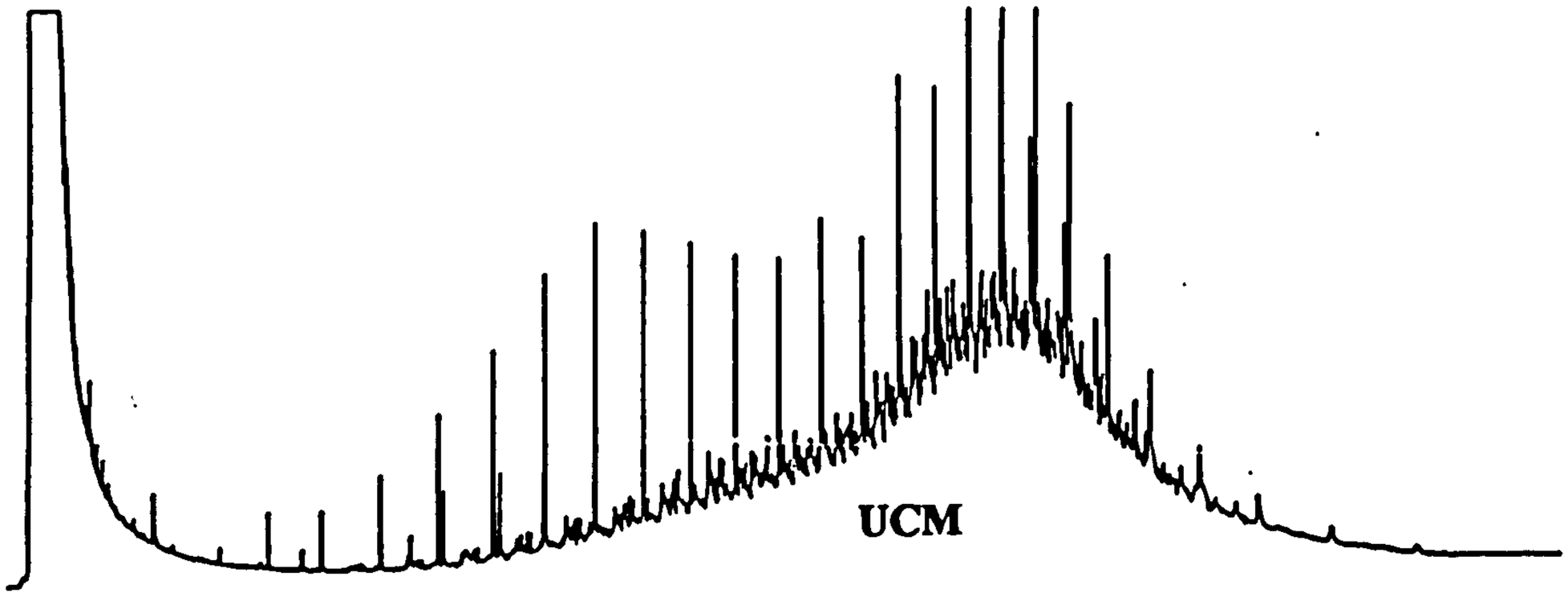
Table 1.1 Estimated world input of petroleum hydrocarbons to the oceans (10^6 t yr^{-1}; Adapted from Clark, 1992)

Unusually, Bomboi and Hernandez (1991) also reported the presence of an aromatic UCM in urban run-off. Although only the resolved polycyclic aromatic hydrocarbons in the aromatic fraction were measured, it is evident from visual inspection of the chromatogram (Figure 1.7) that the aromatic UCM constitutes a significant proportion of the total aromatic hydrocarbons present. However, aromatic UCMs are very rarely measured in sediments and biota and, given that urban run-off is perhaps the principal source of petroleum hydrocarbons to the marine environment, the aromatic UCM may be an environmental contaminant of concern, which at present is overlooked. To date however, the toxicological impact of UCMs of 'aromatic' hydrocarbons remains unknown. The production and environmental fate of used lubricating oil have been reviewed in detail by Vazquez-Duhalt (1989) and GESAMP (1993).

Owing to the relatively inert nature of UCM hydrocarbons, the occurrence of hydrocarbon UCMs in marine sediments and organisms in areas impacted by petroleum hydrocarbons is widely reported and well documented (*e.g.* Fossato and Siviero, 1974; Thompson and Eglinton, 1978; Risebrough *et al.*, 1983; Shaw *et al.*, 1986; Mason, 1988; Aboul-Kassim and Simoneit, 1995; Macias-Zamora, 1996). Indeed, the presence of an UCM in the aliphatic fraction of a sediment or biota extract is widely accepted as a reliable indication of fossil fuel contamination.

Examples of the typical range of UCM hydrocarbon concentrations reported in the literature are presented in Table 1.2. In relatively enclosed areas receiving significant petroleum hydrocarbon inputs (*e.g.* harbours), concentrations of aliphatic UCM

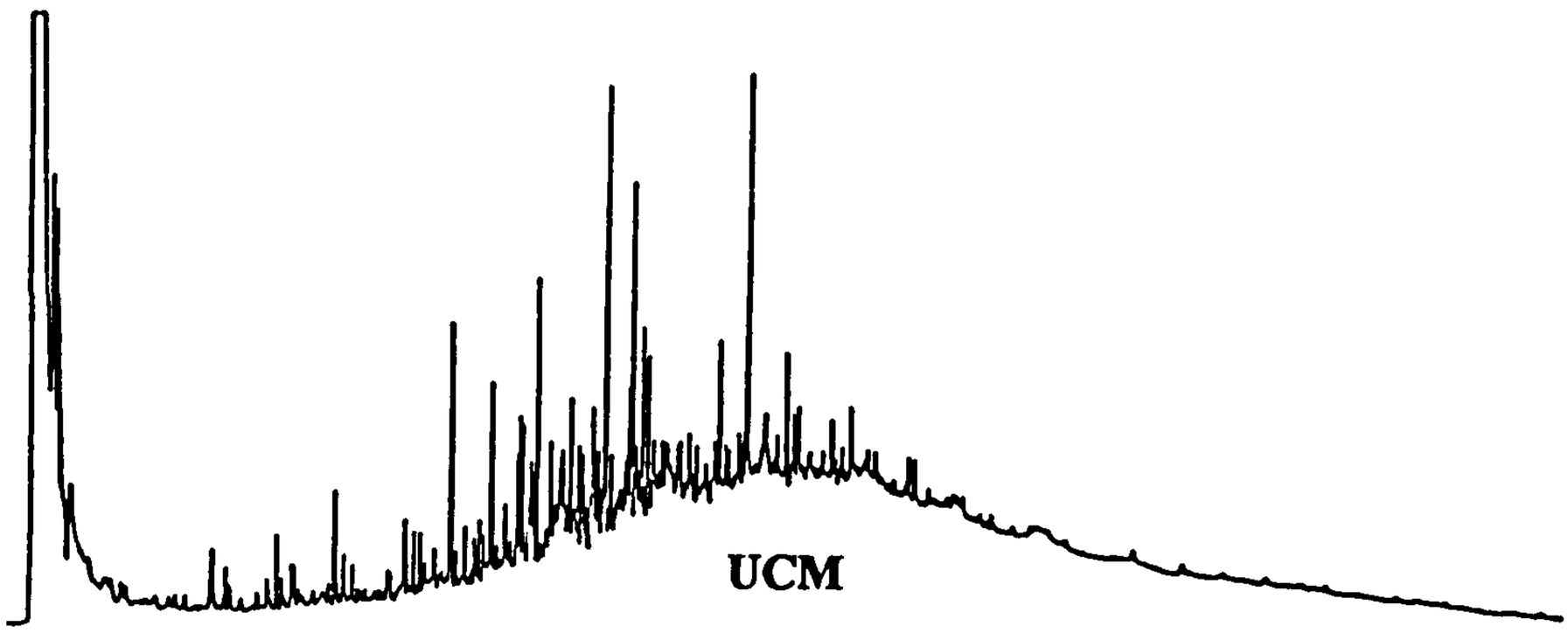
(a)



time (mins)

[GC details; columns,SE-30; Temp program, 50° 1 min, 50° -100°C @ 10° min^{-1}, 100 - 290°C @ 3.5° min^{-1}]

(b)



time (mins)

[GC details; column,SE-54; Temp. program 100°C 1min, 100° - 290°C @ 3.5 min^{-1}]

Figure 1.7 Gas chromatograms of (a) aliphatic hydrocarbons in urban run-off and (b) aromatic hydrocarbons in urban run-off sampled from Principle de Vergara, Madrid, Spain (Bomboi and Hernandez, 1991)

Aliphatic UCM conc. ($\mu g\ g^{-1}$)	*Aromatic UCM conc. ($\mu g\ g^{-1}$)*	*Sample details*	*Reference*
6 - 33	5 - 23	Mussels, legs of an oil production platform	Soler *et al.* (1989)
18 - 740	0.4 - 66	Mussels, Ebro Delta. Spain	Risebrough *et al.* (1983)
10 - 104	-	Sediment from Mersey, Dee & Tamar estuaries , U.K	Readman *et al.* (1986)
< 1 - 898	-	Mussels, Port Valdez. Alaska	Shaw *et al.* (1986)
5 - 21	-	Fish, W. Mediterranean	Albaiges *et al.* (1987)
4 - 390	-	Mussels, California Coast	Risebrough *et al.* (1990)
76 - 275	-	Sediment, Rhone Delta	Lipiatou & Saliot (1991)
4 - 24	-	Sediment, Gulf of Lions, Mediterranean	
2 - 99	-	Mussels, Catalan Coast, .Mediterranean.	Porte & Albaiges (1993)
0.6 - 3	-	Sediment, Mina al Fahal	Badawy *et al.* (1993)
3 - 220	-	Sediment, Baja California Coast	Macias-Zamora (1996)
0.2 - 7	-	Sediment, Montevideo. Uraguay	Moyano *et al.* (1993)
26 - 1792	-	Sediment, Mangroves. Puerto Rico	Klekowski *et al.* (1994)
4317	7172	Mussels, entrance to Cape Town Harbour.	Mason (1988)
1950	489	Mussels, sewage outlet, West coast, South Africa	
380 - 13 832	-	Sediment, Eastern Harbour (inside), Alexandria. Egypt	Aboul-Kassim & Simoneit (1995)
193 - 8738	-	Sediment, Eastern Harbour (outside)	

N.B All values expressed in terms of dry weight

- not reported

Table 1.2 Examples of environmental occurences of UCM hydrocarbons

hydrocarbons measured in mussels ranged from 100 - 4000 μg g^{-1} dry weight mussel tissue (Mason, 1988), occasionally with levels as high as 13,800 μg g^{-1} (Aboul-Kassim and Simoneit, 1995). Whilst in open coastal areas, levels are slightly lower, ranging in general from below the limit of detection in relatively clean coastal sites (*e.g.* Shaw *et al.*, 1986) to approximately 800 μg g^{-1} in the more industrialised and heavily populated coastal areas (*e.g.* Risebrough *et al.*, 1983).

It is interesting to note that whilst the aliphatic UCM is routinely quantified in monitoring programmes as a measure of petrogenic contamination, the presence of an aromatic UCM is often ignored. The few literature reports of aromatic UCMs in marine sediments and mussels suggest that environmental concentrations of aromatic UCMs are comparable with those of the aliphatic UCM. This is discussed further in Chapter 2.

1.4 The use of marine organisms as a tool for environmental monitoring of hydrocarbon pollutants

Bivalve filter feeding organisms, such as mussels, actively concentrate environmental contaminants, including petroleum hydrocarbons, within their tissues to concentrations significantly greater than ambient environmental levels. Pollutants which are present in the water column at levels of 1 ng l^{-1} or less are therefore significantly amplified within organisms. For the analyst, marine bivalves thus represent a substrate in which it is easier to detect and quantify environmental contaminants, reducing the need for costly and demanding water sampling programmes (Burns and Smith, 1981). Mussels are also dominant members of coastal and estuarine communities and have a wide geographical distribution. These attributes have resulted in the widespread use of mussels as 'sentinel' organisms or 'bioindicators', to assess the spatial and temporal trends in chemical contamination of estuarine and coastal areas. The use of mussels as indicator organisms,

known as the 'Mussel Watch' concept, was first established in North America in the mid 1970s (Goldberg *et al.*, 1978). However, mussels are now widely used throughout the world in environmental monitoring programmes. The Mussel Watch concept has been extensively reviewed in a number of publications (*e.g.* NAS, 1980; Widdows and Donkin, 1991).

Measurement of contaminant levels bioaccumulated within the tissues of marine organisms also provides an indication of the biological availability of a particular compound. By comparison with laboratory derived concentration-response data, the impact of measured pollutant levels can be estimated, providing a more meaningful interpretation of concentrations of environmental contaminants reported. This approach has been applied successfully in a number of studies (*e.g.* Burns *et al.*, 1990; Widdows *et al.*, 1990, 1995a,b). For example, Widdows *et al.* (1995a) used mussels (*Mytilus edulis*) to monitor the degree of chemical contamination (petroleum hydrocarbons and organotins) and the associated sublethal biological effects (in terms of Scope for Growth) in the vicinity of the Sullom Voe oil terminal, Shetland Isles over a seven year period. These authors found that the spatial and temporal changes in the concentration of aromatic hydrocarbons within the mussels reflected the major sources of oil inputs (*i.e.* oil spillages during tanker loading operations). A significant correlation was reported between Scope for Growth and the concentration of polycyclic aromatic hydrocarbons (2 and 3 ring ; PAH) in the mussel tissues. The integration of biological and chemical measurements in environmental assessment studies is discussed in greater detail in Section 1.5

1.5 Responses of marine organisms to petroleum hydrocarbons

The effects of both chronic discharges of petroleum hydrocarbons and accidental releases, *i.e.* tanker disasters, upon the marine environment have been studied with a wide variety of organisms and ecosystems and their biological processes. Comprehensive reviews are available (*e.g.* GESAMP, 1977, 1993; NAS, 1985).

The chemical complexity of crude oils and refined petroleum products combined with the very low water solubility of the majority of components significantly complicates the ecotoxicological assessment of petroleum hydrocarbons. Traditionally, tests have involved exposure of test organisms to a range of concentrations of the water accomodated fraction (WAF) of the test product in order to determine the Lethal Loading (LL_{50}, as oil:water ratio) or Lethal Concentration (LC_{50}) which results in 50 % mortality of the organisms (*e.g.* Peterson, 1994). A comprehensive review of this test procedure is given by Betton (1994).

Water accommodated fractions are essentially the fraction of the total products that are present in the aqueous phase following a period of mixing (Betton, 1994). The toxicity of crude oils and petroleum products is generally attributed to this fraction. Theoretically, within an homologous series of hydrocarbons, toxicity is greatest for those compounds with the highest molecular weight (*cf.* Bobra *et al.*, 1983). However, the proportion of crude oil which dissolves in seawater is relatively small compared with the total mass of oil. It is therefore the lower molecular weight components which are considered responsible for acute toxic effects, owing to their greater aqueous solubility and therefore greater bioavailability, enabling penetration to sites of toxic action (Bobra *et al.*, 1983; GESAMP, 1993). Higher molecular weight components such as aliphatic

UCM hydrocarbons are considered insufficiently soluble in water to have much influence upon toxicity (Bobra *et al.*, 1983; Peterson, 1994).

The components of a WAF are principally alkylated benzenes, with smaller amounts of naphthalenes and *n*-alkanes with less than 10 carbons. However, the actual composition and relative concentrations of individual components varies dramatically according to the method of preparation (Bobra *et al.*, 1983; Shiu *et al.*, 1988, 1990). Consequently, comparison of data from different workers is often difficult. Also, analytical data such as the results of qualitative or quantitative water or tissue analyses are rarely provided (Betton, 1994). The variation in LC_{50} values for a range of crude oils and refined petroleum products is illustrated in Table 1.3 (from Betton, 1994).

Product	LC_{50} Range (mg l^{-1})
Gasoline	10 - 100
Lubricants / base oils	> 1000
Kerosene	1 -10
Gas oil/diesel	10 - 100
Fuel oil	
Nos 1,2	10 - 100
Nos 3,5	100 - 1000
Nos 6	> 1000
Crude oil	10 - 100

Table 1.3 Summary data on ecotoxicity of petroleum products (from Betton, 1994)

A further problem associated with this approach is the necessity for a closed test system to prevent losses of toxicant owing to volatilization, as the most water soluble components are also the most volatile (*e.g.* benzene, toluene and xylenes). This is

necessary when toxicity is expressed in terms of aqueous concentration (*i.e.* LC_{50}) as the test organisms must be maintained in a test medium at constant concentration in order to relate the observed toxicity to exposure conditions (Peterson, 1994). Such experimental conditions may not simulate chronic exposure, since in the natural environment these compounds are often rapidly volatilized and may not be bioaccumulated by marine organisms (Betton, 1994). Similarly, there is also the difficulty of matching laboratory exposures qualitatively to the mixtures experienced in the field, owing to the effects of photo-oxidation, microbial activity and general weathering on the composition of oils (Bayne *et al.*, 1982).

Measurements of acute toxicity in terms of mortality, by determination of the LC_{50} or LL_{50} for crude oils, refined petroleum products and individual compounds, provides a relatively simple method of estimating the concentration of test materials that cause direct and irreversible harm to the test organism. However, acute lethal toxicity tests lack the sensitivity required for the early warning of biological harm, as mortality represents a gross biological end-point, only evident when biological harm has already occurred (Axiak, 1991). Consequently, the usefulness of such data for the prediction of the ecological impact of petroleum hydrocarbons in chronically polluted situations is limited.

To address this problem, a wide range of sublethal toxicity tests have been developed, involving measurement of organism responses to contaminants at subcellular, cellular, physiological and whole organism levels. The present discussion will be limited to physiological responses of marine organisms.

Physiological responses are dependent upon the bioavailability, uptake and distribution of contaminants within the body and can therefore be considered as representative of the fitness of the whole organism (GESAMP, 1995). The aim of a sublethal toxicity test is to determine whether exposure to a pollutant under a given set of conditions stresses the

individual to a point which renders it less fit for survival (Axiak, 1991). Marine molluscs exhibit various sublethal responses to petroleum hydrocarbons, *e.g.* decreased feeding rate and absorption efficiency, and increased respiration, (Widdows *et al.*, 1987; Bayne *et al.*, 1982; Widdows and Donkin, 1992). One approach which is increasingly being applied in field programmes is the determination of Scope for Growth (SFG) in marine bivalves.

The rate of growth is a fundamental measure of physiological fitness and provides one of the most sensitive measures of stress in an organism (Widdows, 1994). However, the direct measurement of growth in marine bivalves and many other species is difficult because the accurate measurement of tissue growth or weight change is impracticable on individuals owing to the presence of a shell, and also because a large proportion of the total production can be lost in the form of gametes when spawning (Widdows, 1985). However, the energy budget of an organism can be assessed by integration of physiological responses such as feeding, food absorption, respiration, excretion and production. This is termed 'Scope for Growth' (SFG) and represents the overall bioenergetic status of an organism or population under specific conditions (Widdows, 1985, 1994; Widdows and Donkin, 1992; Smaal and Widdows, 1994). SFG can be considered a measure of the *growth potential* of an animal under specific conditions. Under pollutant-stress conditions, the energy available for growth and reproduction is less than under optimum conditions, and this is reflected in the SFG with values ranging from maximum positive values under optimum conditions, declining to negative values when the animal is severely stressed.

The concept of SFG has been used to assess the sublethal biological effects of pollutants on a variety of marine invertebrates and has proved to be particularly useful when combined with analysis of chemical contaminants. To date mussels have been the most widely used 'bio-indicator' organism. This is principally because of their

established role as sentinel organisms for chemical monitoring programmes (*cf.* Section 1.4). However, they are also ideal organisms for biological monitoring, not only because they are sensitive to a wide range of pollutant levels, but are also relatively unaffected by transplantation and handling stress, making them amenable to both laboratory and field studies (Widdows and Donkin, 1992; Widdows, 1994). Mussel physiology is also well studied and documented. Detailed reviews of the measurement of SFG are given by Widdows (1985, 1994) and Smaal and Widdows (1994).

The coupling of SFG measurements to tissue residue chemistry provides a toxicological interpretation of contaminant concentrations in the tissues and enables identification of the cause of effects observed in the field (Widdows and Donkin, 1989, 1992; Smaal and Widdows, 1994). This approach has been successfully applied in a number of field studies (Gilfillan *et al.*, 1977; Widdows *et al.*,1980-81, 1990, 1995a,b; Martin *et al.*, 1984). For example, Gilfillan *et al.* (1977) measured the long term effects of an oil spill on populations of the clam *Mya arenia* from different sites in Casco Bay, Maine, U.S.A. Two years after the oil spill, Gilfillan and co-workers found that whilst there was no correlation of response with the total body burden of hydrocarbons (dominated by the aliphatic UCM), a significant reduction in carbon flux (effectively SFG) was measured which was found to correlate with elevated body burdens of low molecular weight aromatic hydrocarbons (including the low molecular weight aromatic UCM). This is one of very few studies (perhaps the only) to include the aromatic UCM in the toxicity assessment.

Widdows *et al.* (1987) exposed mussels (*Mytilus edulis*) in a mesocosm study to 28 μg l^{-1} and 125 μg l^{-1} of oil for 8 months and demonstrated a marked reduction in SFG of mussels exposed to both oil concentrations. This was attributed principally to a reduction in feeding rate and food absorption efficiency. Analysis of the body tissues of *M. edulis* revealed a significant correlation between the log tissue concentration of

aromatic hydrocarbons (as 2- and 3- ring aromatics) and SFG. A similar relationship was demonstrated in an eight year field study. Widdows *et al.* (1995a) monitored the temporal and spatial variation in environmental contamination by petroleum hydrocarbons and the associated sublethal biological effects in the vicinity of the North Sea oil terminal at Sullom Voe in the Shetlands over the period 1982-1989. The results again demonstrated a significant negative correlation between SFG and the log hydrocarbon concentration of 2- and 3- ring aromatic hydrocarbons in the mussels tissues. This relationship is illustrated below in Figure 1.8.

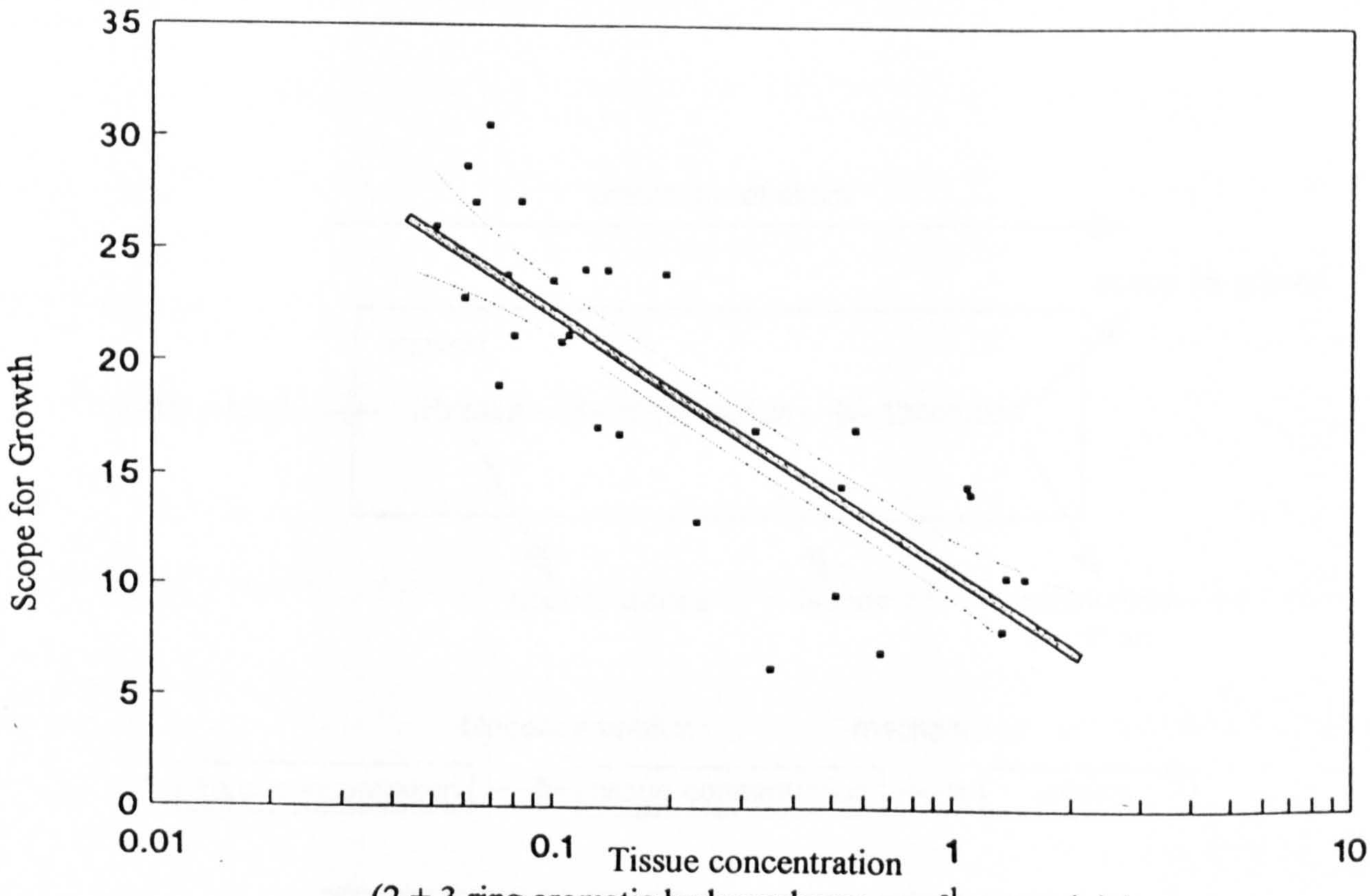


Figure 1.8 Relationship between mean Scope for Growth (SFG) and the log concentration of 2- and 3-ring hydrocarbons in the tissues of *Mytilus edulis* collected from sites in Shetland during the period 1982-1989 (Widdows *et al.*, 1995a)

The examples described above (Gilfillan *et al.*, 1977; Widdows *et al.*, 1995a) relate to environmental situations where petroleum hydrocarbons are the dominant environmental contaminant. However, more commonly, hundreds of environmental contaminants may be present, significantly complicating the toxicological interpretation of tissue residues. Analysis and interpretation of the combined measurement of Scope for Growth and chemical contaminants in the mussels can be considered a two stage process; (i) the detection and quantification of environmental quality, and (ii) the toxicological interpretation and identification of the causes of the observed deleterious effects. This is summarised in Figure 1.9.

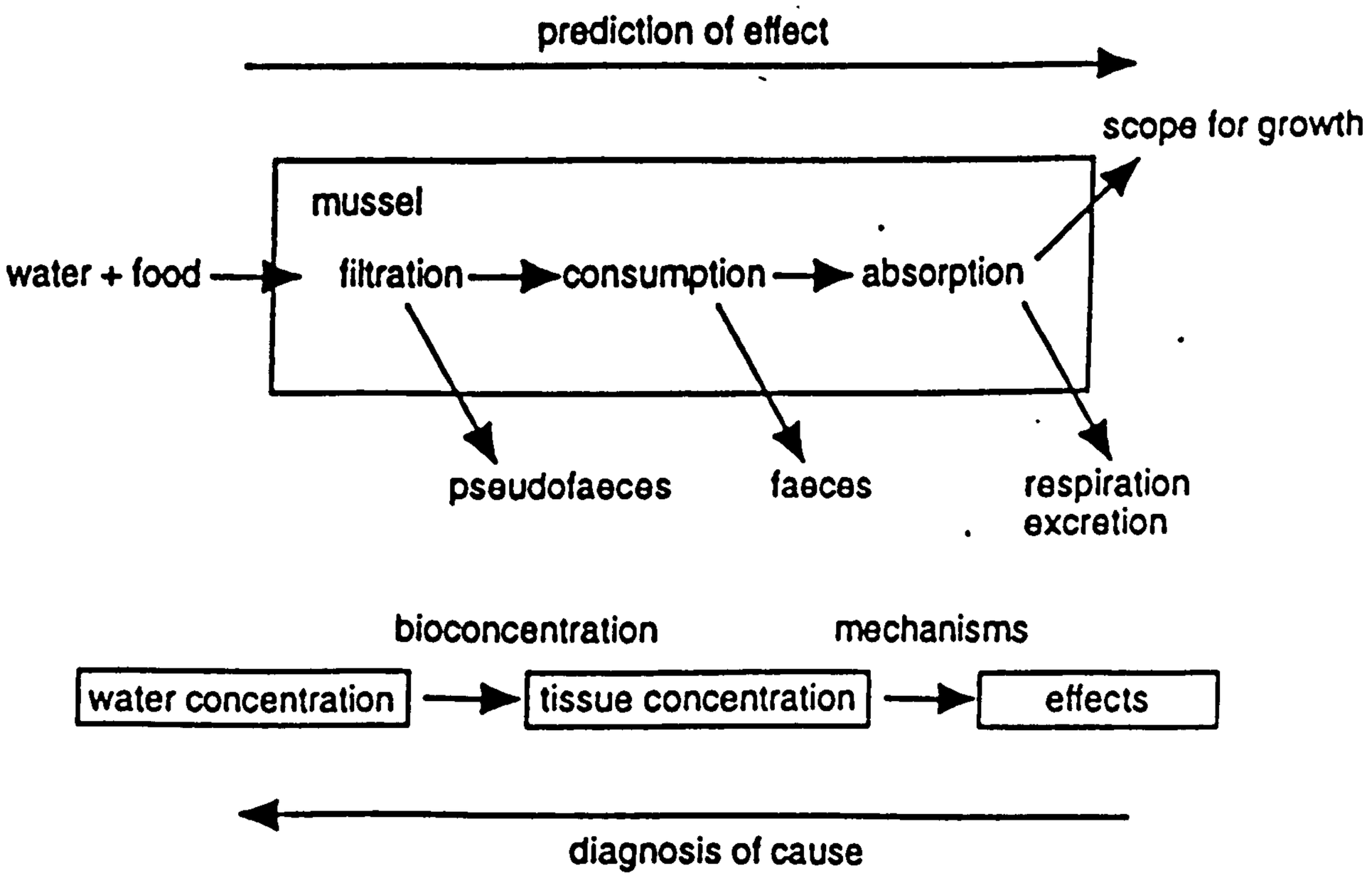


Figure 1.9 Scope for Growth (SFG) as part of the individual energy budget, in an ecotoxicological framework (Smaal and Widdows, 1994)

Laboratory and mesocosm-derived tissue concentration-physiological response relationships are clearly essential to provide the information necessary for toxicological interpretation of chemical and biological measurements in the field (Donkin and Widdows, 1992). This is illustrated in a study by Widdows *et al.*, (1995b). These authors measured the SFG and extent of chemical contamination (2- and 3- ring hydrocarbons, alkyltins, 'polar organics', organochlorines and metals) at a number of coastal and offshore sites in the North Sea, extending from Shetland to the Thames Estuary. At approximately half the sites which showed a marked reduction in SFG, the reduction could be explained using experimentally derived tissue concentration-response relationships. However, at a number of sites, there was a significant 'unexplained component' to the SFG values. A tentative correlation was identified between the unexplained toxicity and concentrations of organochlorines in mussels. However, the authors concluded that as the effects of organochlorines on SFG have not yet been quantified, no firm conclusions could be drawn from this (Widdows *et al.*, 1995b). Considering their quantitative importance, the derivation of concentration-response data for aliphatic and aromatic UCM hydrocarbons may also provide a useful explanation for a proportion of the 'unexplained toxicity'. However, owing to the lack of knowledge of UCM molecular composition until very recently, this has not been possible.

Whilst it is possible to derive tissue concentration-physiological response data for a small number of contaminants *e.g.* tributyltin compounds(Widdows and Page, 1991) and selected hydrocarbons (Widdows *et al.*, 1987; Donkin *et al.*, 1989, 1991), it is clearly impossible to derive individual concentration-response data for each of the thousands of chemicals that enter the environment annually. One approach to overcome this is the use of quantitative structure-activity relationships (QSARs) to predict the toxicological properties of organic compounds from their chemical and structural properties (Donkin *et al.*, 1989, 1991; Donkin and Widdows, 1990; Donkin, 1994; Widdows and Donkin,

1992). The use of quantitative structure-activity relationships is discussed in Section 1.6. Comprehensive reviews on the integration of chemical and biological monitoring methods are provided by Widdows and Donkin (1989, 1991, 1992).

1.6 The use of Quantitative Structure-Activity Relationships in the prediction of aquatic toxicity

Owing to the large number of contaminants entering the marine environment annually (estimates are that over 100,000 substances are in commercial use and this number is continually increasing; Dearden *et al.*, 1994), many ecotoxicological effect assessments are made on the basis of estimation techniques known as Quantitative Structure-Activity Relationships (QSARs). QSARs attempt to relate statistically the biological activity of a chemical to the physico-chemical properties (Cronin and Dearden, 1995). Numerous reviews are available on the use of QSARs in the prediction of aquatic toxicology (*e.g.* Nirmalakhandan and Speece, 1988; Donkin and Widdows, 1990; Donkin, 1994; Cronin and Dearden, 1995) and consequently the present discussion will provide only a brief overview of the subject.

The hydrophobicity of a compound has been widely recognized to be of fundamental importance to its toxicity since the nineteenth century when Overton (1897, cited by Cronin and Dearden, 1995) and Meyer (1899, cited by Cronin and Dearden, 1995) reported correlations between the olive oil-water partition coefficient and narcotic potency of a number of simple organic compounds (reviewed by Donkin and Widdows, 1990; Donkin, 1994; Cronin and Dearden, 1995).

Linear QSARs between a biological response and a single molecular descriptor for hydrophobicity have since been reported by many investigators (*e.g.* Konemann, 1981; Veith *et al.*, 1983; Hermens *et al.*, 1984, 1985; de Wolf *et al.*, 1988; Van Leeuwen *et al.*,

1991). For example, Konemann (1981) established a QSAR for the 14 day LC_{50} (lethal exposure concentration for 50 % of the population) in the guppy for fifty unreactive industrial organic chemicals (aliphatic and aromatic (chloro) hydrocarbons, glycol derivatives and related compounds). This author compared correlations of lethality (in terms of concentration in the aqueous phase to which the organism is exposed) with several expressions of hydrophobicity (octanol-water partition coefficient, solubility data, HPLC retention indices and molecular connectivity indices) and concluded that the octanol-water partition coefficient (log K_{OW}, defined as; $K_{OW} = C_0/C_w$ where C_0 and C_w are the concentrations of the solute in *n*-octanol phase and aqueous phase respectively, when the system is at equilibrium [usually measured at 25°C]) was the best measure of hydrophobicity and could be used to account for a very large part of the variation in observed toxicity for compounds with log K_{OW} values in the range 2 - 6. Comparable relationships have been described by Veith *et al.* (1983) and Hermens *et al.* (1984) and are widely accepted as characteristic QSARs for unreactive non-ionisable organic compounds whose mode of toxic action is termed non-specific narcosis. Figure 1.10 illustrates this relationship.

Whilst log K_{OW} is the most common physicochemical descriptor used in narcosis QSARs, successful QSARs have also been obtained with water solubility (*e.g.* Abernethy *et al.*, 1986) and parameters such as connectivity indices which can be related to hydrophobicity (*e.g.* Govers *et al.*, 1984).

Narcosis is defined as a non-specific reversible disturbance of the functioning of the membrane, caused by the accumulation of pollutants in hydrophobic phases within the organism. The disturbance of membrane function results in decreased activity and a diminished ability to react to stimuli, and can ultimately lead to death (van Wezel and Opperhuizen, 1995). The exact mechanism of narcosis and the site of toxic action remain unknown. However, the favoured sites of toxic action are either the lipid bilayer

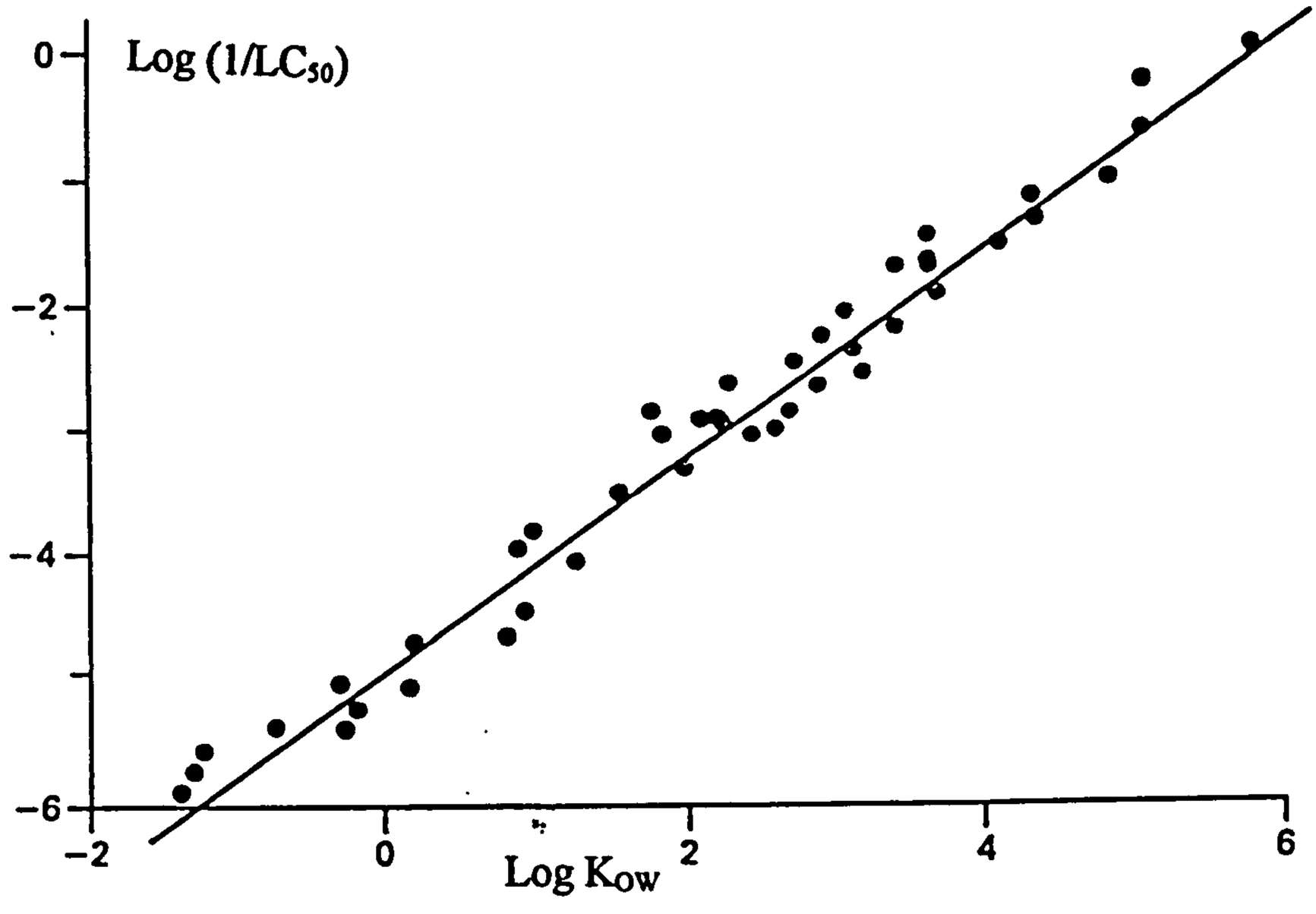


Figure 1.10 QSAR for 50 organic pollutants to the guppy (*Poecilia reticulata)*

[log $1/LC_{50}$ = 0.871log K_{ow} - 4.87 n=50 r=0.988 s=0.237; Konemann, 1981)

of the cell membrane or a specific protein within the membrane.

The lipid or membrane perturbation theory originates from the early studies of Meyer and Overton who demonstrated a correlation between the narcotic potency of a substance and the olive oil/water partition coefficient. This work led to the proposal that narcosis results from membrane perturbation, induced as the toxicant/narcotic chemical partitions into the lipid membrane. Perturbation of the membrane is believed to affect functioning of nerve action, resulting in a diminished nervous response. Ferguson (1939) noted that because a partition process is involved and the narcotic chemical is not irreversibly bound, the toxic action of narcotic chemicals is caused by the physical

presence of the chemical at the target site. Consequently, Ferguson (1939) suggested that chemical activity, as opposed to exposure concentration, was a better index of toxicity. Chemical activity in the aqueous phase was approximated by dividing the concentration in water necessary to produce an effect (*e.g.* LC_{50}) by solubility. In this way it was demonstrated that toxicity as expressed by chemical activity varied significantly less than when expressed by exposure concentration. Equal activity (when expressed as a fraction of chemical solubility) for different toxicants at a specific toxicological end-point also implies approximately equal concentrations of toxicant at the site of toxic action, as activity in the organism is proportional to concentration in the organism (van Wezel and Opperhuizen, 1995). Several studies have since demonstrated that the body burden of toxicant in an organism at a specific toxicological end-point (*e.g.* lethality, loss of equilibrium in fish, reduced feeding rates) is relatively constant (McCarty *et al.*, 1992a; Mackay and Hughes, 1984; Donkin *et al.*, 1989, 1991).

The 'constant concentration' theory was modified by Mullins (1954) who proposed that narcosis was more closely related to molar volume than molar concentration, such that when the volume fraction of chemical in the membrane reached a critical volume, narcosis occurred. Mullins (1954) introduced a correction for molecular size, noting that chemicals of larger molar volume are more potent because at equal molar concentration they occupy a larger volume fraction. Several studies have provided evidence in favour of the critical volume theory. For example, Seeman (1972) estimated both anaesthetic potency (*via* haemolytic activity) and molar concentration of 70 organic chemicals in the membrane phase of erythrocytes. Although a relatively constant concentration of narcotic in the membrane was associated with haemolytic activity, the relationship was improved by correlating the effect concentration to the product concentration in the membrane and molecular volume of the chemical (Seeman, 1972).

Abernethy *et al.* (1988) estimated the volume fraction occupied by a toxicant at the site of action by multiplying LC_{50}, log K_{ow} and molar volume. Data sets describing the toxicity of narcotic chemicals to several species (Fathead Minnow, Golden Orfe, *Daphnia* and *Artemia*) were employed. These authors suggested that acute lethality to aquatic animals occurrs when the volume fraction of narcotic toxicant in membranes reached approximately 0.6 %. A similar approach was applied by Warne *et al.* (1991). These authors compared the critical concentration and critical volume hypotheses using a data set describing the sub-lethal toxicity of a range of narcotic chemicals to marine bacteria. Values for both the critical concentration (CC) and volume fraction (VF) of toxicant were calculated (using toxicity data, molar volumes and log K_{ow} values) and then plotted against log K_{ow}. The slopes of the regression equations for both CC and VF were not significantly different from each other. However, the slopes of both equations were significantly different to zero, indicating that both the hypotheses (CC and VF) were equally unsuccessful in modeling narcotic toxicity (Warne *et al.*, 1991). Warne *et al.* (1991) suggested that observed results may be attributed to the use of K_{ow} values which may overestimate the actual target tissue partition coefficient (*cf.* Abernethy *et al.*, 1988). Equations were re-calculated using K_{PW} (dimyristoylphosphatidylcholine (DMSP)-water partition coefficient), as it was suggested that the phospholipid DMSP would more closely resemble the cell membrane composition than octanol. The authors reported that the resulting equation for VF had a gradient not significantly different from zero, whilst the slope of CC was. This was interpreted as providing support for the critical volume theory.

An alternative theory for the mechanism of narcosis is the protein receptor theory, based upon the hypothesis that one or more proteins serve as narcotic receptor sites. This theory has been advocated principally by Franks and Lieb (1984, 1987, 1990, 1994). These authors compared correlations between the narcotic potency of different

chemicals and their solvent-water partition coefficients using various solvents. A good correlation was reported when partition coefficients were measured using an organic solvent with both polar and apolar characteristics such as *n*-octanol, whilst a non-polar solvent such as *n*-hexadecane gave poor correlations. It was concluded that the site of action must be a single amphiphillic pocket such as a protein (Franks and Lieb, 1984). However lipid bilayers also have both polar and non-polar regions. Franks and Lieb (1983) isolated an enzyme, luciferase from the Firefly *Photyinus pyralis*. The purified (99%) enzyme was incubated with different concentrations of the substrate luciferin, together with various narcotics. Luciferase was demonstrated to be competitively inhibited by narcotic chemicals at approximately the same 50 % concentration that narcoses animals (Franks and Lieb, 1984). Franks and Lieb (1984) proposed that the narcotic chemicals had bound to the pocket in which luciferin normally binds, thereby preventing the substrate from binding with luciferase, analogous to the 'lock and key' hypothesis for enzyme catalysis. However, to date no suitable protein receptor in the nervous system of any organism has been identified. This theory has been reviewed by Franks and Lieb (1990, 1994).

It must be noted that the 'critical volume' theory discussed previously is equally applicable to both a protein or lipid site of action. More detailed discussions regarding the theories and mechanism of narcosis are found in Seeman (1972), Miller (1985), Franks and Lieb (1990) and van Wezel and Opperhuizen (1995).

Non-specific narcosis is considered to be a minimum or 'baseline' effect, as many reactive chemicals are more toxic than predicted from non-specific QSARs owing to additional modes of toxic action. McKim *et al.* (1987) and Bradbury *et al.* (1991) exposed immobilized trout to a range of aquatic pollutants and recorded a variety of physical responses. After statistical analyses, they identified seven common modes of toxic action, which were named Fish Acute Toxicity Syndromes (FATS). These were

nonpolar narcosis, polar narcosis, uncoupling of oxidative phosphorylation, respiratory membrane irritation, respiratory blockers, acetylcholinerase inhibition and central nervous syndrome seizure. With the exception of non-polar narcosis, these modes of action represent chemicals with toxicities greater than baseline (non-polar) narcosis. A review of the classification of modes of toxic action of organic chemicals is given by Verhaar *et al.* (1992).

The strong log K_{ow} influence in toxicity can be explained by the well documented correlation of bioconcentration with log K_{ow} (Hermens *et al.*, 1985). The octanol-water partition coefficient effectively mimics partitioning of organic compounds into lipid and, as such, numerous linear relationships between the bioconcentration factor of a contaminant (BCF, defined as the ratio of compound concentration in the organism to concentration in water at equilibrium) and octanol-water partition co-efficient have been established (*e.g.* Hawker and Connell, 1986). This is illustrated in Figure 1.11. Such linear relationships have been shown to apply for organic compounds with log K_{ow} values in the range 2 - 6. For compounds with log K_{ow} values greater than 6, measured BCF factors are generally lower than predicted (Hawker and Connell, 1986). These authors attributed the deviation from linearity for the more hydrophobic compounds (log $K_{ow} > 6$) to the lengthy time periods required to establish equilibrium, suggesting that for very hydrophobic compounds, a steady-state body burden may not be achieved in the lifetime of the organism (*i.e.* for compounds with log $K_{ow} > 8$).

An alternative hypothesis for the deviation from linearity for the more hydrophobic compounds is that the octanol-water partition coefficient may be a poor model for lipid-water partitioning for larger molecules. Gobas *et al.* (1988) demonstrated that for compounds with log K_{ow} values in the range 1 - 5.5, membrane-water and octanol-water partition coefficients are approximately equal and linearly related. However,

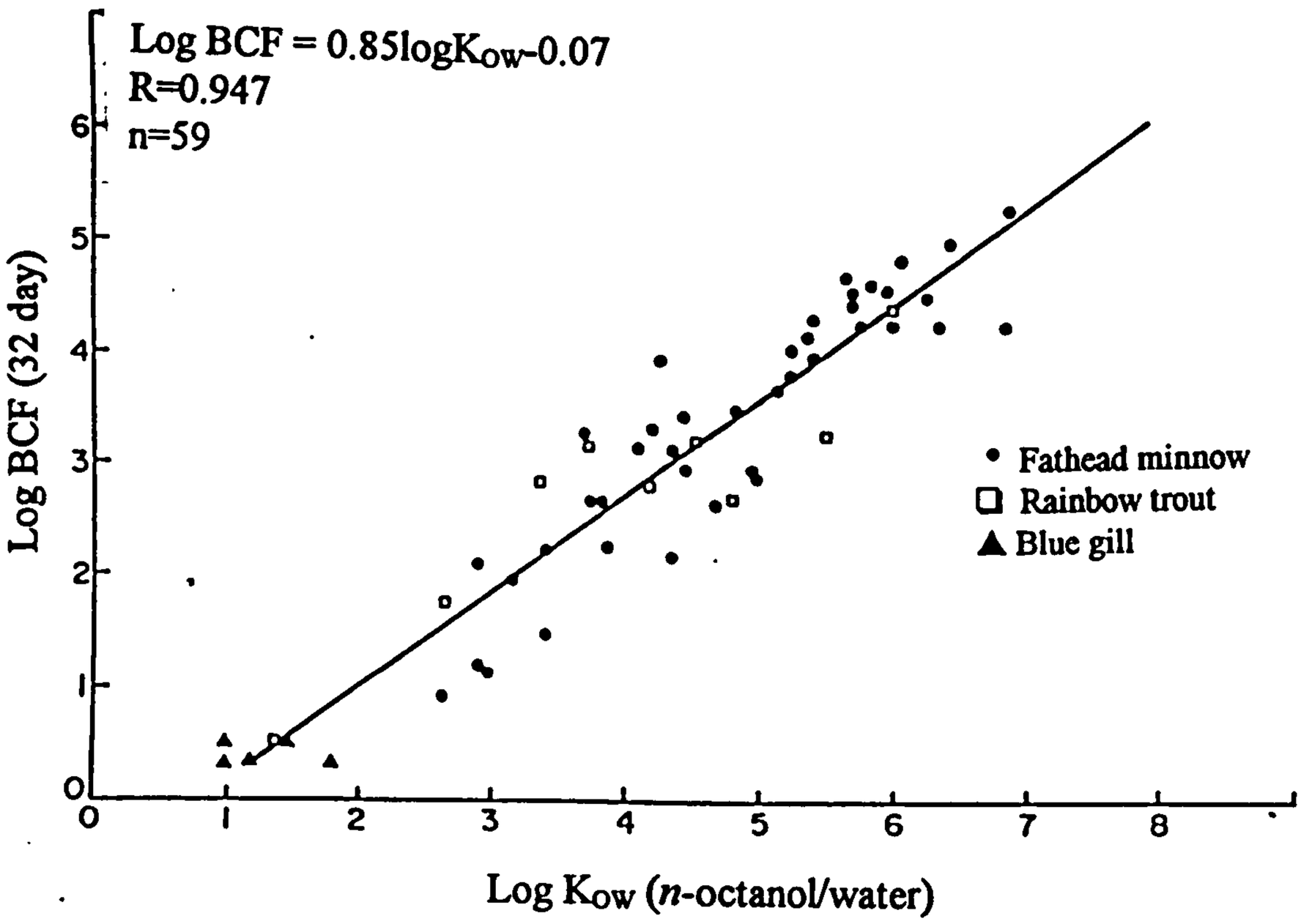


Figure 1.11 Correlation of log BCF with log K_{ow} for organic chemicals (McKim and Schmeider, 1990)

compounds with log K_{ow} values greater than 5.5 partitioned less into lipid membranes than octanol. The relationship between lipophilicity and partitioning is extensively reviewed by Dearden (1985). Bioaccumulation QSARs are also critically reviewed by Donkin and Widdows (1990) and Donkin (1994).

Traditionally, toxicity has been expressed in terms of the aqueous exposure concentration of toxicant. However, as noted by Friant and Henry (1985), expressions of toxicity in terms of the organism toxicant concentration have the advantage of eliminating variations due to bioavailability and kinetics of toxicant uptake. Despite a

number of early studies (*e.g.* Ferguson, 1939) which examined the toxicological residues at pharmacological endpoints, the concept of expressing toxicity in terms of organism concentrations (critical body residue, CBR or body burden, BB) has largely been neglected until recently.

Rogerson *et al.* (1983) and Bobra *et al.* (1983) deduced from aqueous concentration based toxicity data that the lethal body burden associated with ciliate protozoa and *Daphnia* exposed to various hydrocarbons was relatively constant, but unquantified. Similarly, Mackay and Hughes (1984) demonstrated that narcosis in goldfish (as measured by loss of equilibrium) occurred at a mean body burden of 4.4 mmol kg^{-1}. McCarty (1986, 1987a,b) evaluated the use of previously established QSARs between toxicity and log K_{ow}, and bioconcentration factor (BCF) and log K_{ow} to estimate the internal concentrations of narcotic organic chemicals at specific biological endpoints. This author recalculated a series of QSAR regression equations relating aqueous based expressions of toxicity with log K_{ow} using a geometric mean functional regression procedure. These equations were combined with a bioconcentration relationship and manipulated to develop a modified toxicity/bioconcentration relationship. The internal toxic residue was then obtained by multiplying the calculated LC_{50} value by its respective BCF. The relationships obtained are summarised in Figure 1.12. This diagram illustrates a fundamental toxicological principle that, although some organic chemicals have a potency difference of 4-5 orders of magnitude when expressed in terms of molar concentration of the exposure water, they are essentially of equal potency when measured as the organism toxicant concentration at steady state equilibrium (McCarty, 1986). In other words, differences in aqueous based expressions of the toxicity of narcotic organic chemicals are a function of the hydrophobicity and therefore of the kinetics of uptake of a chemical.

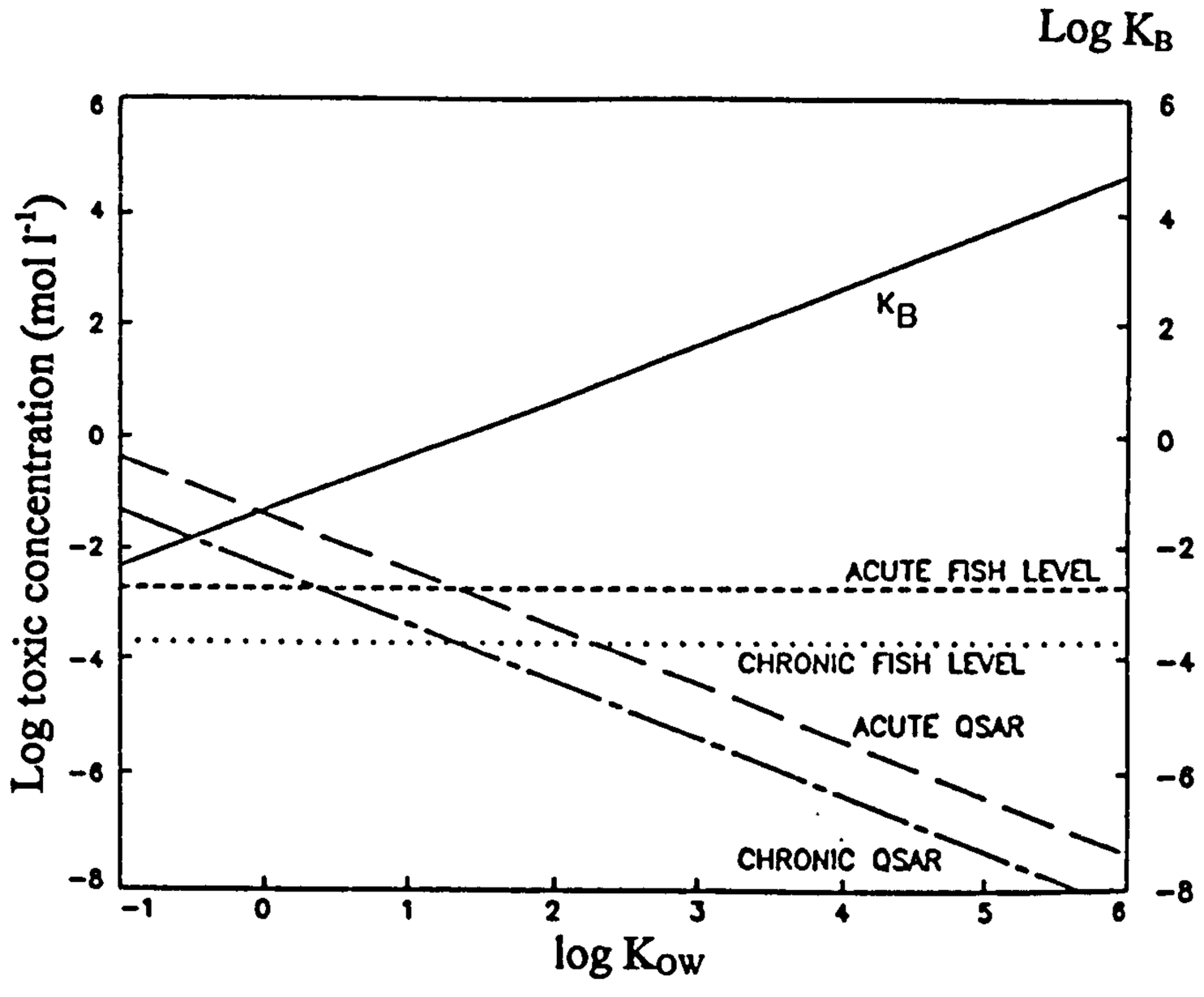


Figure 1.12 Relationship between toxicity, bioconcentration (Log K_B) and octanol/water partition coefficient (log K_{ow}) for some narcotic chemicals (McCarty, 1987a,b)

McCarty *et al.* (1992a) applied a similar approach to a data set for the acute toxicity of a variety of hydrophobic narcotic chemicals examined by the U.S Environmental Protection Agency (Duluth) in tests with juvenile Fathead Minnows. These authors applied a one-compartment first order kinetics (1CFOK) model to the data set, in combination with established BCF/K_{ow} QSAR relationships to develop a relationship between K_{ow} and both threshold acute toxicity (LC_{50}) and whole body toxicant residue. Although this approach has a number of sources of error such as uncertainty in the accuracy of log K_{ow} values and errors in the regression analysis, these problems were acknowledged by the authors and their approach put forward as an 'initial exploratory analysis'. McCarty *et al.* (1992a) estimated the lethal body residue for neutral narcotic

organics to be in the range 2.2 - 8.3 mmol kg^{-1}. Comparison of the predicted range of lethal body burdens with the few available measured lethal residues in fish reported showed reasonable agreement. These authors concluded that the utility of their approach would be increased if it could be determined that certain residue levels are associated with different biological response endpoints. McKim and Schmeider (1990), reviewing the work of McCarty and other workers noted that for non-specific narcotic QSARs, the acute and chronic toxicity regressions are parallel and suggested that the sensitivity and perhaps the accuracy of critical residue estimates could be enhanced by incorporating sublethal or chronic endpoints into such studies.

To date, there are relatively few body residue data for sublethal toxicological endpoints. Donkin *et al.* (1989, 1991) investigated the effect of a number of hydrocarbons and substituted hydrocarbons on the feeding rate of the mussel (*Mytilus edulis*) and measured the toxicant burden in the animals at the end of each feeding rate determination. These authors demonstrated that the tissue concentration required to produce a 50 % reduction in feeding rate (TEC_{50}) was relatively constant (0.1 - 0.3 mmol kg^{-1}), irrespective of compound structure or physico-chemical properties for compounds in the log K_{ow} range 2.5 - 5. This is illustrated in Figure 1.13. The relationships between hydrophobicity and, toxicity and body residues reported by Donkin *et al.* (1989, 1991) are identical to those proposed by McCarty *et al.* (1986, 1987a,b; Figure 1.12 herein). However, whilst McCarty (1986, 1987a,b) estimated internal concentrations of toxicant, Donkin *et al.* (1989, 1991) have actually measured tissue concentrations of toxicant at the toxicological end-point, thereby providing further support to the equipotency of non-specific narcotics at the site of action and demonstrating that non-specific narcotic

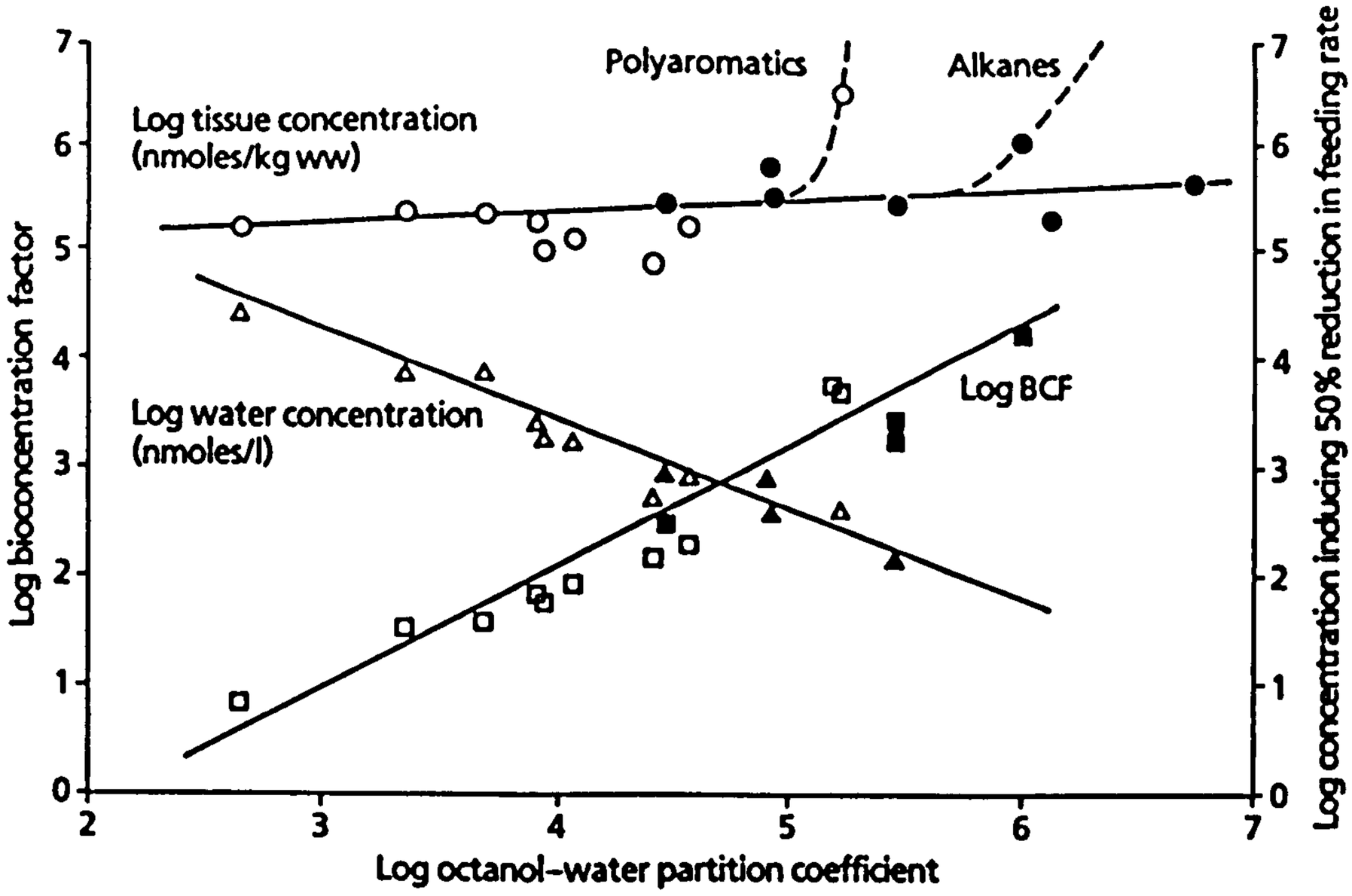


Figure 1.13 Relationship between log K_{ow} of hydrophobic organic chemicals and the log of their bioconcentration factor into the mussel (*Mytilus edulis*), and the log of the concentration of these compounds in water (nmol l^{-1}) and mussel tissue (mmol kg^{-1} wet wt.) which reduce feeding rate by 50%. The symbols on the figure represent the following parameters: water based EC_{50} , (Δ) aromatics, (▲) alkanes and substituted alkanes; tissue based EC_{50} (○) aromatics, (●) alkanes and substituted alkanes; BCF (□) aromatics, (■) alkanes and substituted alkanes. The dashed lines indicate the approximate position of the toxicity cut-off (from Donkin *et al.*, 1989, 1991)

QSARs developed from aqueous exposure concentration data largely reflect differences between bioconcentration factors (Donkin *et al.*, 1989).

Donkin *et al.* (1989, 1991) also found that compounds with log K_{ow} values greater than 5 were non-toxic to mussel feeding rate. The observation of a 'cut-off' in toxicity for compounds with log K_{ow} values greater than approximately 5-6 is commonly reported (*e.g.* Konemann, 1981; Hermens *et al.*, 1984) and is an established characteristic of QSARs with hydrophobic chemicals (Franks and Lieb, 1990; Lipnick, 1990). Since non-specific narcotic QSARs developed from aqueous concentration data largely reflect differences between bioconcentration of toxicants, deviations from

linearity (such as the 'cut-off' in toxicity) may be partly attributed to kinetic factors, *i.e.* the experimental exposure time is shorter than required for sufficient bioaccumulation of toxicant into the organism (Lipnick, 1990). However, Donkin *et al.* (1989, 1991) demonstrated that a cut-off in toxicity occurred, despite efficient accumulation of the compounds tested, as compounds with log K_{ow} values greater than 5-6 were bioaccumulated within the mussels to much greater concentrations than the TEC_{50} values for those compounds which were toxic, with no measurable effect upon feeding rate. For the hydrocarbons studied, these workers suggested that whilst log K_{ow} was a good predictor of toxicity and bioconcentration, aqueous solubility was a better indicator of the cut-off point than log K_{ow} (Donkin *et al.*, 1991). Donkin *et al.* (1991) proposed that a cut-off in toxicity occurs for compounds with aqueous solubilities less than 70 $\mu g\ l^{-1}$. This equates to a cut-off in toxicity for *n*-alkanes greater than *n*-C_{10} (*n*-C_{11} was found to be non-toxic) and at approximately pyrene for aromatic hydrocarbons (Donkin *et al.*, 1989, 1991). Several explanations have been proposed for the observed cut-off effect. Most commonly, it is attributed to the low solubility of larger hydrophobic molecules. Despite the fact that such compounds have a high partition coefficient, their aqueous solubility is so low that insufficient toxicant accumulates at the site of action to induce a response (Veith *et al.*, 1984; Donkin *et al.*, 1989). Alternatively Abernethy *et al.* (1988) postulated that a cut-off in acute toxicity occurs with molecules of molar volumes 300 $cm^3\ mol^{-1}$ or greater. Haydon *et al.* (1977) argued that 'large' compounds (higher homologues) cannot be accommodated in the small hydrophobic regions of the lipid bilayer unless there is an energetically unfavourable distortion of the lipid bilayer.

As discussed previously, Gobas *et al.*, (1988) suggested that log K_{ow} is a poor model for lipid water partitioning of large molecules, owing to the rapidly declining solubility of hydrophobic compounds (log $K_{ow} > 5$) in lipid membranes. It seems plausible, therefore, that the narcotic cut-off occurs as a result of rapidly declining aqueous solubility

combined with declining membrane solubility preventing a compound from achieving a sufficient concentration at the site of toxic action to produce narcosis (Abernethy *et al.*, 1988; Donkin *et al.*, 1991).

As the majority of studies tend to focus upon compounds with log K_{ow} values less than 5, further work with compounds near the toxicity cut-off is required before the toxicity cut-off for narcotic chemicals can be more accurately defined and firm conclusions drawn. However, data available tends to suggest that aqueous solubility may be the most suitable descriptor for delineating the cut-off effect (Donkin *et al.*, 1989, 1991).

Whilst toxicity studies concerned with the effects of single compounds are extremely useful in identifying modes of action and establishing concentration-response curves, contaminated organisms typically contain complex mixtures of a wide range of chemicals. Consequently, consideration must be given to the joint toxicity of mixtures of chemicals to marine organisms.

Several studies (*e.g.* Hermens *et al.*, 1984, 1985; Deneer *et al.*, 1988) have demonstrated that narcotic toxicants are concentration additive when present as a mixture. In other words, the toxicity of a mixture of compounds acting by the same mechanism (in this instance non-specific narcosis) may be calculated by expressing the concentration of each individual compound as a fraction of the effective concentration. Each component of the mixture is expressed as a 'Toxic Unit' (TU) thus;

$$TU_{SUM} = \frac{Cw_1}{LC_{50}1} + \frac{Cw_2}{LC_{50}2} + \cdots\cdots \frac{Cw_i}{LC_{50}i}$$ (McCarty *et al.*, 1992b)

Deneer *et al.* (1988) investigated the joint toxicity of a mixture of 50 narcotic chemicals towards *Daphnia magna* and found that the observed toxicity was accurately predicted using the concentration addition model. These authors investigated the toxicity of

mixtures of narcotics in which some of the components were present in concentrations well below their no-effect concentration, and concluded that in a mixture of narcotic chemicals, any compound will, according to concentration and hydrophobicity, contribute to the toxicity of the mixture, even if it is present at a very low concentration (Deneer *et al.*, 1988).

To date, no systematic studies have been published examining mixture toxicity in terms of body burden. However, considering that a number of single component toxicity studies have demonstrated that narcotic effects occur at a relatively constant body burden of toxicant, it is hypothesized that tissue concentrations of toxicants are also additive. Each molecule of narcotic toxicant is functionally equivalent at the site of toxic action, thereby producing the same toxicological response when equal numbers of molecules reach the site of toxic action. It is anticipated that mixtures of equivalent toxicants would be expected to act in the same manner as an equi-molar amount of any of the individual components (McCarty, 1986).

As mentioned previously (Section 1.5), the QSAR approach may be useful in the toxicological interpretation of tissue residues, with the ultimate aim of identifying those compounds present in a mixture of contaminants which may cause a deleterious response. For example, Gilfillan *et al.* (1977) showed that although there was no correlation between physiological stress and total hydrocarbon concentration, there was an excellent correlation between the concentration of low molecular weight aromatic hydrocarbons in the tissues and SFG. Consideration of the QSAR established by Donkin *et al.* (1989, 1991) provides an explanation for the results of Gilfillan *et al.* (1977), as the majority of the total hydrocarbon body burden will be dominated by aliphatic hydrocarbons > C_{10}, which are non-toxic, owing to their low aqueous solubility. However, the low molecular weight aromatic hydrocarbons are of sufficient solubility to act as non-specific narcotics, hence the correlation with physiological stress.

1.7 The present study

The presence of an hydrocarbon UCM in the 'aliphatic' fraction of petroleum hydrocarbon contaminated sediments and biota is routinely reported and widely accepted as a measure of petrogenic contamination (see Section 1.3), but the toxicological significance of such UCM hydrocarbons remains largely uninvestigated. The presence of an 'aromatic' UCM in environmental samples is commonly ignored and again, the environmental impact of aromatic hydrocarbon UCMs remains unknown.

One reason for the paucity of studies concerning the environmental impact of UCM hydrocarbons may be the lack of knowledge regarding its molecular composition. However, recent attempts to characterise hydrocarbon UCMs isolated from several lubricating oils and crude oils have led to the proposal of a number of model UCM structures (see Section 1.2). Simple 'T'-branched structures have been identified as a component of the aliphatic UCM, whilst alkyl substituted monoaromatic hydrocarbons have been suggested as possible components of the aromatic UCM (Figures 1.2 and 1.5).

As aromatic hydrocarbons are generally more water soluble than aliphatic hydrocarbons, it is probable that a proportion of the aromatic UCM hydrocarbons will have demonstrable narcotic potency. However, first the quantitative significance of the aromatic UCM in environmental samples needs to be established. Therefore an important aim of the present study was to measure both the aliphatic and aromatic UCM concentrations in mussels from a small number of coastal sites, in order to form a preliminary assessment of 'typical' aromatic hydrocarbon UCM concentrations in mussels. A further aim was then to assess the environmental impact of both aliphatic and aromatic UCM hydrocarbons by investigating the toxicity of a number of low molecular weight model UCM hydrocarbons to mussel ciliary feeding activity.

Chapter 2 reports aliphatic and aromatic hydrocarbon concentrations (resolved and unresolved) measured in marine mussels sampled from a small number of U.K. coastal sites

Chapter 3 details the synthesis and characterisation of a low molecular weight model aliphatic UCM hydrocarbon and two model aromatic UCM hydrocarbons

Chapter 4 Describes a number of studies investigating the sublethal effects of the synthetic low molecular weight model aliphatic UCM hydrocarbon

Chapter 5 investigates the effect of the two model aromatic UCM hydrocarbons on mussel ciliary feeding activity

Chapter 6 provides full experimental and instrumental details

Chapter 7 presents conclusions and suggests areas for future work

CHAPTER TWO

Aromatic and Aliphatic hydrocarbon UCM concentrations present in mussels from U.K. coastal sites

2.1 Introduction

The occurrence of UCMs in the aliphatic hydrocarbon fractions of marine sediments and organisms from areas impacted by petroleum hydrocarbons is well documented. Although generally considered to be of little direct toxicological significance, the aliphatic UCM is nonetheless widely accepted as a reliable indicator of fossil fuel contamination (*e.g.* Thompson and Eglinton, 1978; Risebrough *et al.*, 1983; Mason, 1988; Macias-Zamora, 1996; *cf.* Section 1.3). In contrast, and as illustrated in Table 1.2, the presence of an aromatic UCM is often ignored and environmental concentrations of aromatic UCM hydrocarbons in marine sediments and biota are rarely reported.

The paucity of reported aromatic hydrocarbon UCM concentrations is most probably due to the requirement for environmental monitoring schemes to focus upon those compounds which are of known ecological and toxicological concern. Currently, the US Environmental Protection Agency (EPA) priority pollutant list (consisting of over 150 organic compounds) includes only nineteen petroleum-type hydrocarbons, including sixteen polycyclic aromatic hydrocarbons (PAH), and the volatile aromatic hydrocarbons benzene, toluene and xylene. Many PAH, especially the higher molecular weight four- and five-ring PAH, have carcinogenic and/or mutagenic potential (White, 1986; Pahlman and Pelkonen, 1987). Such compounds are typically present in sediments and biota at concentrations in the ng g^{-1} dry weight range. As a consequence, use of the most sensitive analytical techniques, such as selected ion-monitoring GC-MS, is required for accurate quantification of individual PAH in environmental samples. However, this usually precludes the detection of aromatic UCMs.

Some of the shortcomings of the EPA-approved methods in oil spill impact assessments are discussed by Sauer and Boehm (1991). These authors note that the determination of only the nineteen priority pollutant volatile aromatic and polyaromatic hydrocarbons in

environmental samples affected by oil does not provide sufficient data to permit appropriate interpretation of the environmental impact of spilled oil. Many of the four- and five-ring EPA-listed PAH are very minor constituents of most crude or refined oils, whilst alkylated PAH such as C_1-C_4 naphthalenes and phenanthrenes, although not listed in regulatory methods, are more abundant than the parental PAH. Furthermore, many alkylated PAH appear to be more toxic than their parent compounds (Sauer and Boehm, 1991).

Sauer and Boehm (1991) advocate the determination of a number of alkylated PAH (C_1-C_4 naphthalenes, phenanthrenes, dibenzothiophenes and chrysenes) in addition to the EPA listed PAH. Analysis of alkylated PAHs is also valuable for identifying spilled oil, for distinguishing between different sources of hydrocarbons in the environment and providing information on the extent of oil weathering and degradation in the environment (Douglas *et al.*, 1996).

Whilst the carcinogenic properties of PAH molecules are clearly of concern, other mechanisms of toxic action by which petroleum hydrocarbons may act should not be forgotten in environmental assessments. As described previously in Sections 1.5 and 1.6, many low molecular weight hydrocarbons (up to three rings) are non-specific narcotic toxicants to marine organisms such as mussels. Widdows *et al.* (1987,1995a,b) have demonstrated a significant negative correlation between Scope for Growth (SFG) in mussels and the concentration of two- and three-ring aromatic hydrocarbons bioaccumulated by the organisms. SFG is a fundamental measure of the ecological fitness of a population (*cf.* Section 1.5) but monitoring only the EPA priority pollutants, or indeed a selected number of alkylated PAH present in mussels, would not provide sufficient data to enable a meaningful toxicological interpretation. The use of combined chemical and biological monitoring schemes with 'bio-indicator' organisms such as mussels, as advocated by Widdows and co-

workers enables a more meaningful toxicological interpretation of environmental concentrations of petroleum hydrocarbons and other contaminants. However, it is important that existing environmental monitoring requirements continue to be re-examined and updated in the light of new biological and toxicological data.

The few reports of aromatic hydrocarbon UCMs that can be found in the literature indicate that these compounds are present in petroleum hydrocarbon impacted sediments and biota in much greater amounts than PAH, and indeed, appear to dominate the aromatic hydrocarbon body burden of petroleum hydrocarbon impacted sediments and biota. For example, Boehm *et al.* (1982) reported concentrations of aromatic UCM hydrocarbons in *Mytilus edulis* at approximately 900 $\mu g\ g^{-1}$ wet weight, whilst individual PAH, (*e.g.* naphthalene, phenanthrene and alkylated phenanthrenes) were present at concentrations of 0.1 - 0.9 $\mu g\ g^{-1}$.

Until recently, the molecular composition of aromatic UCMs was unknown and thus the environmental impact of aromatic UCM hydrocarbons uninvestigated. The recent proposal of 'average' model structures for aromatic UCM components (Revill *et al.*, 1997; Thomas *et al.*, 1997; *cf.* Section 1.2) may now enable some of the toxicological impacts of aromatic UCM hydrocarbons to be studied. Given the solubility and associated toxicity of a number of resolved low molecular weight aromatic hydrocarbons (Donkin *et al.*, 1989, 1991) it seems very likely that a proportion of the lower molecular weight aromatic UCM components would be of sufficient aqueous solubility to be considered as non-specific narcotics (*cf.* Section 1.6).

A number of recent reports have also highlighted the importance of including hydrocarbon oxidation products in environmental monitoring programmes (Burns *et al.*, 1990; Burns, 1993a). For example, Burns *et al.* (1990) sampled mussels along a contamination gradient

in Hamilton Harbour, Bermuda, and measured concentrations of aromatic and polar hydrocarbons bioaccumulated by the mussels. By integrating the data with a parallel biological measurement programme (Widdows *et al.*, 1990), these authors demonstrated that the polar oxidation products of hydrocarbons were accumulated by mussels in quantities sufficient to induce a significant reduction in the SFG. In a recent study, Thomas *et al.* (1995) demonstrated that whilst the aliphatic UCM isolated from a lubricating oil was non-toxic to mussel feeding rate, oxidation of the aliphatic UCM (using a chemical oxidation technique which produced compounds somewhat analogous to those produced by microbial degradation) resulted in the formation of products of sufficient aqueous solubility to act as narcotic toxicants. Similar oxidation products are thought to be present in used engine lubricating oils, owing to the high temperatures to which the oil is exposed during operation of the engine. Used engine oil enters the marine environment in large quantities each year (Vazquez-Duhalt, 1989; *cf.* Section 1.3) and UCM oxidation products may also be contaminants of environmental concern (Burns, 1993a).

The analysis of petroleum hydrocarbons in environmental matrices is complicated by the broad range of volatilities, molecular weights and polarities of compounds present in fossil fuels and their related degradation products. There is no standard method for the quantification of such contaminants in marine ecosystems and consequently, a range of analytical methodologies are employed (Farrington *et al.*, 1988; Burns, 1993b). Although determinations of hydrocarbons in the marine environment are usually based upon common analytical steps, *i.e.* organic solvent extraction, column chromatographic separation and hydrocarbon detection and identification; variations in equipment and solvents used in the extraction step and columns used in chromatographic separations have led to large variations in reported environmental hydrocarbon concentrations (Awad, 1981). For example, the

International Council for the Exploration of the Sea (ICES) conducted an intercomparison exercise on the determination of petroleum hydrocarbons in mussel homogenate (Farrington *et al.*, 1988). Subsamples of mussel homogenate prepared from *Mytilus edulis* sampled near a municipal sewer outfall were randomly distributed to approximately 50 laboratories world-wide. No particular analytical method was specified, owing to considerable controversy about which parameters to measure in assessing petroleum contamination. However, the use of UV-fluorescence spectroscopy, HPLC, GC or GC-MS for measurement of hydrocarbons after extraction and isolation was suggested. The results demonstrated a large variation in hydrocarbon concentrations reported by different laboratories. Relative standard deviations for reported data for *n*-alkanes, pristane and phytane determined by GC ranged from ± 67 to ± 104 % , with similar results noted for PAH. Intralaboratory variation was somewhat less, with precision of measurements in one laboratory for 5-8 subsamples of tissue homogenate ranging from ± 5-50 % relative standard deviation, depending on the parameter or compound being measured (Farrington *et al.*, 1988). Farrington *et al.* (1988) concluded that results from different laboratories should be compared with caution, and highlighted the need for a certified reference material.

A standard reference mussel homogenate (SRM 1974 [Organics in Mussel (*Mytilus edulis)*]) has since been prepared (Wise *et al.*, 1991), and provides certificated concentrations for only nine PAH based on the combination of measurements by GC-MS and reversed phase HPLC combined with fluorescence detection. However, such a limited range of analytes, covering a fairly narrow range in terms of volatility and polarity is insufficient for the needs of the present study.

2.2 Aims of the present study

Owing to the lack of reported data on environmental concentrations of aromatic UCMs, the aim of the work described in this Chapter was to provide a preliminary assessment of concentrations of aromatic UCMs bioaccumulated by mussels collected from U.K. coastal sites. The lower molecular weight compounds are most toxic to marine organisms owing to their greater aqueous solubility (*cf.* Sections 1.5 and 1.6). However, these compounds are often lost or poorly recovered in analytical schemes because of their volatility (Farrington *et al.*, 1988; Ali, 1994). Emphasis in the present study was therefore on optimum recovery of the lower molecular weight compounds. To achieve this, the study initially focused upon validation of a suitable analytical method. Once a suitable method was established, it was used to isolate and measure the aliphatic and aromatic UCM concentrations in mussels sampled from a small number of coastal locations around the U.K.

2.3 Development and validation of a method for the quantification of aliphatic and aromatic unresolved complex mixtures (UCMs) in mussel tissue

To ensure maximum recovery of lower molecular weight compounds during analysis of the field samples, a number of extraction methods were compared. This was achieved by spiking a range of standards into mussel homogenate and measuring the percentage recovery of the spike. Although a Standard Reference Material (SRM) validated for the measurement of trace organic contaminants is commercially available, SRM was not used for method validation in the present study because the range of compounds for which certificated values were provided in the SRM was too limited. The standard reference material (SRM 1974) provides certificated values for only nine PAH, the lowest molecular weight compound being phenanthrene. As the focus of the present study was to optimize recovery of the toxic lower molecular weight compounds, with much greater volatilities than phenanthrene *i.e.* alkylbenzenes, naphthalenes, and $< C_{12}$ alkanes, it was decided that use of the SRM mussel tissue would not provide the information necessary. Instead, a number of compounds covering a range of volatilities and polarities were spiked into mussel homogenate, and the percentage recovery of each compound, as obtained by a number of different extraction methods, compared.

A further aim of the method development was to extend the analytical method to identify and measure accurately polar hydrocarbon oxidation products bioaccumulated within mussels by GC-MS. Previously, Burns *et al.* (1990) used only UV-fluorescence, a semi-quantitative technique to identify the presence of significant quantities of polar hydrocarbon oxidation products in mussels sampled from Hamilton Harbour, Bermuda.

The following sections detail the authentic reference compounds used and discuss optimisation of each stage in the analytical scheme. Full experimental details are provided in Section 6.3.

2.3.1 Sample pre-treatment

Wet mussel tissue contains a significant amount of water which can complicate the extraction procedure and this must be removed from the sample prior to analysis. Typically this is achieved either by drying the sample in an oven or a freeze dryer. Alternatively, the wet tissue may be ground with anhydrous sodium sulphate prior to extraction.

The influence of sample pre-treatment upon the recovery of analytes of interest has been noted by a number of authors (*e.g.* Awad, 1981; Farrington *et al.*, 1988). For example, Farrington *et al.* (1988), when comparing hydrocarbon concentrations reported in the ICES intercomparison exercise noted that hydrocarbon concentrations reported for freeze dried mussel tissue were approximately half the concentrations reported for wet tissue homogenate. These authors attributed this result to the loss of volatile aromatic hydrocarbons such as alkylated benzenes, naphthalene and alkyl naphthalenes during the freeze drying process. Such compounds may also be lost during oven drying. Therefore in the present study, mussel tissue was extracted as wet homogenate, in order to maximise recovery of the lower molecular weight compounds.

2.3.2 Authentic compounds used for method validation

A variety of aliphatic, aromatic and polar compounds were chosen. Where available, compounds with structural moieties similar to those believed to be present within the UCM were chosen. The purity of each compound was monitored by GC and in each instance

found to be greater than 98 %. A list of compounds is presented in Table 2.1. A gas chromatogram of a mixture of the compounds is presented in Figure 2.1.

Compounds were dissolved in acetone and spiked into wet mussel homogenate, mixed thoroughly and left to equilibrate for 18 hours prior to extraction. Unfortunately, spiking standards into mussel homogenate in this manner probably does not represent all the ways in which contaminants are naturally sorbed into tissues. The latter can possibly best be simulated by incorporation into biological tissues through feeding or exposure studies, but such experiments are time consuming and can rarely be justified on the basis of determining extraction efficiency alone (Wells, 1993). Consequently, spiking of standards into mussel tissue followed by thorough mixing and an equilibration period prior to extraction was the best approximation to true incorporation of contaminants into mussel tissue that could be practicably achieved in the present study.

2.3.3 Sample concentration

The concentration/solvent removal step in any analytical method is a potential source of large losses and consequent variability in the recovery of analytes, particularly of the lower molecular weight, more volatile compounds. For example, low boiling point hydrocarbons such as naphthalene or *n*-decane can be lost selectively during solvent removal by rotary evaporation (Grob and Muller, 1987).

As these compounds are of considerable toxicological significance, the concentration step must be carefully controlled to ensure minimal loss. Ali (1994) reviewed numerous sample concentration methods and apparatus, and developed an optimized procedure for sample concentration using a micro-Kuderna Danish (K.D.) apparatus to

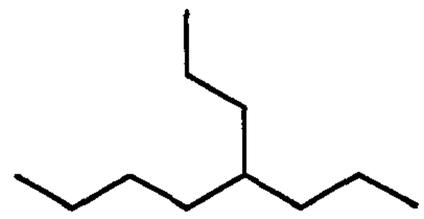
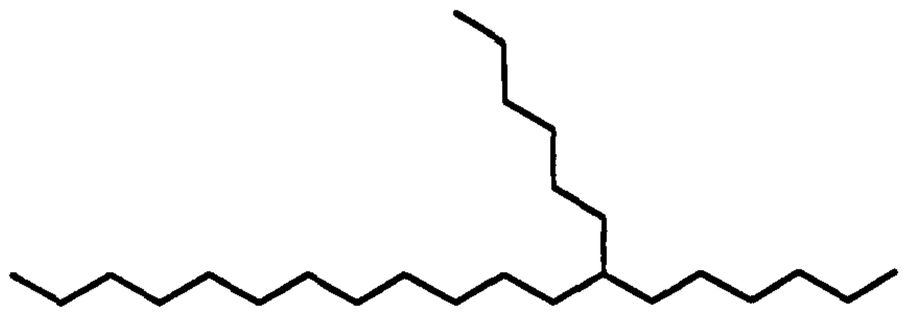
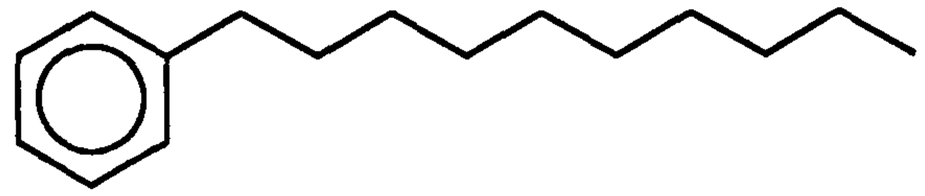
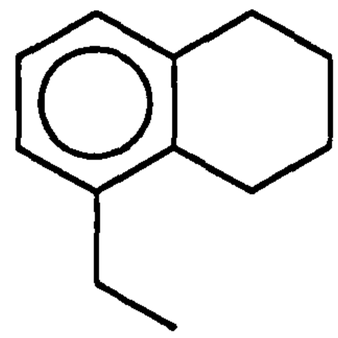
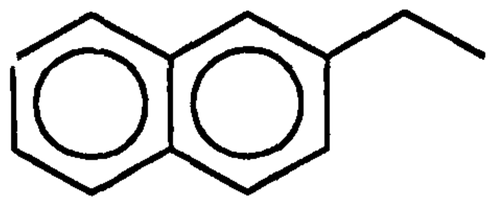
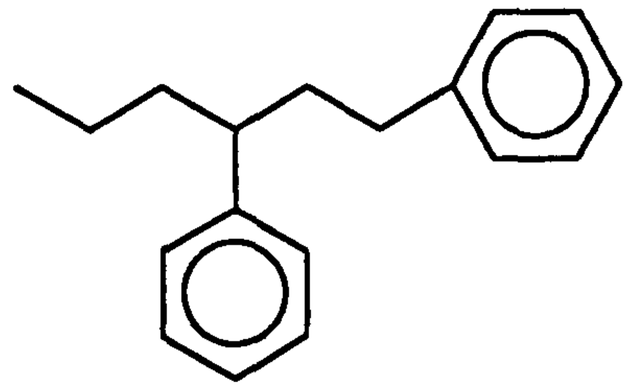
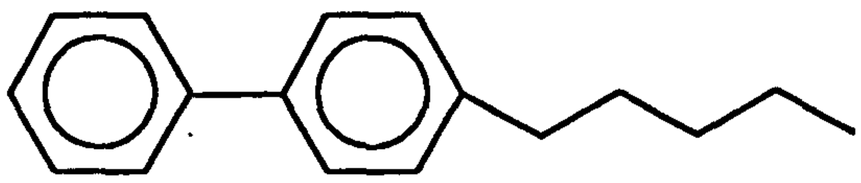
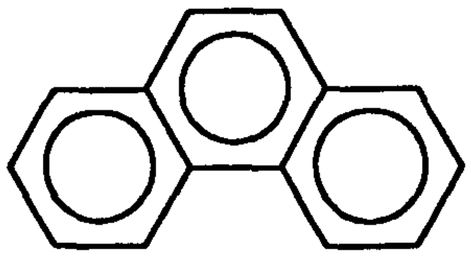
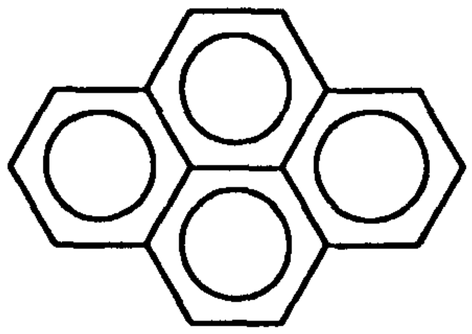
Compound	
4-propyloctane	
7-hexylnonadecane	
phenyldecane	
5-ethyltetralin	
2-ethylnaphthalene	
1,3-diphenylhexane	
4-pentylbiphenyl	
phenanthrene	
pyrene	

Table 2.1 Aliphatic and aromatic compounds used in method validation... *cont'd over*

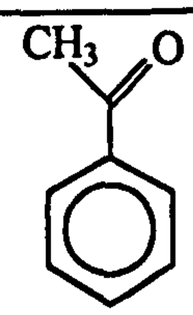
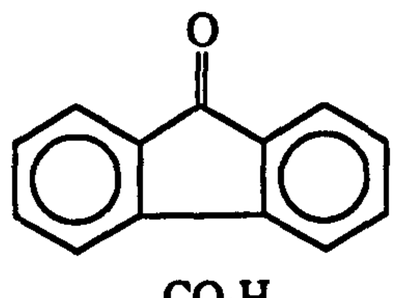
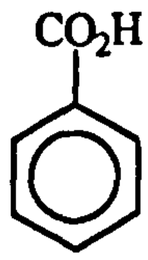
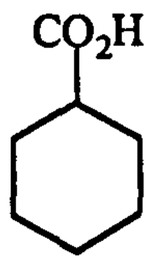
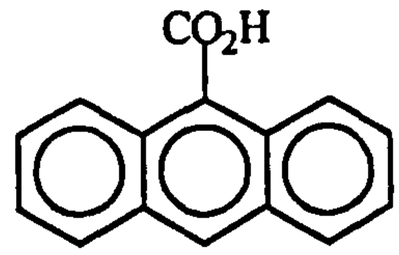
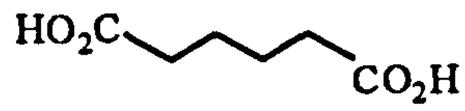
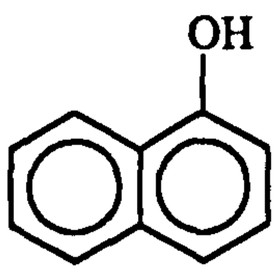
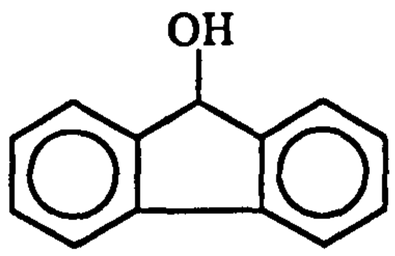
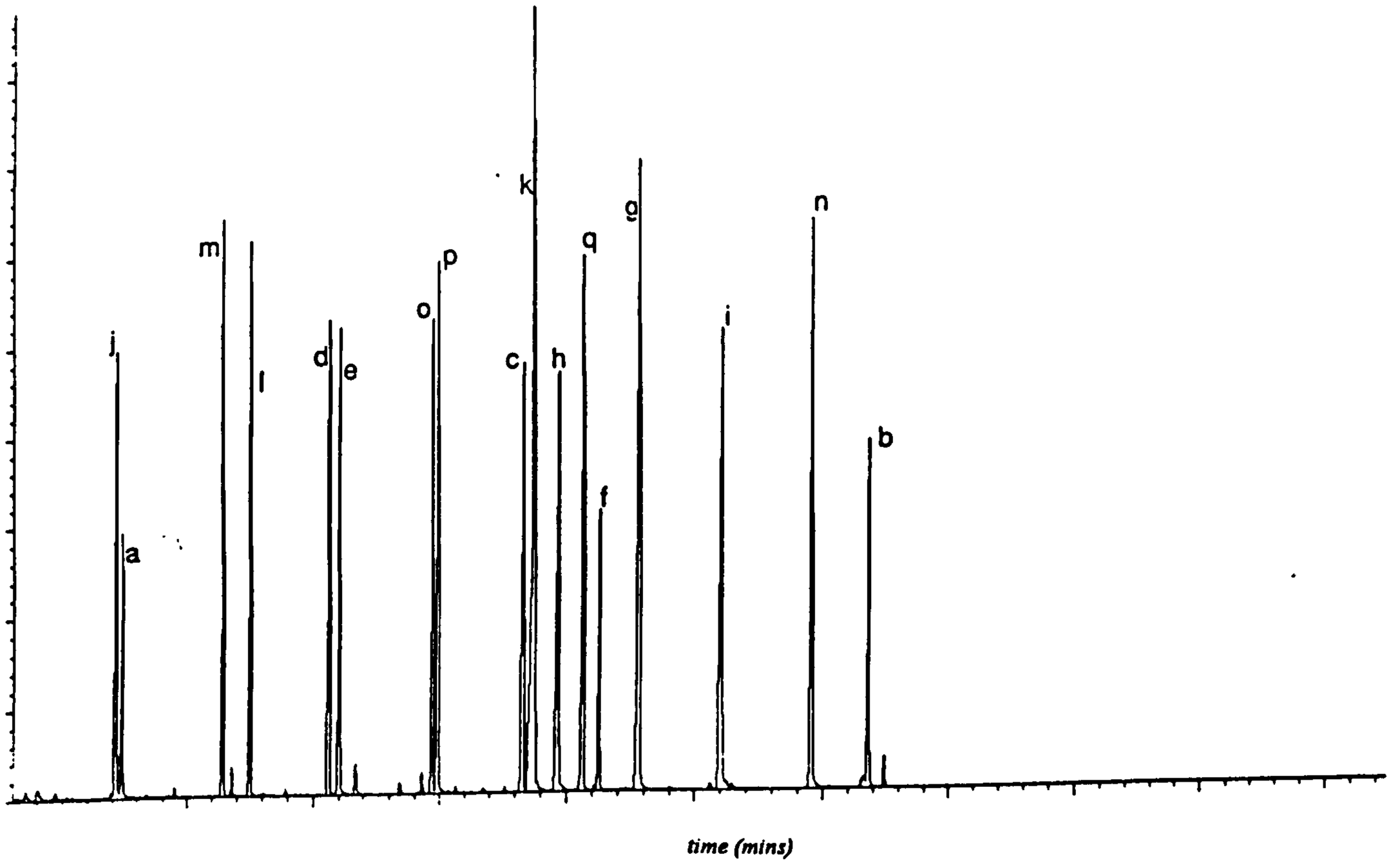
Compound	
acetophenone	
9-fluorenone	
benzoic acid	
cyclohexanecarboxylic acid	
9-anthracenecarboxylic acid	
hexanedioic acid	
1-naphthol	
9-hydroxyfluorene	

Table 2.1 cont'd. Polar compounds used in method validation



[GC details; HP-1(12m x 0.2mm i.d) column; He carrier gas; 40 - 300°C@ 5°C min^{-1}, hold 10 mins .]

Figure 2.1 Gas chromatogram of the mixture of authentic compounds used for method validation (acids present as trimethylsilyl esters; 1-naphthol and 9-hydroxyfluorene present as trimethylsilyl ethers)

Key;	Peak	Compound
	a	4-propyloctane
	b	7-hexylnonadecane
	c	phenyldecane
	d	5-ethyltetralin
	e	2-ethylnaphthalene
	f	1,3-diphenylhexane
	g	4-pentylbiphenyl
	h	phenanthrene
	i	pyrene
	j	acetophenone
	k	9-fluorenone
	l	benzoic acid
	m	cyclohexanecarboxylic acid
	n	9-anthracenecarboxylic acid
	o	hexanedioic acid
	p	1-naphthol
	q	9-hydroxyfluorene

produce reproducible recoveries of a wide range of volatile compounds, (*e.g.* 90 % for d_8-naphthalene; Ali, 1994), compared with severe losses by rotary evaporation.

Solvent removal by controlled evaporation using the K.D. concentrator was therefore the method of choice. However, as the sample volume was much larger in this study (*ca* 250 ml) than that of the optimised procedure (40 ml) of Ali (1994) it was necessary to compare the efficiency of several sample concentration techniques. These were macro-K.D. concentrator, rotary evaporation and rotary evaporation to a small volume (*ca* 35 ml) followed by controlled evaporation using the micro-K.D. concentrator. The methods were compared by spiking the standards (Table 2.1) into 250 ml dichloromethane (DCM) and concentrating the solution to a final volume of 0.5 ml. Percentage recoveries were then determined by GC. Full experimental details are provided in Section 6.4.3. Percentage recovery of the standards (mean ± relative standard deviation, rsd n=6) as obtained by each method are presented in Table 2.2.

No concentration of the sample was achieved after 48 h using the macro-K.D., although the optimised procedure described for the micro-K.D. apparatus was followed (*cf.* Ali, 1994). The use of this apparatus was clearly unsuitable.

Rotary evaporation gave reasonable recoveries for the majority of compounds (60.5% - 94.1 %). However, for the more volatile compounds 4-propyloctane, phenyldecane, 5-ethyltetralin and 2-ethylnaphthalene, the relative standard deviations are typically greater than 10 %. As illustrated in Table 2.2, recovery values were increased, and the variability in the percentage recoveries of the more volatile compounds reduced, by carefully controlling the final stages of concentration with the use of the micro-K.D. apparatus. Recovery values for the aliphatic and aromatic hydrocarbons by each sample concentration method were compared using a one-tailed t-test. This indicated that, with the exception of pyrene,

		% RECOVERY		
Compound	*amount spiked **	*Rotary evaporation*	*Rotary evap + micro K.D*	*macro K.D*
aliphatic				
4-propyloctane	4 µg	60.5 (19.5)	80.5 (7.3)	
7-hexylnonadecane	3 µg	91.9 (4.2)	100.2 (4.6)	
aromatic				
phenyldecane	3 µg	68.4 (6.7)	86.7 (7.3)	
5-ethyltetralin	6 µg	68.1 (13.1)	88.6 (5.6)	
2-ethylnaphthalene	3 µg	73.8 (10.5)	89.5 (8.3)	
1,3-diphenylhexane	3 µg	79.5 (9.3)	94.3 (4.8)	no
4-pentylbiphenyl	3 µg	86.7 (4.7)	95.5 (3.3)	concentration
phenanthrene	2 µg	88.4 (4.6)	97.0 (4.0)	achievable
pyrene	3 µg	94.1 (4.7)	97.3 (3.8)	after 48 h
polar				
acetophenone	4 µg	74.3 (7.5)	95.2 (5.4)	
9-fluorenone	5 µg	88.3 (9.2)	100.9 (6.0)	
benzoic acid	2 µg	77.1 (11.6)	93.4 (8.5)	
cyclohexanecarboxylic acid	2 µg	77.6 (17.2)	92.2 (4.7)	
9-anthracenecarboxylic acid	2 µg	81.6 (7.8)	97.1 (5.1)	
hexanedioic acid	3 µg	75.1 (11.9)	92.3 (11.1)	
1-naphthol	2 µg	74.1 (15.3)	95.9 (6.6)	
9-hydroxyfluorenone	2 µg	82.6 (9.2)	95.1 (5.1)	

values given as mean % recovery (figure in brackets is relative standard deviation, n=6)

** amount spiked into 250 ml DCM*

Table 2.2 Comparison of sample concentration methods

compound recoveries were significantly increased (99 % confidence limit) by the use of a micro-K.D. apparatus for the final stages of sample concentration when compared with rotary evaporation alone. Percentage recoveries were more variable for the polar compounds. However all recoveries were significantly increased at the 95 % confidence level by the use of the micro-K.D. apparatus. All solvent removal/sample concentration was therefore carried out by the method of rotary evaporation followed by micro-K.D. A summary of the raw data statistical test is provided in Appendix A.1

2.3.4 Extraction methods

Extraction techniques vary according to the requirements of the analyst and laboratory facilities available. Currently, the most commonly used techniques are Soxhlet, ultrasonic, blending, column percolation and more recently, supercritical fluid extraction. A comparison of these methods for the extraction of trace organic contaminants from sediments and biological tissue samples is presented in Table 2.3. The relative merits of each extraction method have been reviewed by Wells (1993).

Soxhlet extraction, usually using a fairly polar solvent system such as DCM is the most commonly used technique for the extraction of trace organic contaminants from sediments and biota, since this is a simple, yet efficient, method of extraction for the majority of compounds of interest. More recently, supercritical fluid extraction has become the method of choice where available. However, this extraction method was not available for the present study.

Donkin and Evans (1984) developed a steam distillation method specifically for the determination of lower molecular weight hydrocarbons in mussels. These authors reported recoveries in excess of 80 % for petroleum hydrocarbons in the volatility range encompassed

Method	Advantages	Disadvantages
Soxhlet	Multiple systems possible with banks of 6 or 12 Soxhlets; extraction is automatic once set up; hot extraction to improve recovery	Relatively large volumes of solvent used; loss of some volatiles unless efficient condensers are used; solvent penetration limited if the sample is not completely wetted; thermally labile compounds may decompose; solvent and extraction purity must be checked; thimbles require extraction before use
Supercritical fluid extraction	Relatively fast, depending on the temperature and pressure; uses non-toxic solvents; parallel extractions are possible; can be semi-automated; well controlled, reproducible conditions	SFE gas and modifier purity must be checked; high grade SF essential; limited size of extraction vessel; care on recovery from extraction vessel; losses can occur; blockages through restrictors
Blender/ultrasonication	Simple, inexpensive to purchase and operate; applicable to a wide range of biological tissue and sediments; ambient temperature	Labour intensive; difficult to automate; may not extract contaminants bound to tissue or sediment; separation of extract and matrix debris necessary; filters may clog; limited size of sample
Column percolation	Large sample size can be extracted; cold extraction can be used in parallel; low equipment cost	Very large volumes of solvent used; high solvent blank; labour intensive

Table 2.3 Comparison of the main methods of extraction of sediment and biological tissue for trace organic contaminants (from Wells, 1993)

by toluene to pyrene. However, recoveries of less volatile (>C_{20}) aliphatic hydrocarbons were less satisfactory (*ca* 40 %). Although the focus of the present study was to chose a method with minimal losses of lower molecular weight hydrocarbons, it was hoped to achieve this without compromising the recovery of larger molecules; therefore an alternative method was sought.

A number of different extraction methods were compared in this study and are described in the following sections. Since emphasis was on maximising the recovery of the more volatile lower molecular weight hydrocarbons, the micro-K.D apparatus was employed to minimize losses during solvent removal. Careful consideration was given therefore to the choice of solvents for extraction, as the K.D. apparatus is only suitable for use with low boiling point solvents such as DCM (bpt. 40°C) or *n*-pentane (bpt. 35°C-36°C).

2.3.4.1 Soxhlet extraction (DCM:Methanol)

Initial extractions were carried out by Soxhlet extraction using DCM:methanol (1:1). It was thought that this would prove efficient for the extraction of a wide range of compounds of different polarities. 'Spiked' tissue samples were ground with anhydrous sodium sulphate and Soxhlet extracted using DCM:methanol (1:1, v/v) solvent system for 24 h. As methanol is an unsuitable solvent for sample concentration using the Kuderna-Danish apparatus (bpt. 64.7°C), the extract was washed with acidified water immediately following soxhlet extraction to remove the methanol, and the total organic extract (TOE) dried over anhydrous sodium sulphate. The sample was then concentrated by controlled evaporation (rotary evaporation followed by micro Kuderna-Danish) and the solvent in which the TOE was dissolved, changed from DCM to *n*-hexane under a gentle stream of nitrogen. Experimental details are given in Section 6.3.4.

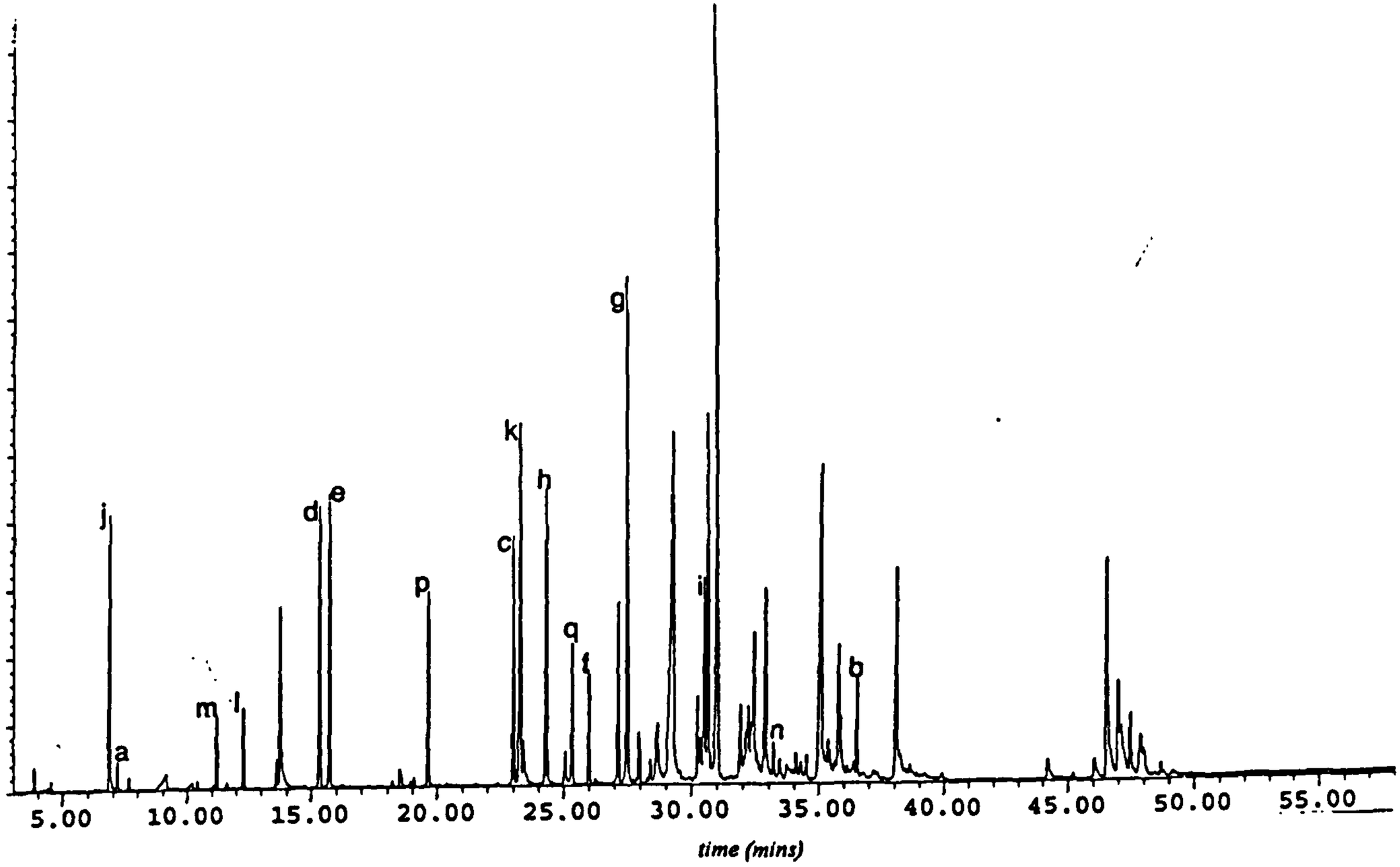
A gas chromatogram of the total organic extract (TOE) obtained (after derivatisation with BSTFA to yield the trimethylsilyl (TMS) derivatives) is illustrated in Figure 2.2. The percentage recovery for each compound, as measured by GC (*cf.* Section 6.3.7) is given in Table 2.4. Values are presented as the mean of six determinations, the standard deviation is given in brackets. Recoveries for the lower molecular weight, more volatile compounds are very low and quite variable. For example, 4-propyloctane (4-PO), a branched C_{11} alkane, has a recovery value of 18.8%, with a relative standard deviation of 22%. Recoveries of the polar compounds are also lower than expected and this is probably attributable to the water-washing stage where the polar compounds may have preferentially partitioned into the aqueous phase despite acidification of the water in order to minimize this.

As a result of the unsatisfactory recoveries obtained by this method, the use of methanol in the extraction procedure was eliminated, and Soxhlet extraction using only DCM was investigated.

2.3.4.2 Soxhlet extraction (DCM)

'Spiked' tissue samples were ground with anhydrous sodium sulphate and Soxhlet extracted with DCM for 24 h. The extract obtained was concentrated by controlled evaporation and the solvent system in which the extract was dissolved changed from DCM to *n*-hexane under a gentle stream of nitrogen (*cf.* Section 6.3.4.2).

The percentage recovery for each standard is given in Table 2.5. A gas chromatogram of the TOE (as TMS derivatives) obtained is presented in Figure 2.3. It is evident that recoveries of the lower molecular weight compounds are considerably higher than recoveries



[GC details; HP-1(12m x 0.2mm i.d) column; He carrier gas; 40 - 300°C@ 5°C min^{-1}, hold 10 mins].

Figure 2.2 Gas chromatogram of the total organic extract of spiked mussel homogenate obtained by DCM:MeOH Soxhlet extraction (acids as trimethylsilyl esters and alcohols as trimethylsilyl ethers).

Key;	Peak	Compound
	a	4-propyloctane
	b	7-hexylnonadecane
	c	phenyldecane
	d	5-ethyltetralin
	e	2-ethylnaphthalene
	f	1,3-diphenylhexane
	g	4-pentylbiphenyl
	h	phenanthrene
	i	pyrene
	j	acetophenone
	k	9-fluorenone
	l	benzoic acid
	m	cyclohexanecarboxylic acid
	n	9-anthracenecarboxylic acid
	o	hexanedioic acid
	p	1-naphthol
	q	9-hydroxyfluorene

Peaks not labelled are naturally occurring lipids

peak	*compound*	*amount spiked**	*[1]mean % recovery*	*RSD*
	aliphatic			
a	4-propyloctane	1 µg	18.8 (4.1)	22.0
b	7-hexylnonadecane	1 µg	88.8 (4.1)	4.7
	aromatic			
c	phenyldecane	3 µg	56.4 (4.3)	7.6
d	5-ethyltetralin	2 µg	68.2 (3.5)	5.2
e	2-ethylnaphthalene	2 µg	69.8 (3.8)	5.4
f	1,3-diphenylhexane	1 µg	71.2 (1.8)	2.5
g	4-pentylbiphenyl	3 µg	77.5 (2.3)	3.0
h	phenanthrene	3 µg	82.2 (2.2)	2.6
i	pyrene	3 µg	92.0 (2.8)	3.0
	polar			
j	acetophenone	4 µg	67.0 (3.4)	5.1
k	9-fluorenone	4 µg	80.1 (5.8)	7.3
l	benzoic acid	2 µg	29.0 (5.0)	17.3
m	cyclohexanecarboxylic acid	2 µg	32.1 (3.2)	9.9
n	9-anthracenecarboxylic acid	2 µg	58.0 (4.4)	7.6
o	hexanedioic acid	1 µg	n.d	-
p	1-naphthol	2 µg	45.6 (3.2)	7.1
q	9-hydroxyfluorene	1 µg	41.1 (3.1)	7.6

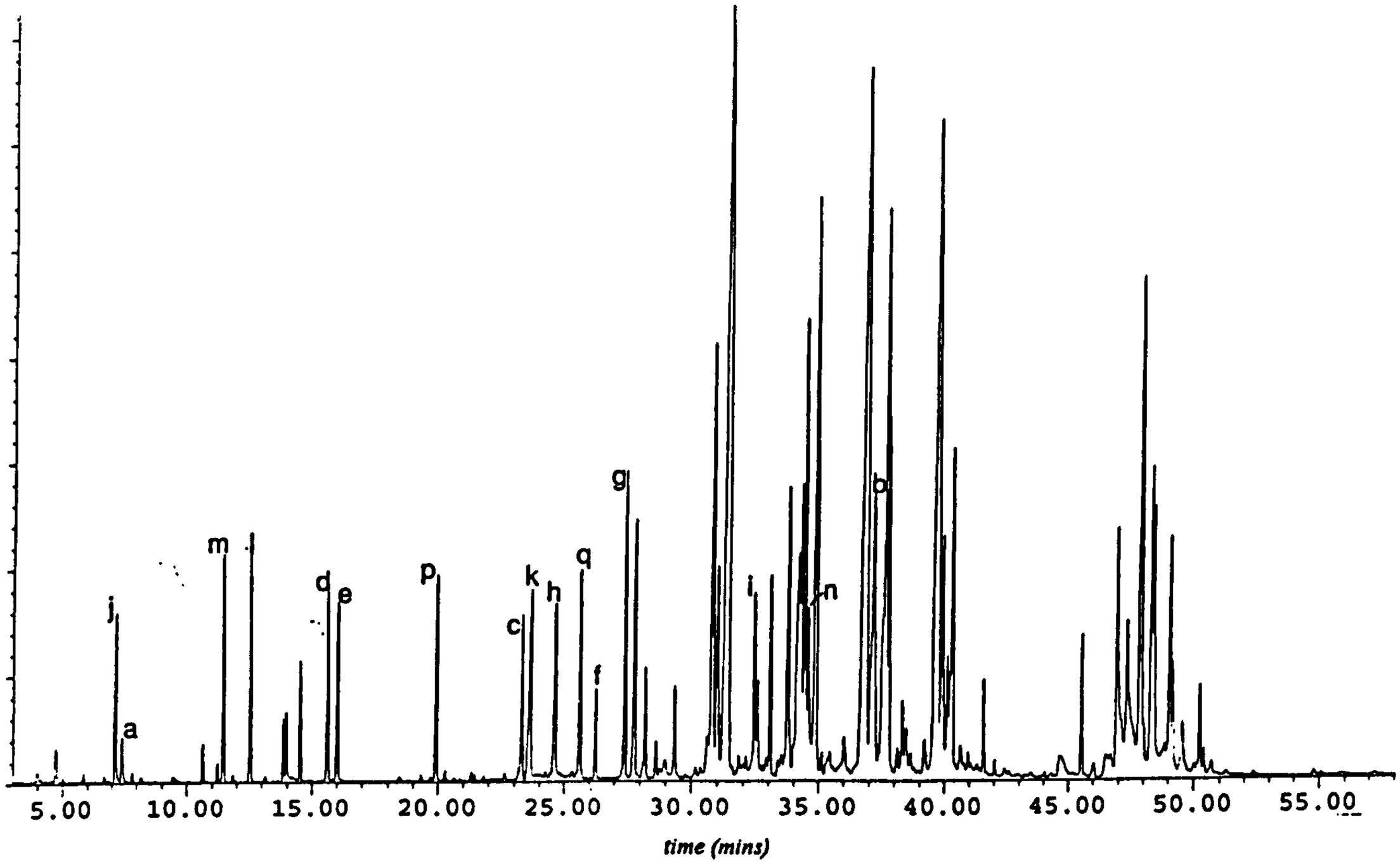
[1]values given as mean of six replicates, standard deviation in brackets

n.d = not detected

RSD = relative standard deviation

** amount spiked into approximately 50 g wet weight tissue homogenate*

Table 2.4 Relative extraction efficiency (*i.e.* percentage recovery of spiked compound) of Soxhlet extraction using DCM:Methanol



[GC details; HP-1(12m x 0.2mm i.d) column; He carrier gas; 40 - 300°C@ 5°C min^{-1}, hold 10 mins .]

Figure 2.3 Gas chromatogram of the total organic extract of spiked mussel homogenate obtained by DCM Soxhlet extraction (acids present as trimethylsilyl esters; 1-naphthol and 9-hydroxyfluorene present as trimethylsilyl ethers)

Key,	Peak	Compound
	a	4-propyloctane
	b	7-hexylnonadecane
	c	phenyldecane
	d	5-ethyltetralin
	e	2-ethylnaphthalene
	f	1,3-diphenylhexane
	g	4-pentylbiphenyl
	h	phenanthrene
	i	pyrene
	j	acetophenone
	k	9-fluorenone
	l	benzoic acid
	m	cyclohexanecarboxylic acid
	n	9-anthracenecarboxylic acid
	o	hexanedioic acid
	p	1-naphthol
	q	9-hydroxyfluorene

peak	*compound*	*amount spiked**	*mean[1] % recovery*	*RSD*
	aliphatic			
a	4-propyloctane	1 µg	39.7 (5.7)	14.4
b	7-hexylnonadecane	1 µg	94.4 (2.9)	3.1
	aromatic			
c	phenyldecane	3 µg	58.8 (5.3)	9.0
d	5-ethyltetralin	2 µg	68.7 (1.7)	2.4
e	2-ethylnaphthalene	2 µg	69.5 (2.5)	3.6
f	1,3-diphenylhexane	1 µg	80.3 (2.7)	3.4
g	4-pentylbiphenyl	3 µg	82.9 (3.4)	4.1
h	phenanthrene	3 µg	83.9 (2.3)	2.7
i	pyrene	3 µg	91.5 (4.8)	5.2
	polar			
j	acetophenone	4 µg	69.8 (2.6)	3.7
k	9-fluorenone	4 µg	80.4 (4.6)	5.8
l	benzoic acid	2 µg	70.6 (4.8)	6.8
m	cyclohexanecarboxylic acid	2 µg	70.9 (5.1)	7.2
n	9-anthracenecarboxylic acid	2 µg	74.5 (3.8)	5.1
o	hexanedioic acid	1 µg	n.d	-
p	1-naphthol	2 µg	71.5 (2.6)	3.6
q	9-hydroxyfluorene	1 µg	68.7 (5.4)	7.8

[1] values given as mean of six replicates, standard deviation in brackets

n.d = not detected

RSD = relative standard deviation

** amount spiked into approximately 50 g wet weight tissue homogenate*

Table 2.5 Relative extraction efficiency (*i.e.* percentage recovery of spiked compound) of Soxhlet extraction using DCM

obtained with the previous method (DCM:methanol), again suggesting that major losses of compounds were occurring during the water washing stage. For the majority of compounds, recoveries are good and correspond to values reported in the literature for similar compounds (*ca* 70-90 %). However, there are still large losses of the volatile compounds such as 4-propyloctane, phenyldecane, 5-ethyltetralin and 2-ethylnaphthalene (*e.g.* 39.7 % recovery for 4-propyloctane). This may be attributed to the necessity of changing the polar solvent system in which the extract is dissolved (DCM) to a non-polar solvent (*e.g.* *n*-hexane) prior to fractionation by column chromatography. The evaporation of DCM is usually carried out under a gentle stream of nitrogen. However, the process has two inherent problems. Firstly it is difficult to ascertain at what point all of the DCM has been removed, and, secondly, as illustrated here, the more volatile compounds are subject to significant and variable losses under the nitrogen stream. To overcome this problem, it is desirable to use a method which would yield the TOE in a non-polar solvent and thus eliminate the need for a 'solvent exchange' step.

Rhead *et al.* (1971) used a two phase extraction method (Dole and Meinhertz, 1960) using a ternary *n*-heptane:propan-2-ol:water mixture to successfully extract lipids from wet sediment samples. As this method yields the analytes of interest in a non-polar solvent (*n*-heptane), it appears to be particularly suited to the requirements of the present study.

2.3.4.3 Two-phase extraction method (pentane:propan-2-ol:H_2O)

The method of Rhead *et al.* (1971) was modified in a number of ways. The original method involves sonication of the acidified tissue sample in a *n*-heptane:propan-2-ol mixture. Addition of a *n*-heptane:water mixture results in the formation of two phases, an aqueous layer and an organic layer containing the analytes of interest. In order to obtain the

TOE in a more volatile solvent which could be removed by controlled evaporation (micro-K.D.), *n*-pentane was substituted in place of *n*-heptane. It was also necessary to adjust the volume of each solvent added to produce an organic phase composed almost entirely of *n*-pentane. Figure 2.4 illustrates the approximate phase relations as a function of composition of the ternary system used. The relative volumes of the three liquid components determines the phase relationship at any one point. The curved line shows the transition from a one-phase to a two-phase system. Mixtures represented by points in the area below the curve form two phases with compositions indicated by intersections of the straight tie line containing the given point and the curved saturation line.

Dole and Meinhertz (1960) advocated a solvent composition close to the centre of the triangle, as below this area pure ternary systems separate more slowly, and in the presence of biological materials the systems tend to form stubborn emulsions. However, using a solvent composition close to the centre of the triangle would yield the TOE in an *n*-pentane/propan-2-ol mixture. This is undesirable as propan-2-ol is a polar solvent with a high boiling point (82°C) unsuitable for use in the K.D. apparatus.

From the phase diagram it was predicted that a solvent ratio of 1:10:9 (propan-2-ol:*n*-pentane:water) would yield an organic phase of greater than 98 % pentane, which could be concentrated using controlled evaporation (micro-K.D). The initial ratio of propan-2-ol:*n*-pentane (4:1) was used (Rhead *et al.*, 1971) to ensure maximum extraction efficiency and the volumes of *n*-pentane and water adjusted to produce the desired solvent ratio. As predicted, an emulsion did form initially after the addition of the *n*-pentane/water mixture, however this was overcome by centrifugation to yield clean separation of the two phases. Full experimental details are given in Section 6.3.4.3.

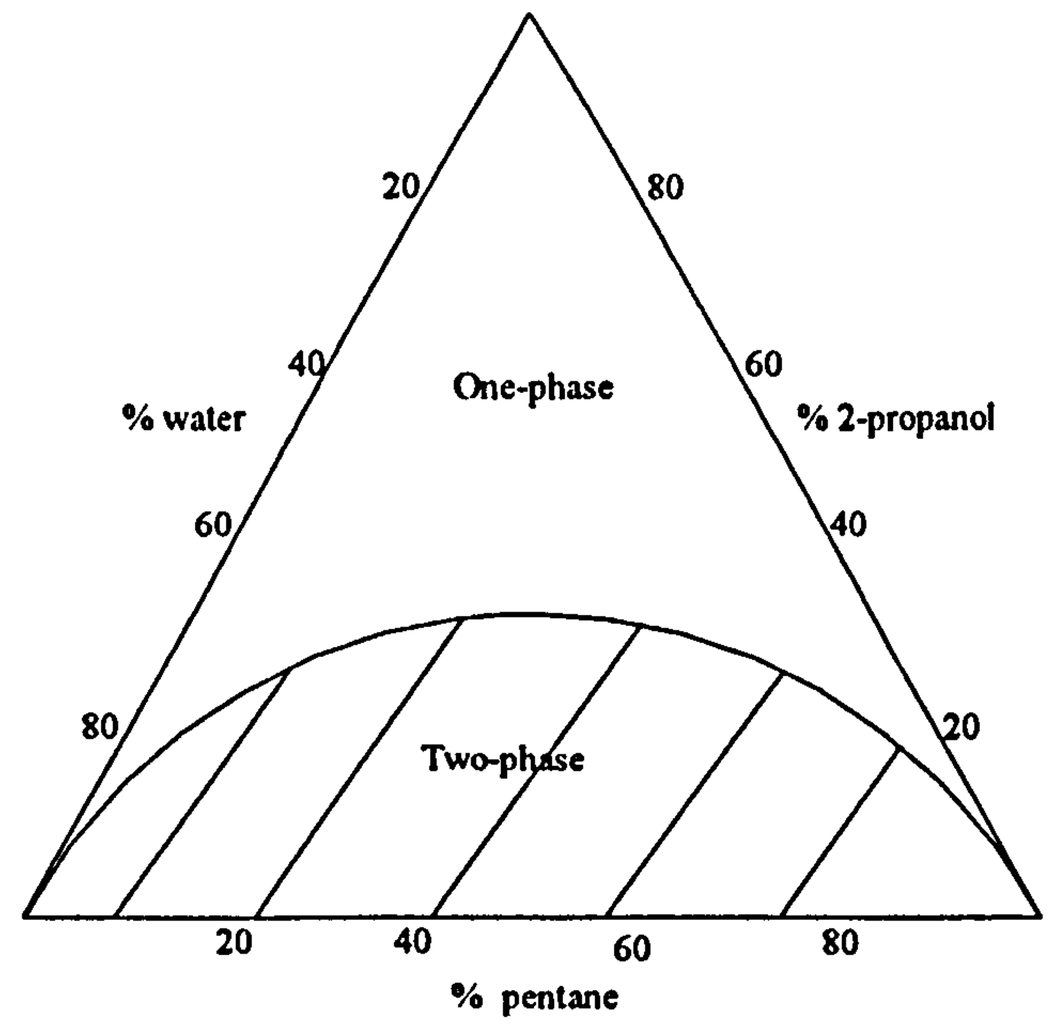
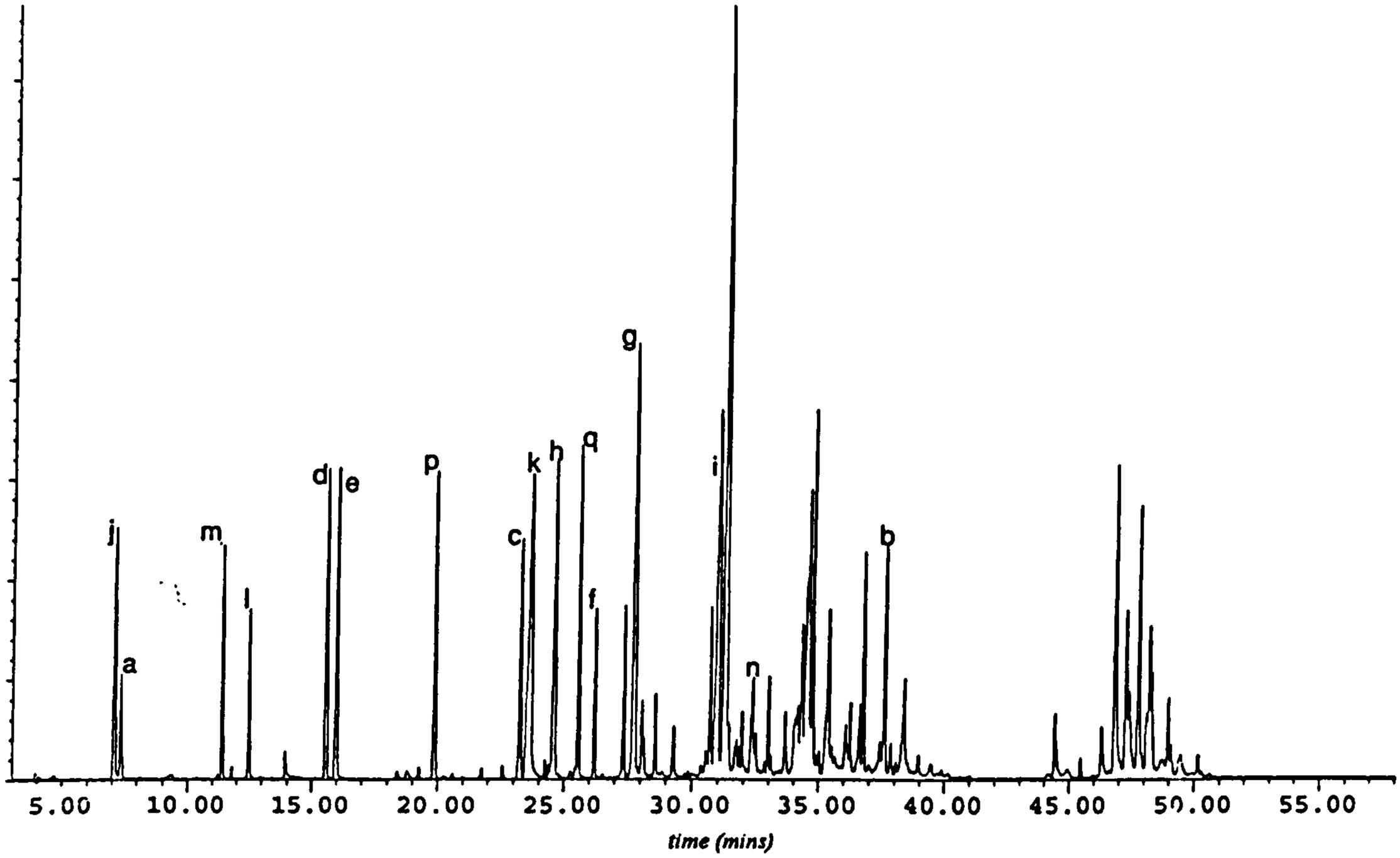


Figure 2.4 An approximate mapping of the phase relations (as a function of composition) of the ternary system *n*-pentane:2-propanol:water. Adapted from Rhead *et al.*(1971)

A gas chromatogram of the TOE (as TMS derivatives) is presented in Figure 2.5. Percentage recoveries for each compound are listed in Table 2.6. Recovery values obtained using the two-phase extraction method for the aliphatic and aromatic hydrocarbons (with the exception of 7-hexylnonadecane and pyrene) are significantly greater (P=0.05; t-test, one tailed) than the recovery values obtained by DCM soxhlet extraction. However, no significant increase was observed for the polar compounds with the use of the two-phase extraction method.

Figure 2.6 compares the percentage recoveries (plotted as mean ± 95 % confidence intervals) as obtained by each extraction method. It is evident that, for the lower molecular weight hydrocarbons, the highest recovery values are obtained using the two-phase extraction method. With regards to the less volatile compounds, recovery values are similar for both Soxhlet extraction using DCM and the two-phase extraction method.



[GC details; HP-1(12m x 0.2mm i.d) column; He carrier gas; 40 - 300°C@ 5°C min^{-1}, hold 10 mins .]

Figure 2.5 Gas chromatogram of the total organic extract of spiked mussel homogenate obtained by the two phase extraction method (acids present as trimethylsilyl esters; 1-naphthol and 9-hydroxyfluorene present as trimethylsilyl ethers)

Key;	Peak	Compound
	a	4-propyloctane
	b	7-hexylnonadecane
	c	phenyldecane
	d	5-ethyltetralin
	e	2-ethylnaphthalene
	f	1,3-diphenylhexane
	g	4-pentylbiphenyl
	h	phenanthrene
	i	pyrene
	j	acetophenone
	k	9-fluorenone
	l	benzoic acid
	m	cyclohexanecarboxylic acid
	n	9-anthracenecarboxylic acid
	o	hexanedioic acid
	p	1-naphthol
	q	9-hydroxyfluorene

peak	*compound*	*amount spiked**	*mean[1] % recovery*	*RSD*
	aliphatic			
a	4-propyloctane	1 µg	57.7 (3.4)	6.0
b	7-hexylnonadecane	1 µg	93.8 (2.8)	3.0
	aromatic			
c	phenyldecane	3 µg	70.9 (2.1)	3.0
d	5-ethyltetralin	2 µg	74.5 (1.7)	2.3
e	2-ethylnaphthalene	2 µg	75.0 (1.9)	2.5
f	1,3-diphenylhexane	1 µg	88.6 (1.8)	2.0
g	4-pentylbiphenyl	3 µg	87.5 (1.8)	2.0
h	phenanthrene	3 µg	90.5 (2.1)	2.3
i	pyrene	3 µg	94.1 (3.7)	3.9
	polar			
j	acetophenone	4 µg	62.5 (4.6)	7.4
k	9-fluorenone	4 µg	94.9 (3.1)	3.3
l	benzoic acid	2 µg	70.3 (4.1)	5.9
m	cyclohexanecarboxylic acid	2 µg	66.8 (6.0)	8.9
n	9-anthracenecarboxylic acid	2 µg	71.6 (5.0)	7.0
o	hexanedioic acid	1 µg	n.d	-
p	1-naphthol	2 µg	67.7 (5.3)	7.8
q	9-hydroxyfluorene	1 µg	68.4 (6.7)	9.8

[1] values given as mean of 6 replicates, standard deviation in brackets
n.d = not detected

RSD = relative standard deviation

** amount spiked into approximately 50 g wet weight tissue homogenate*

Table 2.6 Relative extraction efficiency (*i.e.* percentage recovery of spiked compound) of two-phase extraction method

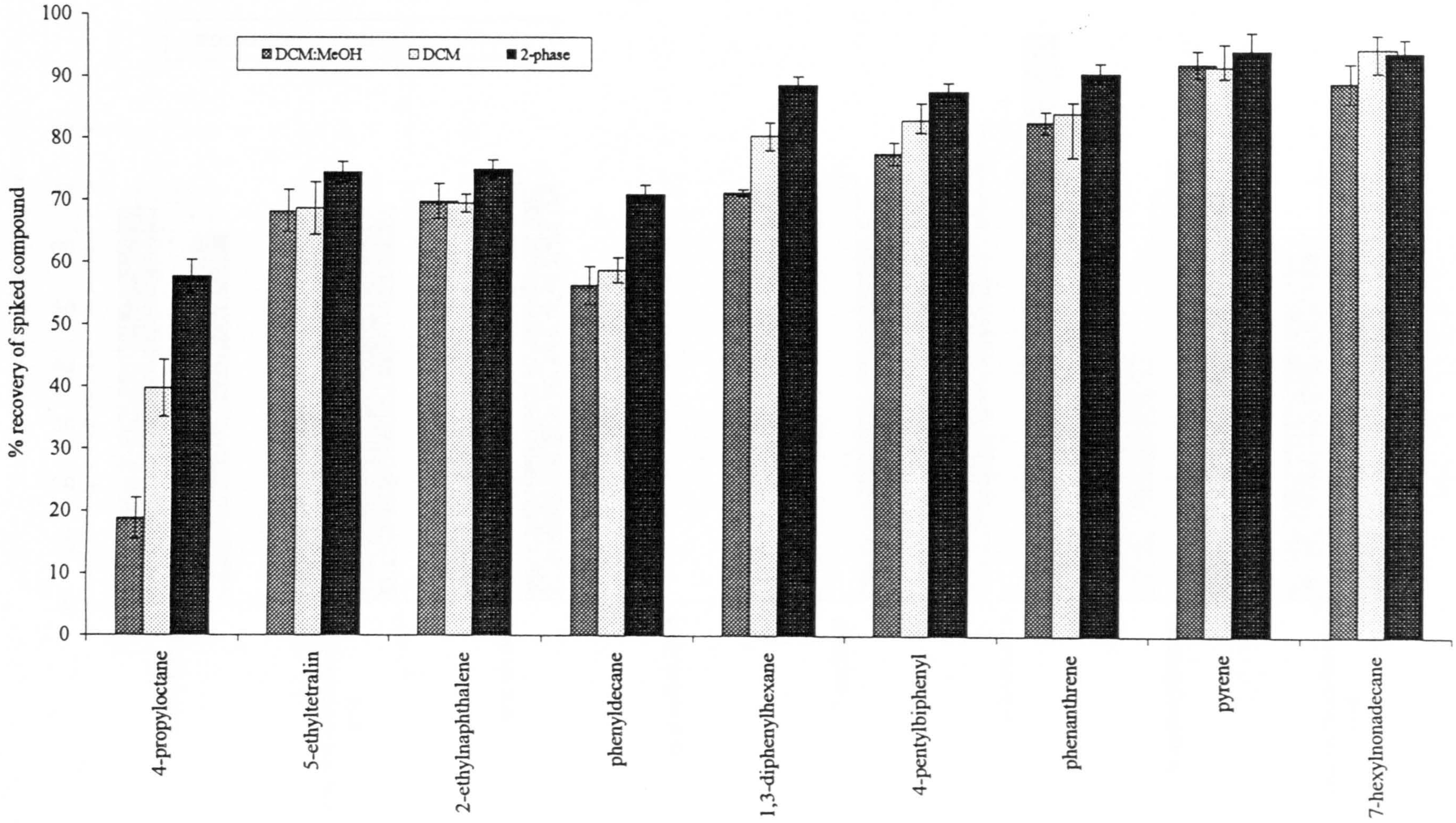


Figure 2.6a Comparison of % recovery of hydrocarbons spiked into mussel tissue as obtained by various extraction methods
values presented as mean ± 95 % confidence intervals (n=6)

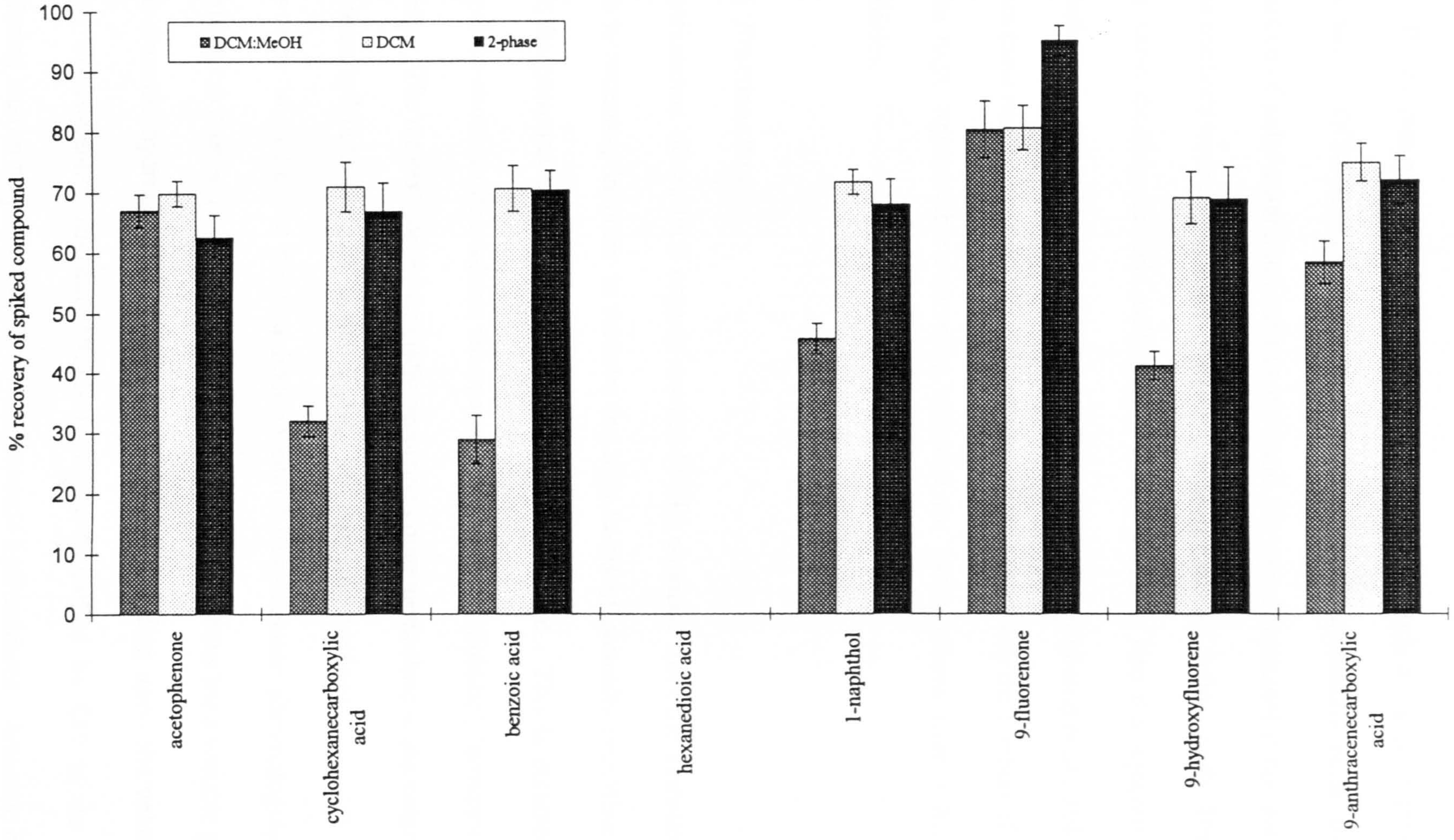


Figure 2.6b Comparison of % recovery of polar compounds spiked into mussel tissue, as obtained by various extraction methods
values presented as mean ± 95 % confidence intervals (n=6)

The extraction method using DCM:methanol yielded the lowest, least reproducible values for all compounds. As mentioned previously, this is most likely owing to the water washing stage. Recoveries of the polar compounds used in this study are greater than 70 % when extracted by DCM Soxhlet, suggesting that DCM is a sufficiently polar solvent for the extraction of polar compounds of this type and the use of methanol is not necessary. None of the methods were successful in extracting hexanedioic acid (adipic acid). However such a short chain dicarboxylic acid will most probably partition into the aqueous phase of an extraction mixture and could be determined separately (*e.g.* Eglinton *et al.*, 1987).

From these results, the two-phase extraction method is clearly the method of choice owing to the high, reproducible recoveries obtained for hydrocarbons over a broad range of volatilities.

2.3.5 Fractionation

Fractionation of the total organic extract (TOE) obtained from the extraction of mussel tissue is necessary in order to remove the high levels of naturally occurring lipids which mask the presence of any petroleum hydrocarbons present. This is commonly achieved using silica-alumina open column chromatography to yield 'aliphatic', 'aromatic' and 'polar' fractions. The majority of the naturally occurring compounds elute in the polar fraction, the petroleum hydrocarbons elute in the aliphatic and aromatic fractions.

The term 'aliphatic' for the first fraction eluted from column chromatography is purely operational as it usually contains not only aliphatic hydrocarbons but a variable proportion of mono-aromatic hydrocarbons, the relative amounts depending upon the volume of eluent used. Consequently, aliphatic UCMs routinely quantified by GC in the majority of monitoring studies may also include mono-aromatic hydrocarbons. Aromatic hydrocarbons

have greater aqueous solubilities than aliphatic hydrocarbons of comparable molecular weight and are more toxic to marine organisms. It is desirable, therefore, to separate, as completely as possible, the aliphatic and mono-aromatic hydrocarbons in order to assess the contribution of each group of compounds to the total 'toxic hydrocarbon body burden'.

Recently, Thomas (1995) has demonstrated that the aromatic UCM is composed principally of mono- and di-aromatic alkyl substituted compounds (*cf.* Section 1.2). This is in agreement with the results of a study by Killops and Readman (1985) who fractionated a number of sediment extracts into aromatic hydrocarbon fractions according to the number of double bond equivalents. These authors found that the bulk of the aromatic UCM was present in the mono- (four double bond equivalents) and di-aromatic ring fractions. Since it is these smaller aromatic compounds (*i.e* mono- and di-aromatic compounds) that are generally the most toxic to mussels (*e.g.* Donkin *et al.*, 1989, 1991), it is important to determine the composition of the aromatic UCM present within mussels. Current chemical-biological monitoring programmes (Widdows *et al.*, 1990, 1995a,b; discussed in Section 1.5) focus upon the two- and three-ring aromatic hydrocarbons as the toxicologically important fractions and tend to ignore the presence of mono-aromatic hydrocarbons, grouping these compounds in the 'non-toxic aliphatic' fraction. Thus, many current monitoring schemes fail to quantify what could be the largest group of aromatic hydrocarbons present. In order to investigate this, various column chromatographic methods were compared herein to obtain the best separation of aliphatic and mono-aromatic compounds. The aromatic fraction obtained from column chromatography was then further fractionated into mono-, di- and tri-aromatic fractions by HPLC in order to assess the contribution of each group of hydrocarbons to the total aromatic body burden.

Fractionation of organic extracts by column chromatography is a well established technique and, as such, the aim of the work described in this section was only to optimise separation of the aliphatic/mono-aromatic hydrocarbon fractions of mussel extracts. This can be achieved by varying the ratio of sample to adsorbent and/or activity of the chromatographic adsorbents used. Typically, the sample to be fractionated is applied to the top of a silica/alumina column and the column sequentially eluted with solvents of increasing polarity (*e.g.* *n*-hexane, DCM, methanol) to obtain aliphatic, aromatic and polar fractions respectively. In the present study, *n*-pentane was used to obtain the first fraction (aliphatic), because of its suitability for use in the K.D. apparatus.

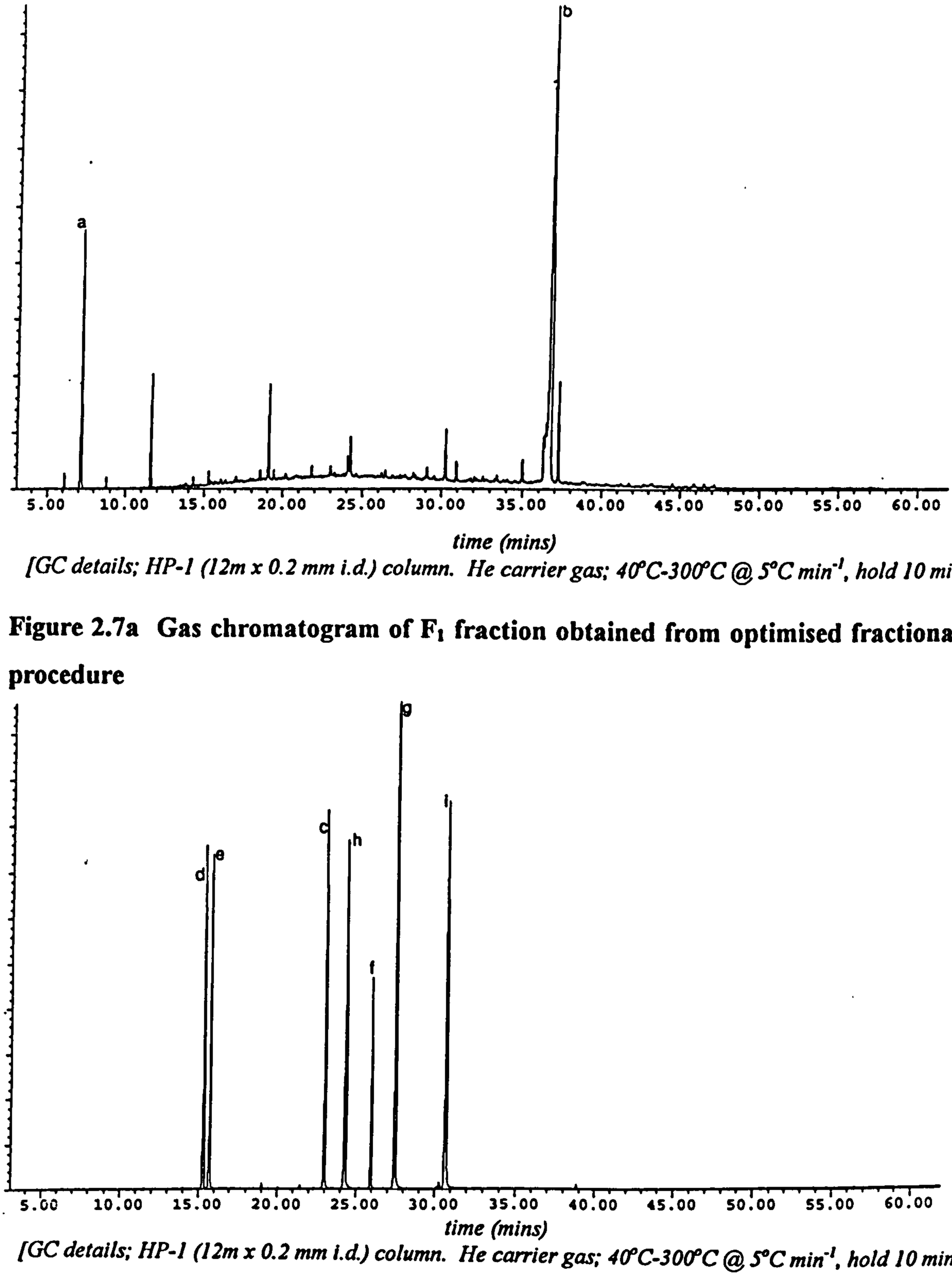
Mussel TOE containing the authentic reference compounds was fractionated using a silica :alumina (5% water deactivated and 1.5% deactivated respectively) column (1:100, sample:adsorbent ratio). The aliphatic fraction (F_1) was obtained by elution with *n*-pentane (1.5 column volumes). Elution with *n*-pentane:DCM (1:1, v/v; 2 column volumes) yielded the aromatic (F_2) fraction. Polar fractions were obtained by elution with DCM (F_3) and DCM:methanol (F_4), (full experimental details are given in Section 6.3.5). Solvent was removed by controlled evaporation and samples analysed by GC and GC-MS. Examination of the F_1 fraction indicated that no separation of aliphatic and mono-aromatic compounds had been achieved. Phenyldecane, 5-ethyltetralin and a proportion of 2-ethylnaphthalene had eluted from the column in the F_1 or 'aliphatic' fraction. This may be attributed to the fact that the TOEs of mussel tissue contain large amounts of naturally occurring lipids which increase the polarity of the sample, thereby reducing the separation of the non-polar aliphatic and aromatic components.

The sample to adsorbent ratio was increased to 1:200 (5% deactivated silica/1.5% deactivated alumina, 1:1) in an attempt to improve the separation. However, although the

separation improved slightly, a significant amount of the mono-aromatic standards still eluted in the F_1 fraction. Increasing the sample:adsorbent ratio further is impractical with respect to the size of column and quantity of adsorbent required. Consequently, the effect of increasing the activity of the adsorbents was investigated. Silica deactivated by 5% with water was replaced with fully activated silica (activated at 180°C, 18 h, *cf.* Section 6.1). An excellent, clean separation was obtained as illustrated in Figure 2.7a and b.

The aromatic fraction was further fractionated into mono-, di- and tri-aromatic ring groups by normal phase HPLC, using a slight modification of the method of Killops and Readman (1985; *cf.* Section 6.3.6) in order to assess the composition (in terms of number of aromatic rings) of the aromatic UCM and the contribution of the aromatic UCM to the total 'toxic hydrocarbon' body burden.

Analysis of the polar fractions F_3 and F_4 by GC revealed significant variation and losses on the recovery of the more polar compounds from the column. Whilst acetophenone and 9-fluorenone were present in the F_3 fraction, the amounts were highly variable and subject to interference from the naturally occurring lipids present. With respect to the more polar acids and alcohols, results were erratic and compounds were often not recovered from the column. As the focus of this study was primarily upon aliphatic and aromatic hydrocarbons, the problems associated with fractionation of the polar compounds were not pursued further. However, analysis of polar compounds present in mussel tissue is clearly an interesting area for future work.



[GC details; HP-1 (12m x 0.2 mm i.d.) column. He carrier gas; 40°C-300°C @ 5°C min^{-1}, hold 10 mins]

Figure 2.7a Gas chromatogram of F_1 fraction obtained from optimised fractionation procedure

[GC details; HP-1 (12m x 0.2 mm i.d.) column. He carrier gas; 40°C-300°C @ 5°C min^{-1}, hold 10 mins]

Figure 2.7b Gas chromatogram of F_2 fraction obtained from optimised fractionation procedure

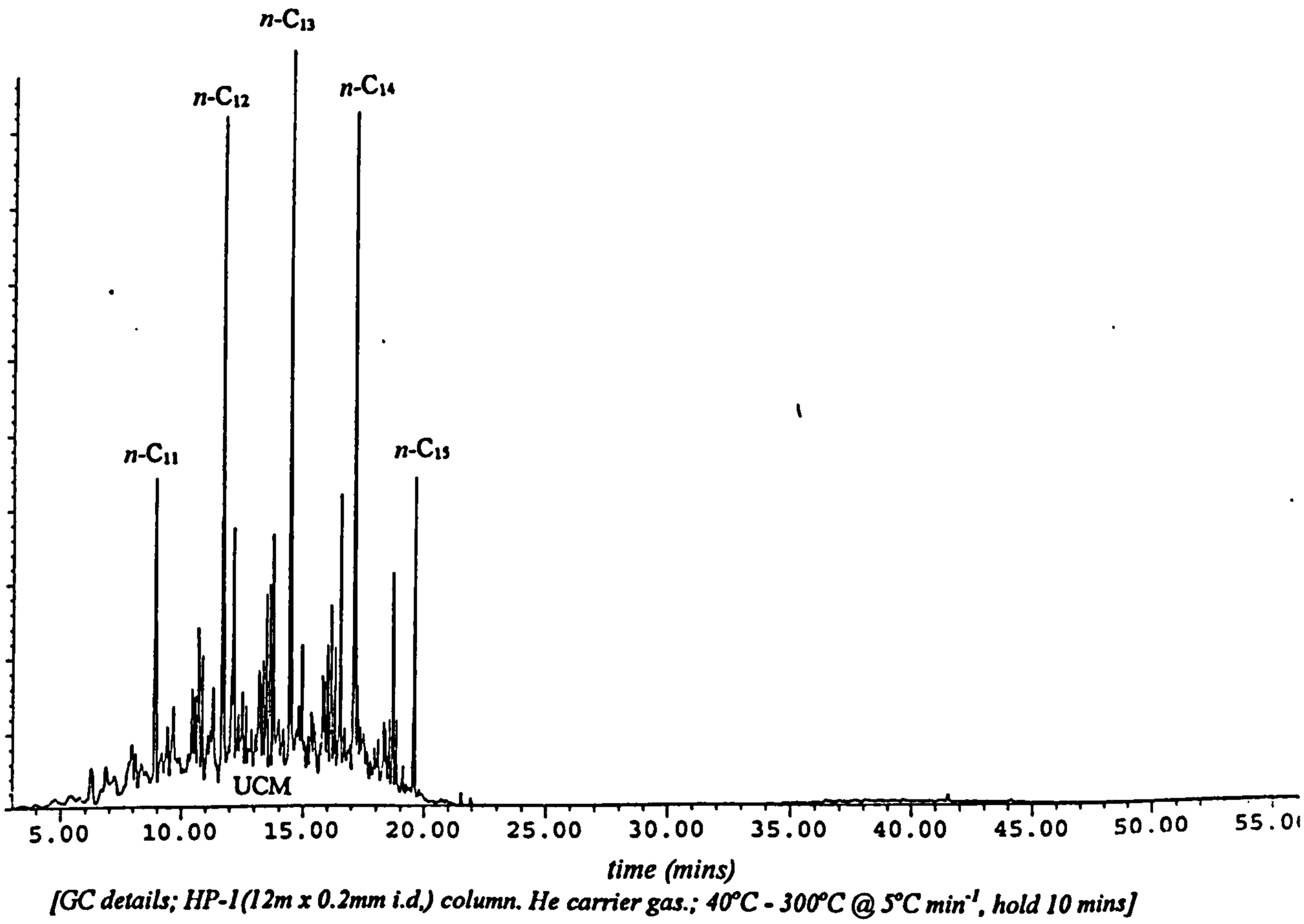
Internal standards; A; 4-propyloctane: B; 7-hexylnonadecane: C; d_{12}-tetralin:

D: d_{10}-1 -methylnaphthalene-d_{10}; E:d_{10}- phenanthrene

2.3.6 Recovery of a low molecular weight UCM spiked into mussel tissue

In order to further validate the method developed for UCM recovery, mussel homogenate was spiked with a lubricating oil (Mobil Velocite) and percentage recovery of the UCM in both the aliphatic and aromatic fractions measured. This oil was chosen as it consists (>55 %) of a low molecular weight UCM (Figure 2.8).

Mussel tissue was spiked with a solution of the oil and a range of internal standards (4-propyloctane, 7-hexylnonadecane, d_{12}-tetralin, d_{10}-1-methylnaphthalene and d_{10}-phenanthrene). The tissue was extracted by the two-phase extraction method and fractionated according to the optimised method.



[GC details; HP-1(12m x 0.2mm i.d,) column. He carrier gas.; 40°C - 300°C @ 5°C min^{-1}, hold 10 mins]

Figure 2.8 Gas chromatogram of Mobil Velocite oil

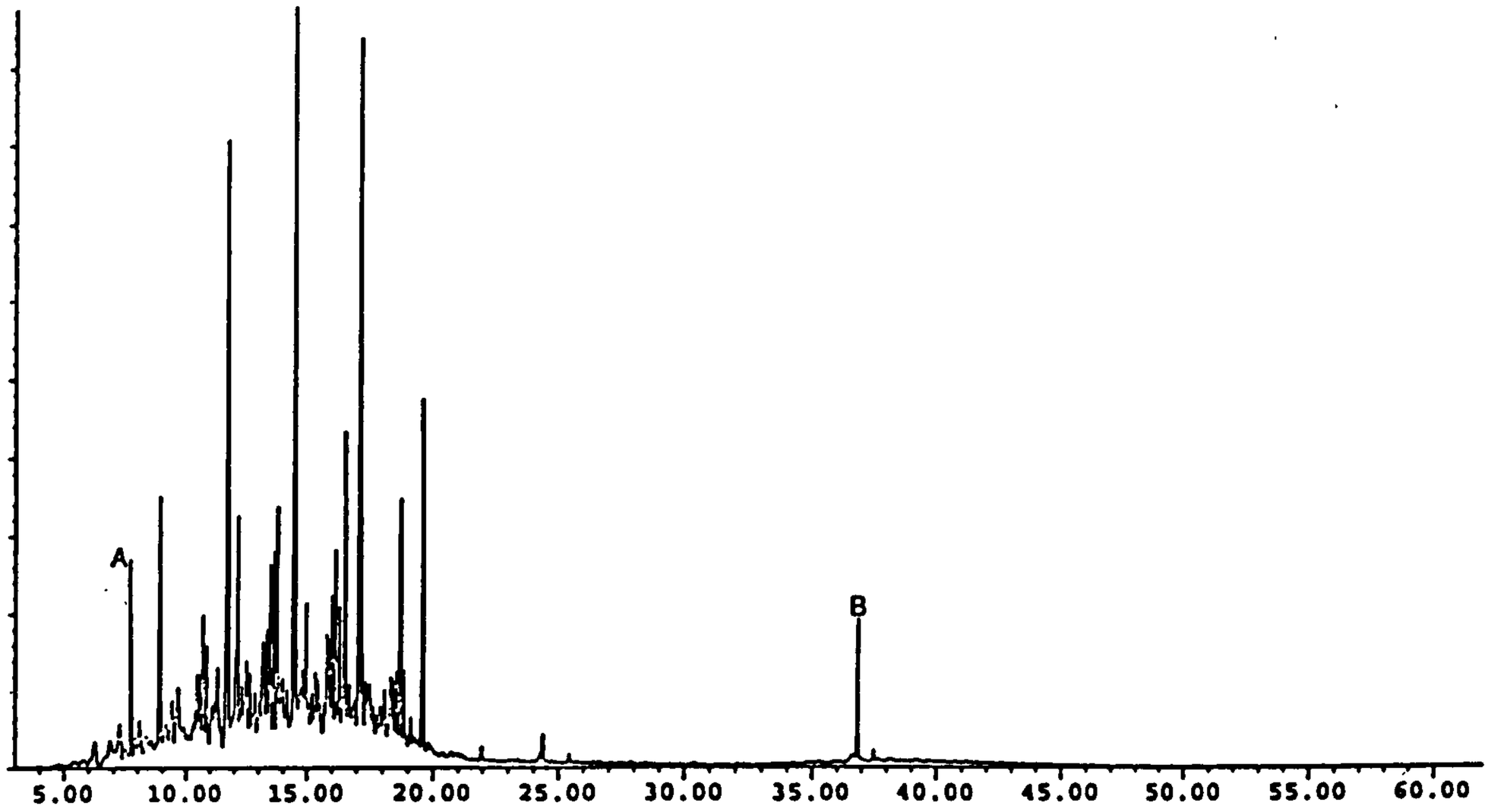
Chromatograms of the F_1 and F_2 fractions are presented in Figures 2.9a and b, respectively. Percentage recoveries of both the internal standards spiked into the mussel tissue and recovery of the UCM (as F_1+F_2) are given in Table 2.7. Recoveries of both the internal standards and the low MW UCM were approximately 70 %. This is reasonable given that the UCM has a low MW range (*ca* *n*-C_{11} - *n*-C_{18}) and is therefore relatively volatile.

2.3.7 Quantification of analytes

Quantification of both authentic compounds and resolved and unresolved hydrocarbon concentrations in mussel tissue was performed by GC-MS using Chemstation (Hewlett Packard) software). Full details are presented in Section 6.3.9

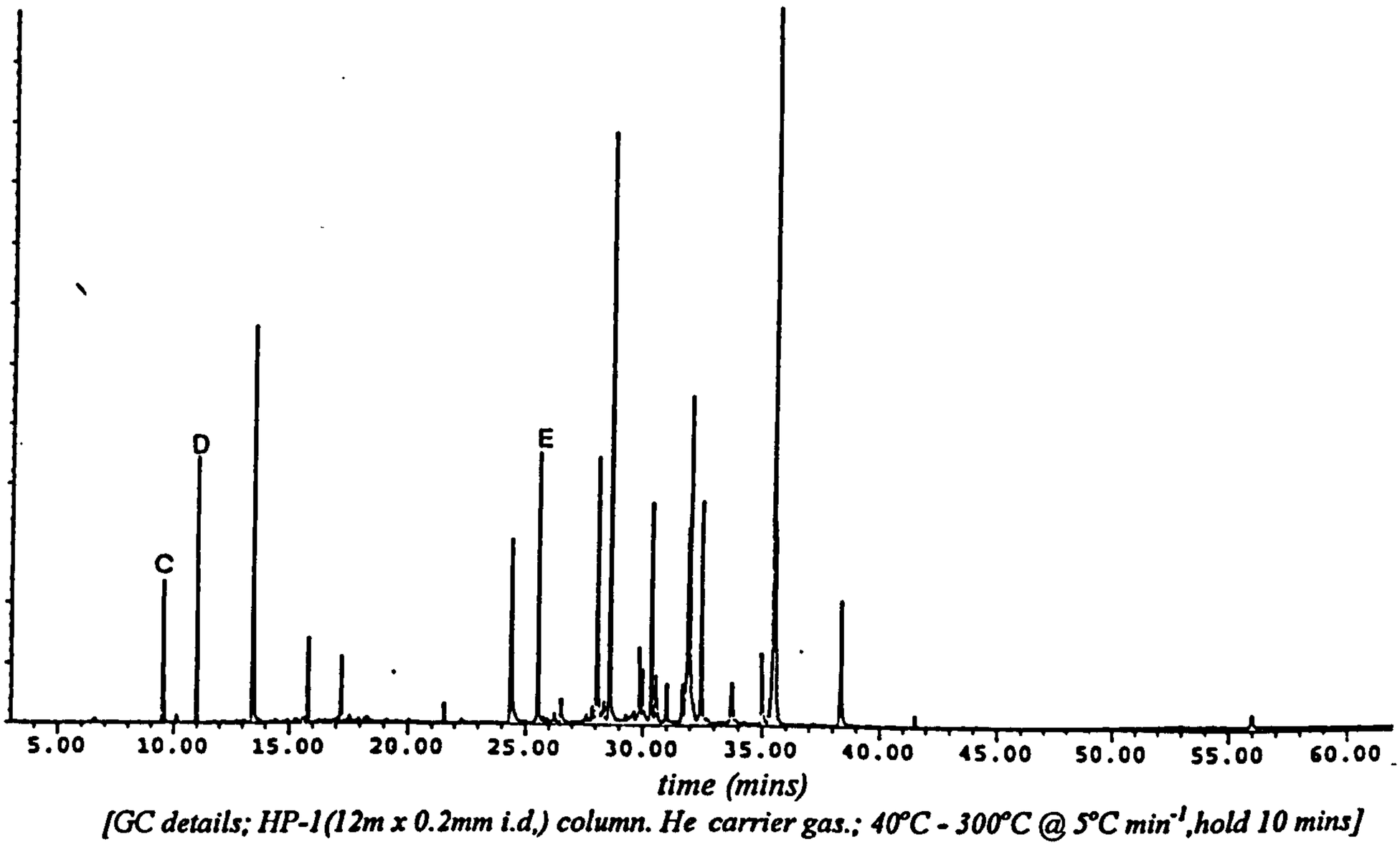
	% recovery (duplicate determinations)
UCM (as F_1 + F_2)	70.1 80.5
4-propyloctane	48.1 64.7
7-hexylnonadecane	90.3 97.1
d_{12}-tetralin	65.8 76.0
d_{10}-1 -methylnaphthalene	70.1 74.7
d_{10}-phenanthrene	82.6 90.8

Table 2.7 Percentage recovery of low MW UCM and internal standards spiked into mussel tissue.



[GC details; HP-1(12m x 0.2mm i.d,) column. He carrier gas.; 40°C - 300°C @ 5°C min^{-1}, hold 10 mins]

Figure 2.9a Gas chromatogram of F_1 (aliphatic) fraction of mussel tissue spiked with Mobil Velocite



[GC details; HP-1(12m x 0.2mm i.d,) column. He carrier gas.; 40°C - 300°C @ 5°C min^{-1},hold 10 mins]

Figure 2.9b Gas chromatogram of F_2 (aromatic) fraction of mussel tissue spiked with Mobil Velocite

Internal standards; A; 4-propyloctane: B; 7-hexylnonadecane: C; d_{12}-tetralin:

D: d_{10}-1 -methylnaphthalene-d_{10}; E;d_{10}- phenanthrene

2.3.8 Conclusions

A reproducible method has been developed and validated for the analysis and quantification of petroleum hydrocarbons in mussel tissue. By carefully controlling the final stages of sample concentration using the micro-Kuderna Danish apparatus the recovery of low molecular weight aliphatic and aromatic hydrocarbons has been significantly increased when compared with rotary evaporation, the most commonly employed method of sample concentration. In addition, the loss of low molecular weight analytes of interest has been minimized by the development of an extraction method which yields the analytes of interest in a non-polar solvent, thereby eliminating the need for changing the solvent prior to fractionation. This method enables good recoveries of the low molecular weight, toxicologically more significant hydrocarbons without compromising recovery of higher molecular weight hydrocarbons which are useful compounds in environmental monitoring schemes for assessing the source of petroleum hydrocarbons.

In the following section, the optimised method was used to measure the aromatic and aliphatic hydrocarbon burdens of mussels (*M. edulis*) from a small number of coastal sites around the U.K.

2.4 Hydrocarbon unresolved complex mixtures (UCMs) in mussels (*Mytilus edulis*) from U.K. coastal sites

The method developed and validated in the previous section (Section 2.3) was used to isolate and measure both the aliphatic and aromatic UCMs in mussels from a small number of impacted sites in coastal locations around the U.K. The aim of the work described herein was not to establish a comprehensive inventory of aromatic UCMs (as that would involve a routine monitoring programme) but simply to establish whether oil-polluted mussels contain an aromatic UCM burden.

2.4.1 Sample sites

Sample sites along the North East coast of the U.K. were selected because this area has been extensively studied in an integrated biological (SFG in mussels) and chemical (aromatic hydrocarbons, organotins, organochlorines and metals) monitoring program (Widdows *et al.*, 1995b).

The sites were chosen to encompass both open water coastal sites and more enclosed locations such as harbours. A mussel population from the Mersey Estuary was also sampled as this area is known to be heavily contaminated with oil (Readman *et al.*, 1986; Davies and Wolff, 1990). Mussels from a location known to be relatively free from petroleum hydrocarbon contamination (Whitsand Bay, Cornwall; P. Donkin, personal communication, 1995) were also sampled and analysed. A map indicating the location of the sample sites is presented in Figure 2.10. Approximately 50 mussels were collected from each site and packed in ice boxes for transportation back to the laboratory.

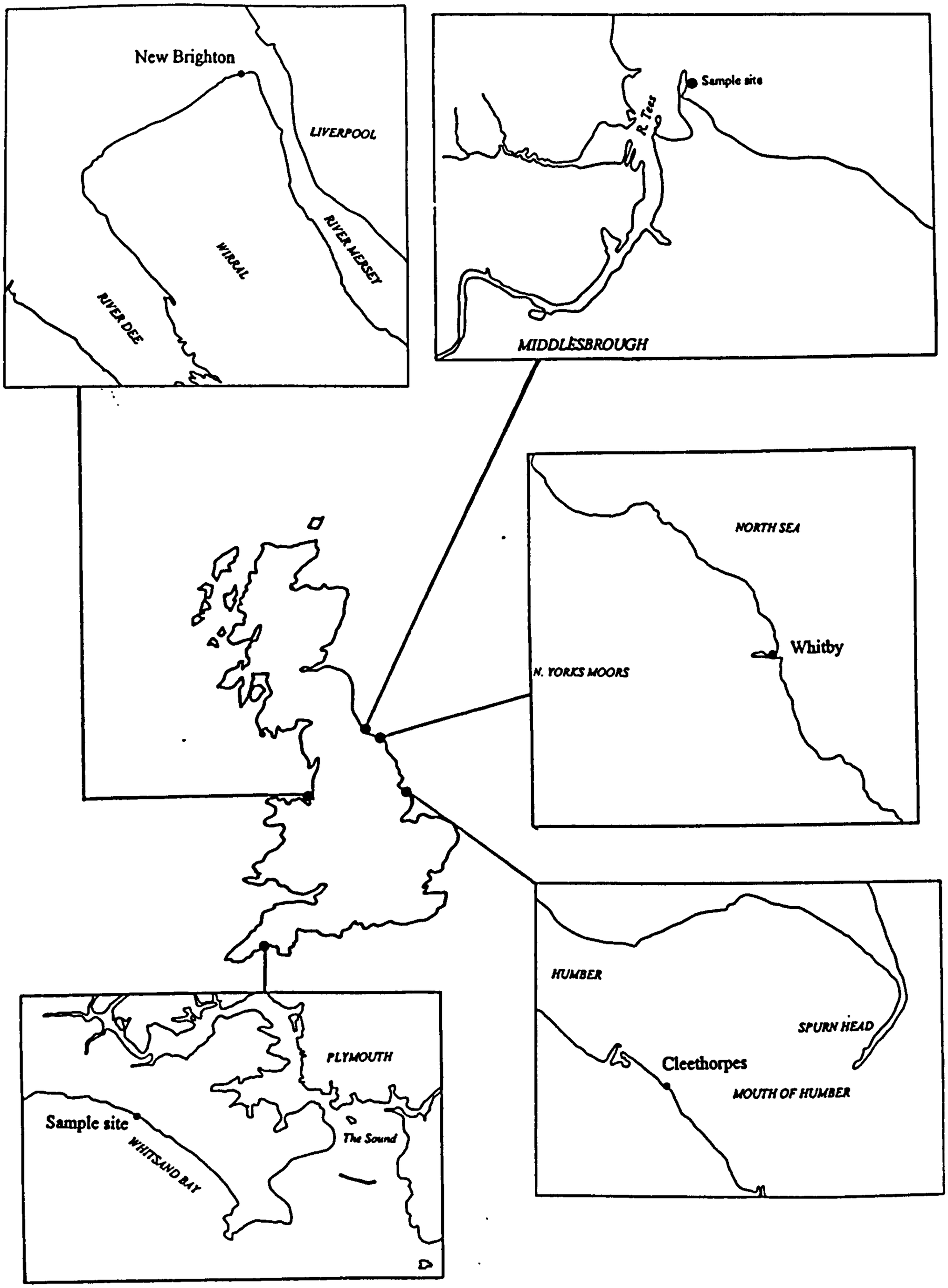


Figure 2.10 Location of sample sites
(not drawn to scale)

2.4.2 Experimental details

Soft tissue was dissected from the shells over ice to minimize losses of volatile analytes. The mussel tissue was then homogenised and stored in solvent rinsed, foil lined glass jars at -20 ^{0}C prior to analysis. Immediately prior to extraction, tissue samples were spiked with the following internal standards; 4-propyloctane (42 µg, synthesised); 7-hexylnonadecane (69 µg, synthesised); d_{12}-tetralin (34 µg, Aldrich); d_{10}-1-methylnaphthalene (63 µg, Aldrich); d_{10}-phenanthrene (61 µg; Aldrich).

Full details of the methods employed are provided in Section 6.3. In summary, approximately 30 g wet weight mussel tissue was extracted using the optimised two-phase extraction method discussed previously (Section 2.3.4.3). A procedural blank was carried out in parallel with each extraction. This involved spiking internal standards into an acidified *n*-pentane:propan-2-ol mixture, followed by extraction and sample concentration in the usual manner. Procedural blanks were monitored by GC.

The total organic extract (TOE) obtained was concentrated to *ca* 1 ml (controlled evaporation) and total extractable lipid (TEL) determined by gently evaporating 20 µl of TOE to dryness under a stream of nitrogen. TEL determinations were carried out in duplicate and a mean TEL value calculated. Dry weight of mussel tissue was determined for each sample by drying approximately 6 g mussel tissue for 48 h at 40°C. Wet to dry weight conversion factors were then calculated from the difference in mass. Dry weight determinations were performed in duplicate. Samples were fractionated by open column chromatography (*cf.* Section 2.3.5) and fractions F_1 and F_2 analysed by GC and GC-MS. The 'aromatic' fraction (F_2) was then further fractionated by normal phase HPLC using the method of Killops and Readman, (1985) to obtain mono-, di- and tri-aromatic fractions and each fraction again analysed by GC and GC-MS. Quantification of the total resolved and

total unresolved components in each fraction was made using an average response factor of the internal standards in each fraction. The area of the unresolved hydrocarbons was calculated by subtraction of the total area of resolved peaks from the total area of the resolved + unresolved peaks as described in Section 6.3.8.

2.4.3 Results and Discussion

The aim of the present study was to make a preliminary assessment of the distribution of aromatic UCMs in mussels around the U.K. coast. Gas chromatograms of the 'aliphatic'[1] (F_1) and 'aromatic'[1] (F_2) mussel fractions for each site are presented in Figures 2.11 - 2.15. The presence of an UCM in both the 'aliphatic' (F_1) and 'aromatic' (F_2) fractions is evident in mussels from all sample sites with the exception of those from Whitsand Bay. The concentrations of total resolved and unresolved hydrocarbons measured in both the aliphatic and aromatic fractions for each site are presented in Table 2.8. For ease of comparison all concentrations are expressed in terms of dry weight of tissue. This is the most common way of expressing similar results in the majority of published literature, and therefore allows easy comparison with the latter. However, it must be noted that the results and those in the literature should be compared with a certain degree of caution as differences in the analytical methodology employed can result in a wide variation in reported hydrocarbon concentrations (Awad, 1981: Farrington *et al.*, 1988). Concentrations in terms of wet tissue weight and total extractable lipid are provided in Appendices B.1 and B.2.

As shown in Table 2.8, total hydrocarbon concentrations measured in this study range from 16 μg g^{-1} dry weight tissue (Whitsand Bay), representative of a relatively uncontaminated site, up to *ca* 4000 μg g^{-1} dry weight tissue (Whitby Harbour). These

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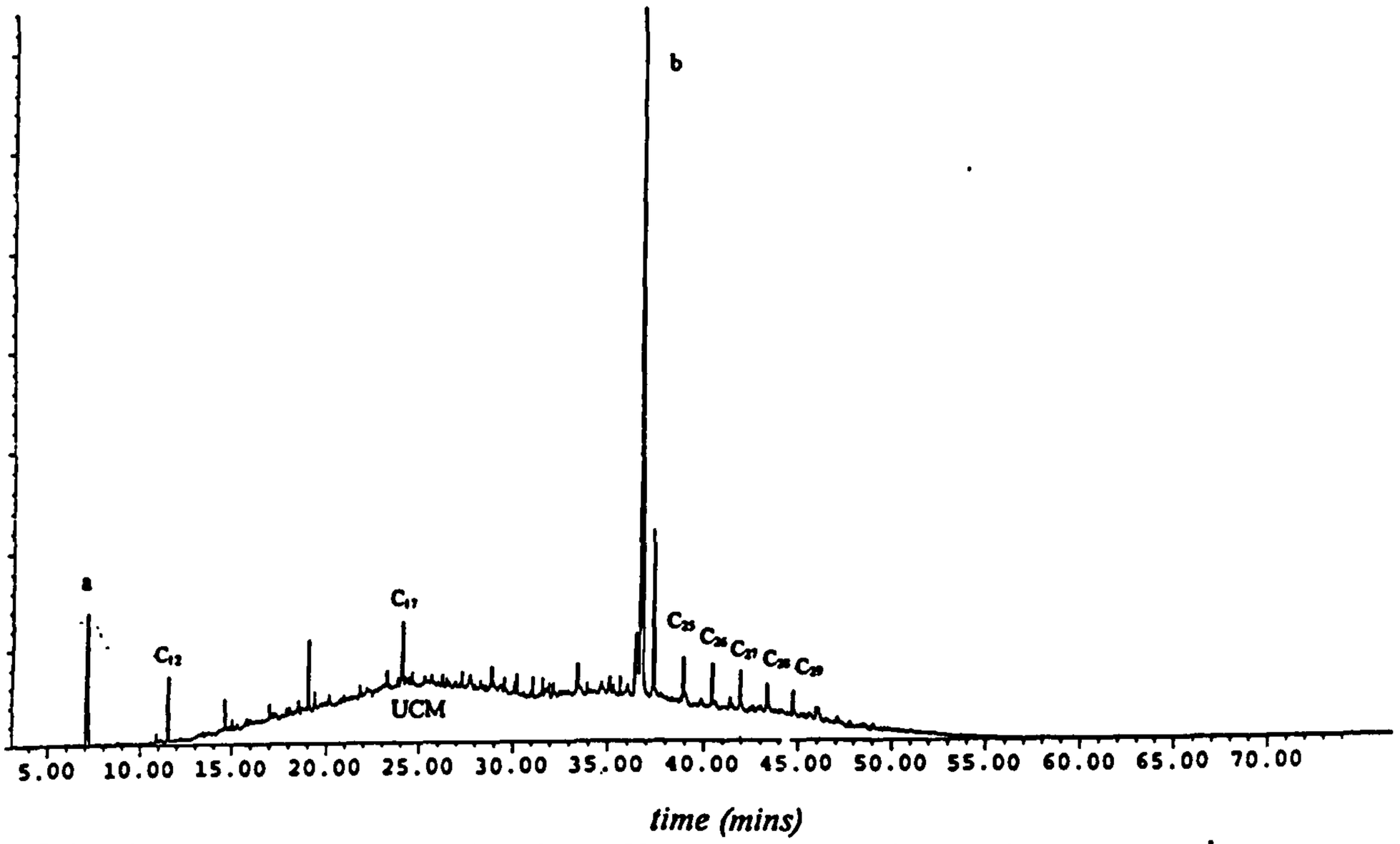
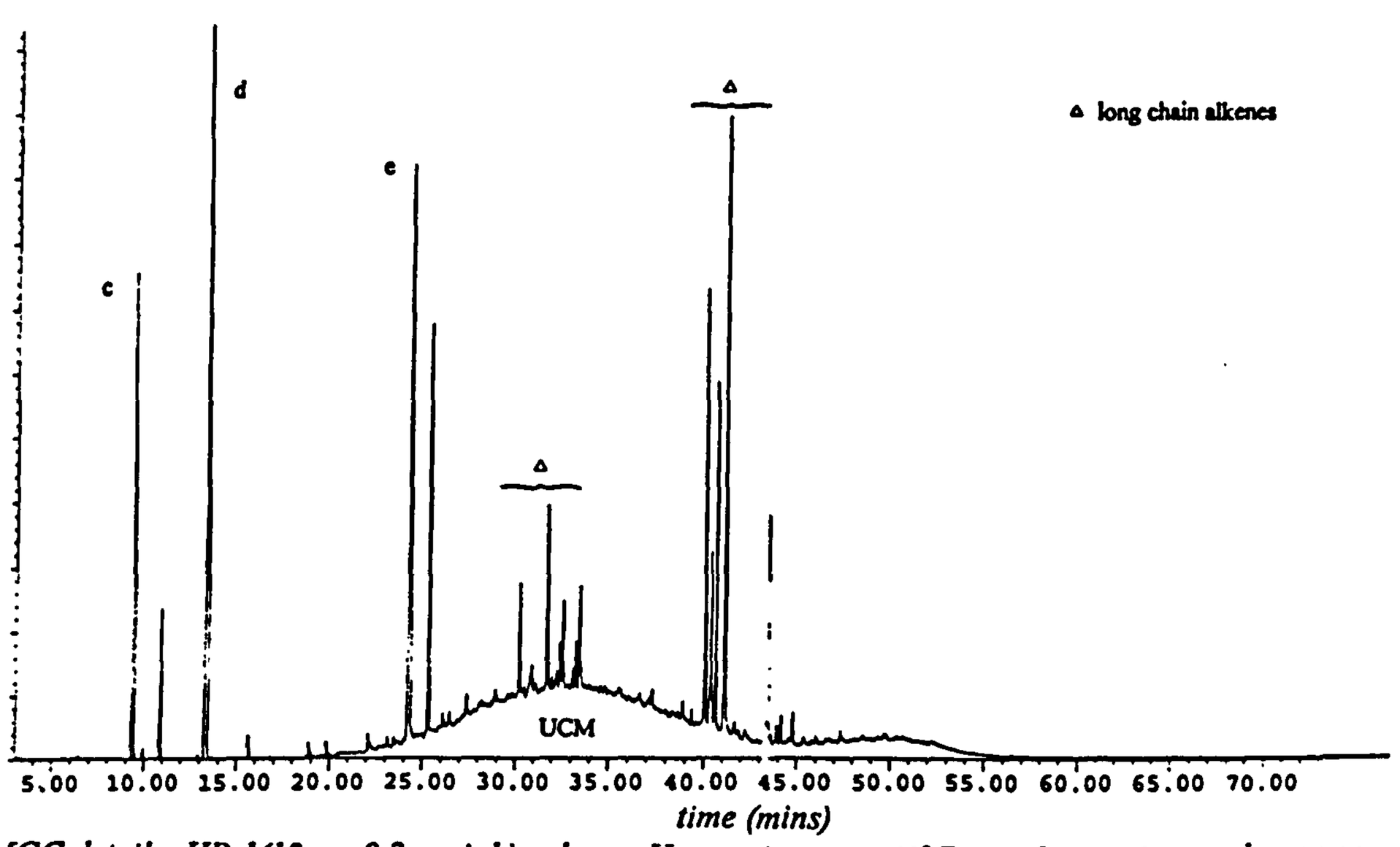
[1] The terms 'aliphatic' and 'aromatic are operationally defined according to the fractionation procedure employed herein (See Section 2.3.5)



[GC details; HP-1(12m x 0.2mm i.d,) column. He carrier gas.; 40°C - 300°C @ 5°C min^{-1} hold 10mins]

Figure 2.11a Gas chromatogram of aliphatic fraction of mussels (*M. edulis*) from New Brighton (Mersey estuary)

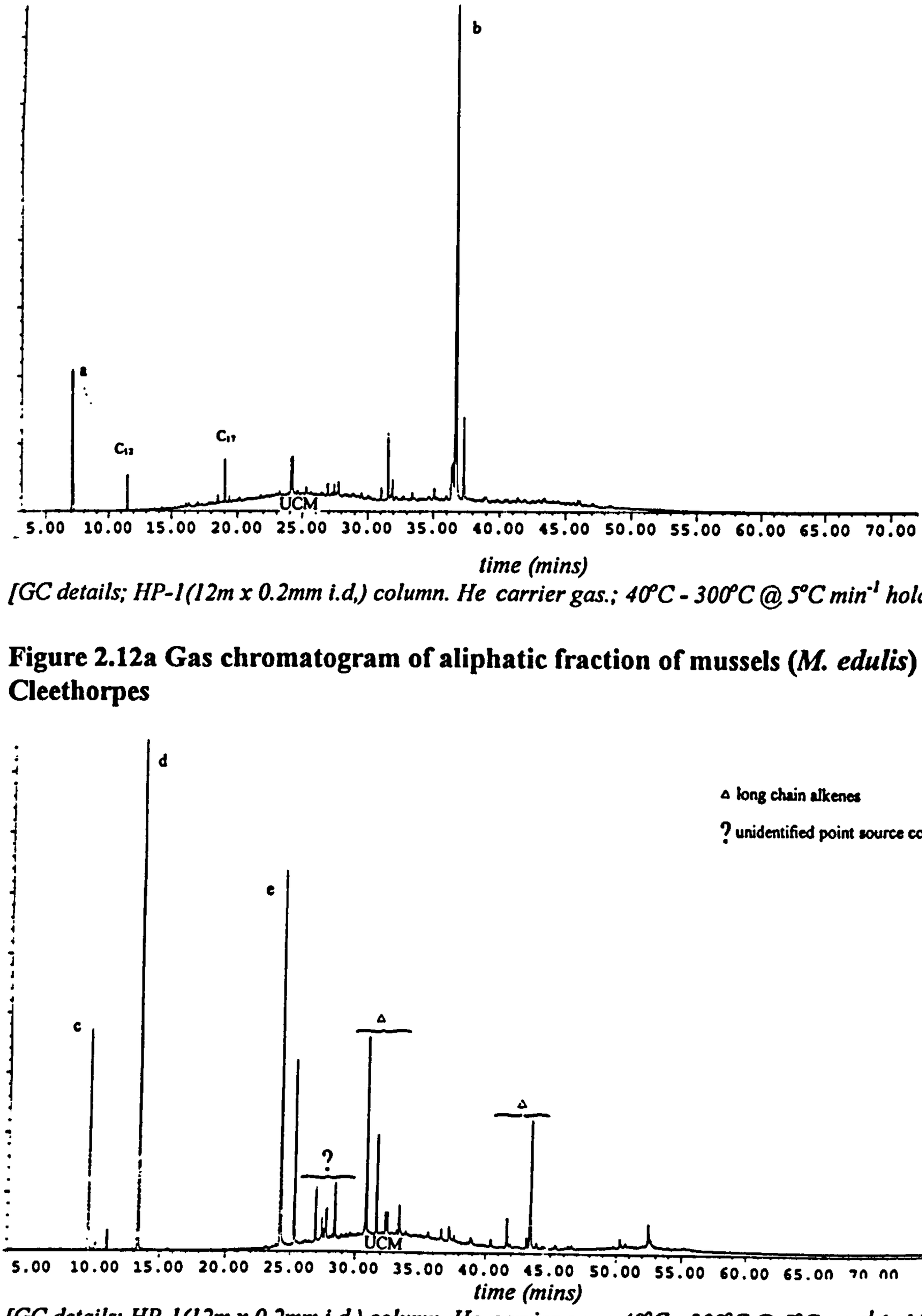


[GC details; HP-1(12m x 0.2mm i.d,) column. He carrier gas.; 40°C - 300°C @ 5°C min^{-1} hold 10mins]

Figure 2.11b Gas chromatogram of aromatic fraction of mussels (*M. edulis*) from New Brighton (Mersey estuary)

Internal standards; a; 4-propyloctane: b; 7-hexylnonadecane: c; d_{12}-tetralin:

d: d_{10}-1 -methylnaphthalene-d_{10}; e;d_{10}- phenanthrene



[GC details; HP-1(12m x 0.2mm i.d,) column. He carrier gas.; 40°C - 300°C @ 5°C min^{-1} hold 10mins]

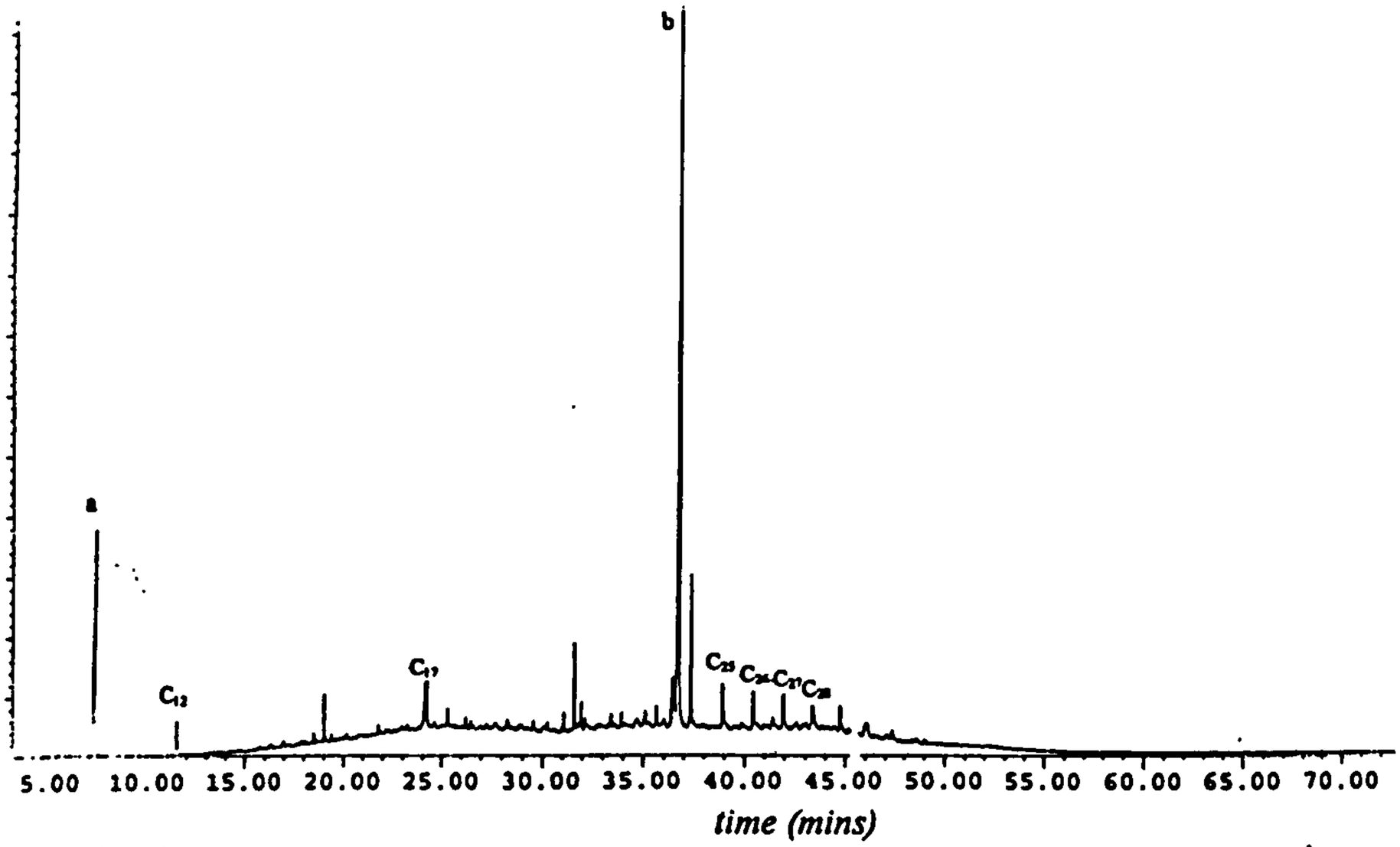
Figure 2.12a Gas chromatogram of aliphatic fraction of mussels (*M. edulis*) from Cleethorpes

[GC details; HP-1(12m x 0.2mm i.d,) column. He carrier gas.; 40°C - 300°C @ 5°C min^{-1} hold 10mins]

Figure 2.12b Gas chromatogram of aromatic fraction of mussels (*M. edulis*) from Cleethorpes

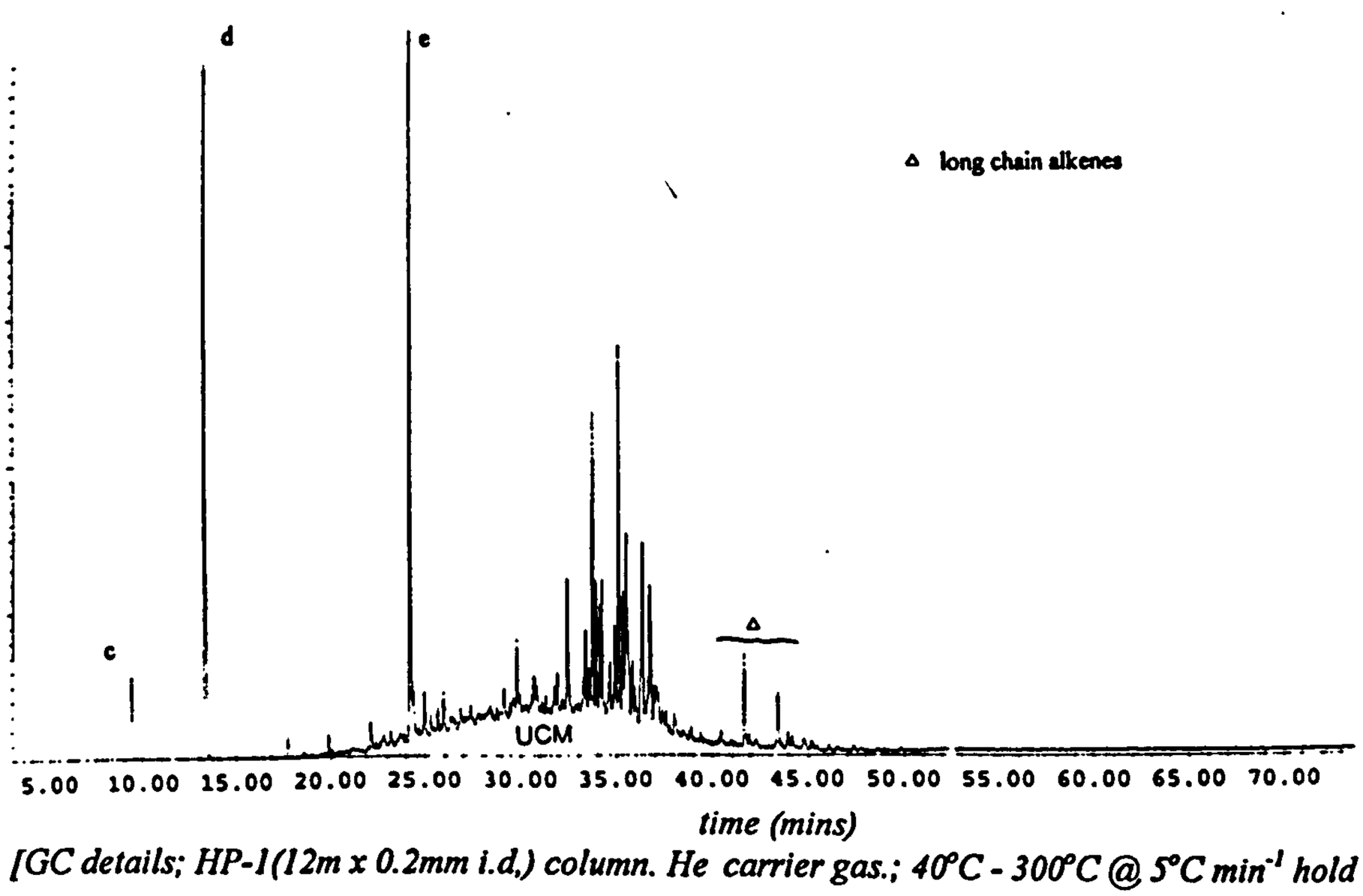
Internal standards; a; 4-propyloctane: b; 7-hexylnonadecane: c; tetralin-d_{12}:

d: 1-methylnaphthalene-d_{10}; e; phenanthrene-d_{10}



[GC details; HP-1(12m x 0.2mm i.d,) column. He carrier gas.; 40°C - 300°C @ 5°C min^{-1} hold 10mins]

Figure 2.13a Gas chromatogram of aliphatic fraction of mussels (*M.edulis*) from Teesmouth



[GC details; HP-1(12m x 0.2mm i.d,) column. He carrier gas.; 40°C - 300°C @ 5°C min^{-1} hold 10mins]

Figure 2.13b Gas chromatogram of aromatic fraction of mussels (*M. edulis*) from Teesmouth

Internal standards; a; 4-propyloctane: b; 7-hexylnonadecane: c; d_{12}-tetralin:

d: d_{10}-1 -methylnaphthalene-d_{10}; e;d_{10}- phenanthrene

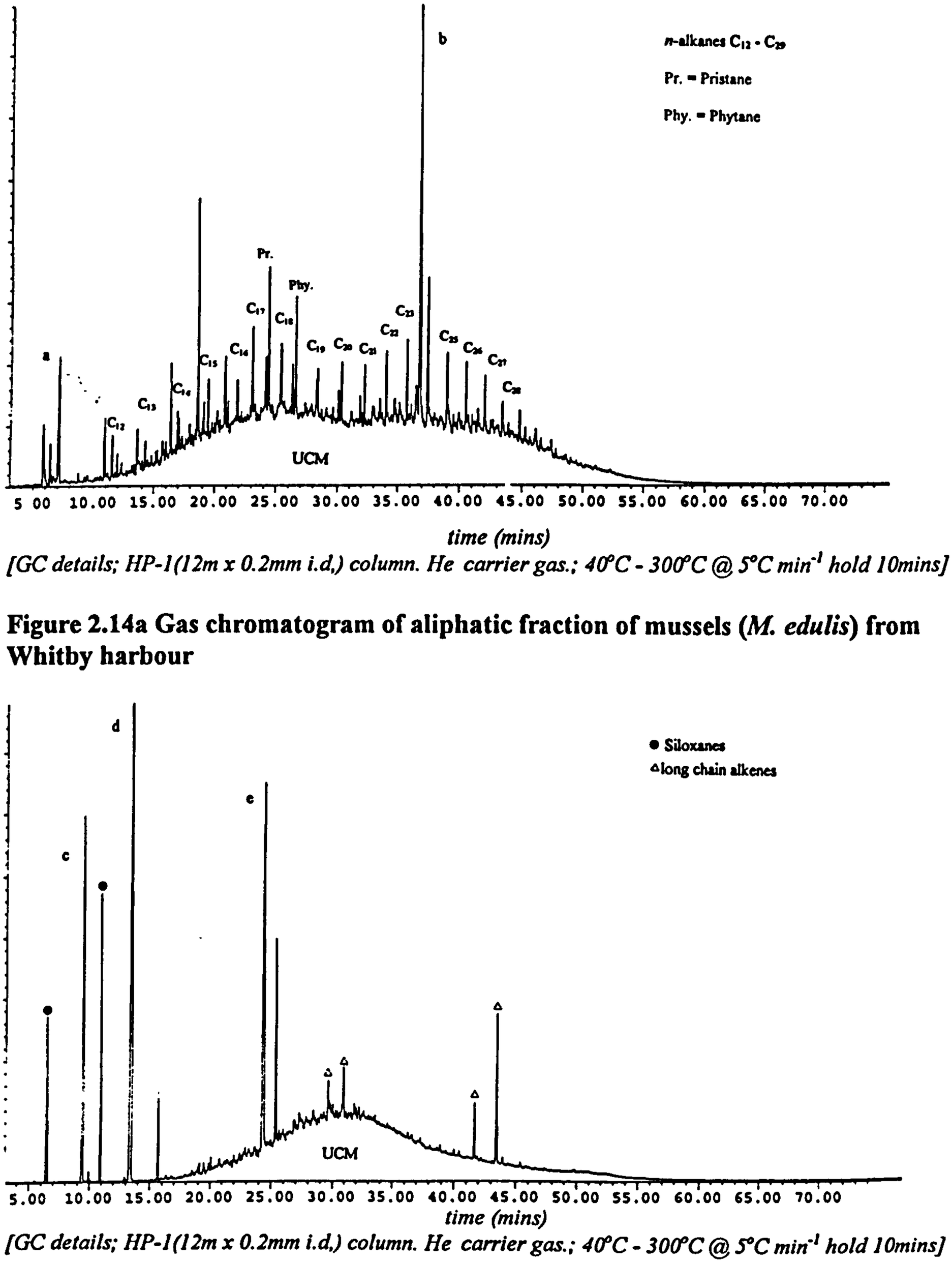
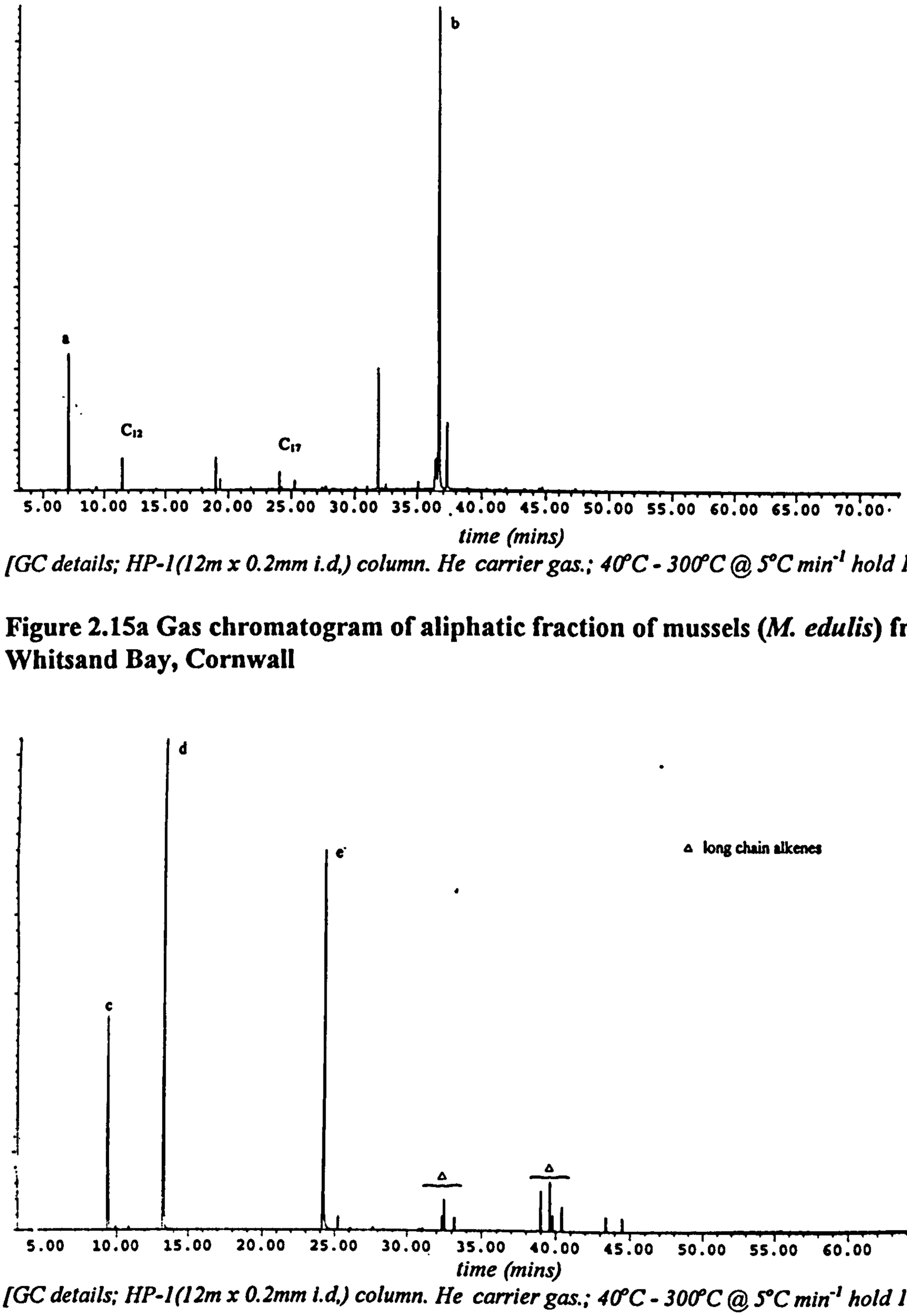


Figure 2.14a Gas chromatogram of aliphatic fraction of mussels (*M. edulis*) from Whitby harbour

Figure 2.14b Gas chromatogram of aromatic fraction of mussels (*M. edulis*) from Whitby harbour

Internal standards; a; 4-propyloctane: b; 7-hexylnonadecane: c; d_{12}-tetralin:

d: d_{10}-1 -methylnaphthalene-d_{10}; e;d_{10}- phenanthrene



[GC details; HP-1(12m x 0.2mm i.d,) column. He carrier gas.; 40°C - 300°C @ 5°C min^{-1} hold 10mins]

Figure 2.15a Gas chromatogram of aliphatic fraction of mussels (*M. edulis*) from Whitsand Bay, Cornwall

Figure 2.15b Gas chromatogram of aromatic fraction of mussels (*M. edulis*) from Whitsand Bay, Cornwall

Internal standards; a; 4-propyloctane: b; 7-hexylnonadecane: c; d_{12}-tetralin:

d: d_{10}-1 -methylnaphthalene-d_{10}; e;d_{10}- phenanthrene

Sample Site		Aliphatic		Aromatic		
		total resolved ($\mu g\ g^{-1}$ dry wt)	total unresolved ($\mu g\ g^{-1}$ dry wt)	total resolved ($\mu g\ g^{-1}$ dry wt)	total unresolved ($\mu g\ g^{-1}$ dry wt)	total hydrocarbons ($\mu g\ g^{-1}$ dry wt)
New Brighton (Mersey)	(i)	13	739	19	114	885
	(ii)	4	408	31	152	595
Cleethorpes	(i)	25	401	23	102	551
	(ii)	22	170	18	136	346
Teesmouth	(i)	8	188	19	83	298
	(ii)	11	275	19	94	399
Whitby Harbour	(i)	114	3610	64	365	4153
	(ii)	123	3280	57	496	3957
Whitsand Bay	(i)	10	9	2	n.d	12
	(ii)	7	5	4		16

n.d; not detected

total hydrocarbons are defined as total (resolved + unresolved) aromatic and aliphatic hydrocarbons

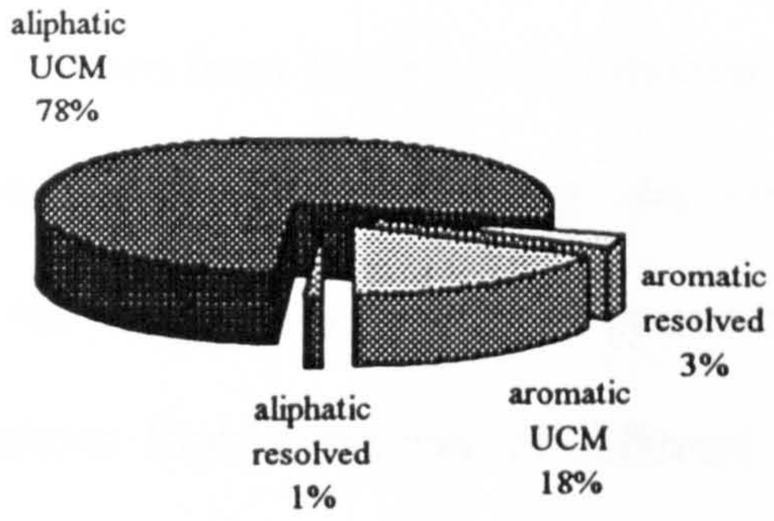
Table 2.8 Hydrocarbon concentrations in mussels from selected U.K. coastal sites

values are within the typical range of values reported in the literature. For example, Mason (1988) reported petroleum hydrocarbon concentrations in mussels around the Cape Pennisula (South Africa) ranging from 10 - 100 μg g^{-1} dry weight tissue at relatively unpolluted sites with concentrations as high as 5000 μg g^{-1} dry tissue weight at sample sites inside Cape Town Harbour.

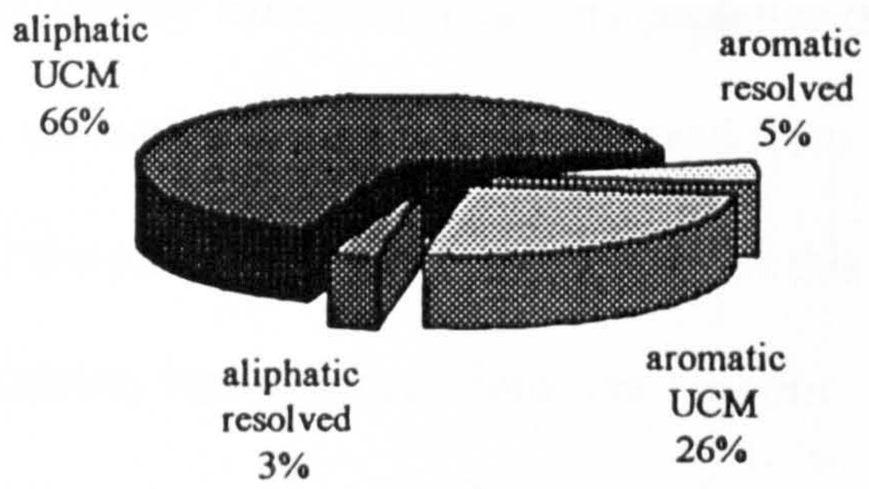
The total hydrocarbon concentrations reported in Table 2.8 includes both hydrocarbons from fossil fuels and also hydrocarbons of biogenic origin such as a number of alkanes and alkenes of algal origin. Concentrations of biogenic alkanes/alkenes in marine sediments are typically in the range of < 1 μg g^{-1} to approximately 10 μg g^{-1}. Total hydrocarbon concentrations higher than this are generally attributed to petroleum inputs (Volkman *et al.*, 1992). Similar total hydrocarbon concentrations are reported for mussels from sampling locations considered to be relatively free from petroleum hydrocarbon contamination (*e.g.* Mason, 1988). The compositional features used to distinguish between natural and anthropogenic sources of hydrocarbons are reviewed by Volkman *et al.* (1992). For example, as discussed previously (Section 1.3), the presence of an UCM in a water, sediment or biota sample is used as a reliable indication of fossil fuel contamination.

The relative contributions of resolved and unresolved, aliphatic and aromatic resolved compounds to the total hydrocarbon body burden in mussels from each sample site are illustrated in Figure 2.16. It is apparent that, with the exception of mussels from Whitsand Bay, the aliphatic UCM constitutes the largest proportion of the total hydrocarbon body burden, typically 64-85 %, indicating that these sites are impacted by petroleum hydrocarbons. The distributions of hydrocarbons in each of the aliphatic and aromatic fractions analysed are discussed below.

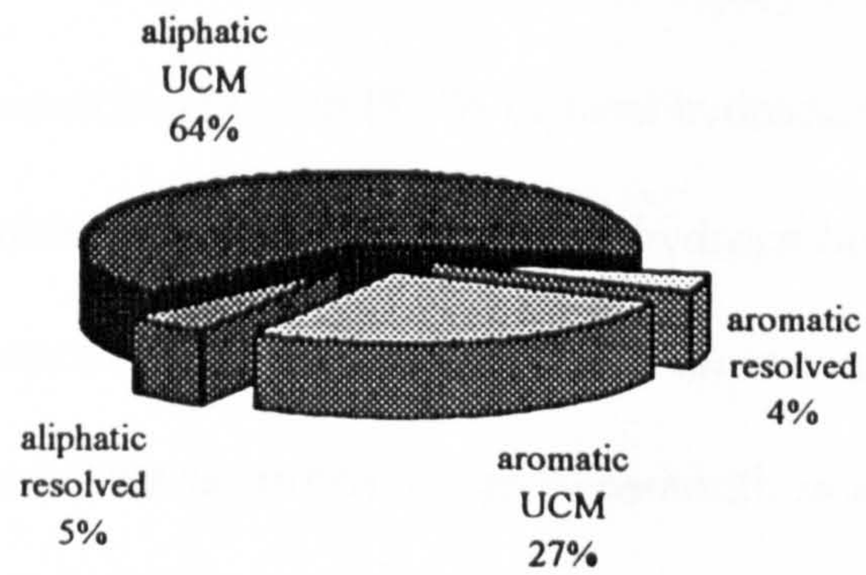
(a) New Brighton



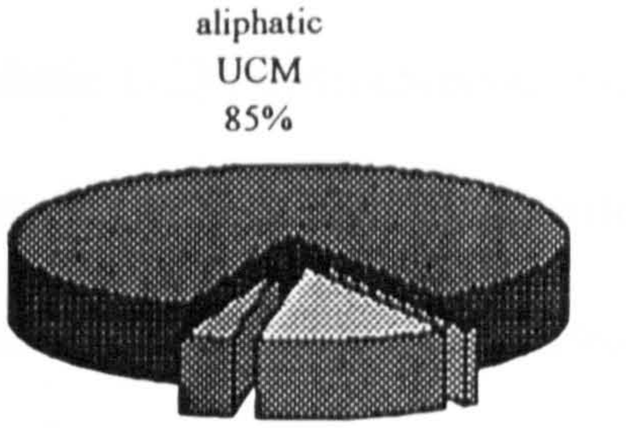
(b) Teesmouth



(c) Cleethorpes



(d) Whitby Harbour



(e) Whitsand ('clean' reference site)

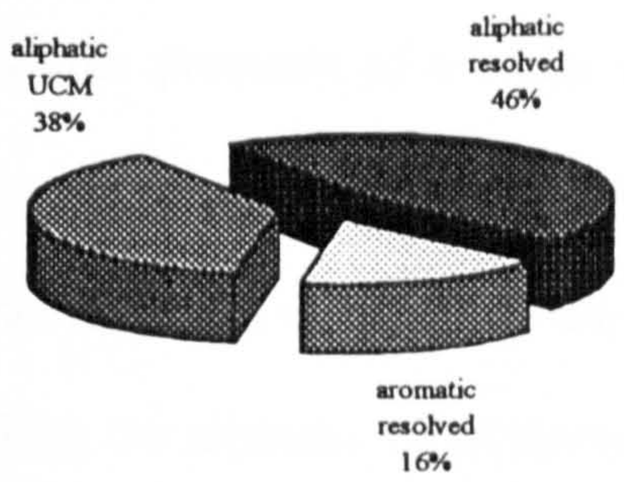


Figure 2.16 The relative contributions of resolved and unresolved aliphatic and aromatic hydrocarbons to the total hydrocarbon body burden of mussels (*M. edulis*) from selected U.K. coastal sites

2.4.3.1 Aliphatic hydrocarbons

It can be seen from Table 2.8 that mussels sampled from Whitby Harbour contained much higher concentrations (> 3000 $\mu g\ g^{-1}$ dry weight tissue) of aliphatic UCM hydrocarbons than any of the other sites. This is presumably owing to the large inputs of petroleum hydrocarbons from a number of different sources, including diesel and engine oil from boating activity, urban run-off and the disposal of used petroleum products such as engine lube oil into a relatively enclosed area. The gas chromatogram of the F_1 fraction of mussels from Whitby harbour reveals the presence of a series of *n*-alkanes ranging from *n*-C_{12} to *n*-C_{31}. As *n*-alkanes are rapidly removed from the environment by microbial action (*cf.* Section 1.1) the presence of such compounds in this fraction may suggest a relatively recent input of petroleum hydrocarbons into the harbour. Alternatively, the presence of a series of *n*-alkanes may simply reflect a chronic input such as diesel from boating activity. Quantitatively, however, the resolved aliphatic components represent only 3 % of the total hydrocarbon burden in mussels from Whitby Harbour, whilst the aliphatic UCM dominates the hydrocarbon burden (85 % of total hydrocarbons).

Concentrations of aliphatic UCM hydrocarbons in mussels from both Teesmouth and Cleethorpes are approximately 10 - 12 times lower than those found in mussels from Whitby Harbour. This is surprising, as Teesmouth is a heavily industrialised area. The sampling location is also situated close to a major shipping route and is therefore subject to chronic inputs of oil from passing shipping traffic. Similarly, Cleethorpes is a heavily urbanised area and is situated close to Grimsby, an extremely busy fishing port. Inputs of petroleum hydrocarbons to both sites were therefore anticipated to be fairly high. However, both Teesmouth and Cleethorpes are open coastal sites and consequently receive more diffuse inputs of petroleum hydrocarbons relative to an enclosed area such as Whitby Harbour.

Elevated levels of petroleum hydrocarbons in harbours have been reported previously by a number of authors (*e.g.* Mason, 1988; Burns *et al.*, 1990). For example, Burns *et al.* (1990) reported petroleum hydrocarbon levels seven times higher in mussels sampled from a Bermudan harbour compared with mussels from a nearby coastal site.

Aliphatic UCM concentrations measured in mussels from New Brighton (Mersey estuary) were in the range 408 - 739 μg g^{-1} dry weight, higher than concentrations measured in mussels from Teesmouth and Cleethorpes. A number of authors have previously identified this area as one of relatively high petroleum contamination (Readman *et al.*, 1986; Davies and Wolff, 1990). Several oil refineries are situated on the banks of the River Mersey, and therefore tanker loading operations will most probably result in chronic inputs of oil to the river. In addition, this is a heavily populated area, and consequently a significant source of hydrocarbons will be *via* urban run-off (*cf.* Section 1.3).

The presence of an UCM in the aliphatic fraction of mussels from Whitsand Bay is barely discernible above the baseline of the chromatogram (Figure 2.15), suggesting little or no petroleum hydrocarbon contamination at Whitsand. The resolved peaks present in this fraction were identified by mass spectrometry (by comparison of spectra with those in the NBS 54 K library) as *n*-dodecane (*n*-C_{12}), 1-tridecene and *n*-heptadecane (*n*-C_{17}). As these peaks are absent from the procedural blank, but also present in the aliphatic fractions of the other samples, these hydrocarbons are most probably of algal origin.

By optimising the analytical method to ensure maximum recovery of the low molecular weight, more volatile hydrocarbons (Section 2.3), it has been possible to assess a wide molecular weight range of environmental aliphatic UCMs bioaccumulated by mussels. Clearly, as can be seen from Figures 2.11a to 2.14a low molecular weight UCMs from *ca* *n*-C_{12} are present in environmental samples. A small fraction of the low molecular weight

aliphatic UCM may be of toxicological significance, and this is investigated further in Chapter 4.

2.4.3.2 Aromatic hydrocarbons

The gas chromatograms of aromatic fractions from all of the petroleum hydrocarbon impacted sites (Whitby Harbour, Cleethorpes, Teesmouth, New Brighton) are also dominated by an UCM (Figures 2.11b - 2.14b) suggesting that the presence of an aromatic UCM in marine biota such as mussels is also a characteristic feature of petroleum hydrocarbon contamination. As discussed previously in Section 2.1, the presence of an aromatic UCM in environmental samples is seldom reported, owing to the use of selected ion monitoring GC-MS to focus upon those compounds which are known to be toxicologically significant, (*e.g.* PAH and alkylated PAH). However, as illustrated in Figure 2.16, the results presented herein show that the aromatic UCM may also be considered a quantitatively important environmental burden, constituting approximately 20 % of the total hydrocarbon body burden in mussels from the petroleum hydrocarbon contaminated sites. The highest concentrations of aromatic hydrocarbons are present in mussels from Whitby harbour (*ca* 400 $\mu g\ g^{-1}$ dry weight tissue), whilst mussels sampled from the other petroleum hydrocarbon impacted sites (New Brighton, Cleethorpes and Teesmouth) all contained approximately 100 $\mu g\ g^{-1}$ dry weight tissue aromatic UCM hydrocarbons. No aromatic UCM was observed in mussels from Whitsand Bay.

The concentrations of aromatic UCMs reported in Table 2.8 are comparable with the few existing reports of aromatic UCMs in environmental samples. For example, Risebrough *et al.* (1983) analysed mussels from a range of sampling locations in the Ebro Delta (Catalonian coast, Spain) and reported concentrations of aromatic UCMs in the range

0.44-66μg g^{-1} dry weight tissue. Mason (1988) analysed the hydrocarbon body burden of mussels from several locations on the West coast of South Africa and reported aromatic UCM concentrations of 489 μg g^{-1} dry weight tissue in mussels near a sewage outlet. This author also reported concentrations of aromatic UCMs in mussels from the entrance to Cape Town Harbour of approximately 7000 μg g^{-1} dry weight.

The aromatic UCM therefore appears to be a quantitatively important environmental burden. Concentrations of resolved PAH compounds, more routinely quantified in environmental monitoring programmes are much lower than the unresolved aromatic hydrocarbon concentrations reported herein, and are typically present in the range of approximately 1 - 10 μg g^{-1} (dry wt) in mussels from impacted sites. To illustrate this, the concentrations of naphthalene and alkylnaphthalenes in the most severely impacted mussels (Whitby Harbour) were measured. Ion chromatograms for m/z 128, 142, 156 and 170 (naphthalene, methylnaphthalenes, dimethylnaphthalenes and trimethylnaphthalenes) are presented in Figure 2.17. The measured concentrations of each compound are provided in Table 2.9. Clearly, concentrations of resolved aromatic hydrocarbons are significantly lower (approximately 1 μg g^{-1} dry weight for the total naphthalene and alkylnaphthalenes) than concentrations of di-aromatic UCM hydrocarbons (approximately 75 μg g^{-1}), highlighting the aromatic UCM as a quantitatively important environmental burden. Indeed the presence of naphthalene and alkylnaphthalenes are masked in the total ion chromatogram by the aromatic UCM. Resolved hydrocarbons in each of the aromatic fractions were identified as predominantly long chain alkenes by the presence of a series of ions characteristic of these compounds (*m/z* 41, 55, 69, 81, 90). Long chain alkenes of microalgal origin have previously been reported in both marine sediments and marine biota (Rowland and Robson,

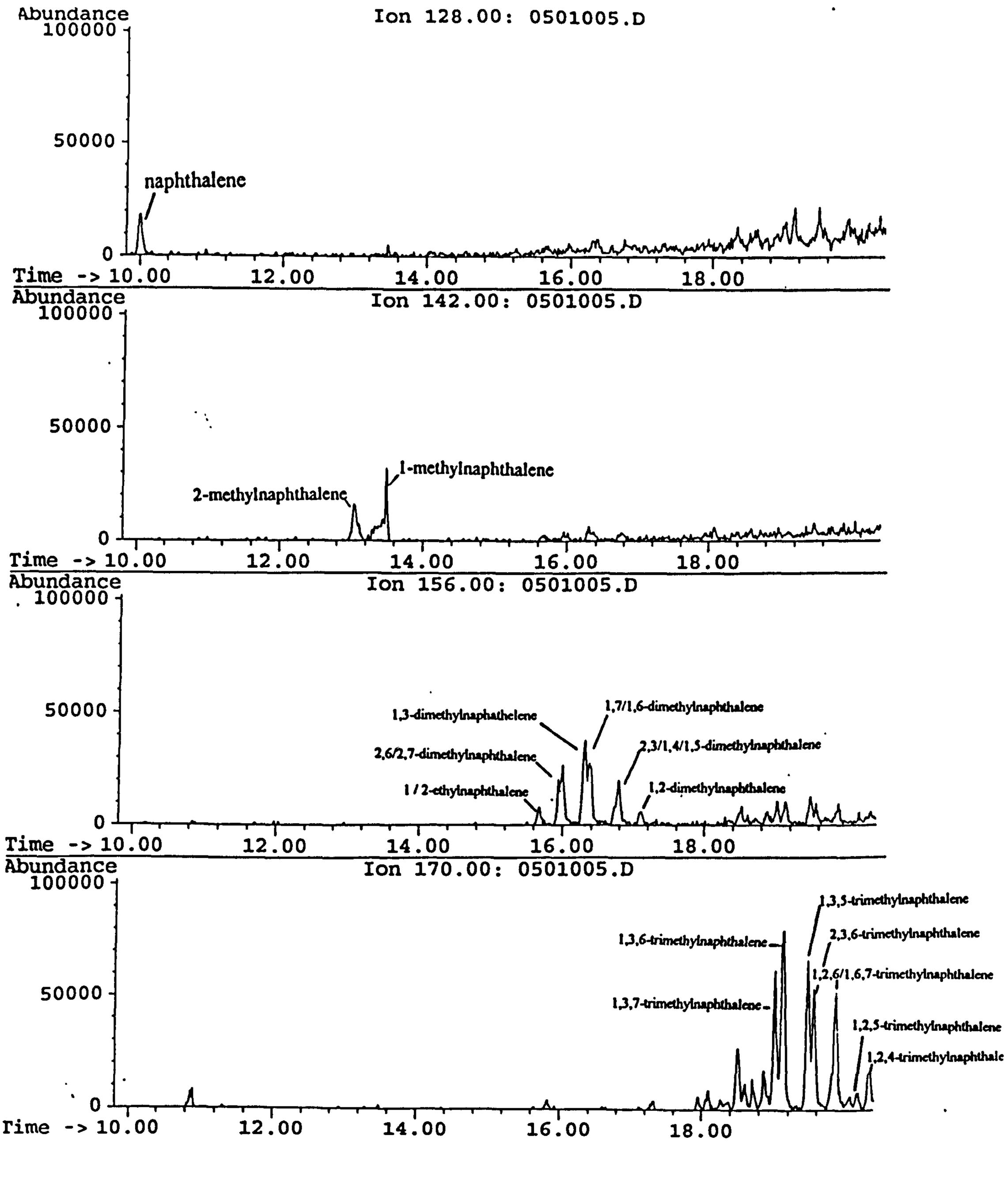


Figure 2.17 Ion chromatograms showing naphthalene and alkylnaphthalenes present in the aromatic fraction of mussels (*M. edulis*) from Whitby harbour

[GC details; HP-1(12m x 0.2mm i.d) column; He carrier gas; 40 - 300°C@ 5°C min^{-1}, hold 10 mins .]

Compound	Concentration ($\mu g\ g^{-1}$ dry weight tissue)
naphthalene	0.02
	0.03
methylnaphthalenes	0.05
	0.07
dimethylnaphthalenes	0.12
	0.13
trimethylnaphthalenes	0.34
	0.35
di-aromatic UCM	89.9
	58.7

Table 2.9 Concentrations of naphthalene and alkylnaphthalenes present in the 'aromatic' fraction (F_2) of mussels (*M. edulis*) from Whitby Harbour (the concentration of di-aromatic unresolved hydrocarbons is provided for comparison)

1990; Volkman *et al.*, 1992). In addition, a number of point source contaminants were identified in several of the fractions. Mussels from Whitby Harbour contained octamethylsiloxane and decamethylcyclopentasiloxane, identified by comparison of their mass spectra with those of the authentic compounds (Van den Heuvel *et al.*, 1972). These compounds were not present in the procedural blank and their source remains unknown. Both Teesmouth and Cleethorpes samples also contained a series of apparently anthropogenic compounds, but these could not be identified.

	UCM concentration in mussel tissue (µg g^{-1} dry weight)			
SAMPLE SITE	**mono-aromatic**	**di-aromatic**	**tri-aromatic**	**> 3 ring**
New Brighton	69.8 111.2 (91 %)	12.7 4.1 (9 %)	n.d.	n.d.
Cleethorpes	94.0 79.8 (85 %)	18.0 6.8 (12 %)	1.4 5.4 (3 %)	n.d.
Teesmouth	36.9 28.3 (74 %)	12.8 3.4 (18 %)	2.0 5.0 (8 %)	n.d.
Whitby Harbour	385.8 336.8 (79 %)	89.9 58.7 (16 %)	29.5 11.7 (5 %)	n.d.
Whitsand Bay	n.d.	n.d.	n.d.	n.d.

n.d; not detected

Table 2.10 UCM concentrations in mono-aromatic, di-aromatic and tri-aromatic fractions of mussels (*M. edulis*) from U.K. coastal sites

To examine the bulk composition of aromatic UCM hydrocarbons bioaccumulated by mussels, aromatic fractions were further fractionated by normal phase HPLC according to ring size, and then examined by GC. The concentration of unresolved hydrocarbons in each ring fraction is given in Table 2.10. Gas chromatograms of the mono-, di- and tri-aromatic hydrocarbon fractions of mussels from Whitby harbour are presented in Figure 2.18. It is evident from Table 2.10 that the bulk of unresolved aromatic hydrocarbons in the mussels sampled are mono-aromatic in nature. This is in agreement with Killops and Readman (1985) who fractionated a number of sediment extracts in the same manner and found the majority of the aromatic UCM to be present in the mono-aromatic fraction.

As discussed in Section 1.6, a 'cut-off' in toxicity for non-specific narcotics has been identified which is thought to be governed by the aqueous solubility of compounds. For example, aromatic hydrocarbons greater than three rings can be accumulated by mussels without inhibiting ciliary feeding activity (Donkin *et al.*, 1989,1991). The present study has demonstrated that the bulk of the aromatic UCM is comprised of mono- and di-aromatic hydrocarbons. It is probable that a proportion of these components will be of sufficient aqueous solubility to be classed as non-specific narcotics. To date, the toxicity of the aromatic UCM remains uninvestigated, most probably owing to the relatively unknown composition of the aromatic UCM. However, as discussed in Section 1.2, a number of model aromatic hydrocarbon structures have recently been proposed (Figure 1.5), thus enabling the toxicity of the aromatic UCM to be studied. The toxicity of a number of model aromatic compounds is investigated in Chapter 5.

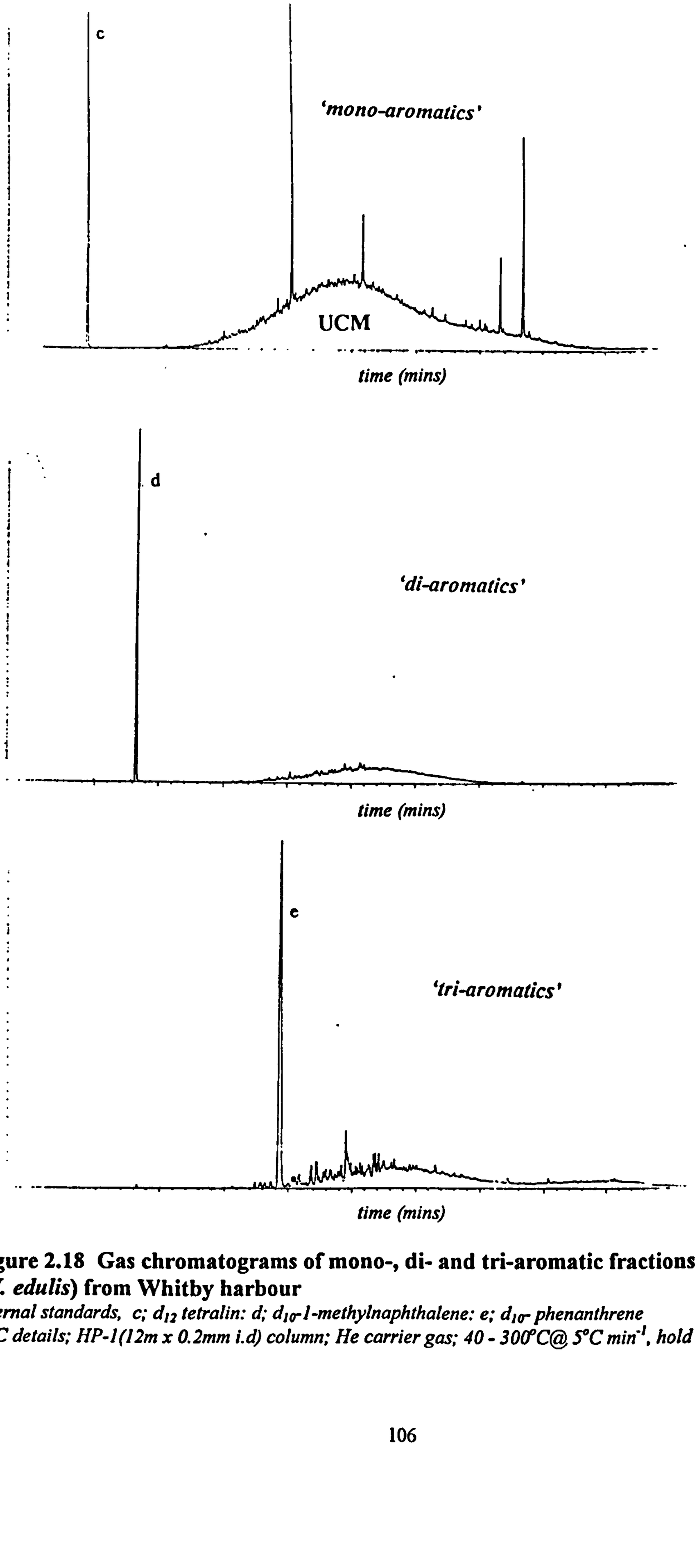


Figure 2.18 Gas chromatograms of mono-, di- and tri-aromatic fractions of mussels (*M. edulis*) from Whitby harbour

Internal standards, c; d_{12} tetralin: d; d_{10}-1-methylnaphthalene: e; d_{10}- phenanthrene
[GC details; HP-1(12m x 0.2mm i.d) column; He carrier gas; 40 - 300°C@ 5°C min^{-1}, hold 10 mins .]

2.4.6 Conclusions

The work described in this Chapter has clearly demonstrated that aromatic hydrocarbon UCMs form a significant proportion of the total hydrocarbon body burden of mussels from petroleum hydrocarbon-contaminated areas (11 - 26%). In the samples examined a large proportion of aromatic UCMs bioaccumulated by mussels consists of mono-aromatic (74 - 91%, with lesser amounts of di-aromatic (9 - 16%) hydrocarbons. Given the toxicological properties of a number of resolved aromatic hydrocarbons (*cf.* Section 1.5) it is likely that a proportion of the aromatic UCM hydrocarbons bioaccumulated by mussels is toxic. Thus, existing methodologies may have failed to provide a measure of an environmentally important burden.

CHAPTER TIIREE

Synthesis of model aliphatic and aromatic UCM hydrocarbons

3.1 Introduction

It has been clear for some time that the pollutant hydrocarbon burden of marine organisms such as bivalve molluscs is often dominated by UCM hydrocarbons (reviewed in Section 1.3) including both aliphatic hydrocarbons and, as demonstrated in Chapter 2, aromatic UCM components. However, the unknown molecular composition of hydrocarbon UCMs has limited the number of toxicological studies of these quantitatively important fractions. The recent proposal of 'average' or model hydrocarbon structures for both aliphatic and aromatic UCMs based on spectroscopic and oxidative degradation of lubricating oils and crude oils (Gough, 1989; Gough and Rowland, 1990, 1991; Killops and Al-Juboori, 1990; Revill, 1992; Thomas, 1995; Revill *et al.*, 1997; Thomas *et al.*, 1997) now affords the possibility of conducting such toxicological studies, once the pure compounds are made available. Proposed model compounds include so-called 'T-branched' alkanes for aliphatic UCMs and substituted naphthalenes for the aromatic UCMs, as illustrated in Figures 1.2 and 1.5, respectively (*cf.* Section 1.2). However, these compounds are not available from commercial sources and only small amounts of one or two relevant compounds have been synthesised previously (Gough, 1989). The aim of the present study was therefore to synthesise suitable quantities of selected, pure, well-characterised model aliphatic and aromatic UCM hydrocarbons for toxicological tests on the mussel *Mytilus edulis*. Individual test compounds were selected on the basis of both UCM characterisation data (*cf.* Gough, 1989; Thomas, 1995) and the known toxicity of simpler hydrocarbons (*cf.* Donkin *et al.*, 1989, 1991).

3.2 Synthesis of a low molecular weight model aliphatic UCM compound; 4-propyloctane

The model aliphatic UCM hydrocarbon chosen as the target for synthesis and toxicological testing was the 'T-branched' alkane, 4-propyloctane, illustrated in Figure 3.1. This compound fits the structural requirements of what has been shown by a number of characterisation and biodegradation studies (*e.g.* Gough, 1989; Gough and Rowland, 1990, 1991) to be an important fraction of several lube and crude oil UCMs.

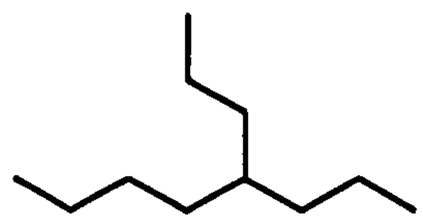


Figure 3.1 A low MW aliphatic UCM model compound, 4-propyloctane (4-PO)

As discussed in Chapter 1, compounds with log K_{ow} values greater than 5-6 are considered to be non-toxic (with respect to the mechanism of non-specific narcosis). For straight chain hydrocarbons, a cut-off in toxicity has been identified between *n*-decane (C_{10}) and *n*-undecane (C_{11}) (Donkin *et al.*, 1989, 1991). Larger *n*-alkanes than *n*-decane are non-toxic, most probably because of their limited aqueous solubility, which prevents sufficient bioaccumulation at the site of toxic action, as reviewed in Section 1.6. However, branched compounds usually have greater aqueous solubility than their straight chain analogues (*e.g.* Leo, 1993, Hansch and Leo, 1995). Consequently, a proportion of the 'T-branched' alkanes identified as aliphatic UCM components may have sufficient solubility to have a narcotic effect upon mussels. Demonstrable toxicological activity of a branched C_{11} compound such as 4-propyloctane compared to non-toxic *n*-undecane would effectively extend the molecular weight range of known toxic action. In other words, aliphatic UCM hydrocarbons may be more toxic than

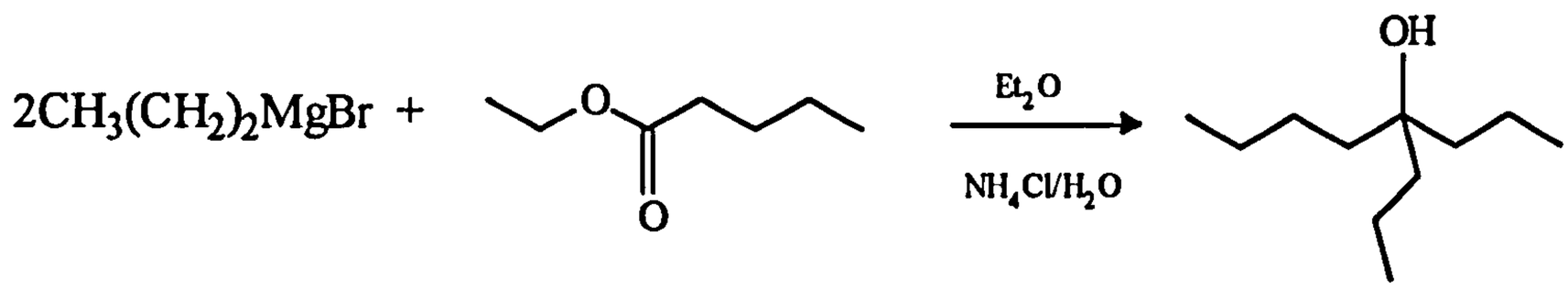
previously reported because their branched structures will increase their aqueous solubilities. Demonstration of such phenomena first requires synthesis of the pure compounds.

3.2.1 Synthetic scheme for 4-propyloctane (4-PO)

The overall reaction scheme for the preparation of 4-propyloctane (4-PO) is summarised in Figure 3.2. The synthesis involved coupling of 1-bromopropane to ethylpentanoate *via* a Grignard reaction to produce 4-propyloctan-4-ol. Dehydration of the alcohol to a mixture of three alkenes, followed by hydrogenation, yielded the target alkane. This method has proved to be successful for a previous synthesis of a C_{25} model UCM hydrocarbon, 7-*n*-hexylnonadecane (Gough, 1989). Particular emphasis was placed on ensuring adequate purity of the final product since the toxicity of the alkane was to be determined. Therefore, the intermediates at each stage of the synthesis were purified and fully characterised. Full experimental details are given in Section 6.4.

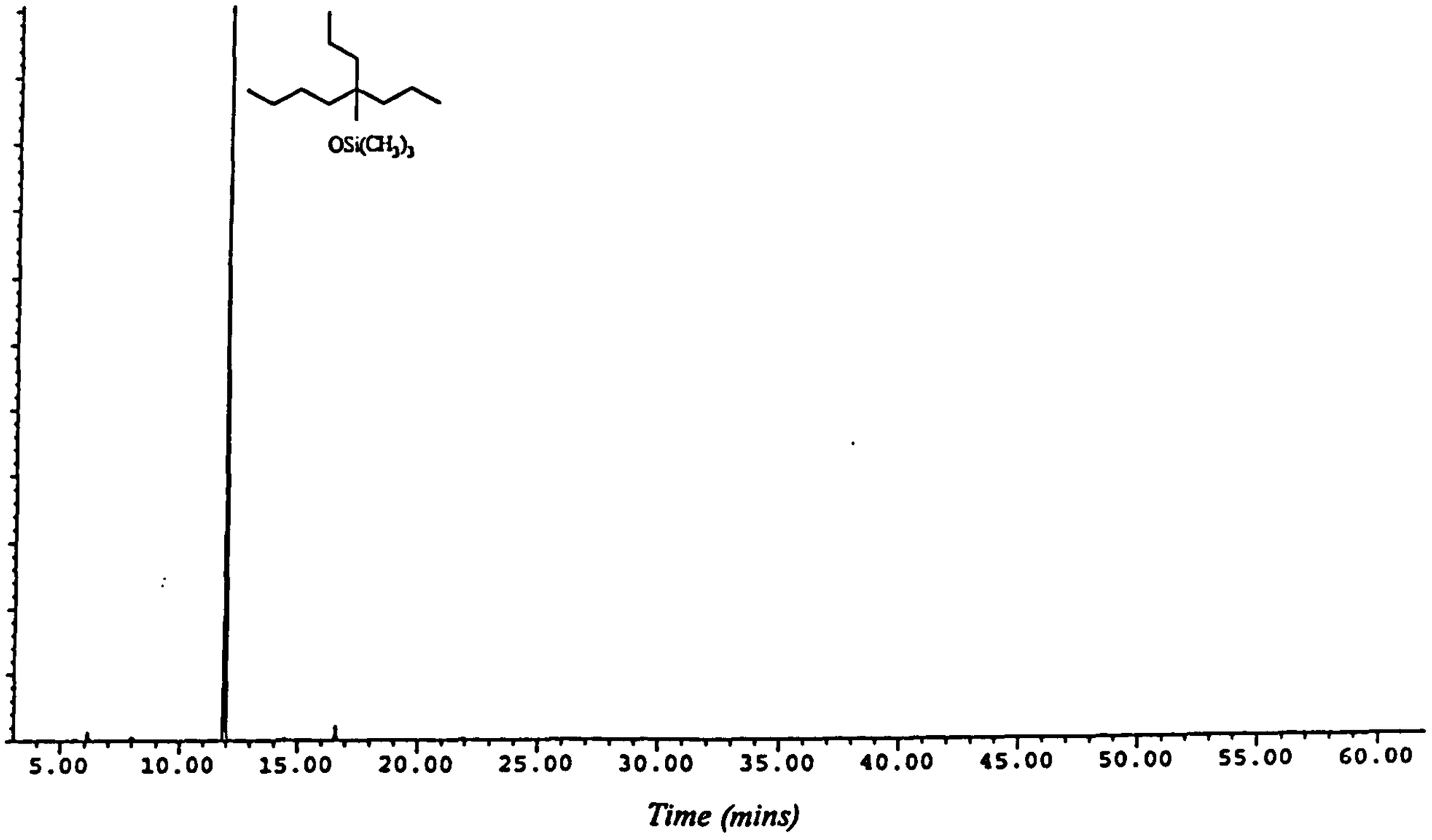
Figure 3.2 Reaction scheme for the synthesis of 4-propyloctane

3.2.2 Synthesis of 4-propyloctan-4-ol



1-Bromopropane was converted to the corresponding Grignard reagent, propylmagnesium bromide, then coupled to ethylpentanoate *via* a Grignard reaction. Vacuum distillation of the crude products yielded 4-propyloctan-4-ol (purity > 98 % by GC, Figure 3.3) in moderate yield (44 %).

The electron impact mass spectrum of the alcohol (Figure 3.4) showed ions characteristic of the fragmentation of tertiary alcohols (*m/z* 129, $[M^{+\cdot}\text{-}C_3H_7]^+$; *m/z* 115, $[M^{+\cdot}\text{-}C_4H_9]^+$) with preferential fragmentation at the tertiary centre (Kemp, 1991). The presence of the hydroxy group was confirmed by infra-red spectroscopy (IR) (Figure 3.5); the broad absorbance at 3400 cm^{-1} is characteristic of a hydrogen-bonded hydroxyl group. Examination of the alcohol by ^{13}C nuclear magnetic resonance spectroscopy (NMR; Figure 3.6a) showed a total of 8 resonances, all aliphatic type carbons. Analysis *via* the DEPT sequence (Distortionless Enhancement by Polarisation Transfer) which allows differentiation between CH, CH_2 and CH_3 carbons, revealed 2 methyl (2 of the methyl groups are presumably co-resonating) and 5 methylene peaks, and by difference, a single quaternary carbon, providing further confirmation of the branching position (Figure 3.6b). The ^1H NMR spectrum (Figure 3.7) consisted of a triplet (δ 0.8, 9H) due to the three methyl groups and a multiplet (δ1.2, 15 H, which includes the hydrogen of the hydroxy group). A summary of the ^{13}C NMR spectral assignments is provided in Appendix C.1.



[GC details; HP-1 column. He carrier gas. Temp program; 40°C - 300°C @ 5° min^{-1}, hold 10 mins]

Figure 3.3 Gas chromatogram of 4-propyloctan-4-ol as TMS ether

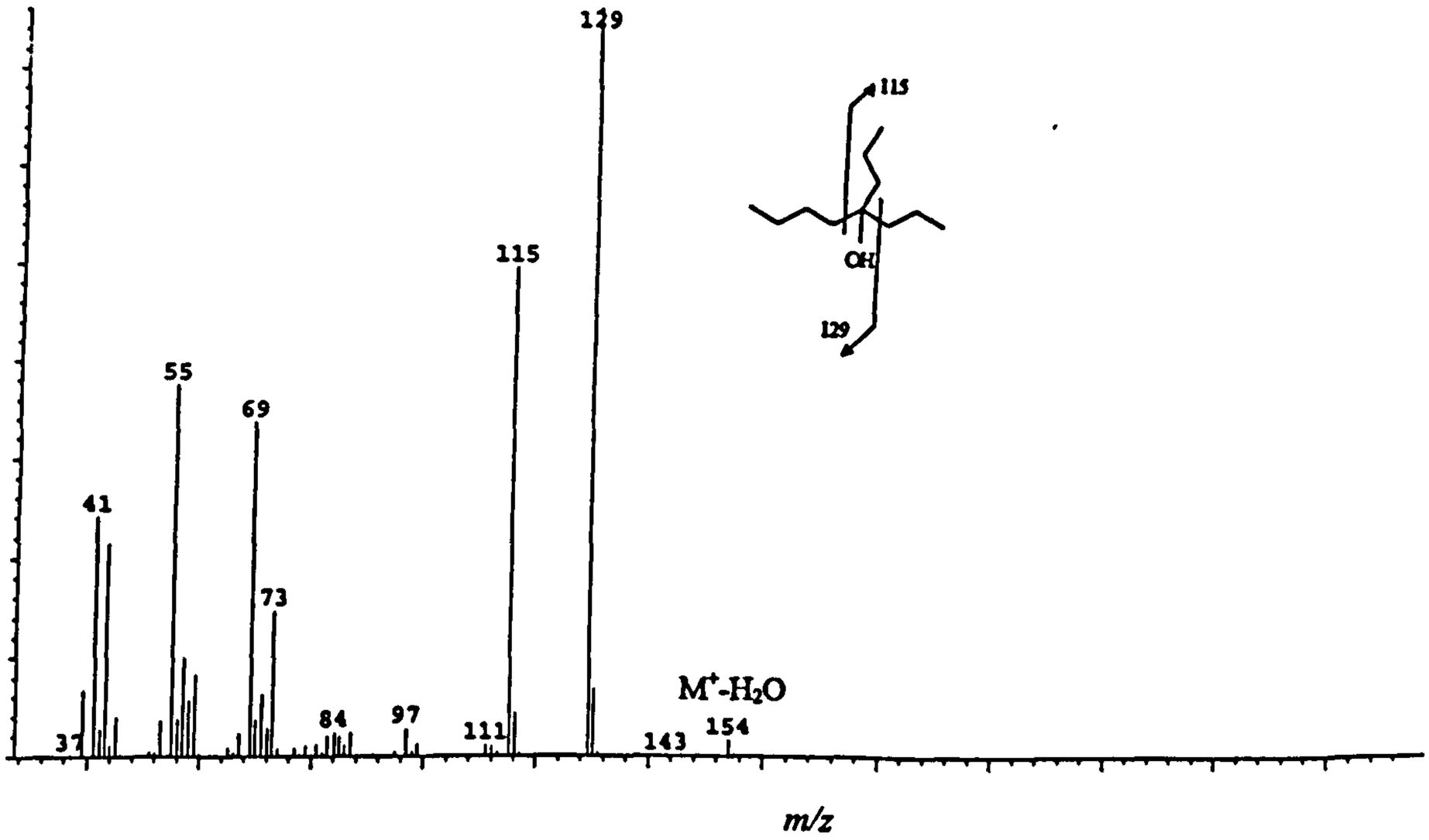


Figure 3.4 Mass spectrum of 4-propyloctan-4-ol

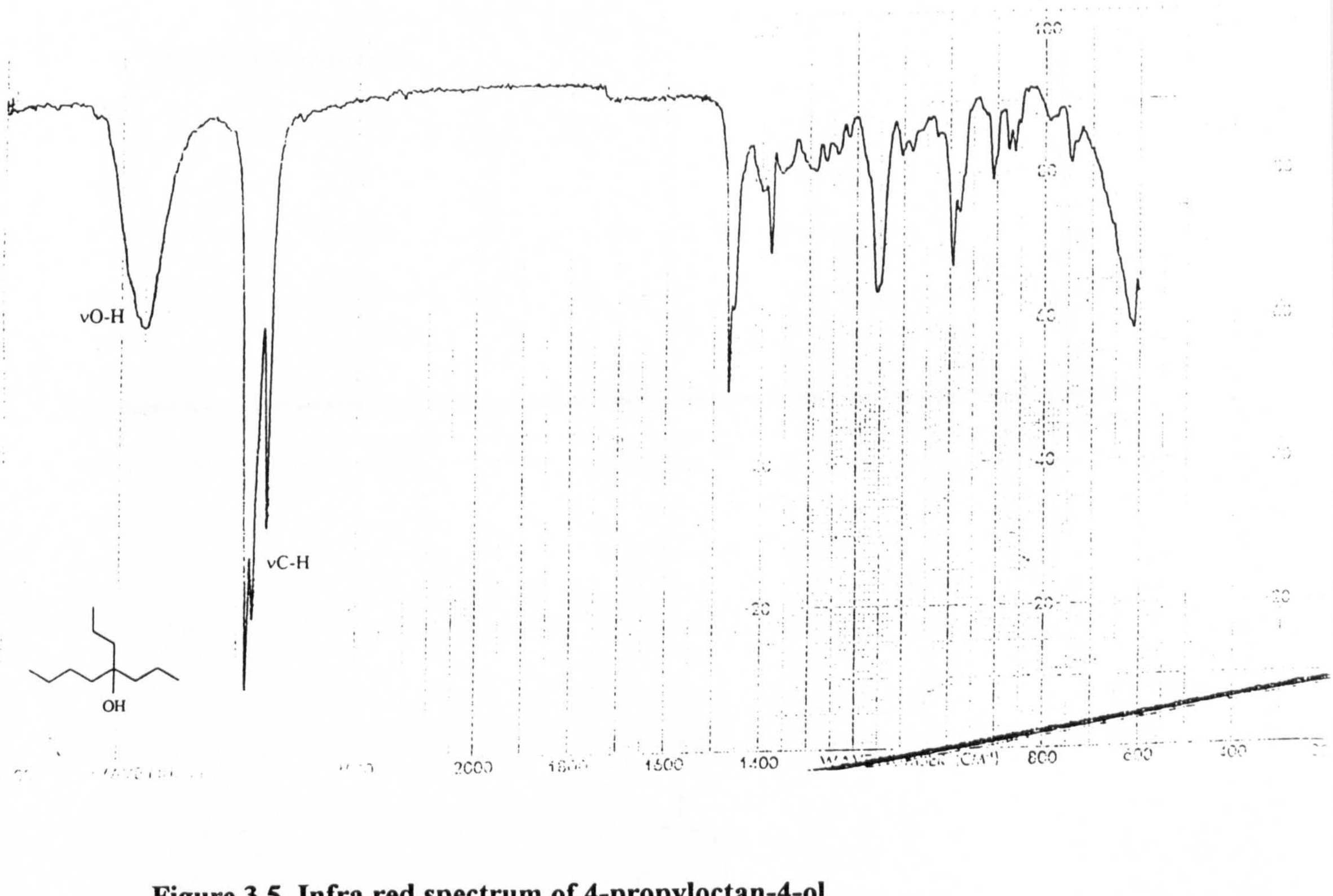
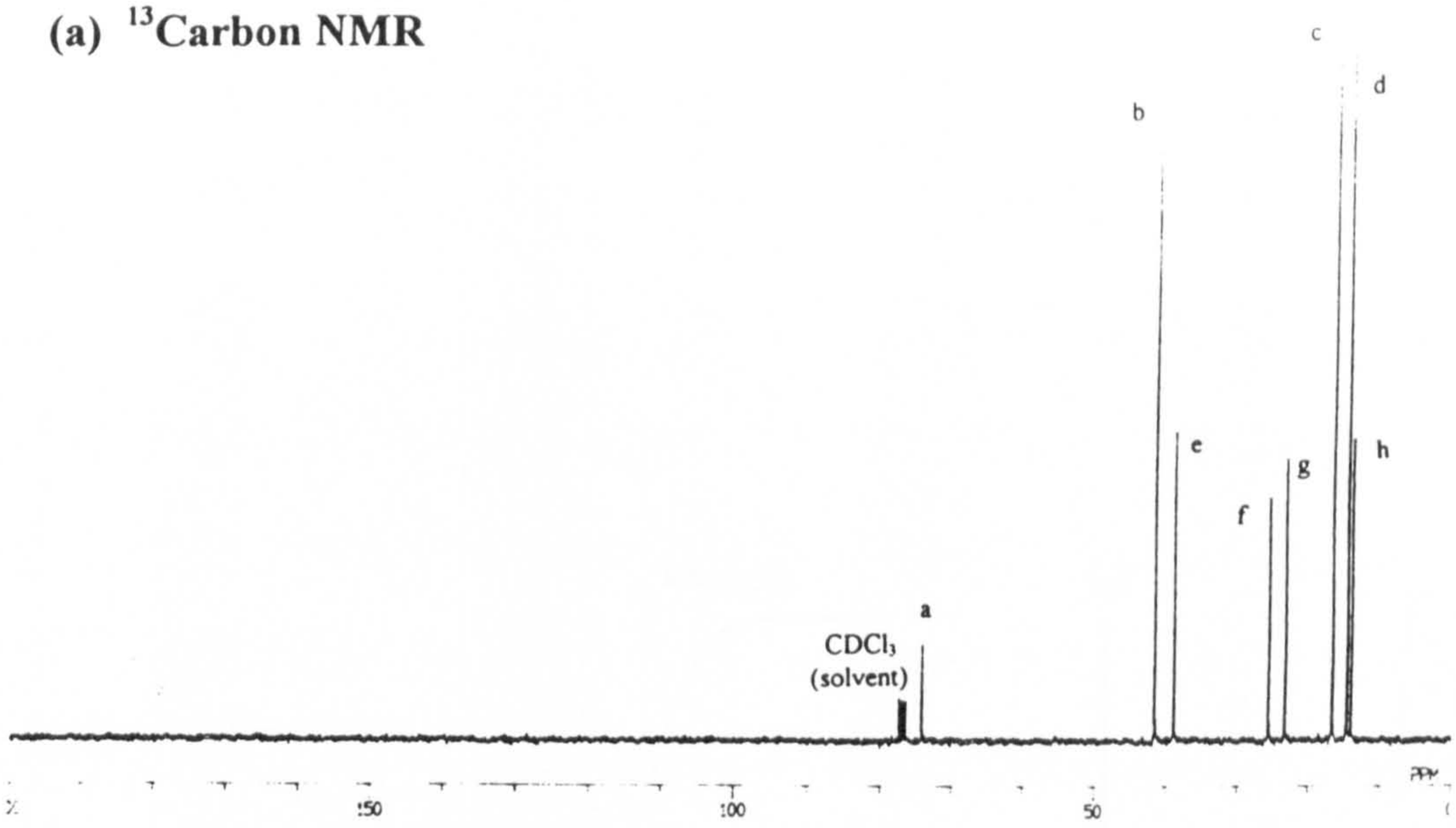
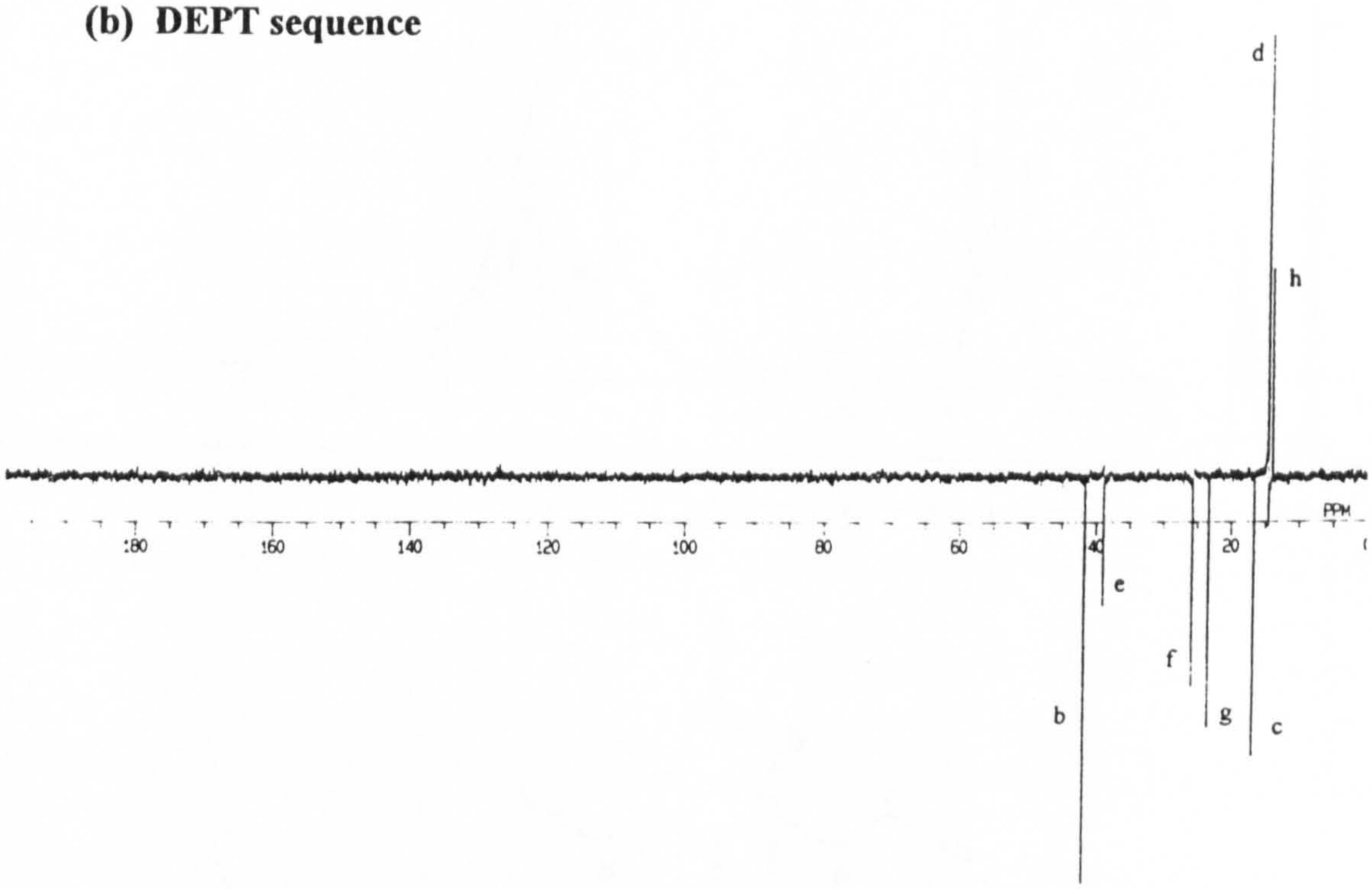


Figure 3.5 Infra red spectrum of 4-propyloctan-4-ol

(a) ^{13}Carbon NMR



(b) DEPT sequence



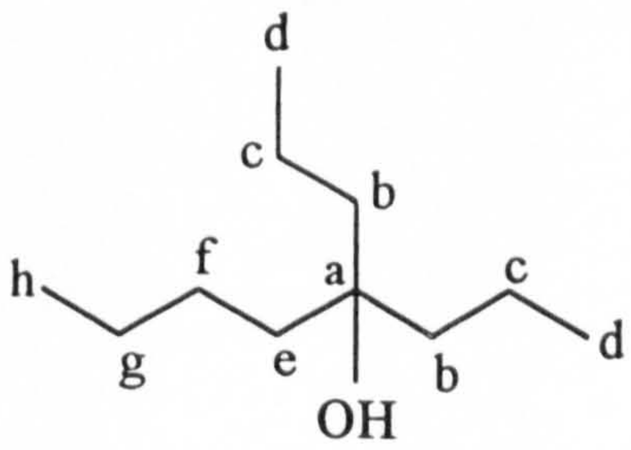
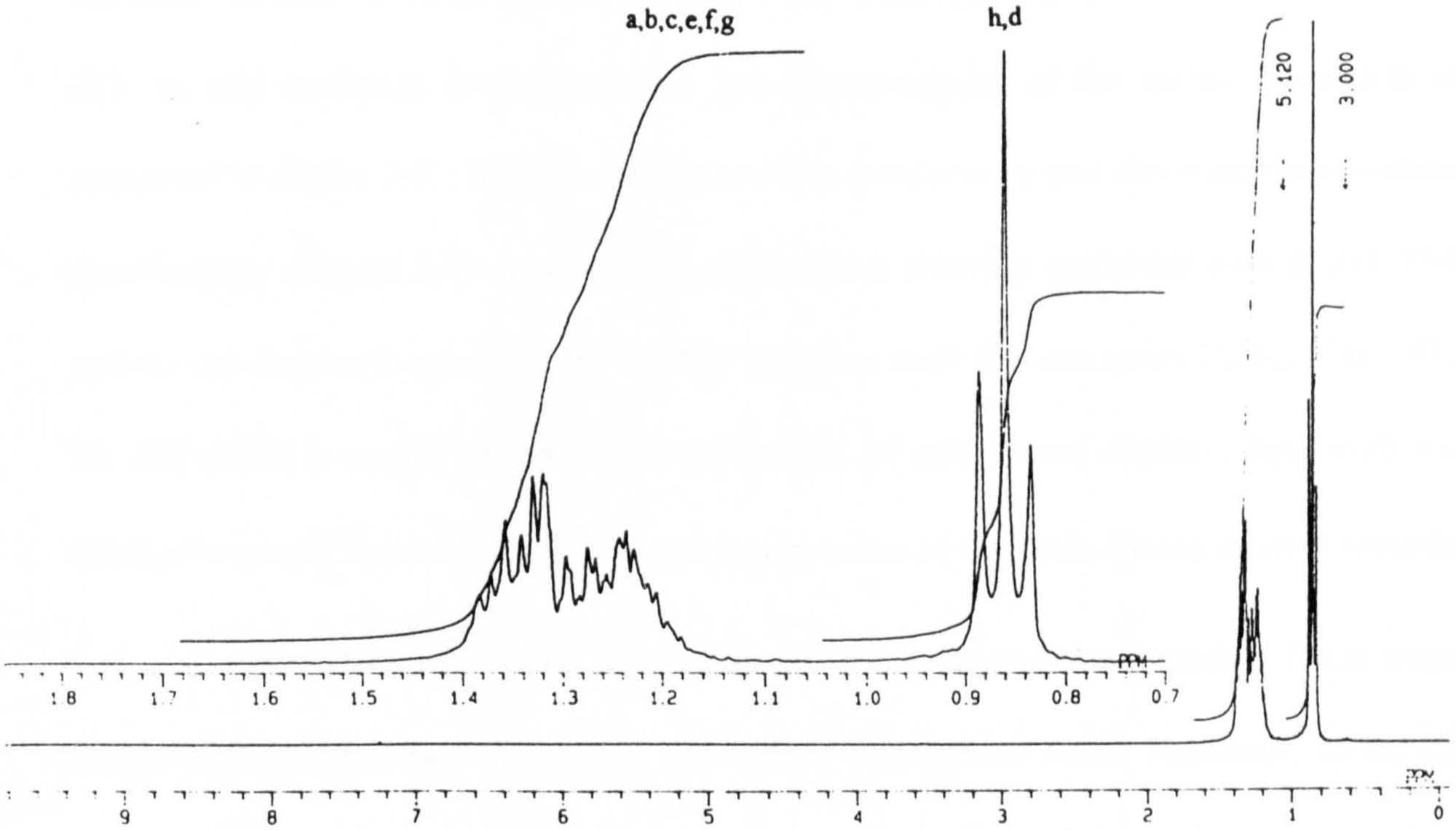


Figure 3.6. ^{13}Carbon and DEPT NMR spectra of 4-propyloctan-4-ol



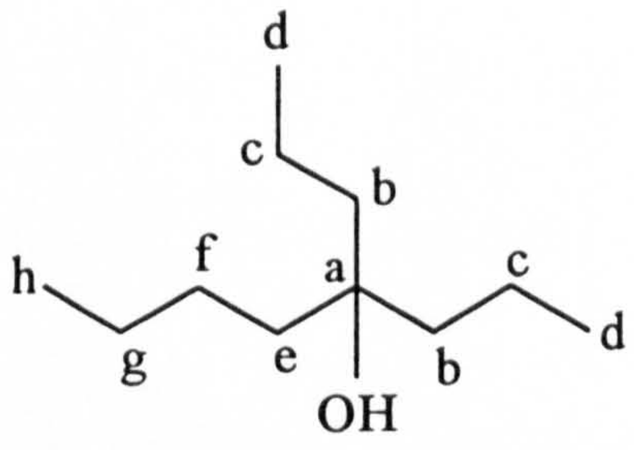
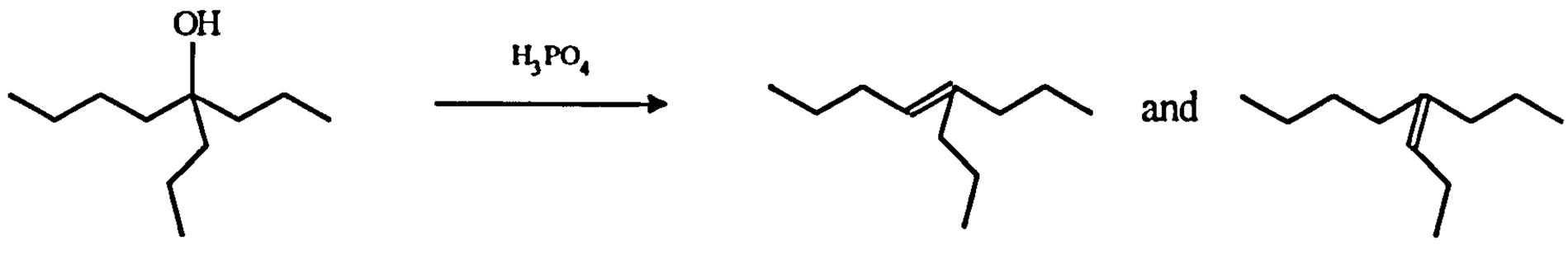


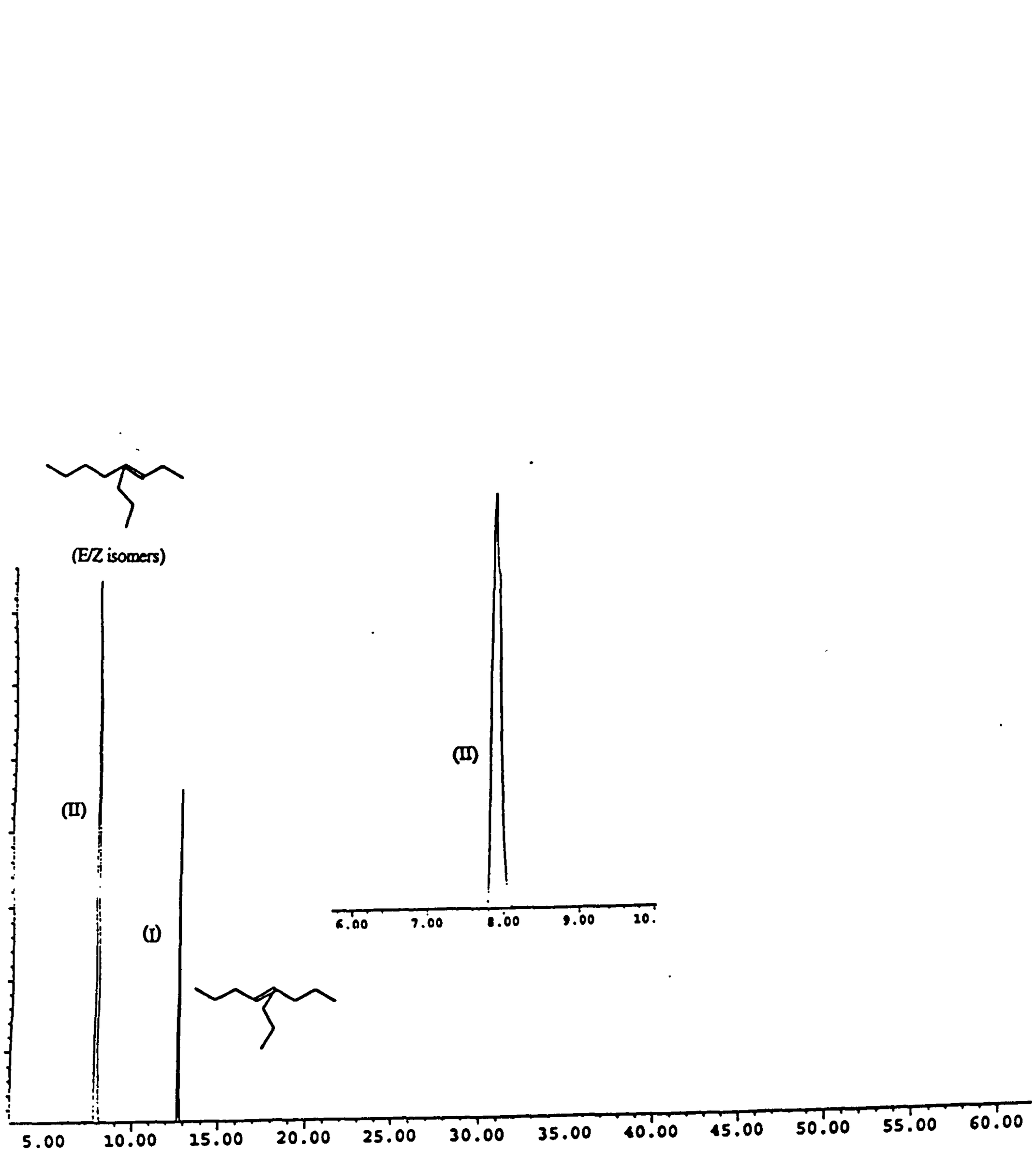
Figure 3.7 ^{1}H NMR spectrum of 4-propyloctan-4-ol

3.2.3 Synthesis of isomeric mixture of 4-propyloctenes



(I) (II)

The alcohol was dehydrated in the presence of orthophosphoric acid (H_3PO_4), to an isomeric mixture of three alkenes (4-propyloct-4-ene (I) and E/Z 4-propyloct-3-enes (II)) by acid catalysed dehydration. A gas chromatogram of the reaction products is presented in Figure 3.8. Analysis of the reaction products by gas chromatography-mass spectrometry (Figure 3.9) revealed two peaks, both showing molecular ions at *m/z* 154 and an ion fragment *m/z* 111 $[M^{+\cdot}\text{-}C_3H_7]^+$ together with the ion series C_nH_{2n-1} [*m/z* 41, 55, 69] which is usually prominent in the spectra of unsaturated aliphatic hydrocarbons (McLafferty and Tureček, 1993). Closer examination of the peak eluting at *ca* 8 minutes indicates a shoulder to the peak, suggesting co-elution of two compounds. These were tentatively identified as the E and Z isomers of 4-propyloct-3-ene. However, owing to virtually identical mass spectra unequivocal assignment of each isomer was not possible.



[GC details; HP-1 column. He carrier gas. Temp. Program; 40°C - 300°C @ 5°C min^{-1}, hold 10 mins]

Figure 3.8 Gas chromatogram of isomeric mixture of 4-propyloctenes

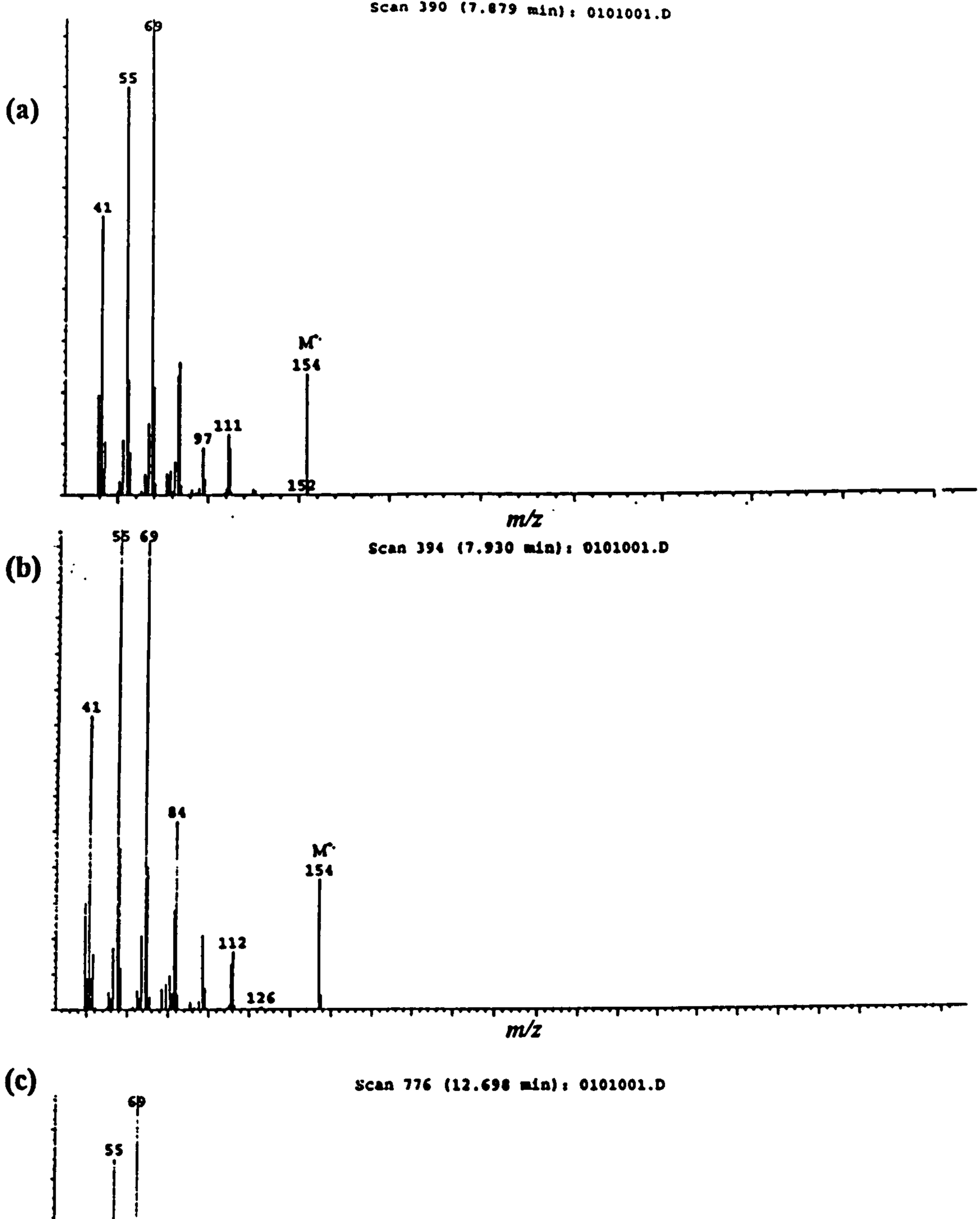
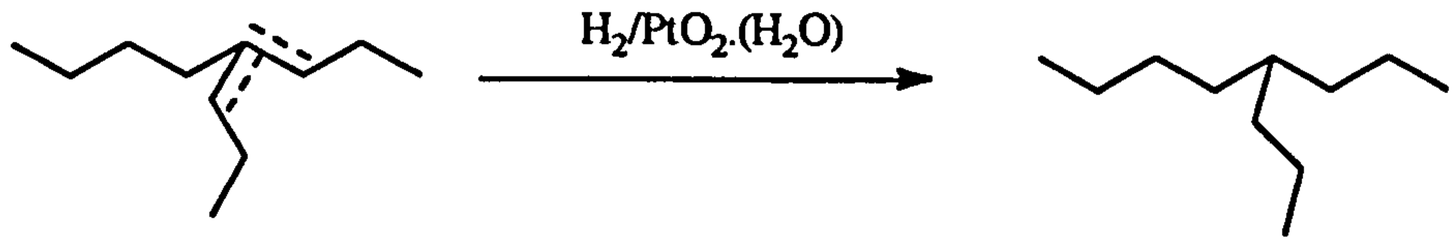


Figure 3.9 Mass spectra of isomeric mixture of 4-proplyoctenes. (a) and (b) are tentatively identified as co-eluting E/Z 4-propyloct-3-enes and (c) as 4-propyloct-4-ene.

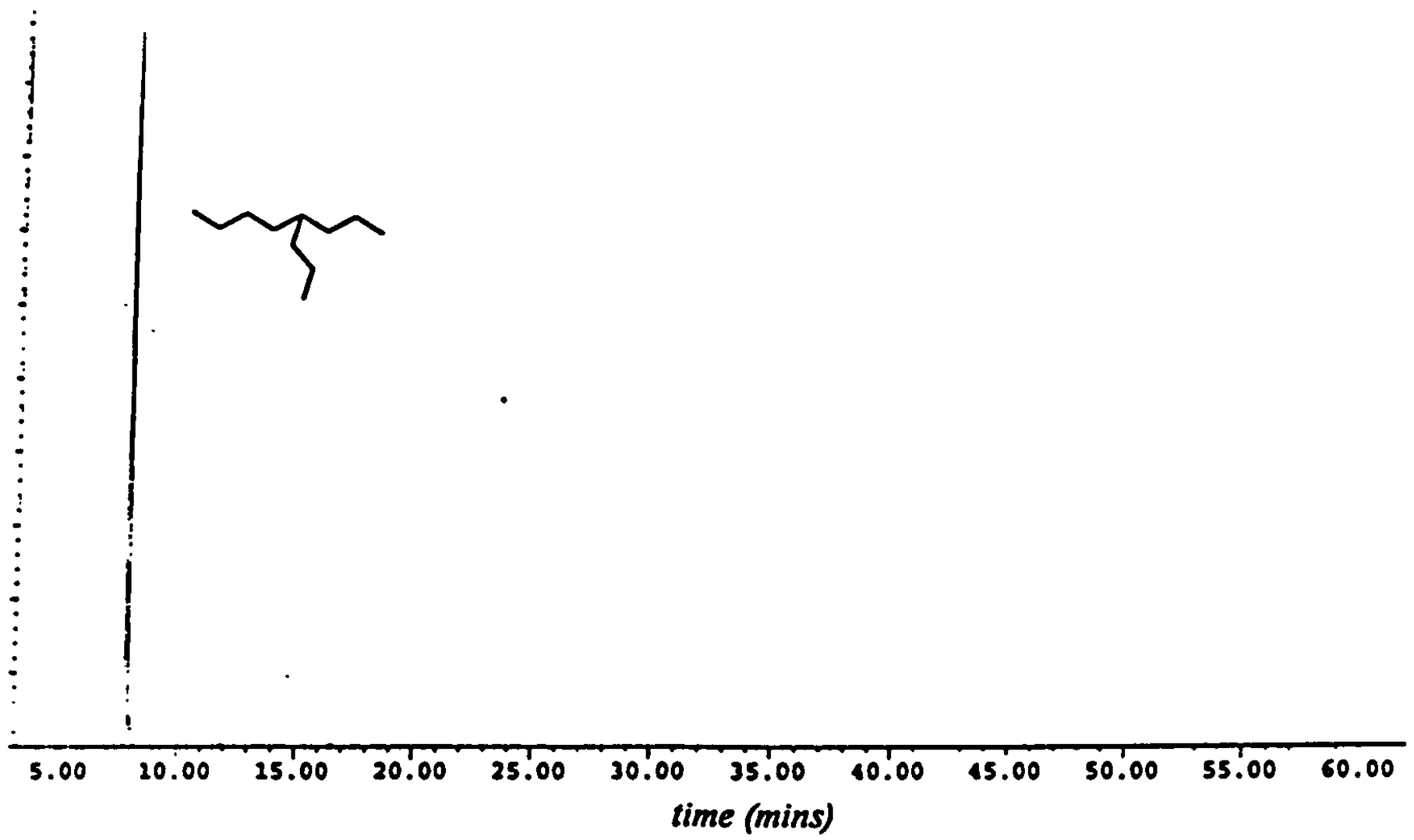
3.2.4 Synthesis of 4-propyloctane



The mixture of alkenes was hydrogenated to 4-propyloctane by bubbling hydrogen gas gently through a solution of the alkenes in hexane, in the presence of a small amount of Adams catalyst (monohydrate). Column chromatography (Ag^+/silica) yielded the desired pure product (> 99 % by GC, Figure 3.10; yield 85%) which was then examined by GC-MS and NMR.

The mass spectrum (Figure 3.11) showed the molecular ion (*m/z* 156) and fragment ions ($C_4H_9^+/C_4H_{10}^+$ [m/z 99/98], $C_3H_7^+/C_3H_8^+$ [m/z 112/113]) derived from α -cleavage about the tertiary centre, confirming the branch position at C-4. The doublet odd:even ions are typical of branched alkanes and are thought to arise from secondary transfer involving a cyclic intermediate (McCarthy *et al.*, 1968)

^{13}C NMR (Figure 3.12a) in conjunction with the DEPT sequence (Figure 3.12b) indicated the presence of 8 aliphatic resonances; 2 methyl carbons, 5 methylene and 1 methine carbon as expected, which in conjunction with the MS data confirmed synthesis of the target compound, 4-propyloctane. ^{13}C-NMR spectral data (chemical shift/intensity/multiplicity) and assignments are presented in Appendix C.2. The ^1H-NMR spectrum of 4-propyloctane together with assignments is presented in Figure 3.13.



[GC details; HP-1 column. He carrier gas. Temp. Program; 40°C - 300°C @ 5°C min^{-1}, hold 10 mins]

Figure 3.10 Gas chromatogram of 4-propyloctane

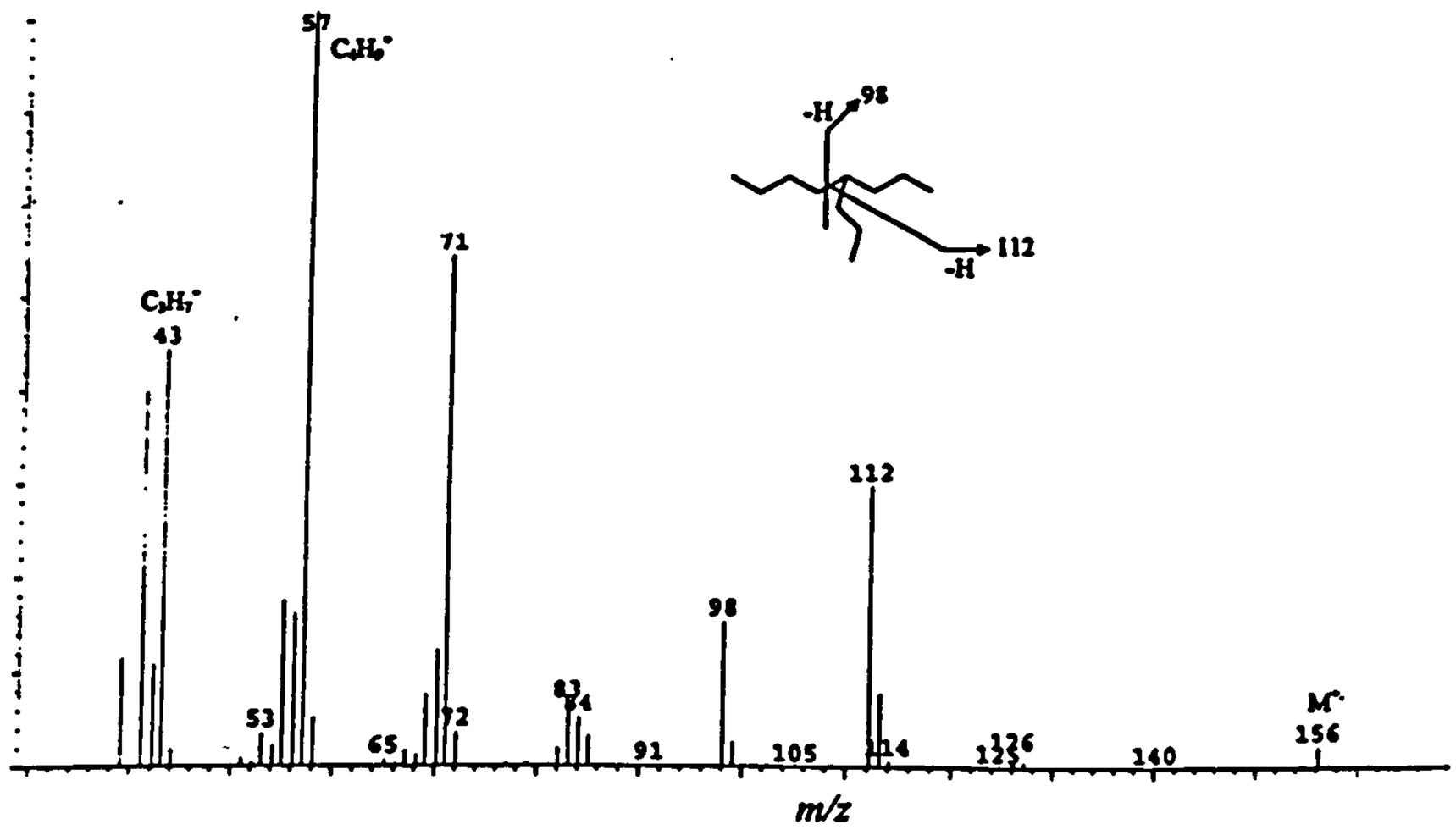
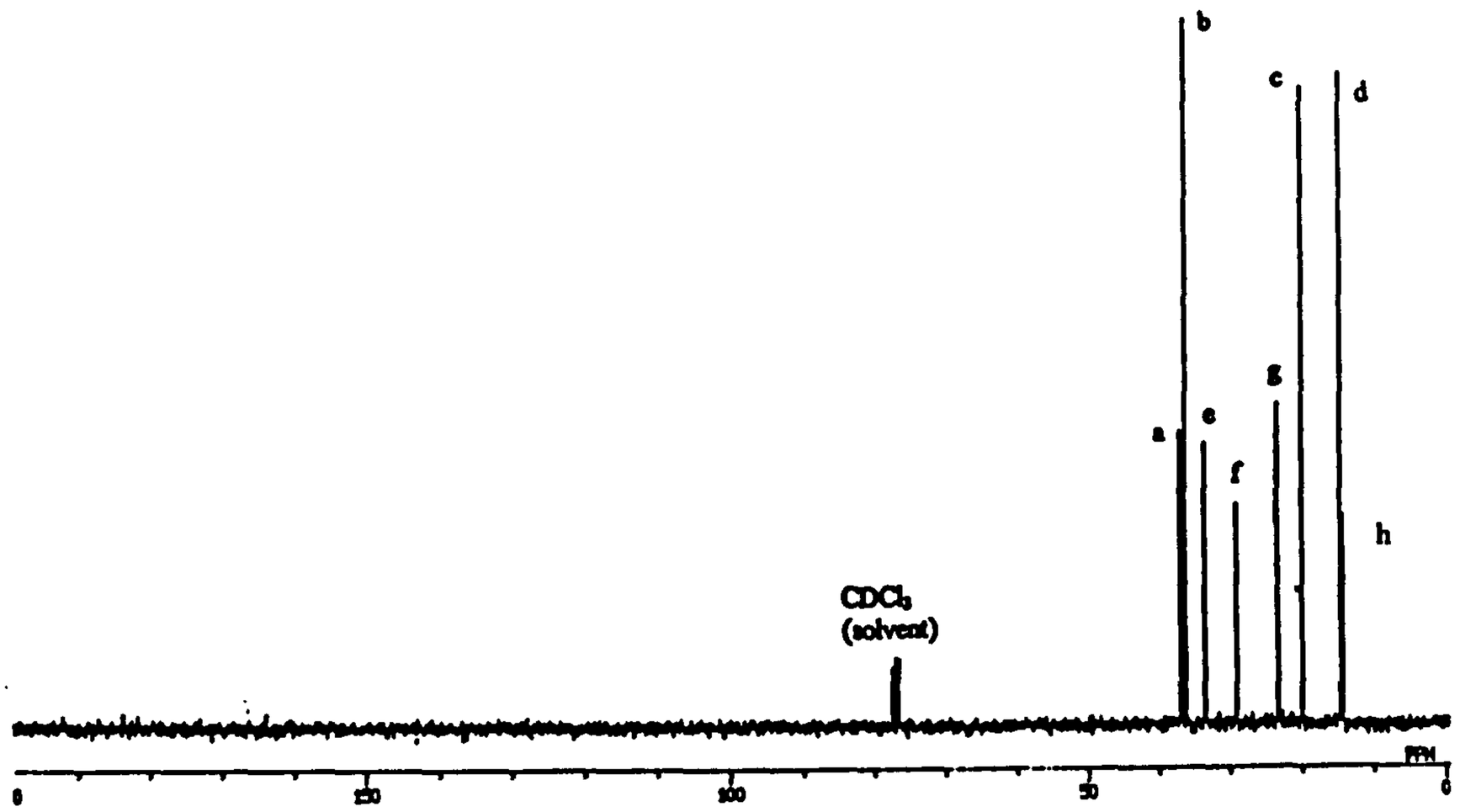
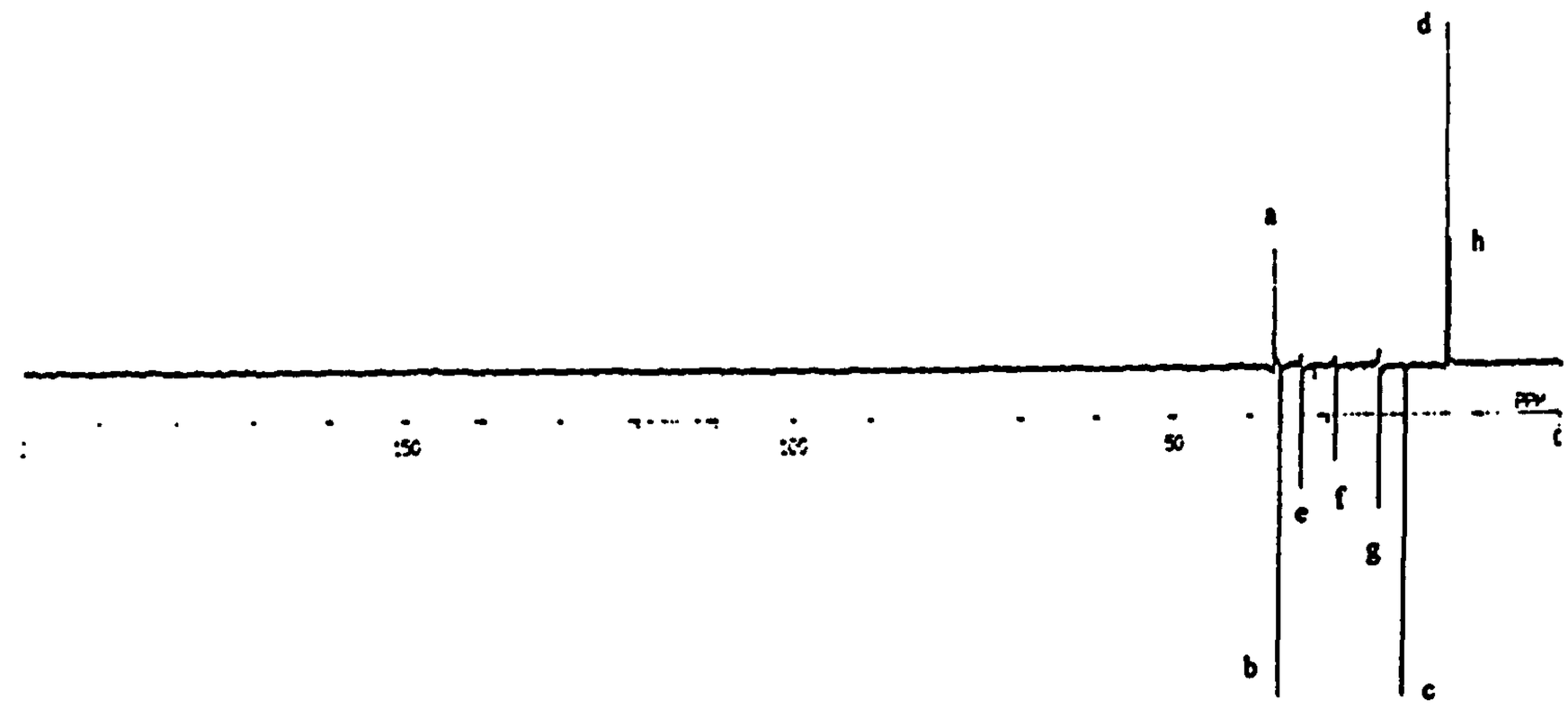


Figure 3.11 Mass spectrum of 4-propyloctane

(a) ^{13}C NMR



(b) DEPT



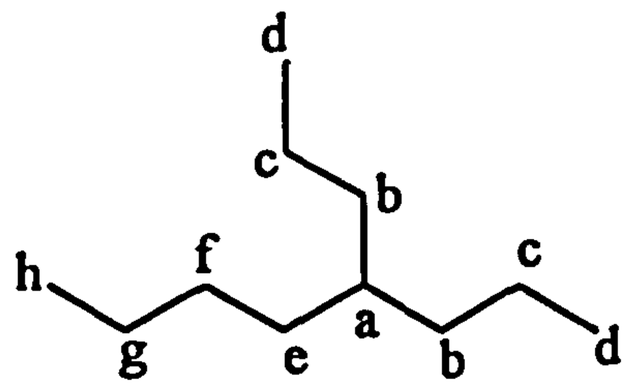
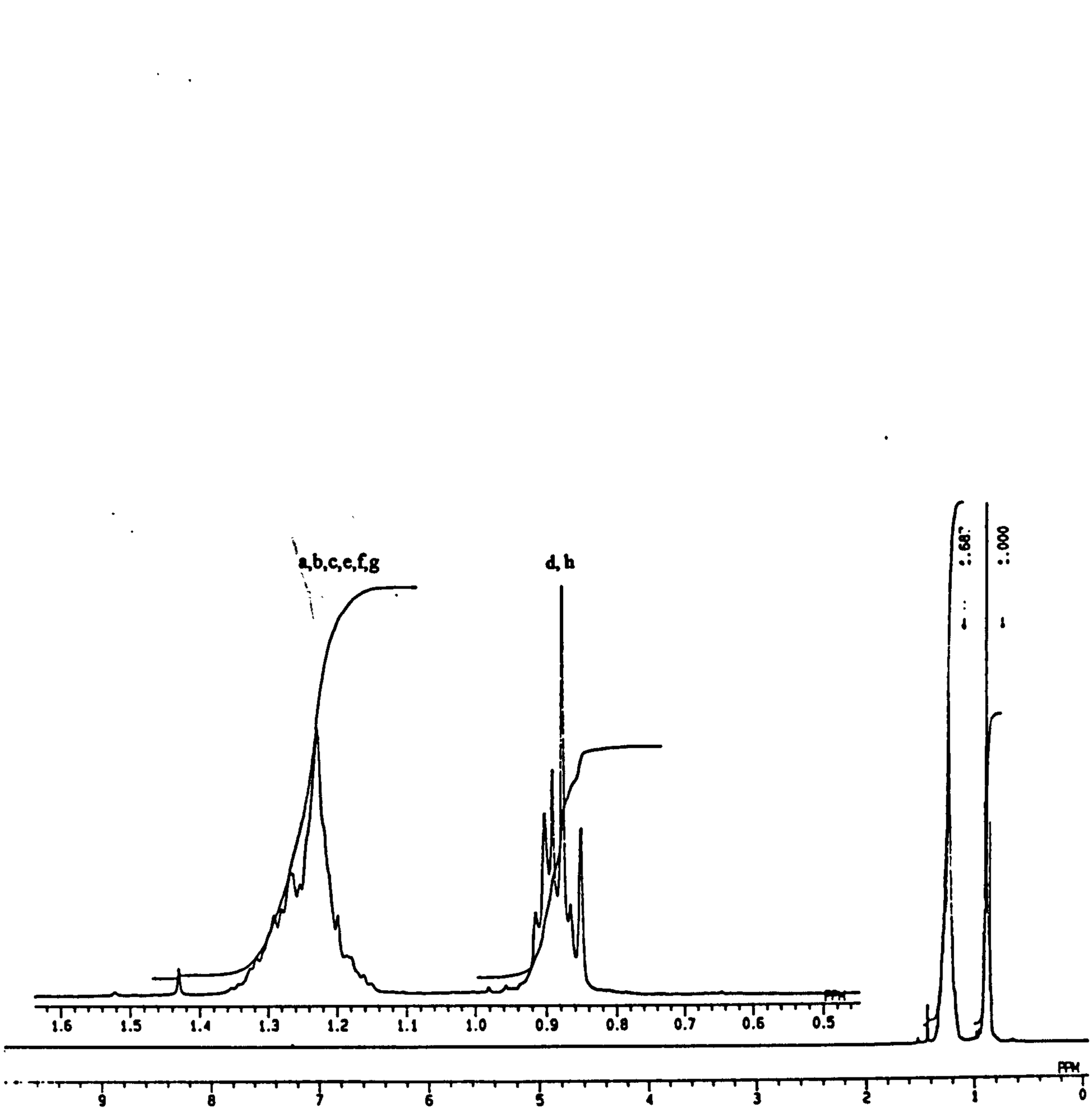


Figure 3.12 ^{13} Carbon and DEPT NMR spectra of 4-propyloctane



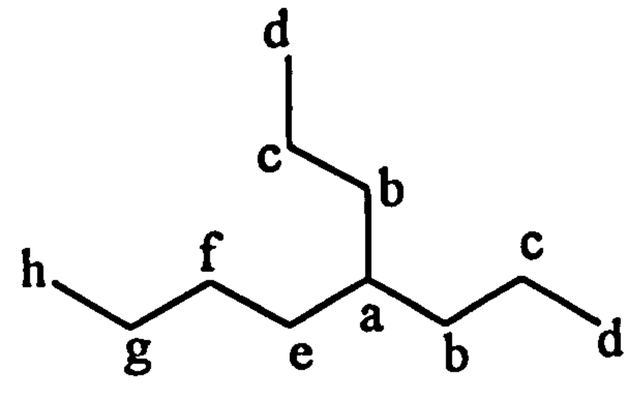


Figure 3.13 ^{1}H NMR spectra of 4-propyloctane

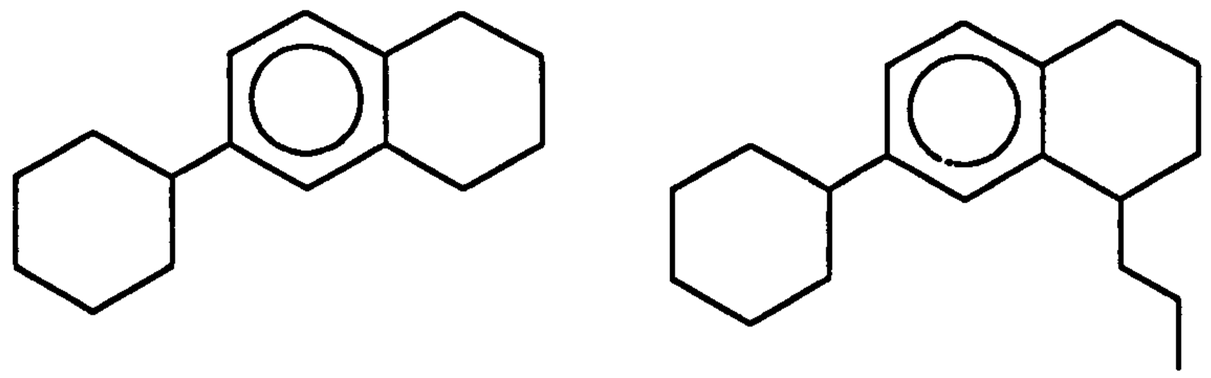
3.2.5 Conclusion

The proposed model aliphatic UCM alkane, 4-propyloctane has been obtained in good purity (>99%) in sufficient quantity (838 mg) for future toxicological tests on the mussel *Mytilus edulis* as detailed in Chapter 4.

3.3 Synthesis of model aromatic UCM compounds

The model aromatic UCM hydrocarbons chosen for synthesis and toxicity testing were the alkyl tetralins, 7-cyclohexyltetralin and 7-cyclohexyl-1-propyltetralin (Figure 3.14 structures I and II, respectively). Such compounds have recently been proposed as 'average' aromatic UCM components on the basis of degradative oxidation studies and ultra high resolution mass spectral (FT-ICR) studies of crude oils and oil refinery fractions (Thomas, 1995; Thomas *et al.*, 1997; reviewed in Section 1.2). As with the aliphatic UCM components, these compounds are not available from commercial sources. Little is known about the solubility or the toxicity of hydrocarbons of this type, but it has been demonstrated in Chapter 2 that mussels in impacted sites contain significant levels of aromatic UCMs, with concentrations typically in the range 100 - 600 $\mu g\ g^{-1}$ dry weight tissue, although values as high as 3500 $\mu g\ g^{-1}$ have been measured in heavily contaminated areas such as harbours (Mason, 1988).

An estimate of the physical behaviour of the alkyl tetralins chosen for synthesis can be computed from methods for octanol/water partition coefficient (K_{OW}) estimation (*e.g.* Hansch and Leo, 1995 and references therein).



I

II

Figure 3.14. Model aromatic UCM compounds; 7-cyclohexyltetralin (I) and 7-cyclohexyl-1-propyltetralin (II)

3.3.1 Estimation of octanol/water partition coefficients

A number of methods for the estimation of octanol/water partition coefficients have been developed. These include the use of substituent/fragment constants, estimation *via* atomic contribution and/or surface area, a method based on calculated molecular properties and also the use of solvatochromic parameters. Detailed reviews of the application and use of each individual estimation method are available in a number of publications (Leo *et al.*, 1971; Leo and Hansch, 1979; Lyman 1990; Leo, 1993; Hansch and Leo, 1995). Estimation of log K_{OW} *via* the fragment method of Leo and Hansch (1979) is the most widely used and well developed method and appeared most suitable for the needs of the present study.

The method involves the use of empirically derived atomic or group fragment constants (f) and structural factors (F). All calculations are carried out in terms of log K_{OW}. Thus;

$$\text{Log } K_{OW} = \text{sum of fragments (f)} + \text{factors (F)}$$

A comparable method also involving the use of fragment constants has been reported by Nys and Rekker (1973, 1974), but more fragment constants are available for the Leo method and the 'rules of fragmentation' for the solute of interest are less ambiguous than

those of Nys and Rekker (1973) where the fragmentation of the solute is operator-defined (Lyman, 1990; Leo, 1993).

Fragment values (f) used in this study are taken from Lyman (1990). Different factors are considered, taking into account molecular flexibility, unsaturation, branching, halogenation and interaction with H-polar fragments. It must be noted that for hydrophobic compounds with large log K_{ow} values, (greater than 6), errors in the estimation of log K_{ow} for a particular compound may be large. Hansch and Leo (1980, cited by Lyman, 1990) note that estimates of log K_{ow} greater than 6 may be overestimates of the measured log K_{ow} value by perhaps one or more log units. However, as the compounds of interest in this study have yet to be synthesised and there are no log K_{ow} values for similar/comparable compounds reported in the literature (Leo *et al.*, 1971; Leo and Hansch, 1979) an estimate of log K_{ow} by Leo's fragment method will, at the least, provide an indication of the log K_{ow} of the alkyltetralins and guide the choice of particular alkyltetralin model aromatic UCM components to be synthesised. A summary of the rules employed in Leo's fragment method and the calculations employed herein are presented in Appendix D.1.

Estimates of the log K_{ow} and aqueous solubility of the model aromatic UCM compounds to be synthesised were also calculated using several established computer programs. Log K_{ow} values were calculated using the MedChem ClogP program, which calculates log K_{ow} *via* the fragment method discussed above and the Syracuse Corporation Software (SRC). MicroQSAR and SRC software were used to obtain estimates of the aqueous solubility data (values provided by Prof. J. Dearden, Liverpool John Moores University). All estimated log K_{ow} values, as obtained by the various methods are summarised in Table 3.1 No indication of the error in the estimates obtained using ClogP and SRC software was available and consequently, the accuracy of these values is unknown. However, all of the estimates of log K_{ow} obtained for the

model aromatic UCM compounds by the various different estimation methods were similar, but, as noted previously, estimates of log K_{OW} greater than 6 may be significantly overestimated. The measured values of log K_{OW} used to derive fragment constants are themselves mainly compounds with log K_{OW} values less than 5-6, owing to the inherent difficulties in accurately measuring the octanol/water partition coefficients of highly hydrophobic compounds (*i.e.* log $K_{OW} > 6$).

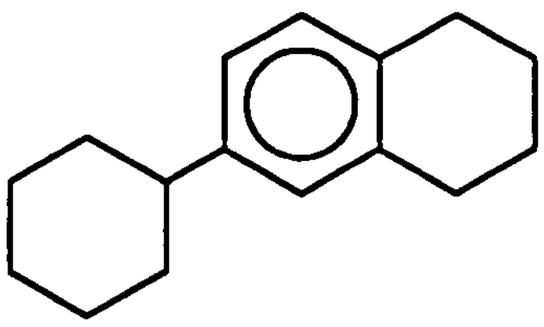
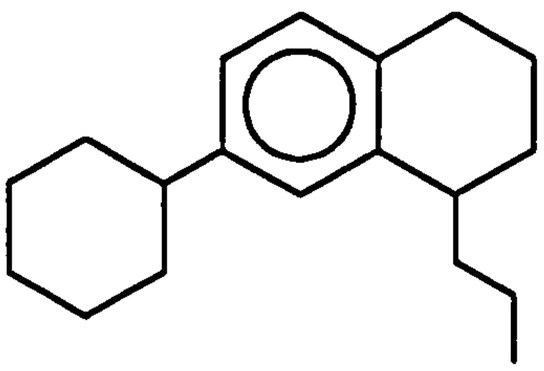
Compound	log K_{OW}			Aqueous solubility	
	estimate (this study)	ClogP	SRC	microQSAR	SRC
	6.61	6.33	6.77	151 µg l^{-1}	60 µg l^{-1}
	7.73	7.91	8.17	3 µg l^{-1}	2 µg l^{-1}

Table 3.1 Estimates of log K_{OW} and aqueous solubility for model aromatic UCM compounds (provided by Prof. J. Dearden, Liverpool John Moores University)

3.3.2 Synthetic scheme for alkyltetralins

Two model aromatic UCM compounds, 7-cyclohexyltetralin and 7-cyclohexyl-1-propyltetralin (Figure 3.14) were synthesised using a modification of the Haworth synthesis (March, 1985; Vogel, 1989, Figure 3.15). This synthetic pathway has been

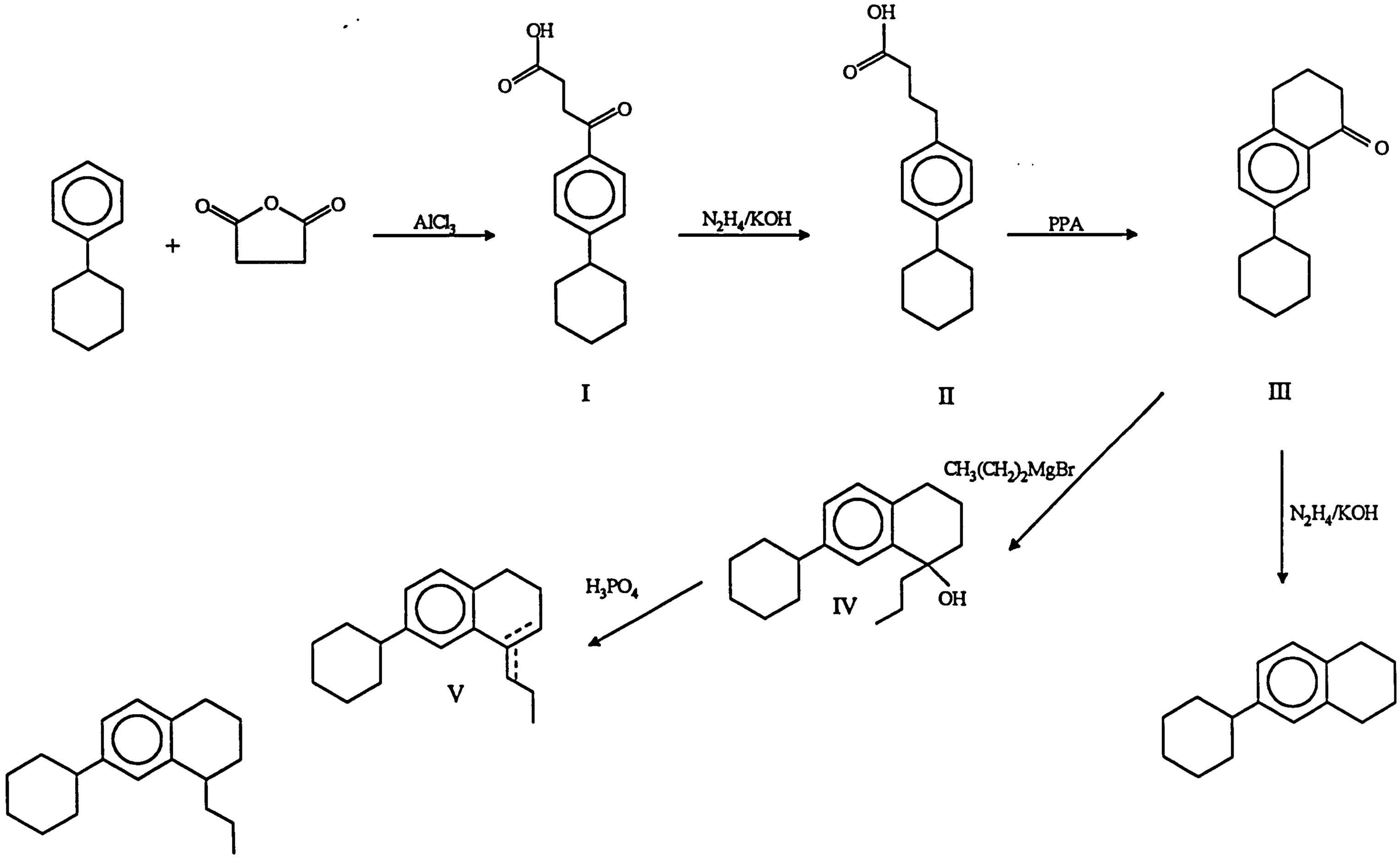


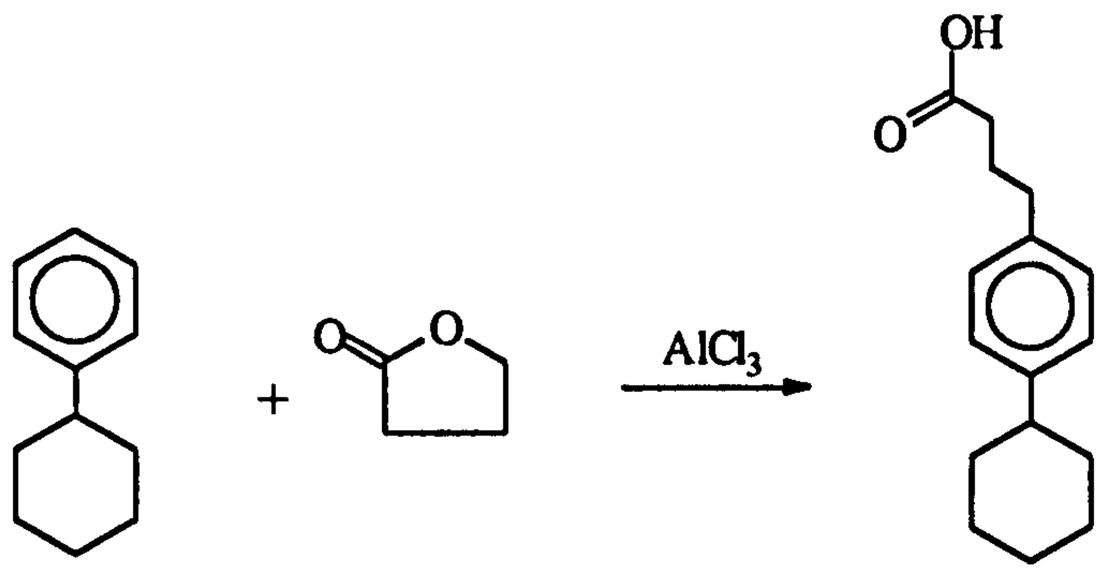
Figure 3.15 Reaction scheme for the synthesis of model aromatic UCM compounds

extensively studied previously and is commonly used for the synthesis of a wide range of aromatic hydrocarbons.

Typically, the Haworth synthesis involves formation of a keto acid from an aromatic hydrocarbon reactant using the Friedel-Crafts acylation reaction. The keto acid is then reduced by the Clemmensen reduction and the reduced acid converted to a cyclic ketone using polyphosphoric acid (PPA). The cyclized intermediate may then be converted into a polycyclic hydroaromatic or aromatic compound by standard procedures such as dehydrogenation using, for example, palladium or platinum with a charcoal catalyst (Berliner, 1949). Alkyl substituents may be added by means of a Grignard reaction with the cyclized ketone.

The synthetic route for both compounds was the same for the first three stages, to the cyclic ketone (7-cyclohexyl-1-tetralone). For the 'base' compound 7-cyclohexyltetralin, the keto group was reduced to an alkyl group, whilst the second model compound, 7-cyclohexyl-1-propyltetralin was synthesised by addition of a propyl chain to the tetralone using a Grignard reaction. Dehydration of the alcohol product with phosphoric acid followed by catalytic hydrogenation yielded the target alkyltetralin.

3.3.3 Attempted synthesis of 4-phenyl(4'-cyclohexyl)butanoic acid. Friedel-Crafts alkylation of phenylcyclohexane using γ-butyrolactone



4-phenyl(4'-cyclohexyl)butanoic acid

The conventional route for the step-wise synthesis of aromatic hydrocarbons *via* the Haworth synthesis involves coupling of an aromatic hydrocarbon and an aliphatic dibasic acid by a Friedel-Crafts acylation reaction. However, a number of authors have reported syntheses utilizing lactones in Freidel-Crafts alkylation reactions, (Mosby, 1952; Truce and Olsen, 1952, Eisenbraun *et al.*, 1971). This synthetic route has the advantage of producing an acid (Figure 3.15, II) as opposed to the keto-acid (Figure 3.15, I), thereby eliminating one stage in the synthetic route.

Mosby (1952) reported a successful condensation of γ-valerolactone with each of the isomeric xylenes in the synthesis of various polymethylnaphthalenes. Good yields of pure xylylvalearic acids were obtained if the reaction mixture was hydrolysed immediately after production of hydrogen chloride had ceased. However, varying amounts of trimethyl-1-tetralone were obtained if the reaction mixture was heated longer than necessary or allowed to stand. Mosby (1952) suggested that additional aluminium chloride may cause cyclization at this stage but this was not investigated.

Truce and Olsen (1952) also reported successful condensation reactions using lactones. These authors reported the condensation of γ-butyrolactone with benzene in the presence of aluminium chloride and demonstrated that by varying the molar ratio of aluminium

chloride/lactone, either a mixture of γ-phenylbutyric acid and γ-tetralone or simply γ-tetralone as the sole product (66 % yield) could be obtained.

These studies suggest that Friedel-Crafts alkylation reactions using lactones may provide a quicker and shorter synthetic route than the conventional acylation route using aliphatic anhydrides.

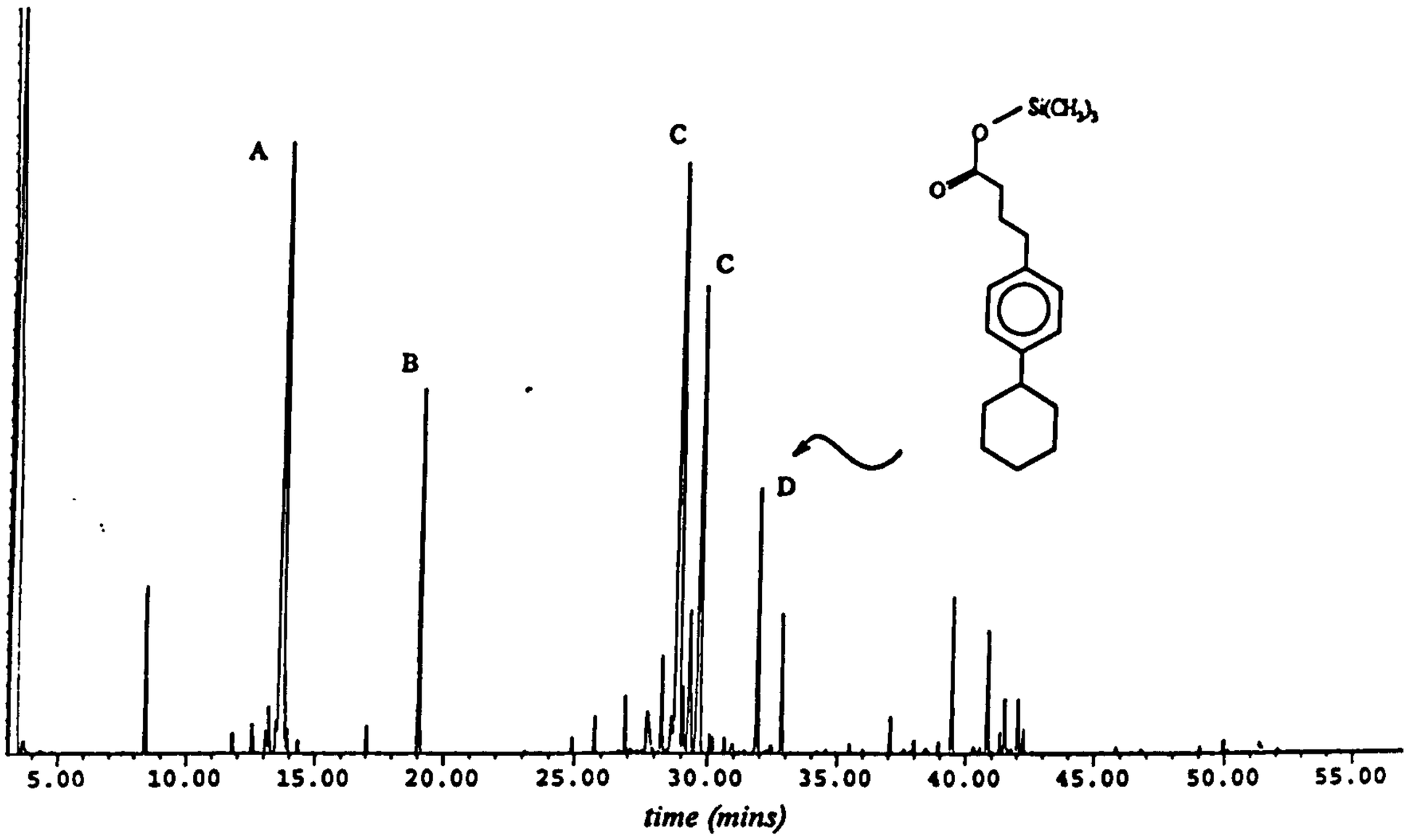
In contrast, in a study to assess and re-examine the stepwise synthesis of a number of polyalkylnaphthalenes, Eisenbraun *et al.* (1971) compared the acylation route with alkylation syntheses utilizing lactones. Variable results were reported for reactions involving either γ-butyrolactone or γ-valerolactone. For example, mixtures of acids in varying yields (23% - 81% according to reaction conditions) were produced. Pronounced isomerization was also evident (*i.e.* 7 % *m*-xylene was present in the recovered *p*-xylene in the reaction of γ-butyrolactone and *p*-xylene). These results were attributed to the difference in reactivity and stability of intermediate species generated from lactones by aluminium chloride and the authors concluded that although Friedel-Crafts alkylation of an aromatic hydrocarbon with a γ-lactone is a shorter route than the acid anhydride acylation, the latter, in certain cases is to be preferred.

With respect to the current synthesis, the presence of a cyclohexyl group on the benzene ring should promote the substitution reaction rate to a greater extent when compared with benzene owing to the activating inductive effect. Consequently, the coupling of phenylcyclohexane and γ-butyrolactone *via* a Freidel-Crafts alkylation reaction was investigated.

Initially, alkylation of phenylcyclohexane with γ-butyrolactone using excess phenylcyclohexane as a solvent was investigated. However, only a very low yield (4 %) of the target acid was obtained. The total reaction products were dominated by three compounds which were tentatively identified as dimers of phenylcyclohexane and

4-phenylbutyric acid. A gas chromatogram of the total reaction products is presented in Figure 3.16, together with mass spectra of the principal reaction products (Figure 3.17). Several further attempts in which the order of addition of reactants was varied yielded similar results.

The synthesis was repeated using nitrobenzene as a solvent. It was thought that this would moderate the activity of the aluminium chloride and possibly suppress any undesirable side reaction (J. Braven, pers comm). Analysis of the total organic reaction products by GC-MS indicated that using nitrobenzene as a solvent had indeed suppressed the side reactions. However yields of the desired acid were still very low (13 %). In light of these various unsuccessful attempts to alkylate phenylcyclohexane using γ-butyrolactone, it was decided to follow the more conventional route of Friedel-Crafts acylation using succinic anhydride.



[GC details; HP-1 column. He carrier gas. Temp. program; 40°C - 300°C @ 5° min^{-1}, hold 10 mins]

Figure 3.16 Gas chromatogram of total organic reaction products (derivatised with BSTFA) from alkylation of phenylcyclohexane using γ-butyrolactone

(a) Peak A

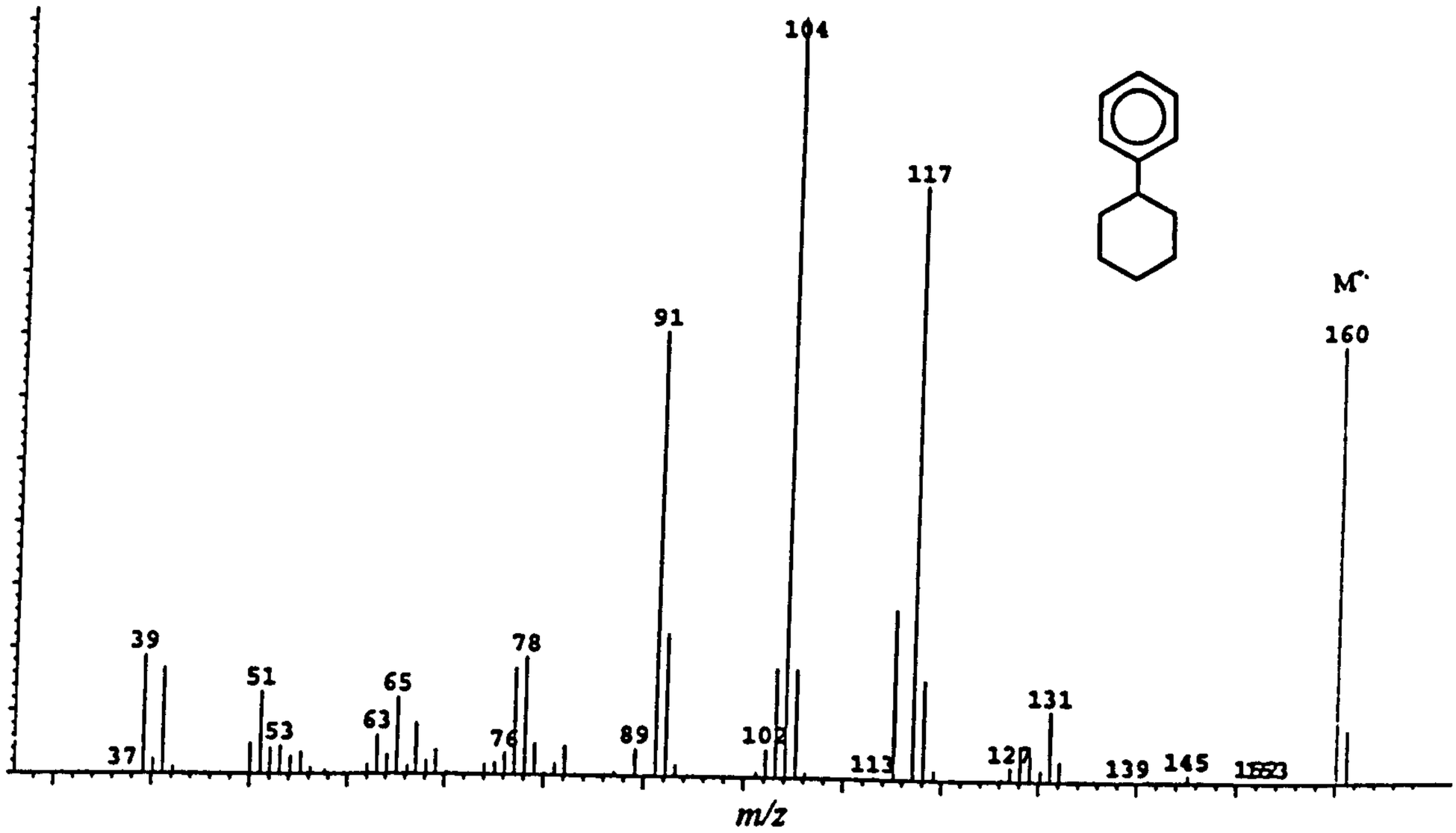
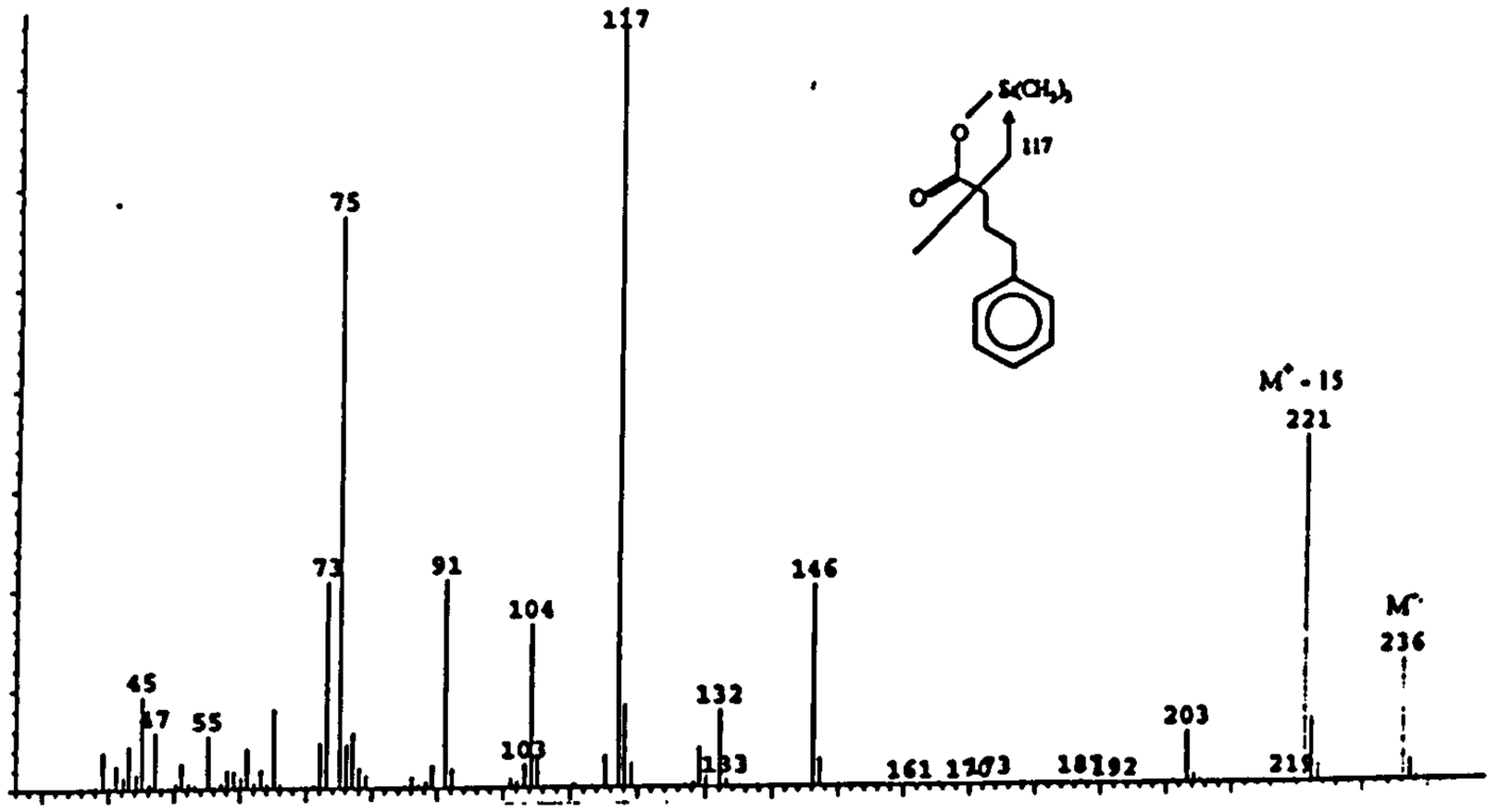
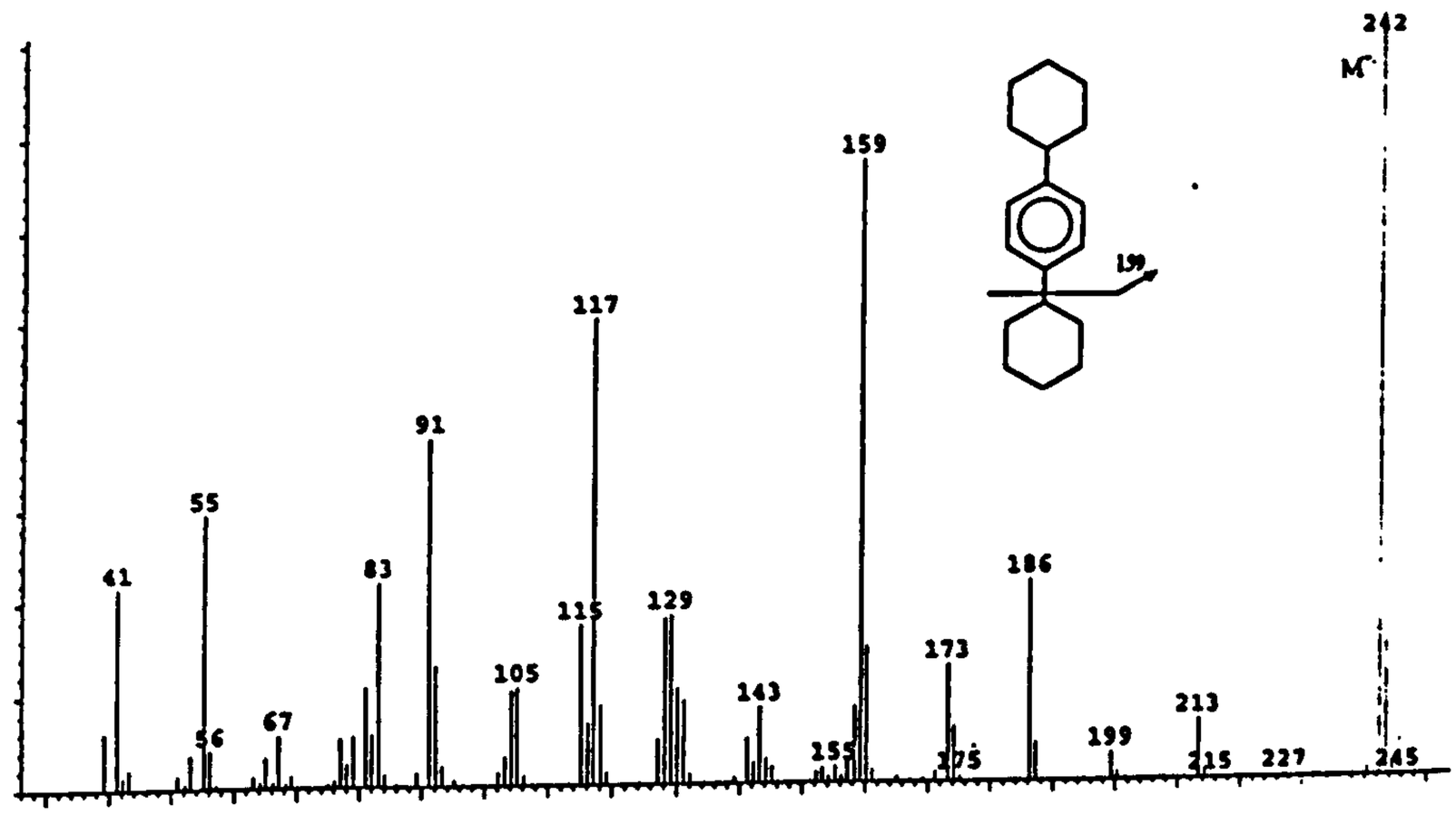


Figure 3.17 Mass spectra of principal components of the total organic reaction products (derivatised with BSTFA) from alkylation of phenylcyclohexane using γ-butyrolactone *Cont'd over*

(b) Peak B



(c) Peak C



(d) Peak D

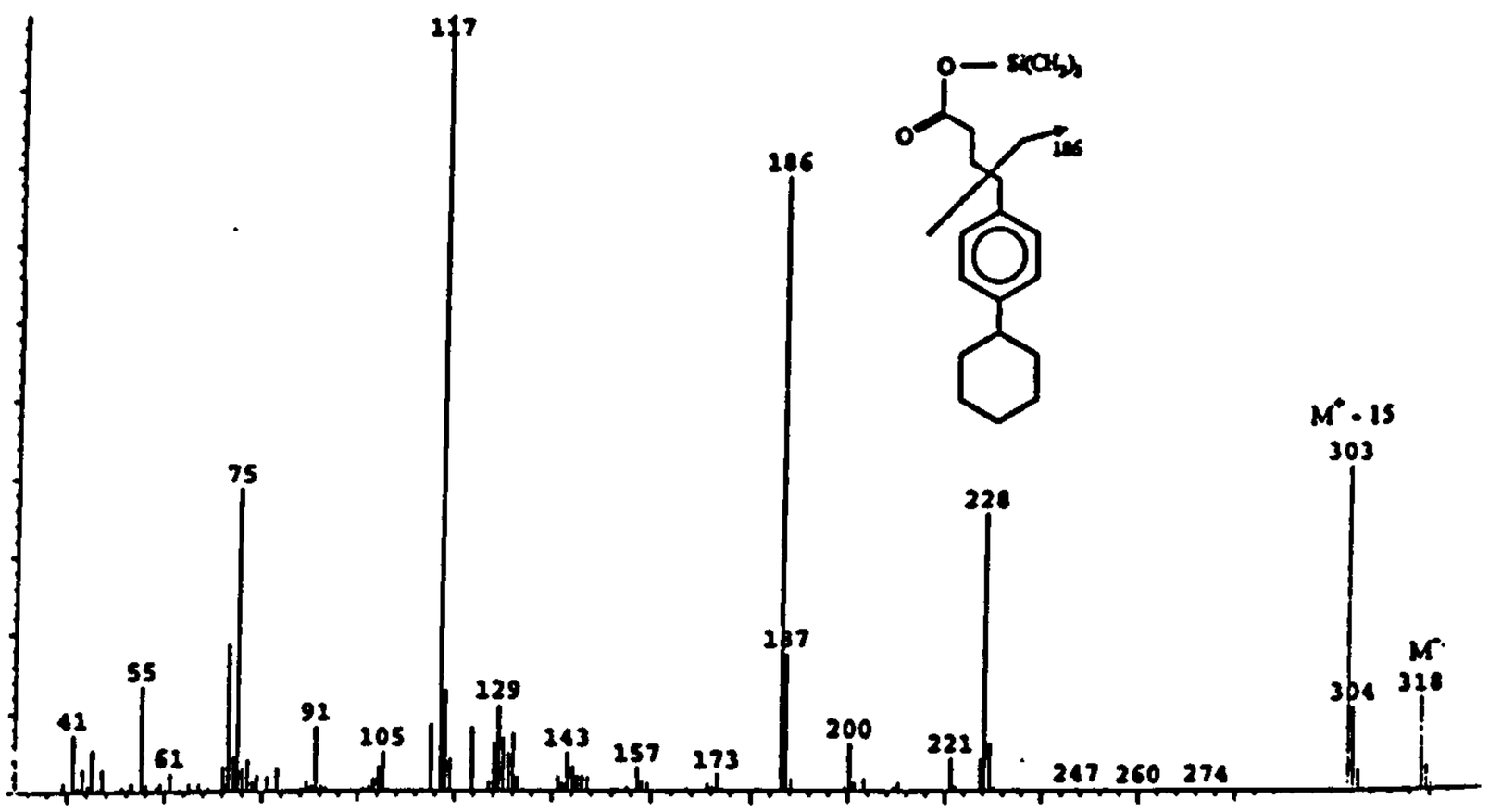
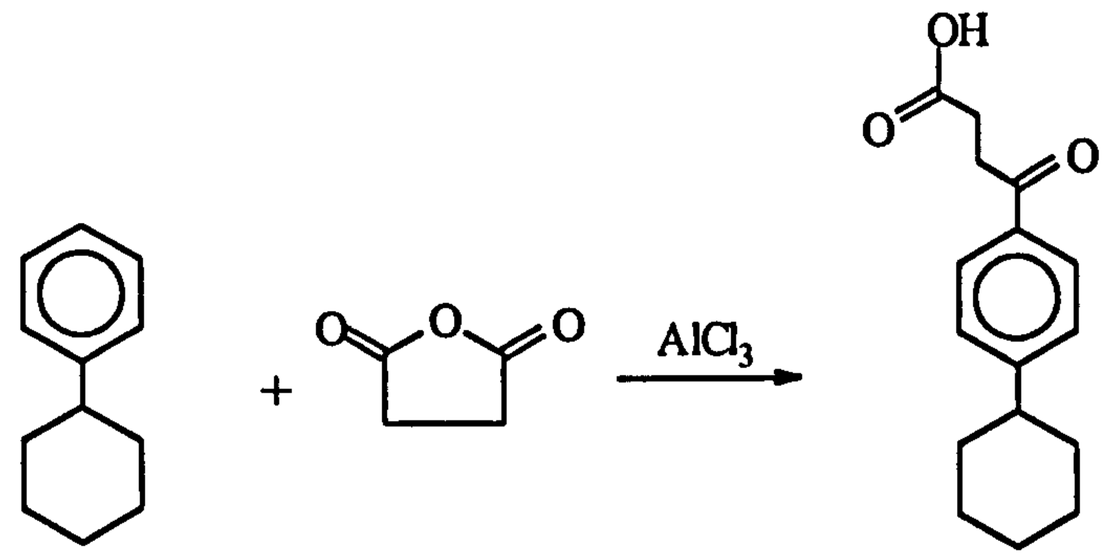


Figure 3.17 *Cont'd* Mass spectra of principal components of the total organic reaction products (derivatised with BSTFA) from alkylation of phenylcyclohexane using γ-butyrolactone

3.3.4 Synthesis of 3-benzoyl(4'-cyclohexyl)propanoic acid. Freidel-Crafts acylation using succinic anhydride.



3-benzoyl(4'-cyclohexyl)propanoic acid

Comprehensive reviews of Friedel-Crafts acylations using succinic anhydride are provided by Berliner (1949) and Olah (1963). These authors discuss experimental conditions and notes that, along with the usual requirements for anhydrous conditions of the aluminium chloride and reagents, the choice of solvent in which the reaction is carried out is also important as this often determines the yield, and, in some reactions, the position of substitution. For example, nitrobenzene, a commonly used solvent in such reactions, forms a complex with the aluminium chloride, promoting *para*-substitutions owing to the steric bulk of the complex which hinders *ortho*- substitution.

Acylations of benzene and toluene generally employ excess of the compound to be acylated as the solvent. However, nitrobenzene is the solvent of choice in the majority of acylations of larger aromatic hydrocarbons. The formation of a complex between nitrobenzene and aluminium chloride also modifies the destructive action of aluminium chloride on many aromatic hydrocarbons (Berliner, 1949; Olah, 1963).

3.3.4.1 Nitrobenzene as a solvent

Succinic anhydride was coupled to phenylcyclohexane by means of a Friedel-Crafts acylation reaction using aluminium chloride as a catalyst (Section 6.5.2.1). Nitrobenzene was employed as the solvent. The crude reaction products were steam distilled twice to ensure maximum removal of nitrobenzene. Recrystallisation of the crude keto-acid (ethanol-water) yielded pure 3-benzoyl(4'-cyclohexyl)propanoic acid in good yield (63 % by GC). The pure keto-acid was characterised by MS, NMR (^{13}C and ^{1}H) and IR.

3.3.4.2. 1,1,2,2- Tetrachloroethane as a solvent

The use of tetrachloroethane as a solvent in the acylation of alkylated benzenes is recommended by Barnett and Sanders, (1933, cited by Berliner, 1949). The most obvious advantage of using tetrachloroethane in preference to nitrobenzene is the ease of solvent removal. Nitrobenzene is particularly difficult to remove from the reaction products owing to the high boiling point of the solvent (210-211°C). In comparison, tetrachloroethane has a lower boiling point (147°C), and can be removed more quickly and more efficiently by steam distillation.

The synthesis was thus repeated with 1,1,2,2-tetrachloroethane as the solvent. Pure keto-acid (>99 % by GC) was obtained in good yield (85 %) and fully characterised by GC-MS, NMR and IR (see below).

As predicted from the boiling points, 1,1,2,2-tetrachloroethane (bpt. 147°C) was removed much more rapidly and efficiently from the crude reaction products than nitrobenzene (bpt. 210-211°C). Consequently, 1,1,2,2-tetrachlorochloroethane was the solvent of choice for further syntheses.

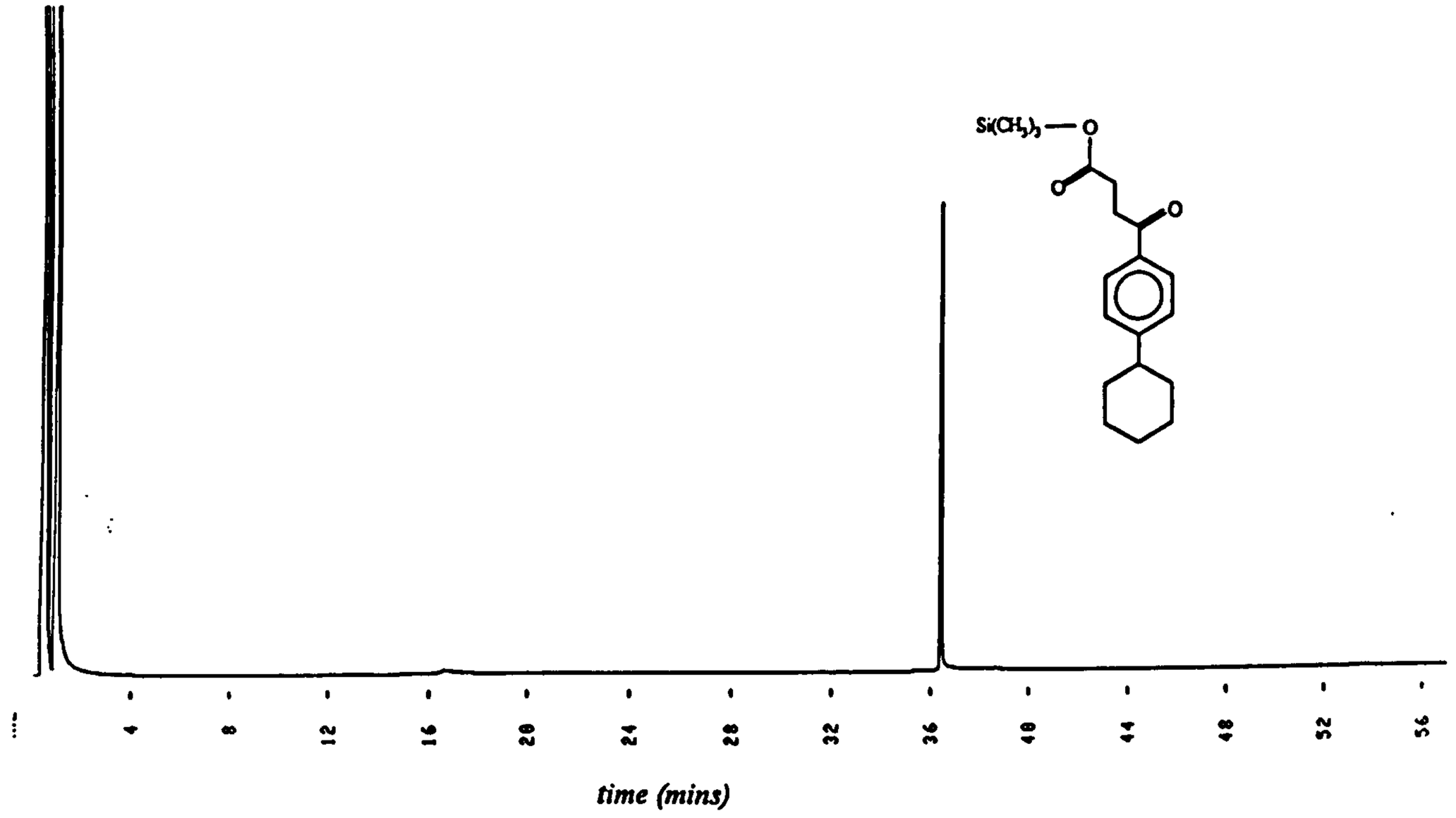
A gas chromatogram of the synthetic 3-benzoyl(4'-cyclohexyl)propanoic acid (as the trimethyl silyl ester, TMS) is presented in Figure 3.18.

The electron impact mass spectrum of the TMS ester (Figure 3.19) showed the presence of the molecular ion (*m/z* 332; 4%) supported by M^+- CH_3 at *m/z* 317, typical of TMS esters. Benzylic cleavage of the molecule, as illustrated in Figure 3.19 yields a base peak ion, *m/z* 187.

The presence of both the keto- and carboxylic acid groups was confirmed by infra-red (IR) spectroscopy of the free acid. The IR spectrum (Figure 3.20) showed absorptions at 1709 cm^{-1} and 1679 cm^{-1}, corresponding to the C=O stretches of the carboxylic acid and keto group, respectively. Conjugation of the double bond of the keto group with the adjacent aromatic ring resulted in absorption at a lower frequency (1679 cm^{-1}) than the unconjugated carboxylic acid group (1709 cm^{-1}). A broad absorption band centered around 3400 cm^{-1} corresponds to the O-H stretch of the carboxylic acid group.

Absorptions due to CH_2, CH_3 (aliphatic) were present at 2924 cm^{-1} and 2853 cm^{-1}, whilst a much weaker absorption band corresponding to the aromatic νC-H was evident at 3038 cm^{-1}.

The ^{13}C NMR spectrum (Figure 3.21a) showed a total of 12 resonances for the 16 carbon atoms; two carbonyl (d,a), four aromatic (e,f,g,h) and 6 aliphatic (b,c,i,j,k,l). These were assigned by comparison of experimental chemical shift values with published tables (Prestch, 1989). The carbonyl carbons (C=O and CO_2H) exhibit distinctive chemical shifts at 178 ppm (keto group, d), and 197 ppm (CO_2H, a). The *ipso*- carbons of the aromatic ring are evident at 154 ppm, (e) and 134 ppm, (h). The deshielding effect of the carbonyl carbon (d) adjacent to carbon (e) is apparent in the higher frequency chemical shift of (e) relative to carbon (h). The remaining four aromatic carbons resonate at 128 ppm and 127 ppm (f,g). Six aliphatic carbons are present. The tertiary



[GC details; DB-5 column. H_2 carrier gas. Temp. program; 40°C - 300°C @ 5° min^{-1}, hold 10 mins]

Figure 3.18. Gas chromatogram of 3-benzoyl(4'-cyclohexyl)propanoic acid, (as TMS ester)

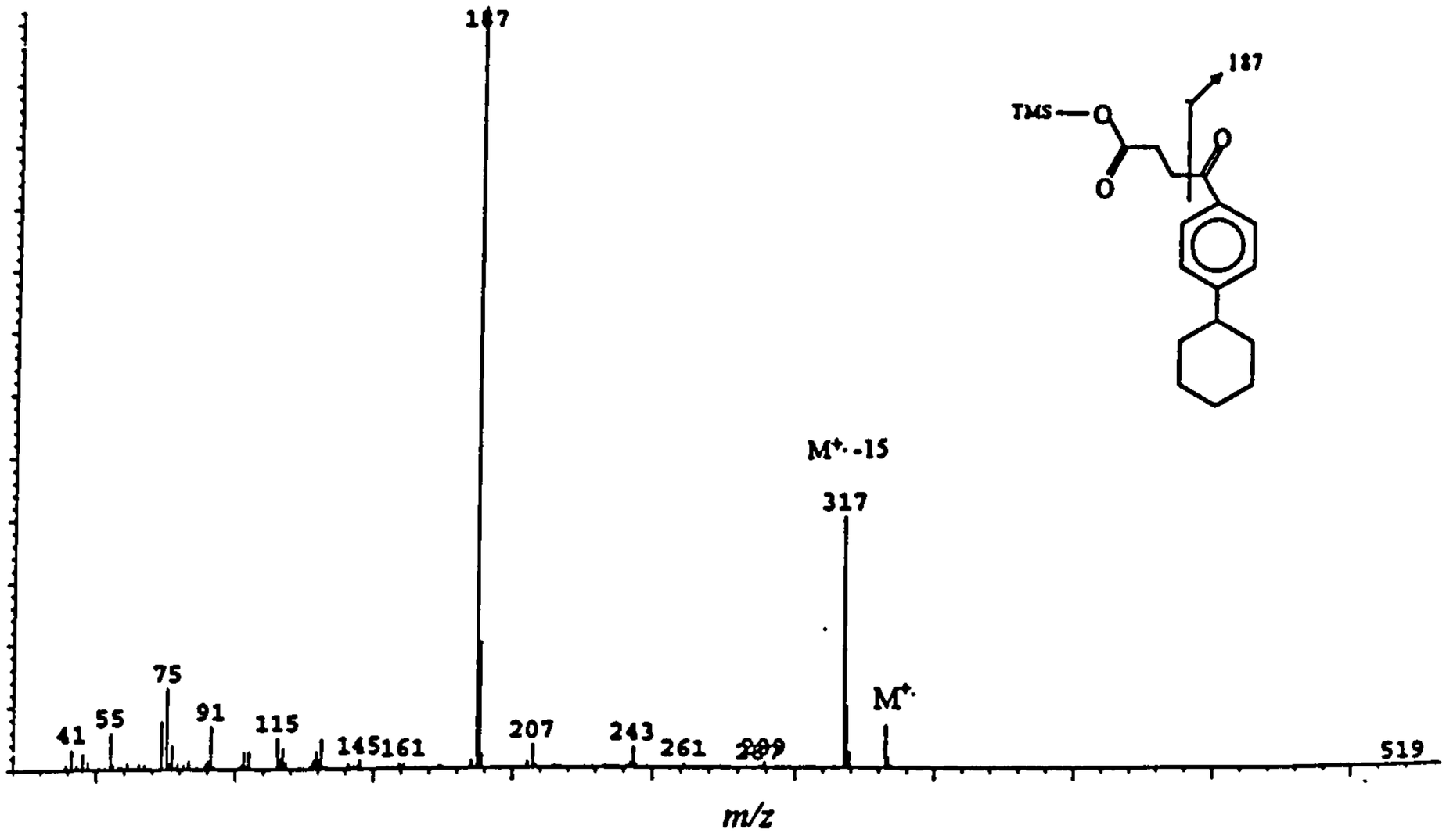


Figure 3.19 Mass spectrum of the TMS ester of 3-benzoyl(4'-cyclohexyl)propanoic acid

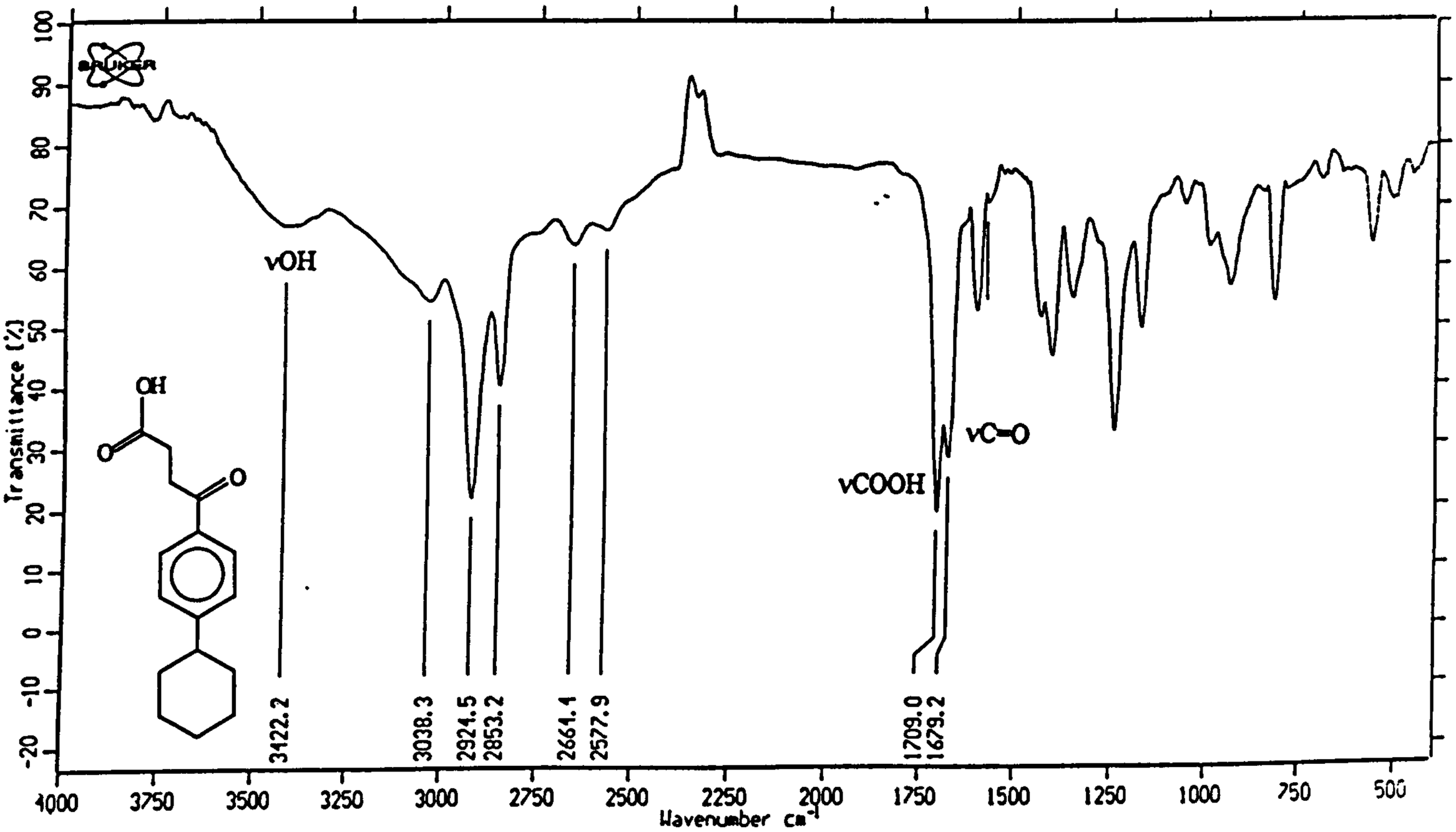
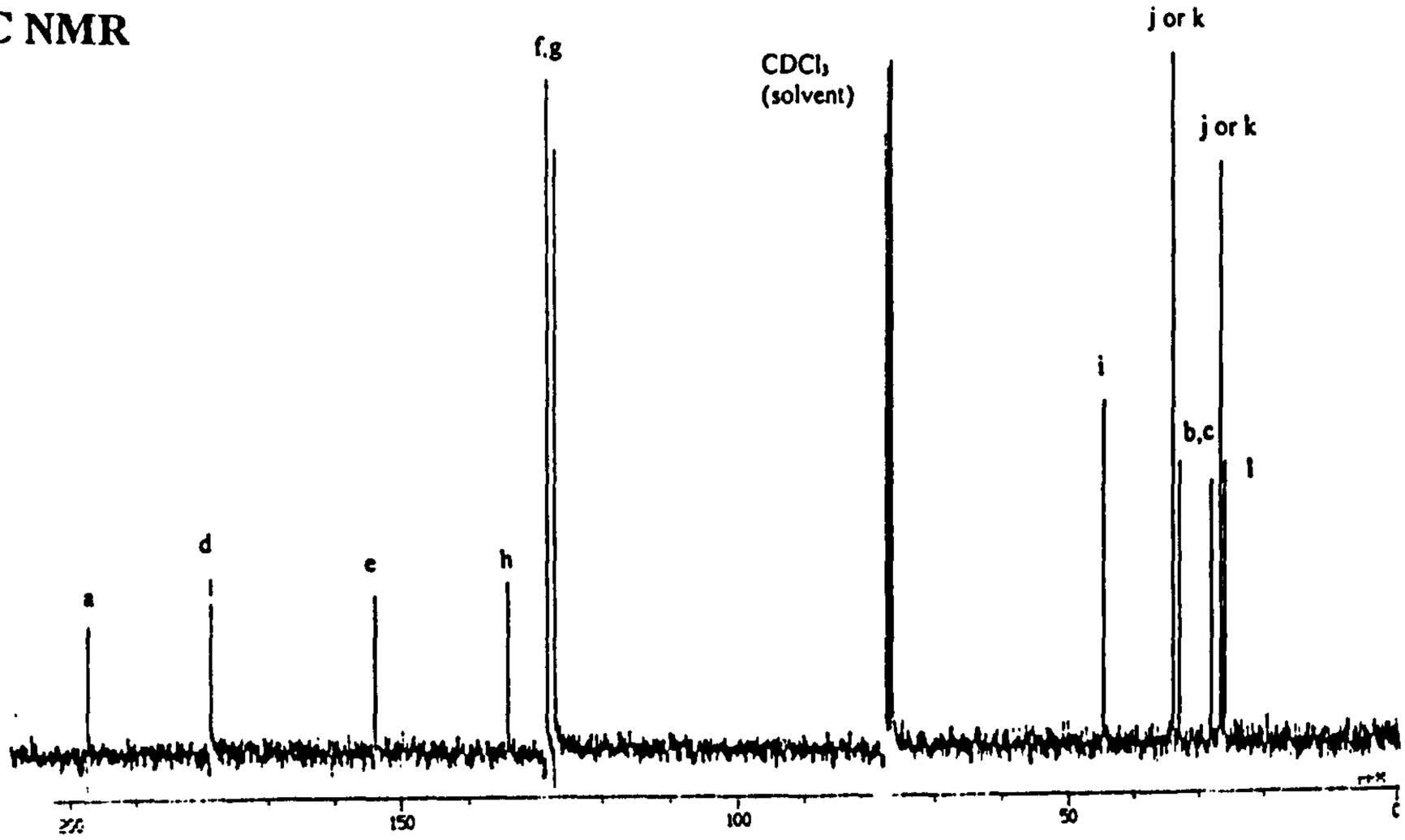


Figure 3.20 Infra red spectrum of 3-benzoyl(4'-cyclohexyl)propanoic acid

(a) ^{13}C NMR



(b) DEPT sequence

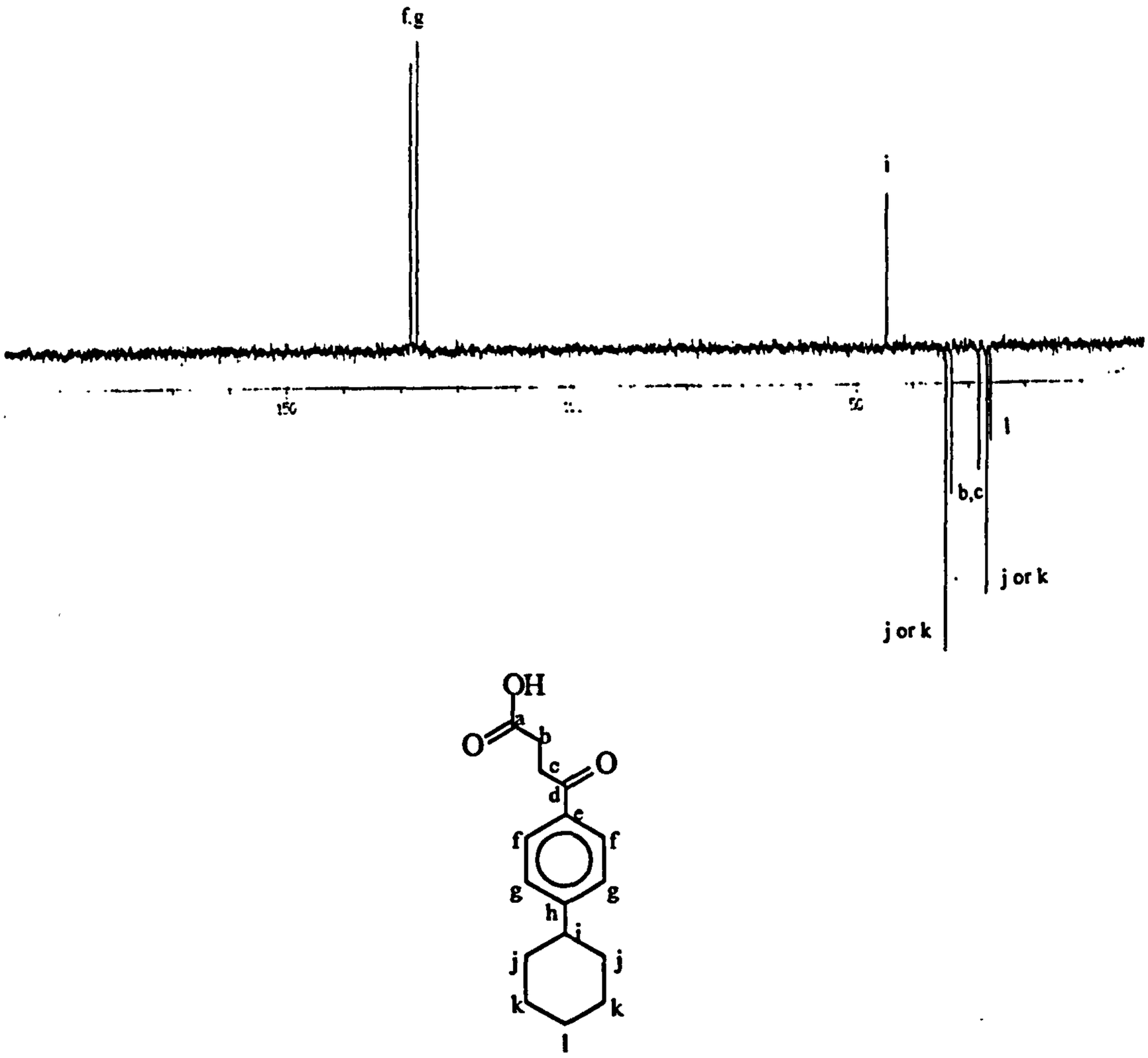
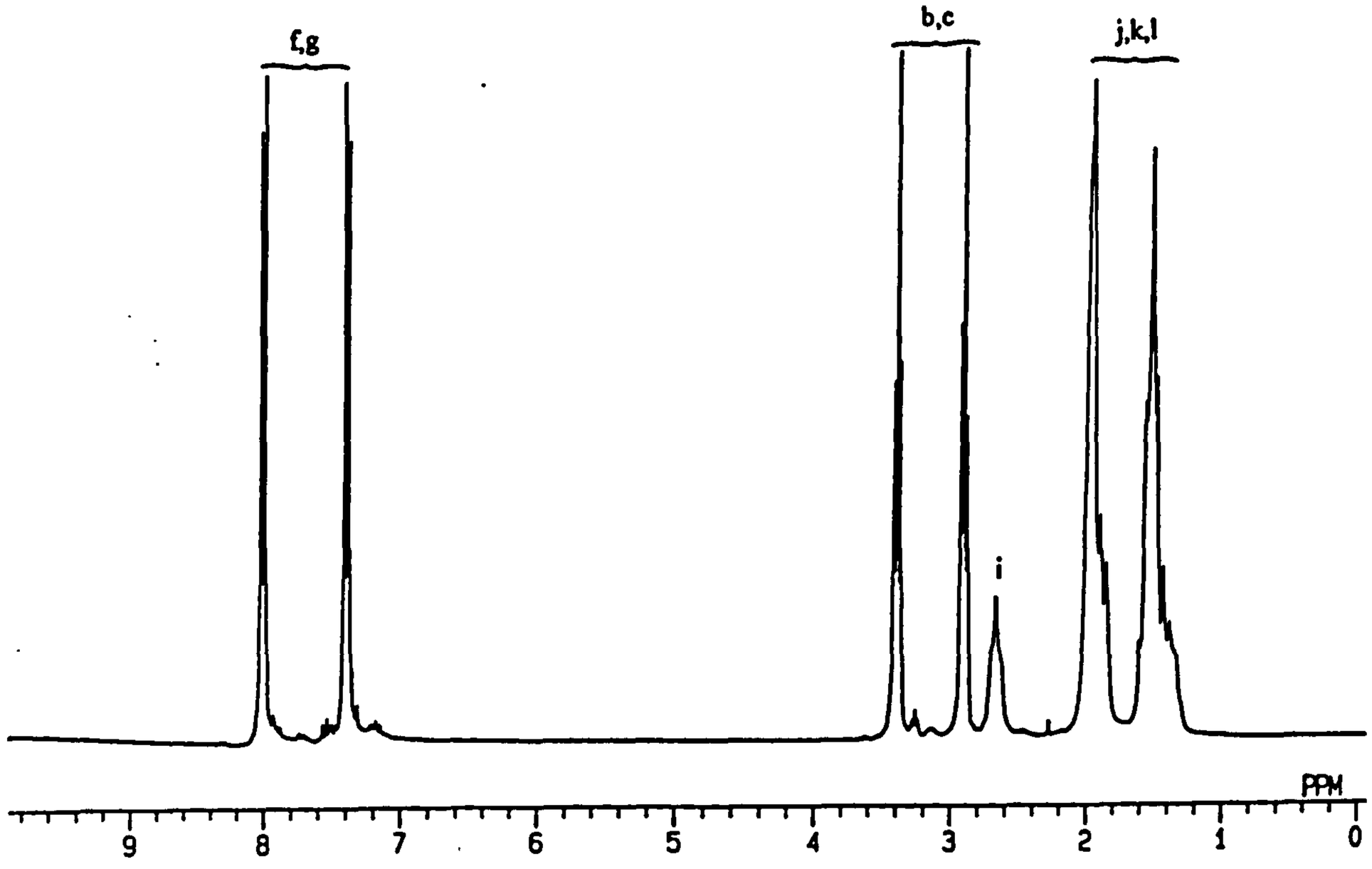


Figure 3.21. ^{13}Carbon and DEPT NMR spectra of 3-benzoyl(4'-cyclohexyl)-propanoic acid



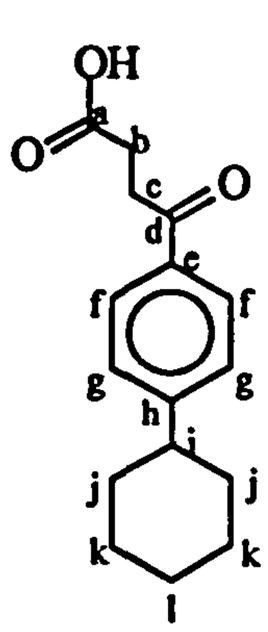


Figure 3.22 ^{1}H NMR spectrum of 3-benzoyl(4'-cyclohexyl)propanoic acid

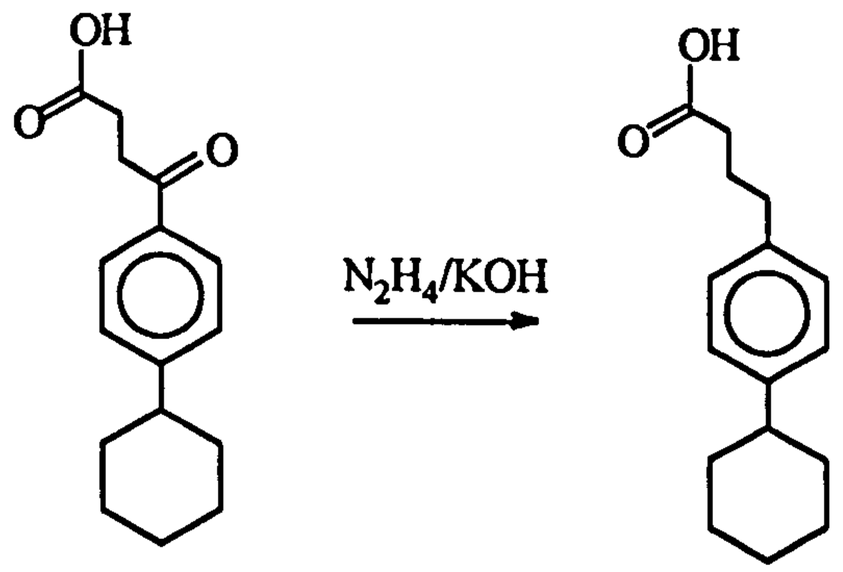
carbon in the cyclohexyl ring is evident at 44 ppm. With respect to the remaining carbons, the methylene carbons labelled (j) and (k) have been assigned on the basis of the intensity of the signals at 34 ppm and 26 ppm, whilst the methylene carbons of the butanoic side chain are evident at 33 ppm and 28 ppm. The remaining methylene carbon of the cyclohexyl ring (l) is present at 25 ppm. As unequivocal assignment of each individual carbon is not possible from the data, signals in the spectra (Figure 3.21 a,b) are labelled in groups with all possible assignments.

Analysis *via* the DEPT sequence supports these assignments (Figure 3.21b), revealing the presence of five resonances due to methylene carbons (b,c,j,k,l), three methine (f,g,i) and four quaternary carbons (a,d,e,h).

The proton NMR spectrum (Figure 3.22) contains two doublets (7.9 ppm and 7.3 ppm, J 8 Hz) corresponding to the aromatic protons (f) and (g). A coupling constant (J) of 8 Hz is characteristic of *ortho* protons, confirming the presence of a disubstituted benzene.

The methylene protons of the acid side chain (b,c) are evident as two triplets (3.3 ppm and 2.8 ppm, J 6 Hz). A broad multiplet at 2.5 ppm corresponds to carbon (i) of the cyclohexyl group. The deshielding influence of the adjacent aromatic ring causing this proton to have a higher chemical shift than the other protons of the cyclohexyl ring which are present as two multiplets at 1.8 ppm and 1.3 ppm because of the axial and equatorial protons.

3.3.5 Synthesis of 4-phenyl(4'-cyclohexyl)butanoic acid

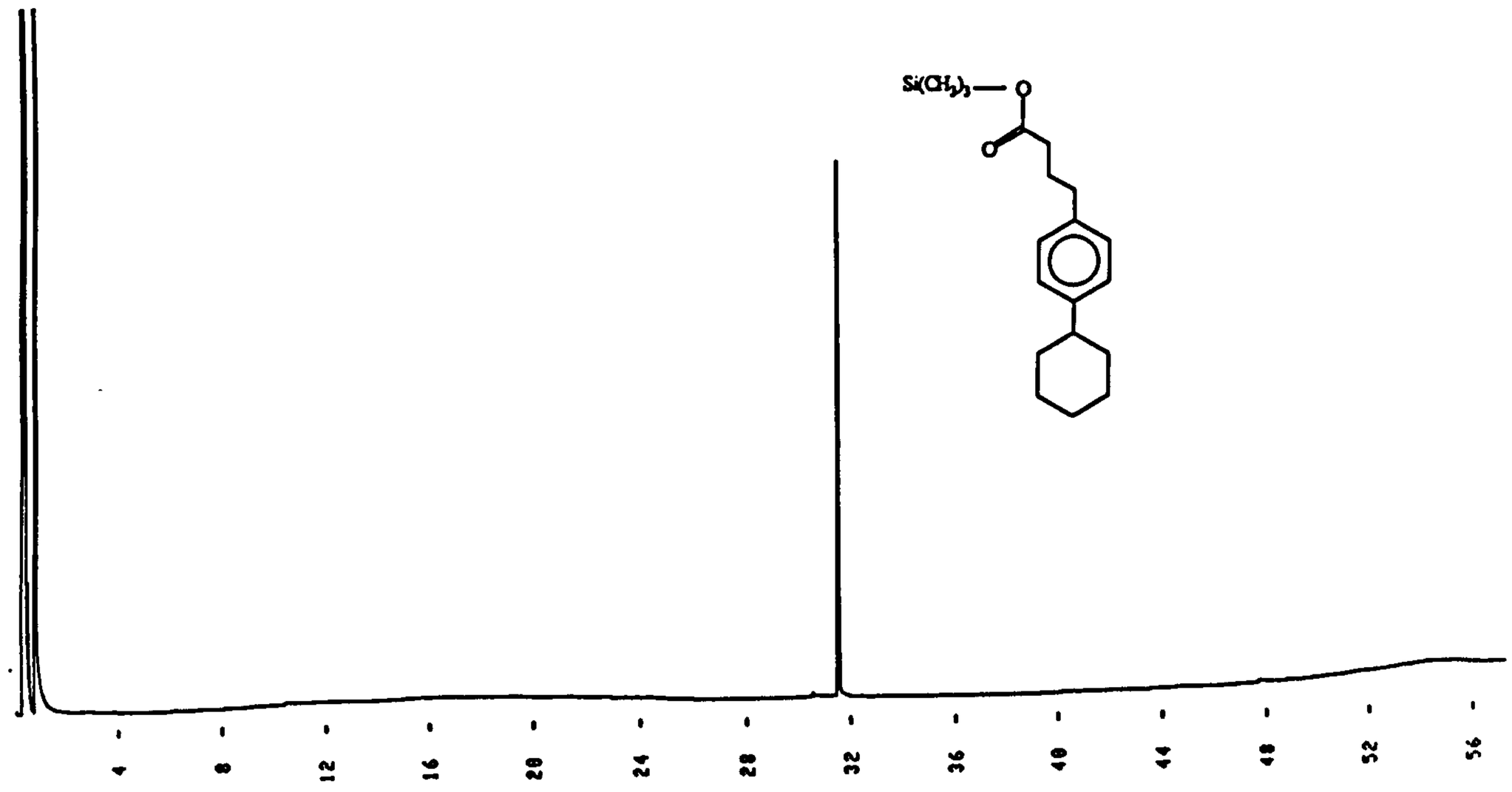


4-phenyl(4'-cyclohexyl)butanoic acid

There are various methods of reducing the carbonyl group of aldehydes and ketones. The most widely used methods are the Clemmensen reduction and the Wolff-Kischner reduction. The Clemmensen reduction involves heating the ketone or aldehyde with zinc amalgam and aqueous hydrochloric acid (Vedejes, 1975). However, this method is unsuitable for high molecular weight substrates owing to their low solubility in the reactants, and the formation of side products such as pinacols (March, 1985; Vogel, 1989). The Wolff-Kischner reduction involves heating the substrate with hydrazine hydrate and a base (usually potassium hydroxide). The Huang-Minlon modification of this method (Huang-Minlon, 1946, 1949) has now completely replaced the original procedure (March, 1983; Vogel, 1989). Consequently 3-benzoyl(4'-cyclohexyl)propanoic acid was reduced to 4-phenyl(4-cyclohexyl)butanoic acid using the Huang-Minlon modification (Durham *et al.*, 1963).

Examination of the crude reaction products by GC indicated no further purification steps were necessary at this stage (purity > 98 % by GC). A gas chromatogram of 4-phenyl(4'-cyclohexyl)butanoic acid (as the TMS ester) is presented in Figure 3.23.

A mass spectrum of the TMS ester of 4-phenyl(4'-cyclohexyl)butanoic acid is presented in Figure 3.24. The molecular ion ($M^{+\cdot}$) is evident at *m/z* 318. Diagnostic peaks at *m/z* 186 (base peak) and *m/z* 117 have probably resulted from γ-H rearrangement followed by



[GC details; DB-5 column. H_2 carrier gas. Temp. program; 40°C - 300°C @ 5° min^{-1}, hold 10 mins]

Figure 3.23. Gas chromatogram of 4-phenyl(4'-cyclohexyl)butanoic acid, (as TMS ester)

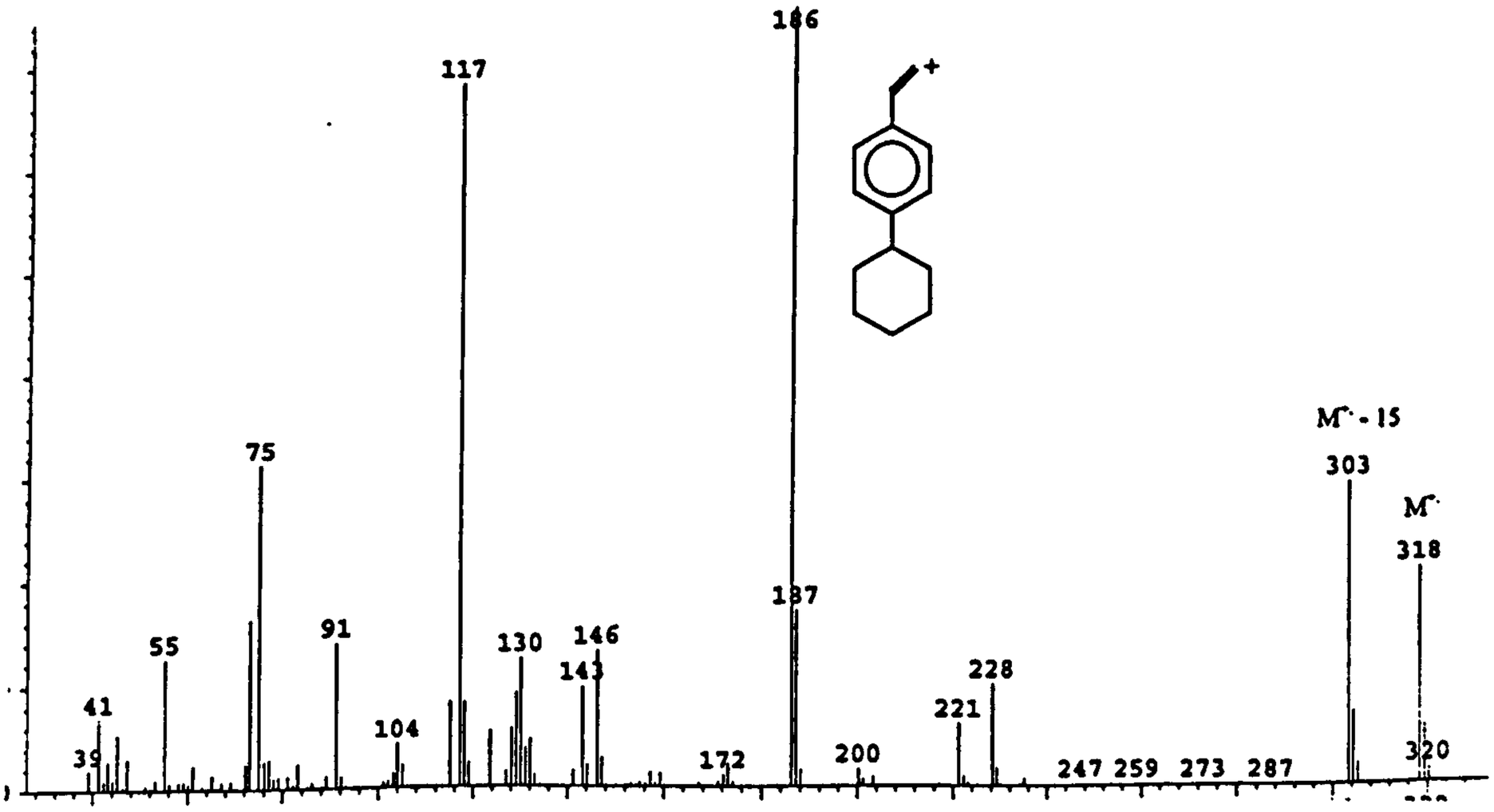


Figure 3.24 Mass spectrum of the TMS ester of 4-phenyl(4'-cyclohexyl)butanoic acid

β-cleavage, which is a common fragmentations in compounds containing an unsaturated functionality, *i.e.* the carbonyl group. This arrangement is commonly known as the McLafferty re-arrangement (McLafferty and Turečeck, 1983).

Examination of the product by IR spectroscopy (Figure 3.25) indicated that reduction of the keto group had indeed occurred. The carbonyl absorption at 1679 cm^{-1} was absent, leaving only a single absorption at 1708 cm^{-1} corresponding to νC=O of the carboxylic acid group.

Further confirmation of the reduction of the keto acid was provided by ^{13}C NMR. The spectrum is presented in Figure 3.26a and shows a total of 10 resonances. Most notable is the absence of the downfield carbonyl carbon (d) which was present in the ^{13}C spectrum of the keto acid (Figure 3.21). The chemical shift of the *ipso* carbon (e) has also moved upfield to 148 ppm (compared with 154 ppm in the keto acid) as it is no longer experiencing a deshielding effect from an adjacent carbonyl carbon. In the aliphatic region of the spectrum there are only 5 resonances instead of the expected seven. The methine carbon of the cyclohexyl ring is easily distinguished using the DEPT sequence as the signal at 44 ppm. However, overlap of the resonances of the methylene carbons of the cyclohexyl ring with the methylene carbons of the side chain has resulted in four resonances instead of the expected six. An additional 2-D NMR experiment (^{13}C ^{1}H correlation) confirmed overlap of several of the methylene resonances and allowed the signals in the ^{13}C spectrum (Figure 3.26) to be assigned.

The ^{1}H spectrum together with assignments is presented in Figure 3.27. The singlet in the aromatic region of the spectrum represents protons (f)and (g), whilst the protons of the butanoic acid side chain are clearly seen as two triplets at 2.6 ppm and 2.3 ppm ((b) and (d)) and a quintet at 1.9 ppm (c). A broad multiplet at 2.4 ppm is due to the proton

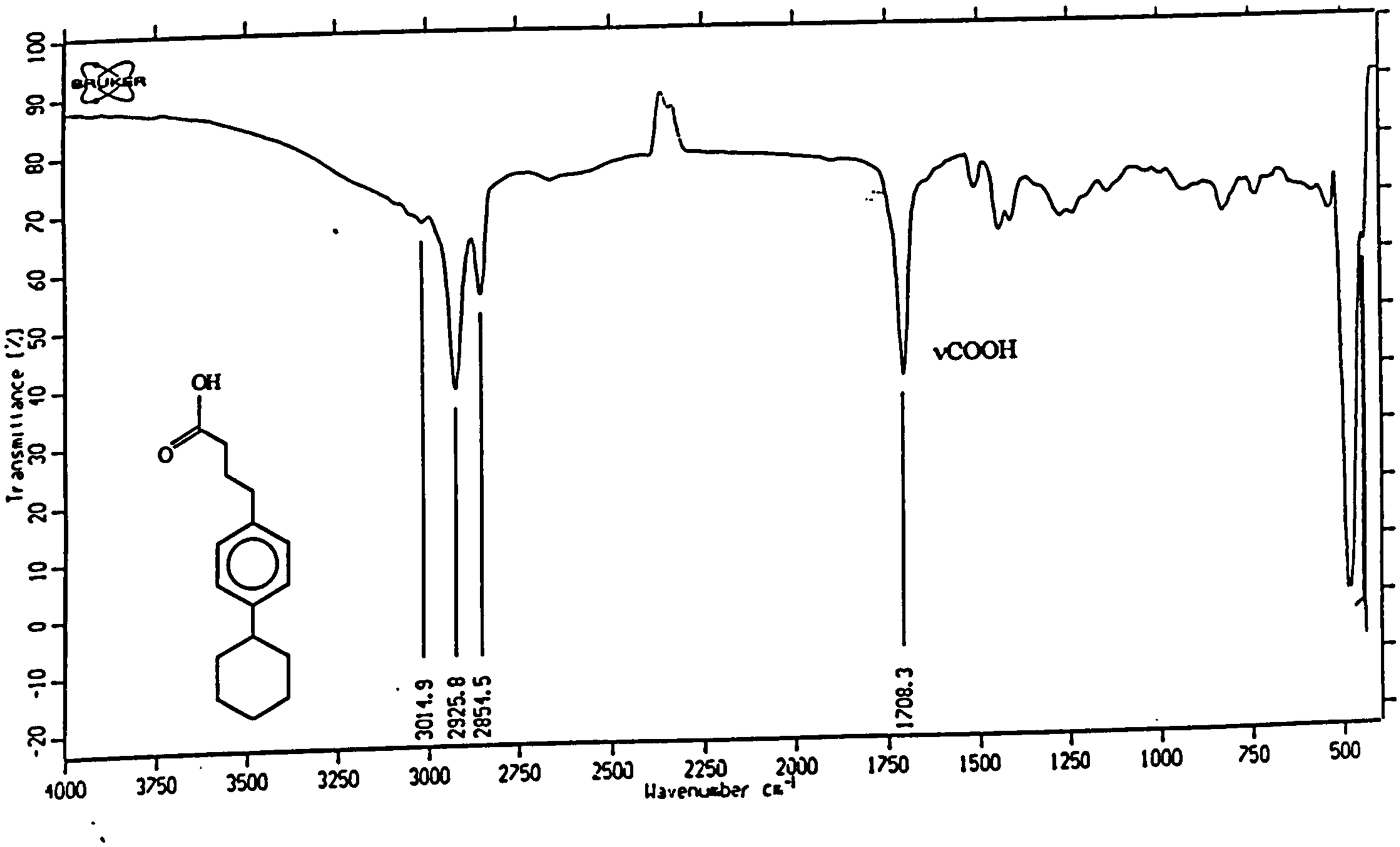
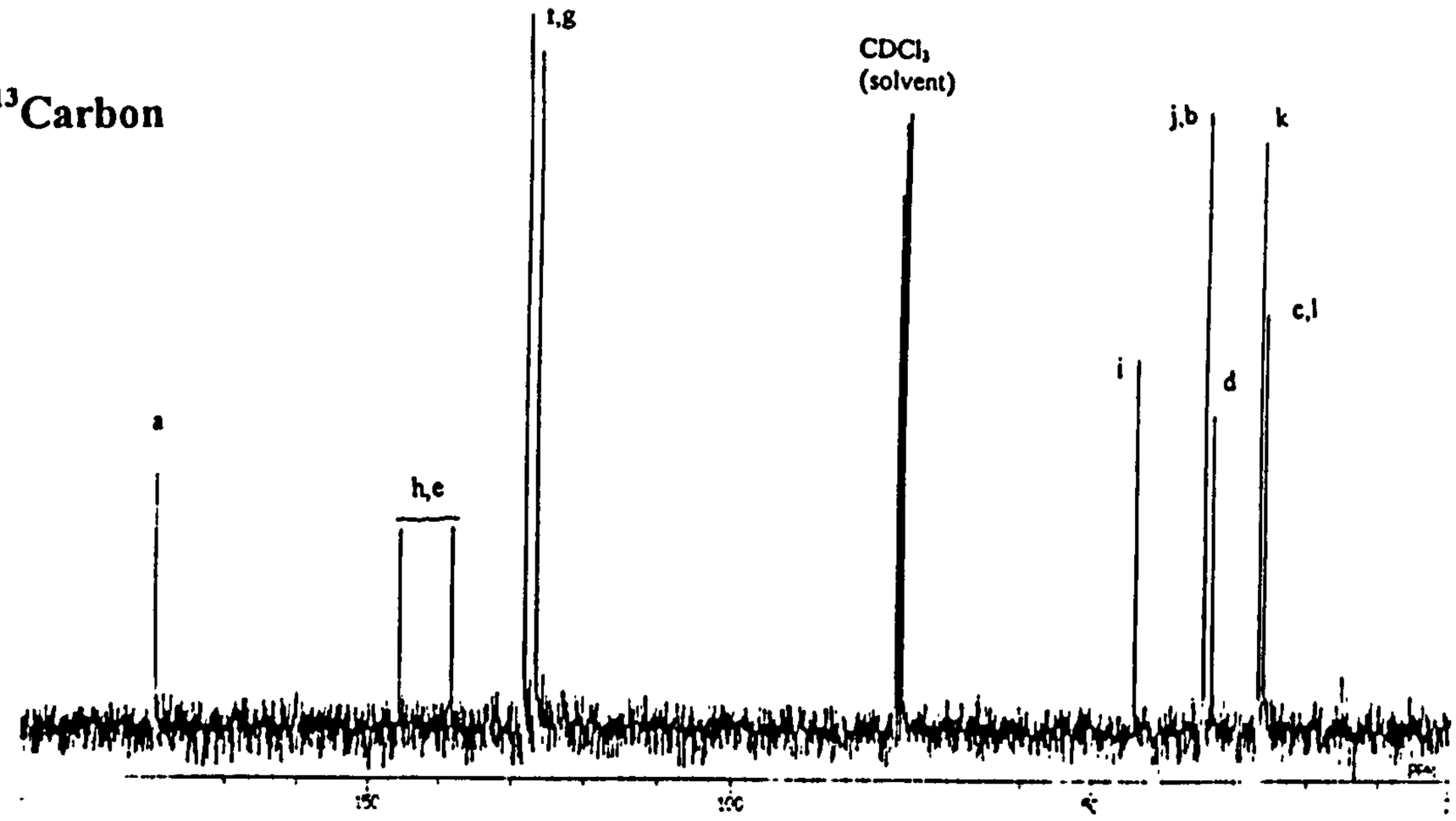
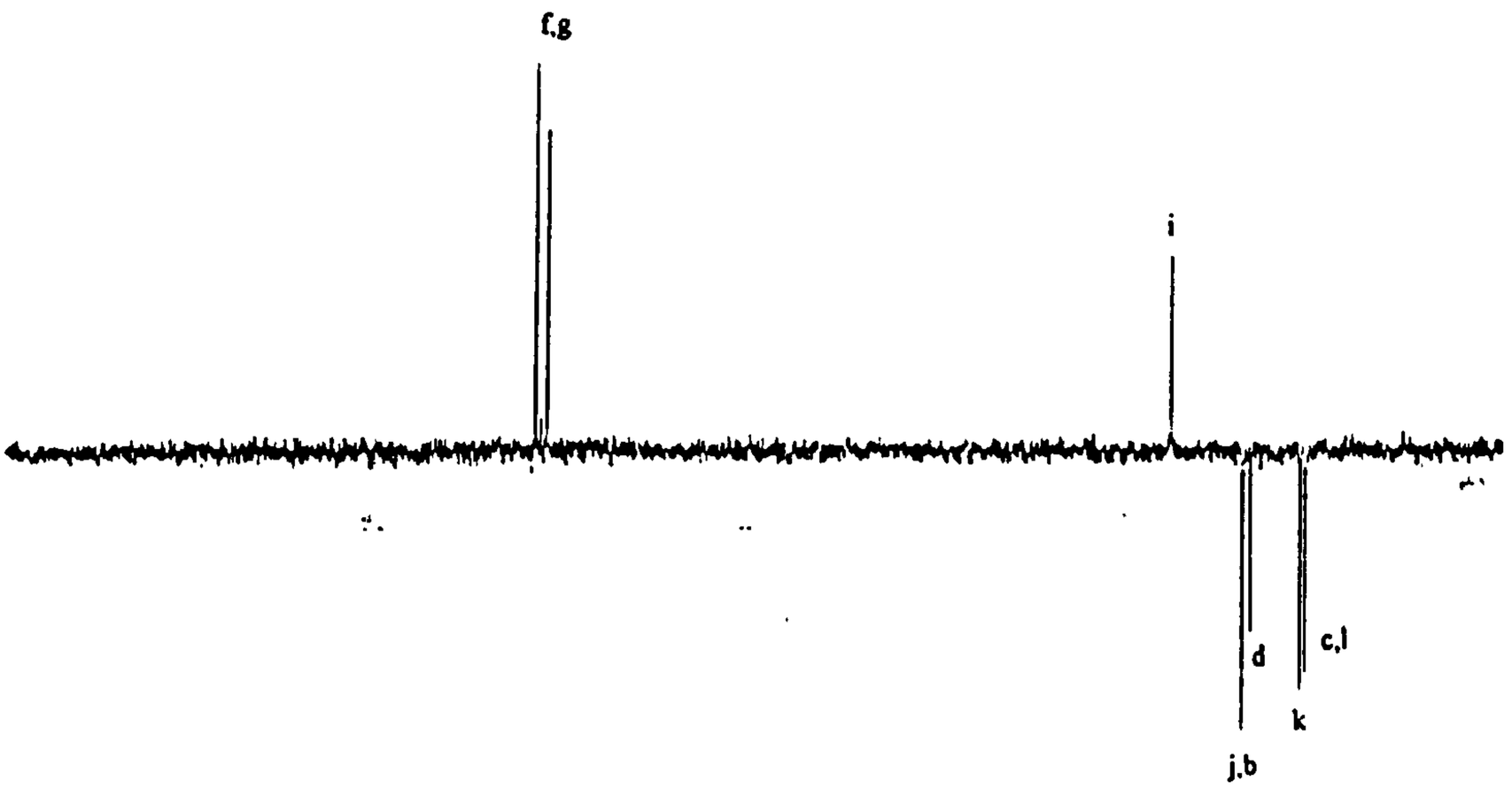


Figure 3.25 Infra-red spectrum of 4-phenyl(4'-cyclohexyl)butanoic acid

(a) ^{13}Carbon



(b) DEPT sequence



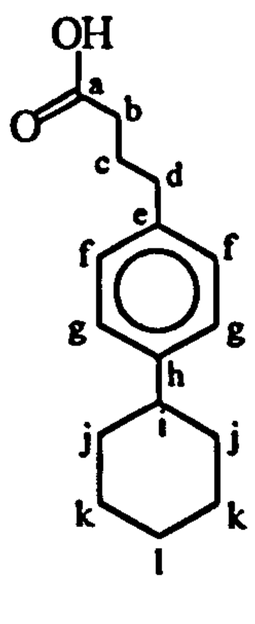
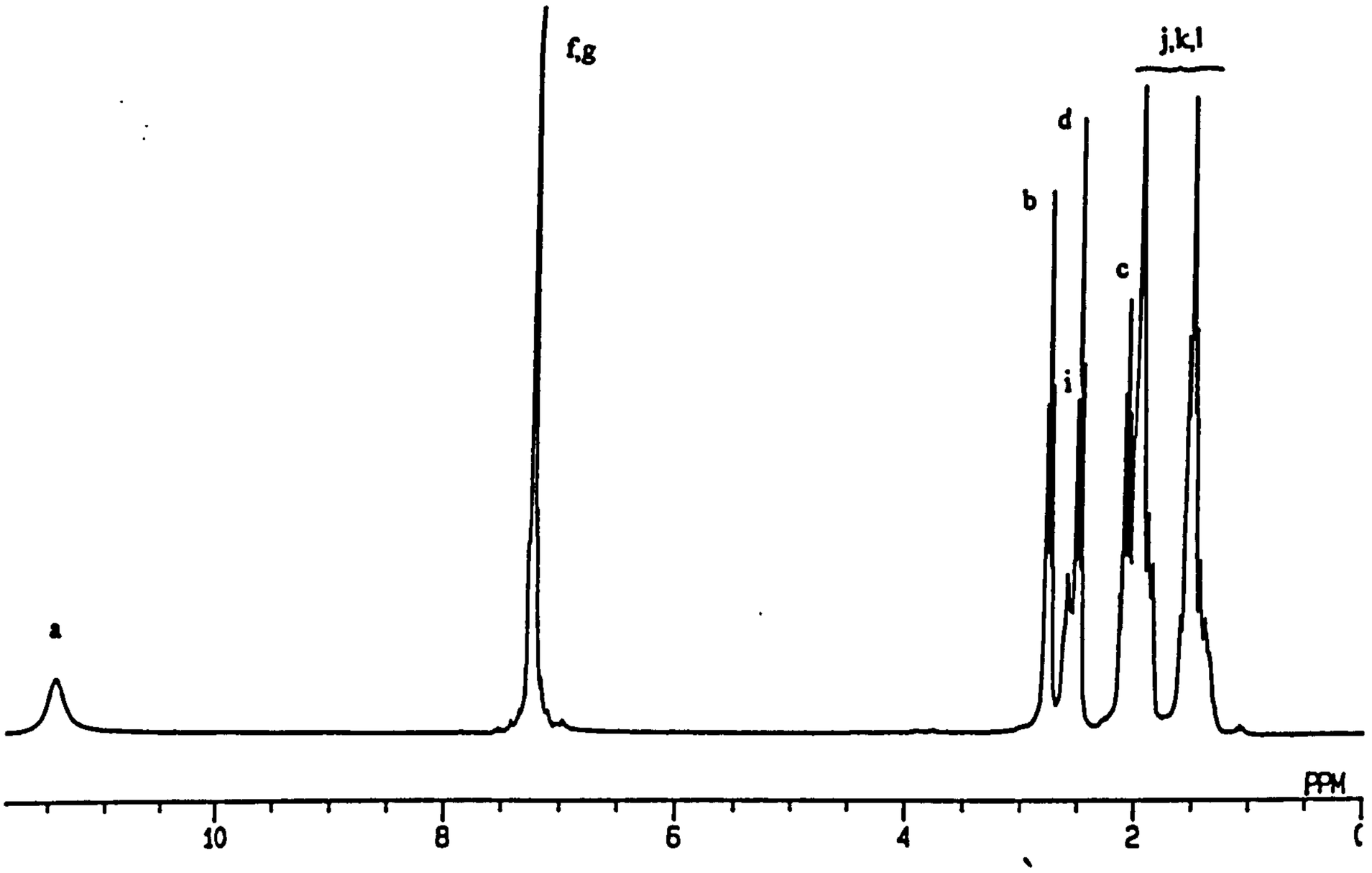


Figure 3.26 ^{13}Carbon and DEPT NMR spectra of 4-phenyl(4'-cyclohexyl)butanoic acid



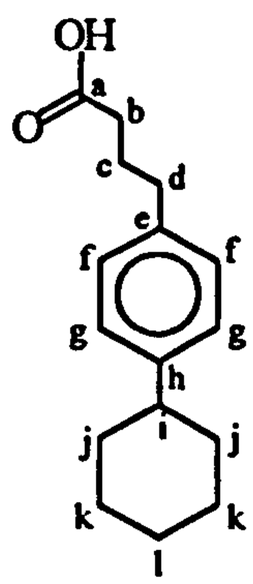
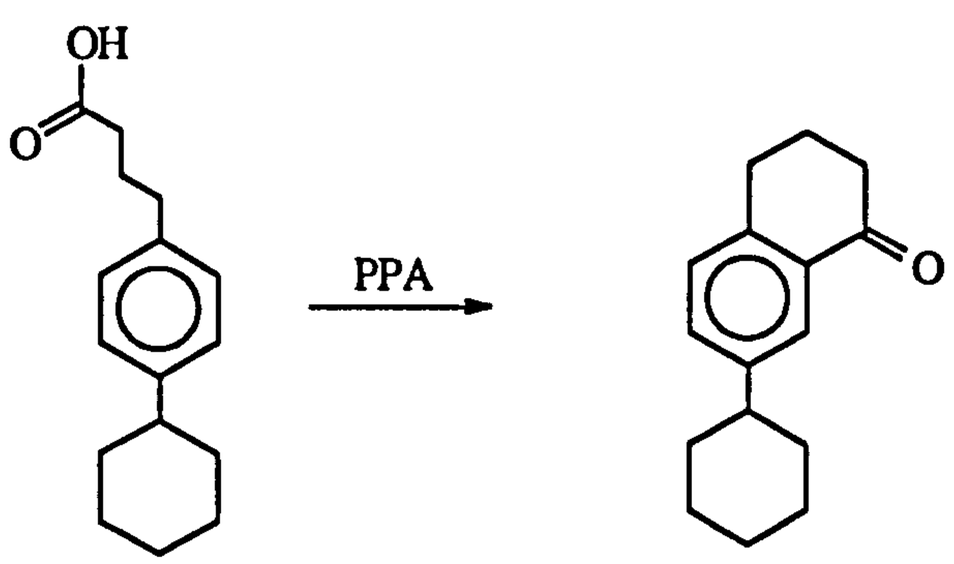


Figure 3.27 ^{1}H NMR spectrum of 4-phenyl(4'-cyclohexyl)butanoic acid

(i) of the cyclohexyl ring whilst the remaining protons of the cyclohexyl ring are seen as two multiplets at 1.8 ppm and 1.4 ppm.

3.3.6 Synthesis of 7-cyclohexyl-1-tetralone

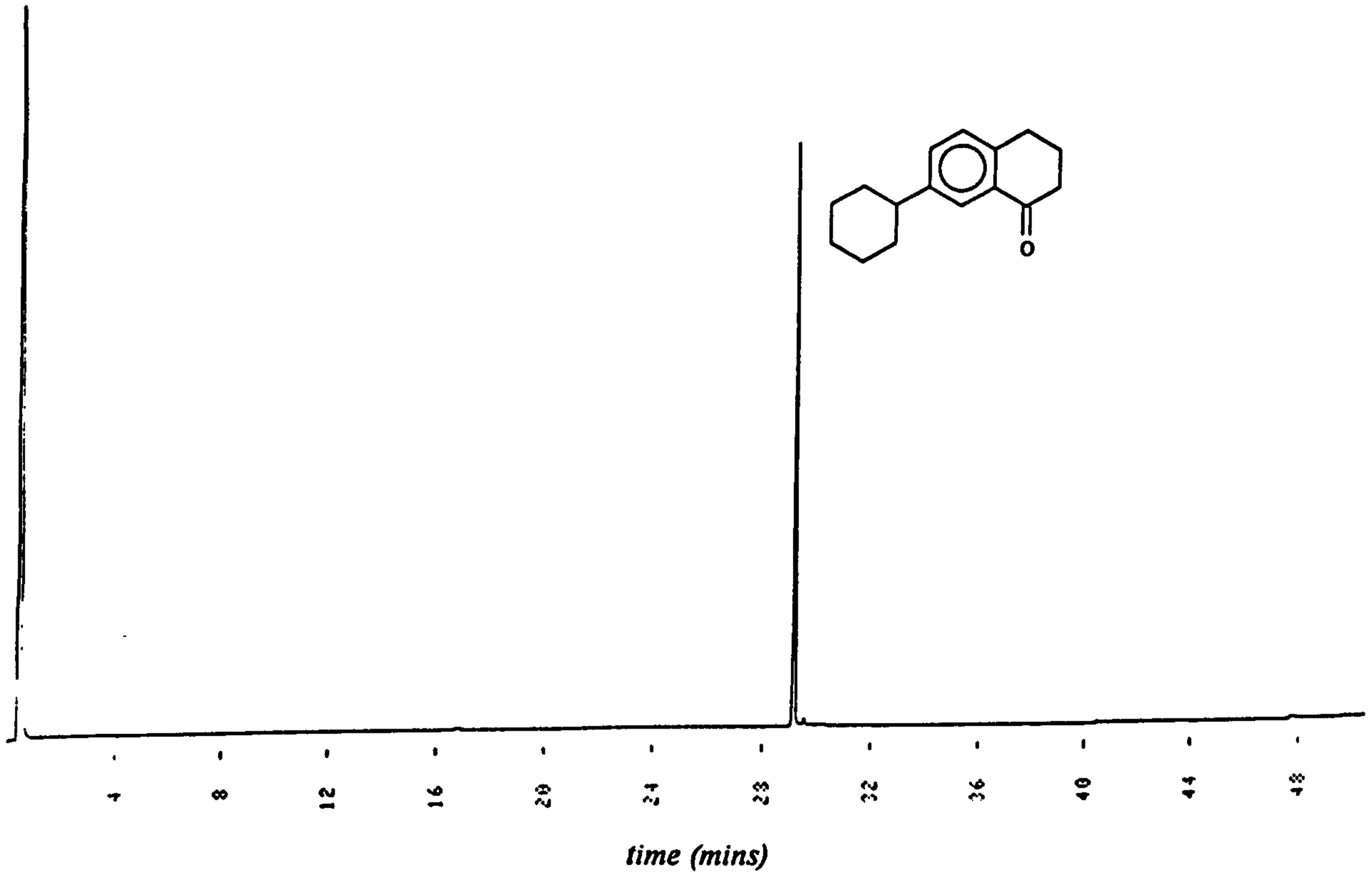


7-cyclohexyl-1-tetralone

4-Phenyl(4'-cyclohexyl)butanoic acid was cyclised with hot polyphosphoric acid using the method of Snyder and Webber (1955). Base extraction of the crude reaction products provided pure (>98 % by GC) 7-cyclohexyl-1-tetralone in good yield (88 %).

A gas chromatogram of 7-cyclohexyl-1-tetralone is presented in Figure 3.28. The mass spectrum (Figure 3.29) showed an intense molecular ion ($M^{+\cdot}$, *m/z* 228), and diagnostic ion fragments *m/z* 200 [$M^{+\cdot}$- CO]; *m/z* 185 [$M^{+\cdot}$-C_2H_3O] and *m/z* 172 [$M^{+\cdot}$-56, possibly loss of C_2H_4 from $M^{+\cdot}$- CO *via* a *retro*-Diels-Alder reaction].

^{13}C NMR spectroscopy revealed the presence of 13 resonances which were assigned in conjunction with the DEPT sequence. The spectra, together with assignments are presented in Figure 3.30. The carbonyl carbon (a) is evident furthest downfield at 198 ppm, whilst the aromatic carbons are present as five resonances between 120 ppm and



[GC details; DB-5 column. H_2 carrier gas. Temp program; 40°C - 300°C @ 5°C min^{-1}, hold 10 mins]

Figure 3.28 Gas chromatogram of 7-cyclohexyl-1-tetralone

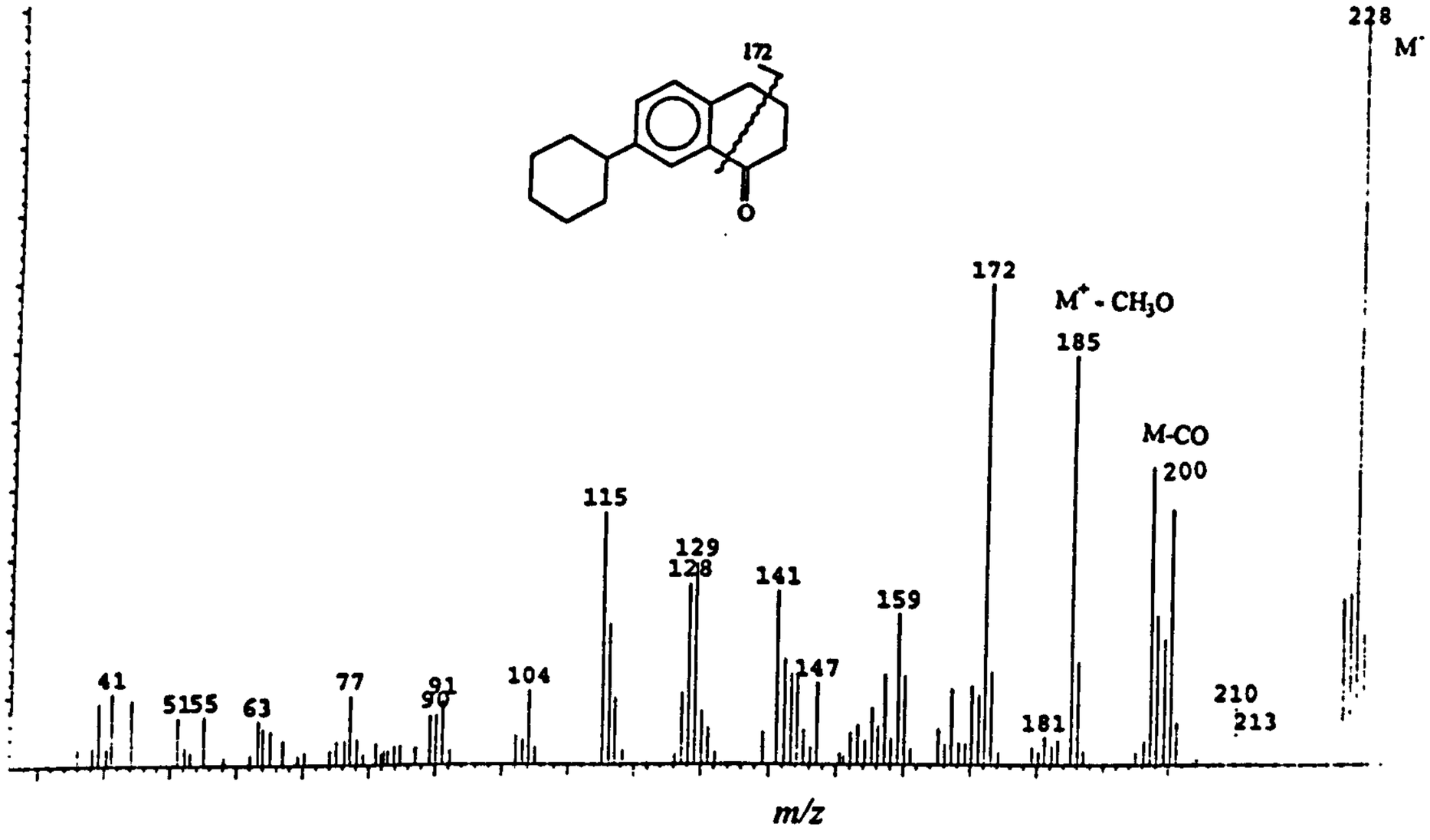
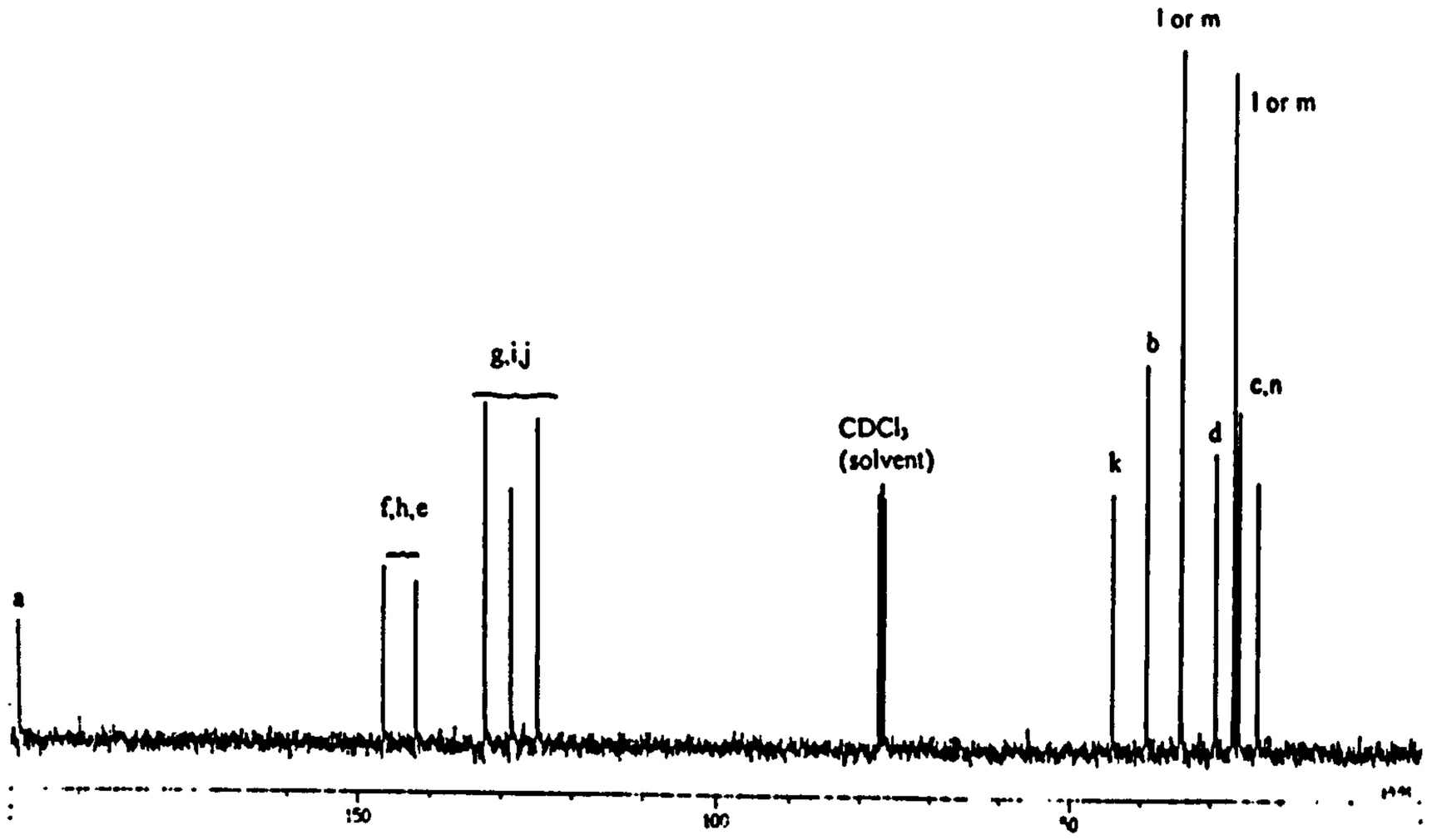
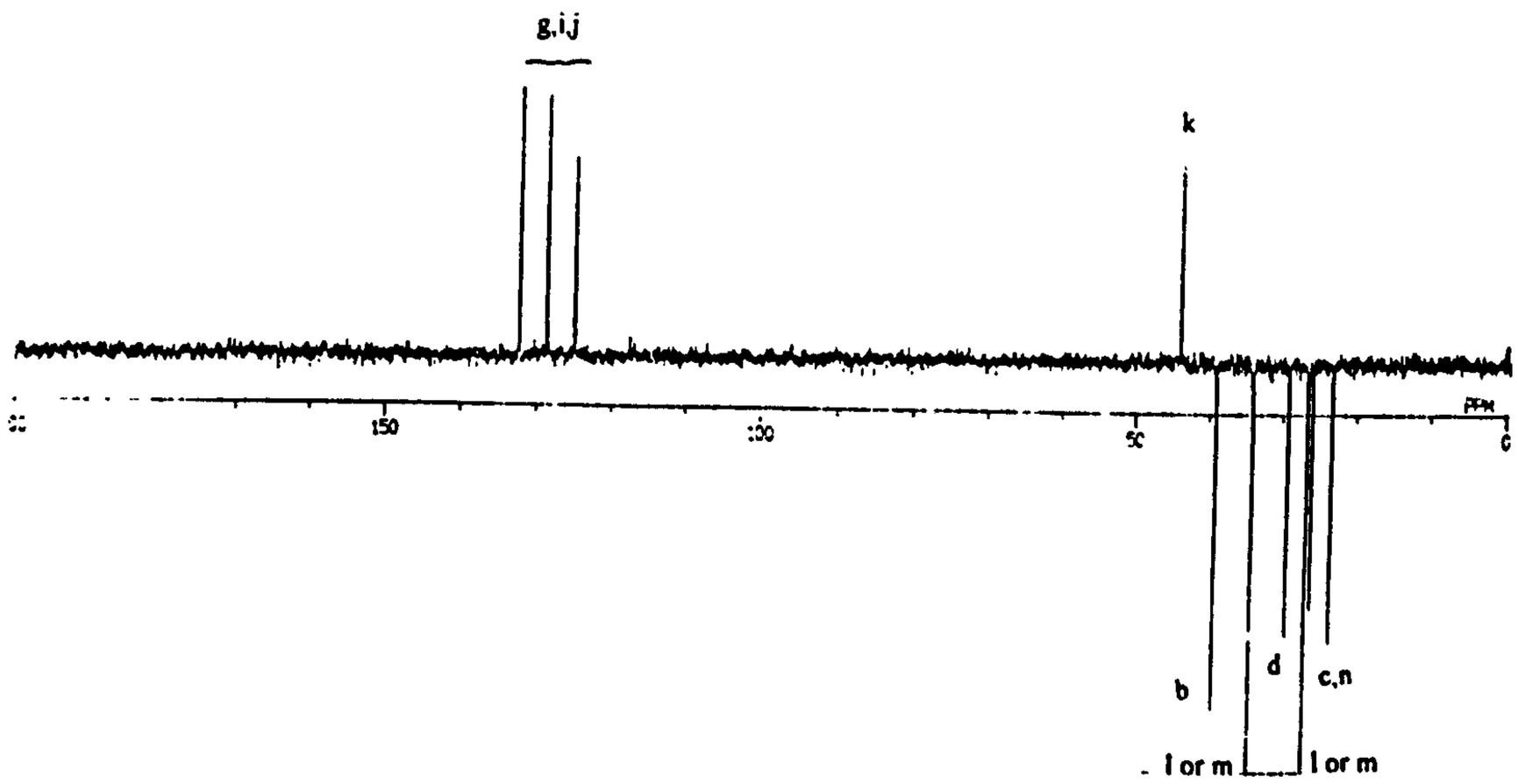


Figure 3.29 Mass spectrum of 7-cyclohexyl-1-tetralone

(a) ^{13}C



(b) DEPT



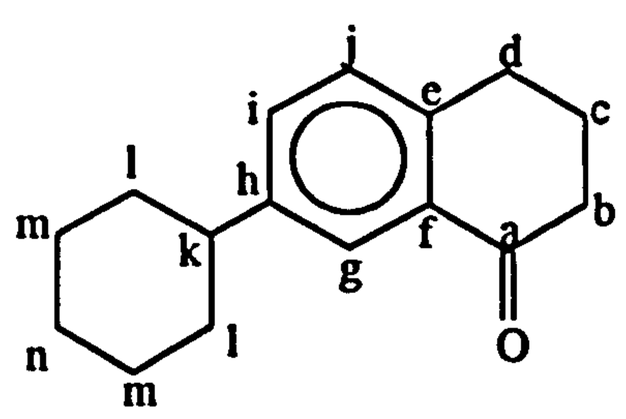
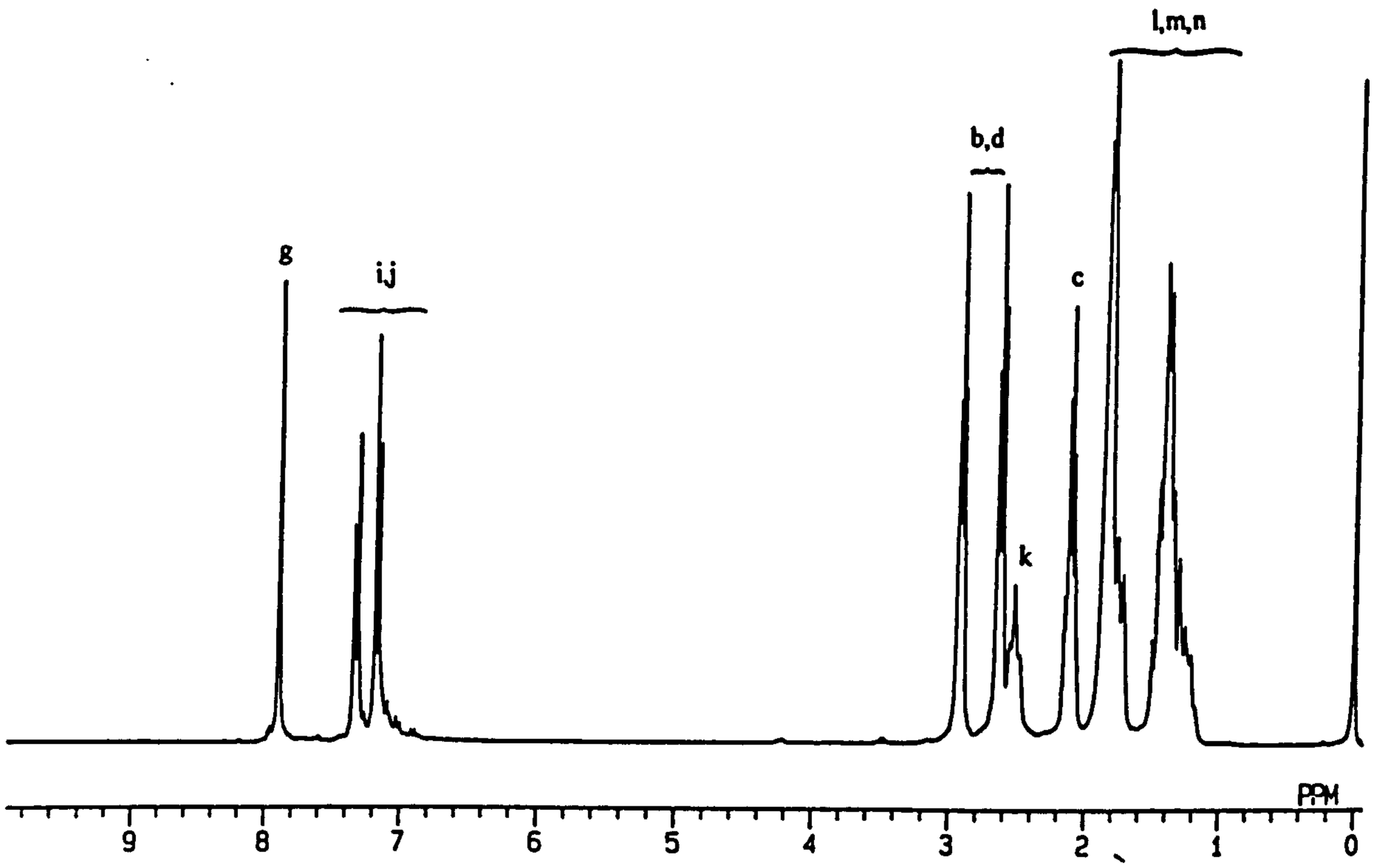


Figure 3.30 ^{13}Carbon and DEPT NMR spectra of 7-cyclohexyl-1-tetralone

150 ppm. By comparison with the DEPT spectrum (Figure 3.30 b), the quaternary carbons (e,f,h) are identified as the resonances at 146 ppm and 141 ppm. Thus the signals at 132 ppm, 128 ppm and 124 ppm correspond to the remaining aromatic carbons (g,i,j). The methine carbon of the cyclohexyl moiety (k) is also identifiable from the DEPT spectrum as the signal at 44 ppm, whilst the remaining methylene carbons of the tetralone and cyclohexyl groups are represented by six resonances between 20 ppm and 40 ppm. Carbons (l) and (m) are assigned to the two most intense signals at 34 ppm and 36 ppm, whilst the highest frequency methylene carbon resonance is tentatively assigned to carbon (b), adjacent to the carbonyl group. It is anticipated that this carbon will experience a large deshielding effect from the adjacent carbonyl group. Similarly, (d) is assigned to the signal at 29 ppm owing to its position next to the aromatic ring.

The ^{1}H NMR spectrum is presented in Figure 3.31 together with assignments. The aromatic protons are evident in the region 7 - 8 ppm. A singlet corresponding to proton (g) is found at 7.9 ppm, whilst (i) and (j) are evident as two doublets at 7.3 ppm and 7.1 ppm. The aliphatic region of the spectrum shows two triplets at 2.8 ppm and 2.6 ppm corresponding to protons (b) and (d) respectively. It is assumed that (b) will be further downfield owing to its position next to the carbonyl group. The quintet at 2.1 ppm corresponds to protons (c), owing to coupling with neighbouring protons (d) and (b). The broad multiplet at 2.5 ppm represents the proton (k), present further downfield than the remaining cyclohexyl protons because of the deshielding effect of the adjacent aromatic ring. The two multiplets centering at 1.8 ppm and 1.4 ppm correspond to the remaining protons of the cyclohexyl moiety.

The IR spectrum of 7-cyclohexyl-1-tetralone (Figure 3.32) confirms the presence of the C=O group in the molecule, with a characteristic absorption at 1680 cm^{-1}.



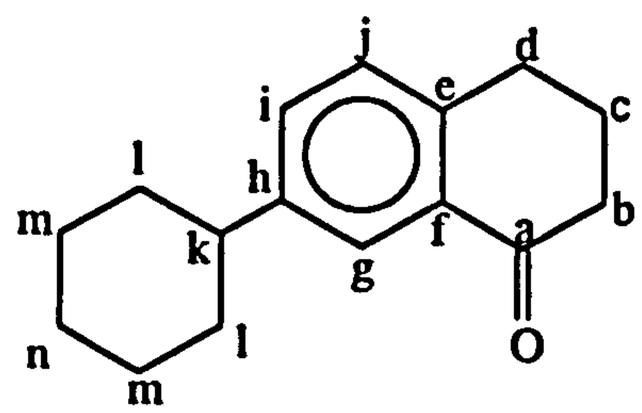


Figure 3.31 ^{1}H NMR spectrum of 7-cyclohexyl-1-tetralone

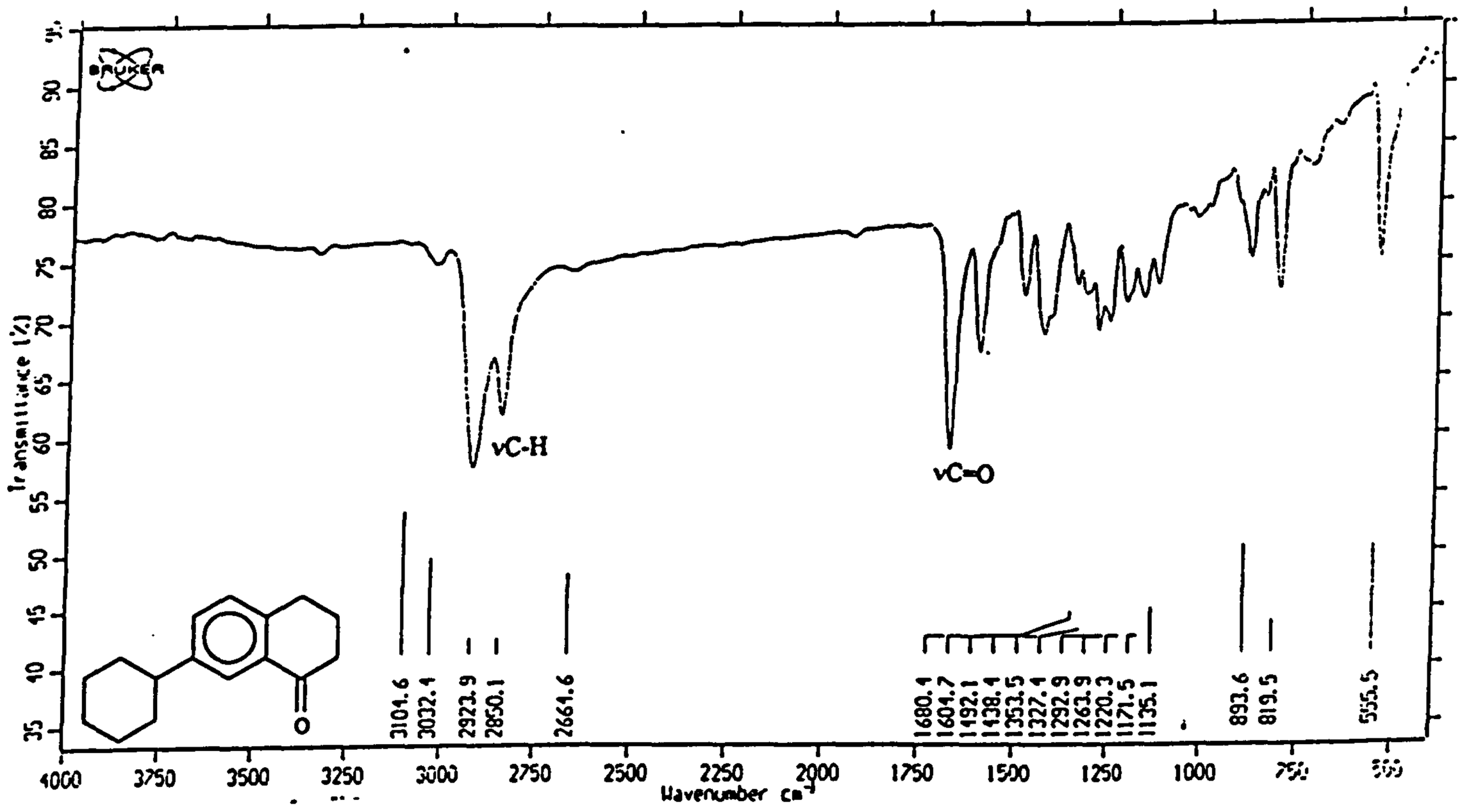
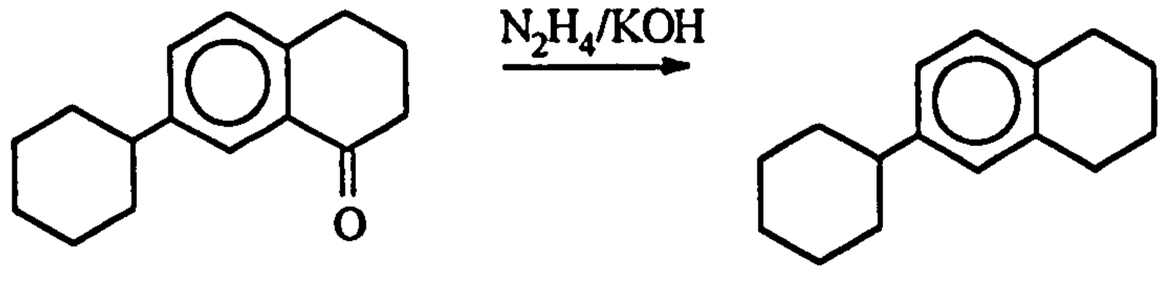


Figure 3.32 Infra-red spectrum of 7-cyclohexyl-1-tetralone

3.3.7 Synthesis of 7-cyclohexyltetralin

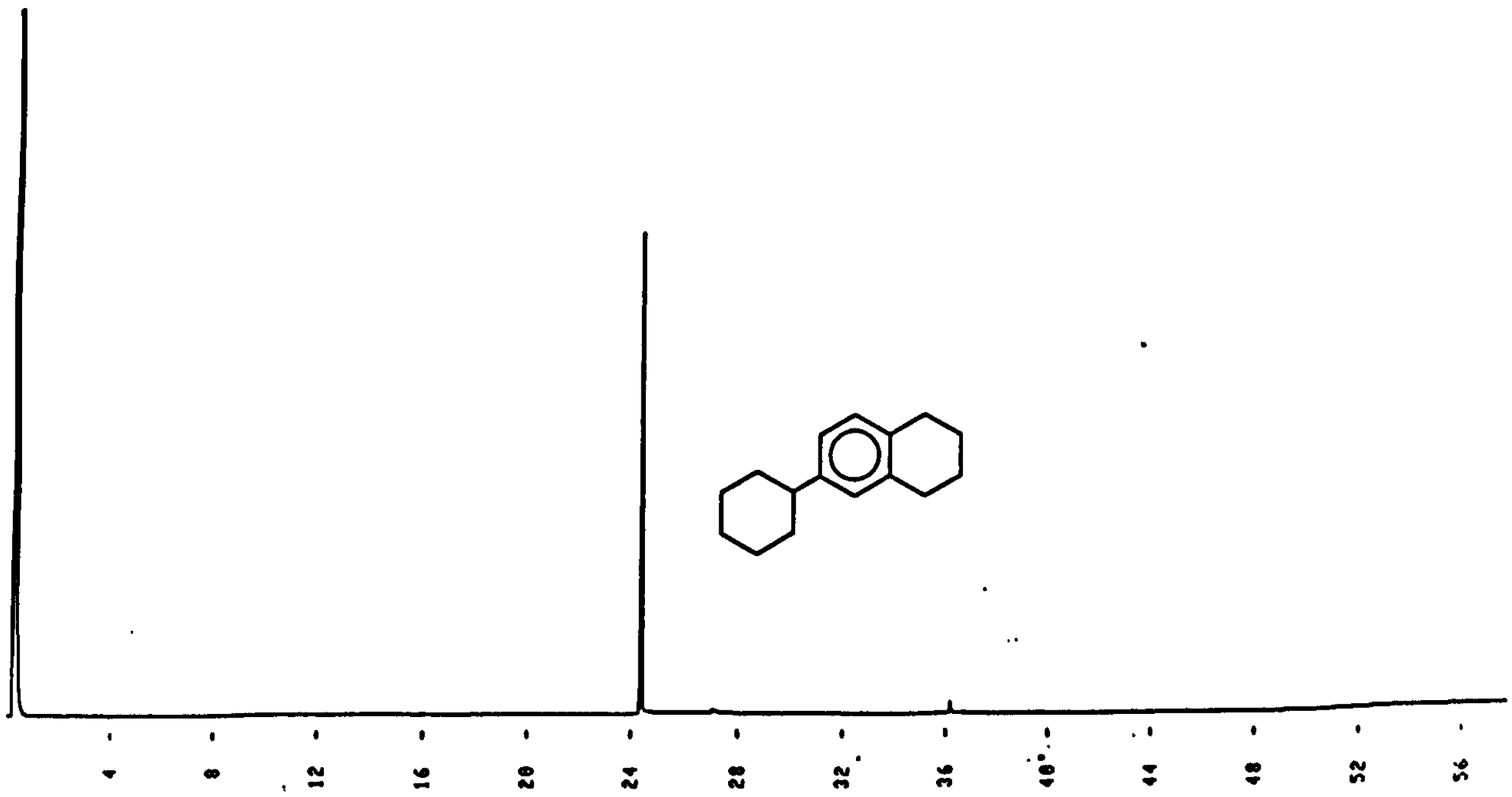


7-cyclohexyltetralin

Reduction of the tetralone *via* the Huang-Minlon modification of the Wolff-Kischner reaction (*cf.* Durham *et al.*, 1963) yielded the first of the two target compounds, 7-cyclohexyltetralin. The crude reaction products were purified by column chromatography to yield pure (> 99 % by GC, Figure 3.33) 7-cyclohexyltetralin in good yield (87 %). The mass spectrum of 7-cyclohexyltetralin (Figure 3.34) showed an intense molecular ion (*m/z* 214, 100%) and a fragment ion at *m/z* 171 [$M^{+\cdot}$ - 43].

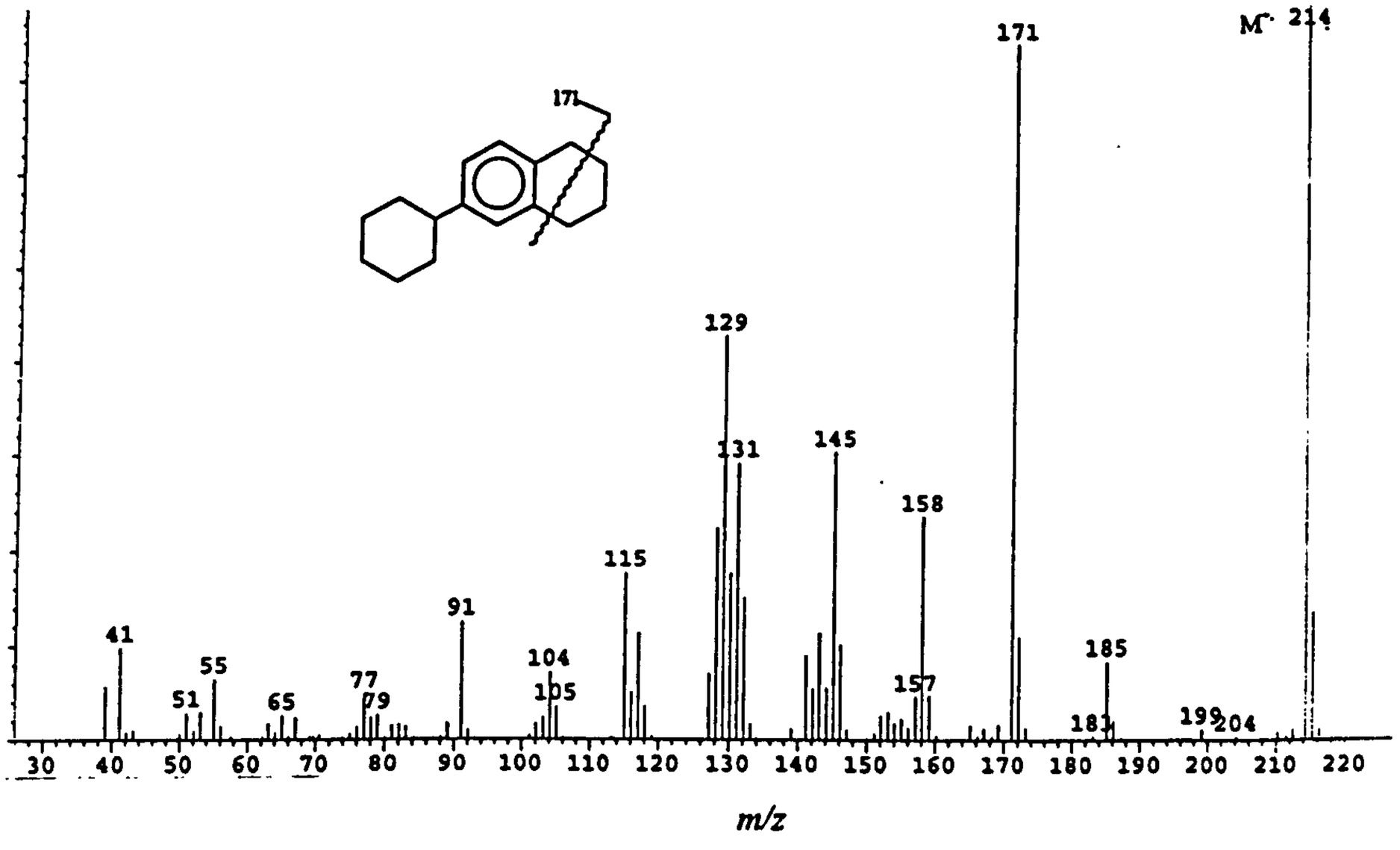
The ^{13}C-NMR and DEPT spectra of 7-cyclohexyltetralin are presented in Figure 3.35 (a) and (b) respectively. The ^{13}C spectrum showed a total of 13 resonances; 6 aromatic carbons, of which the CH and quaternary carbons were distinguished by the DEPT sequence, and 7 aliphatic resonances. The methine carbon (k) of the cyclohexyl group was assigned *via* the DEPT sequence, whilst carbons (l) and (m) were assigned according to the intensity of the signals. The two resonances at 29 ppm have been assigned to carbons (a) and (d) of the ring structure, as these carbons are adjacent to the aromatic ring and will therefore experience a degree of deshielding, resulting in a higher chemical shift than the remaining methylene carbons.

The ^{1}H-NMR spectrum of 7-cyclohexyltetralin is presented in Figure 3.36. The aromatic protons are evident at 6.9 ppm, whilst in the aliphatic region of the spectrum, protons (a) and (d) are farthest downfield at 2.7 ppm. The proton (k) of the cyclohexyl group is shifted downfield relative to the other protons of the cyclohexyl group owing to



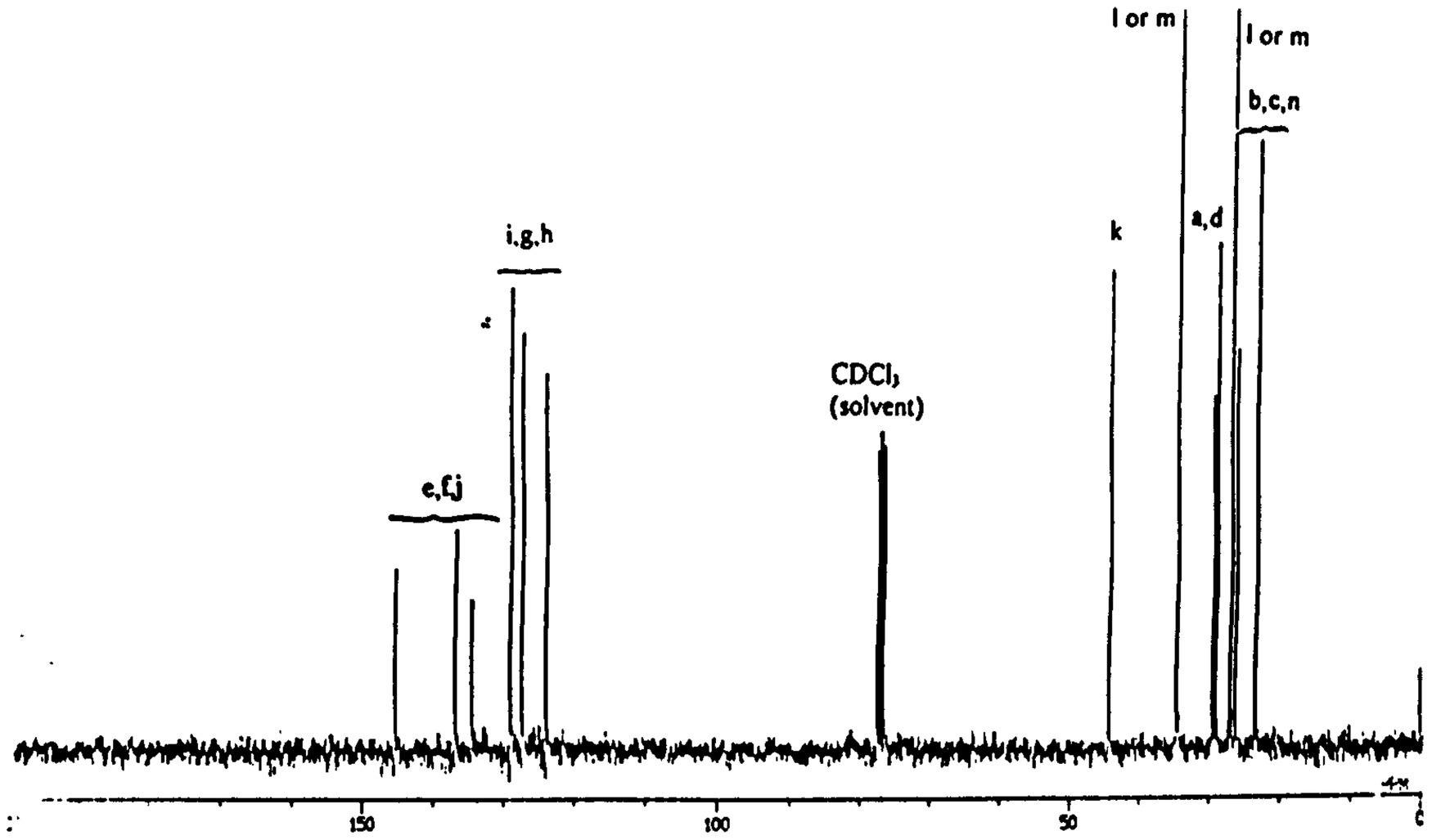
[GC details; HP-1 column. He carrier gas. Temp program; 40°C - 300°C ' 5°C min^{-1} , hold 10 mins]

Figure 3.33 Gas chromatogram of 7-cyclohexyltetralin



3.34 Mass spectrum of 7-cyclohexyltetralin

(a) ^{13}C



(b) DEPT

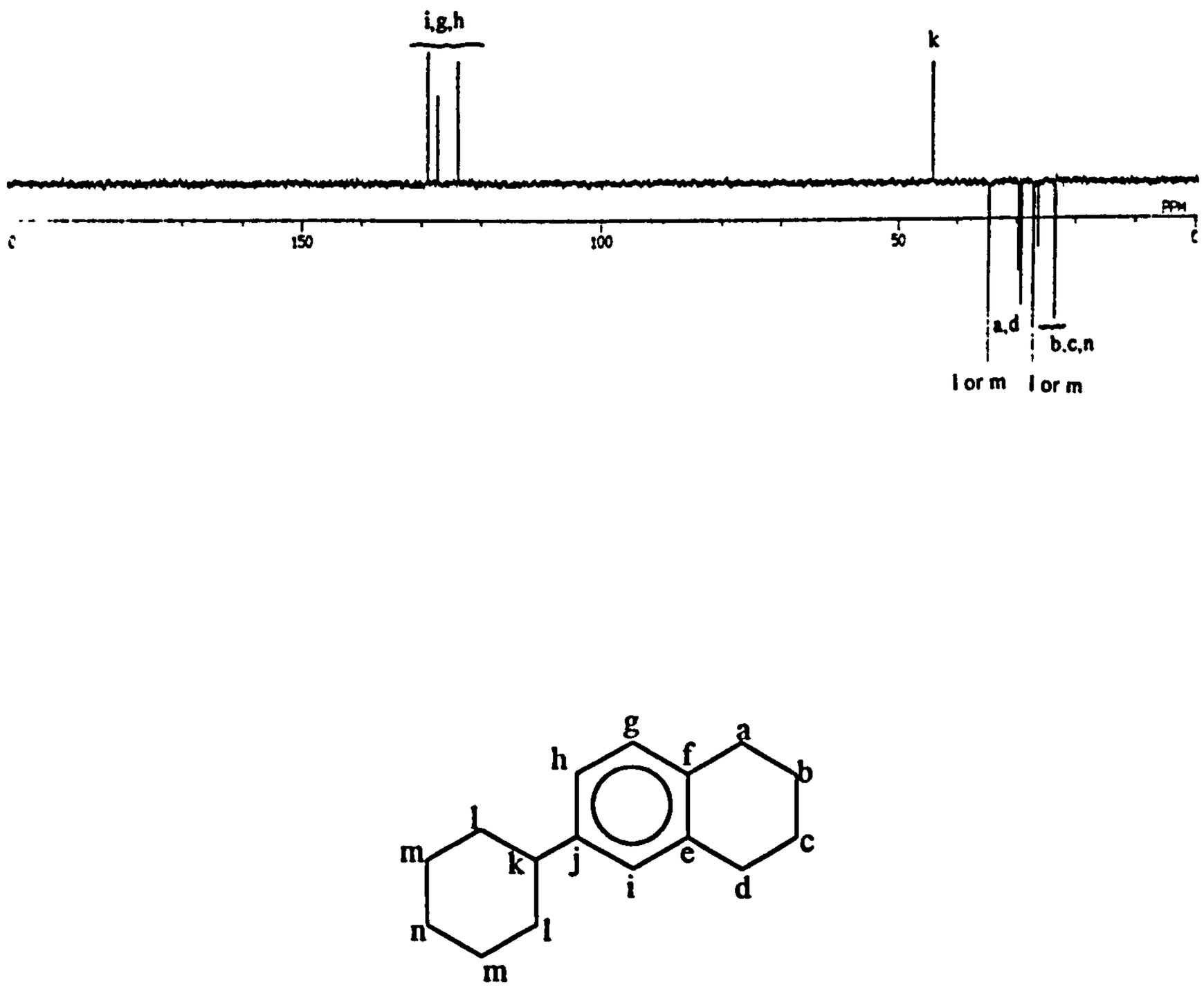
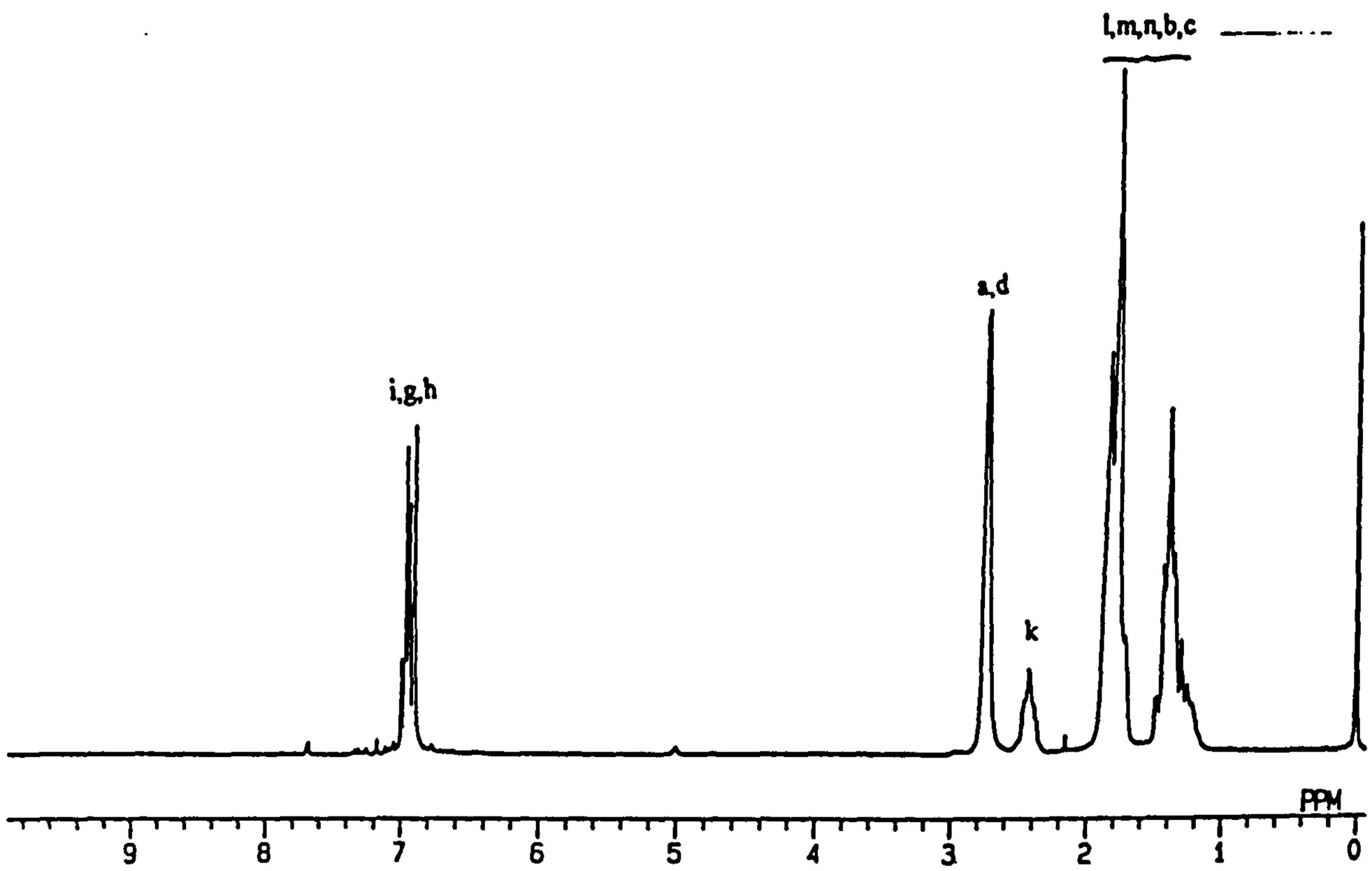


Figure 3.35 (a) ^{13}Carbon and (b) DEPT NMR spectra of 7-cyclohexyltetralin



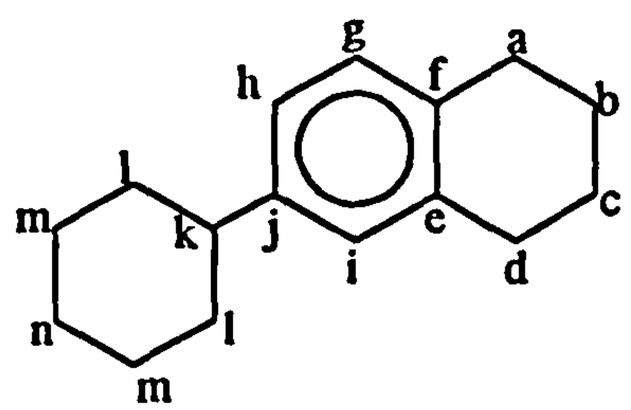


Figure 3.36 ^{1}H NMR spectrum of 7-cyclohexyltetralin

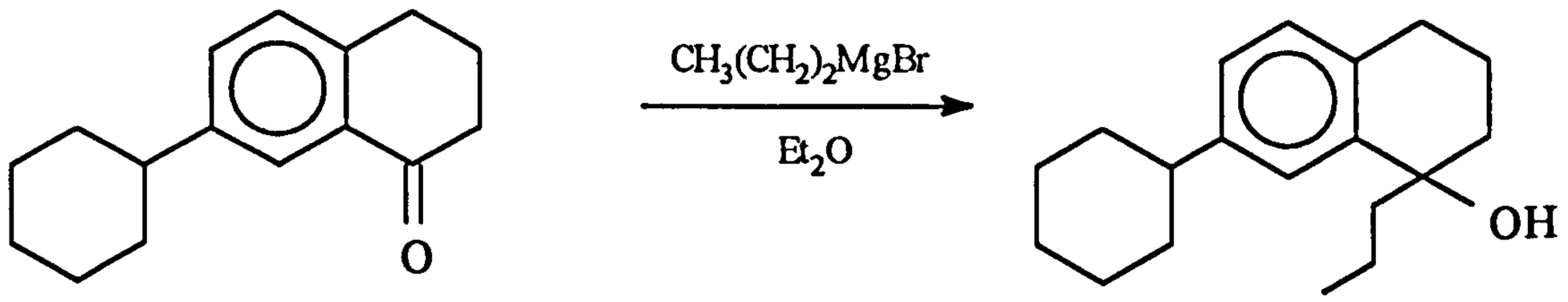
it's position adjacent to the aromatic ring. The remaining protons (l,m,n,b,c) are represented by two multiplets at 1.7 ppm and 1.4 ppm.

Examination of the product by IR confirmed successful reduction of the keto- group, as illustrated in Figure 3.37 by the absence of a strong absorption at approximately 1680 cm^{-1}.

3.3.8 Synthesis of 7-cyclohexyl-1-propyltetralin

Synthesis of 7-cyclohexyl-1-propyltetralin followed an identical synthetic pathway as for 7-cyclohexyltetralin to yield 7-cyclohexyl-1-tetralone (Sections 3.3.4 - 3.3.6), a propyl chain was then introduced *via* a Grignard reaction to yield 7-cyclohexyl-1-hydroxy-1-propyltetralin. The alcohol was dehydrated to an isomeric mixture of alkenes which were subsequently hydrogenated to the target alkane 7-cyclohexyl-1-propyltetralin. The reaction scheme is illustrated in Figure 3.15.

3.3.9 Synthesis of 7-cyclohexy-1-hydroxy-1-propyltetralin



1-bromopropane was coupled to 7-cyclohexyl-1-tetralone *via* a Grignard reaction. Examination of the crude reaction products by GC-MS (sample derivatised with BSTFA at 60°C for 30 minutes) revealed the presence of three major reaction products (Figure

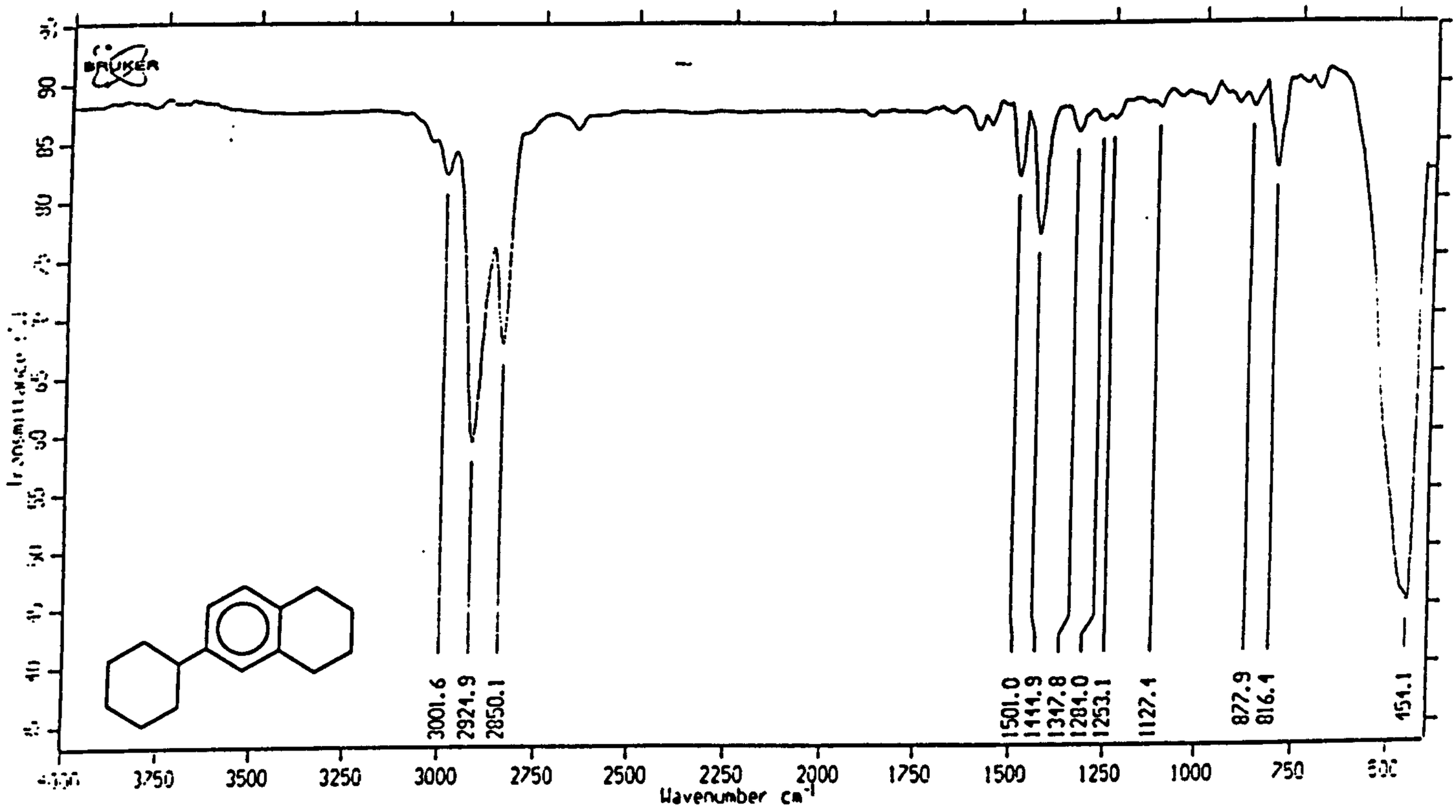
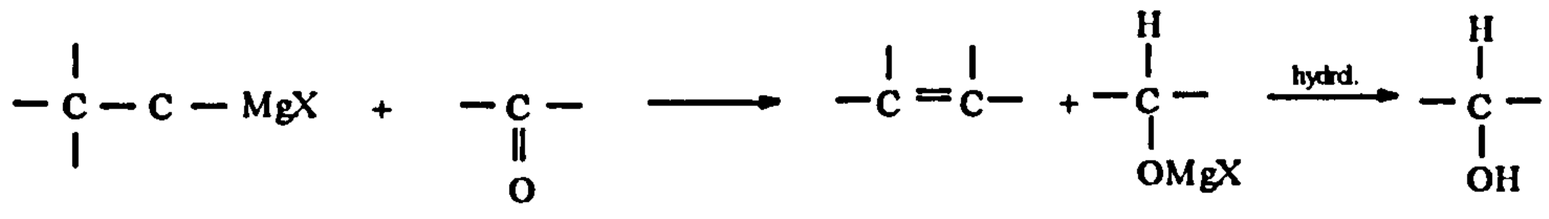


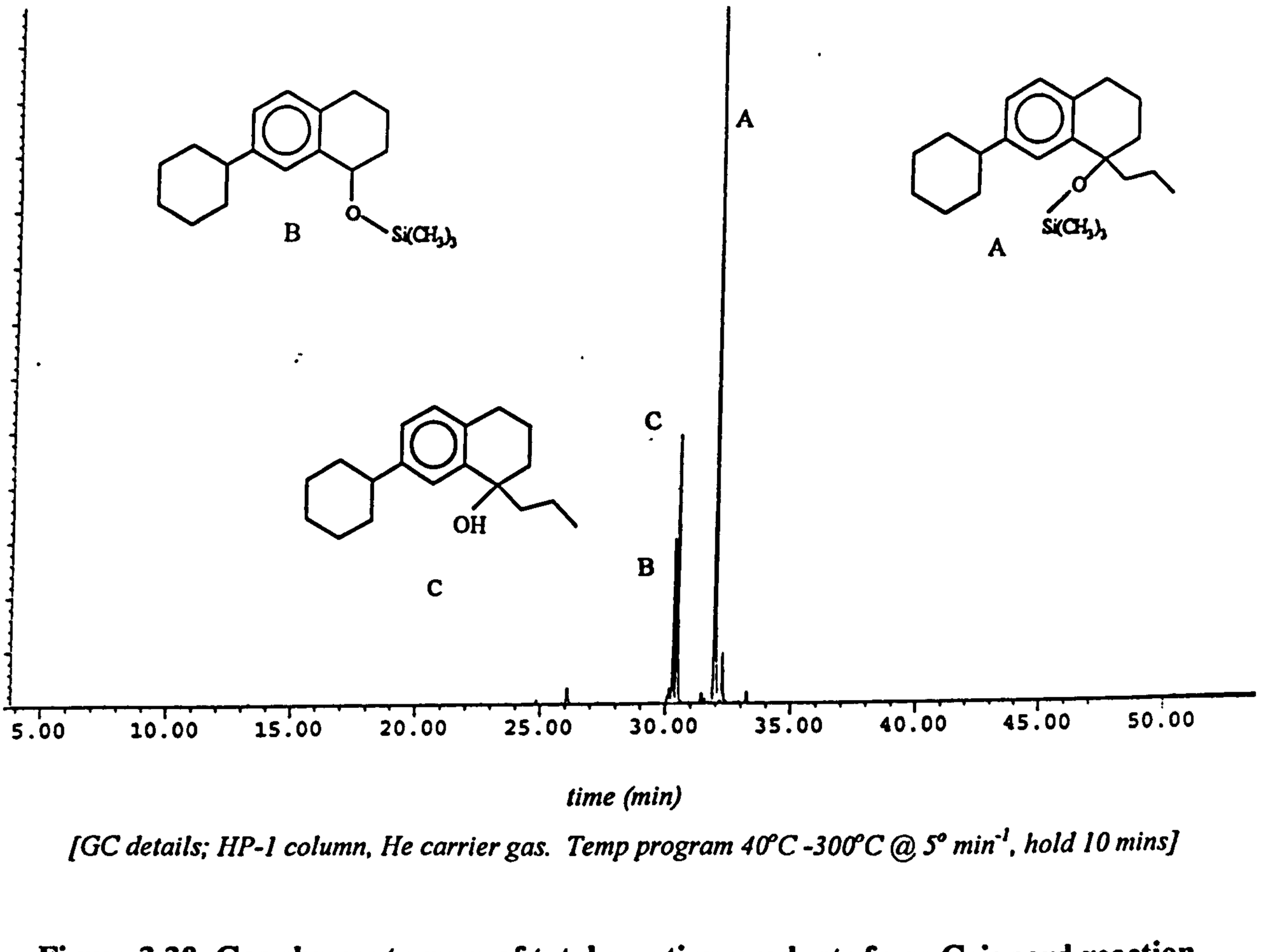
Figure 3.37 Infra-red spectrum of 7-cyclohexyltetralin

3.38). These compounds were tentatively identified by mass spectrometry (Figure 3.39) as the target alcohol (peak A; 65 % of total reaction products); identified by diagnostic ions *m/z* 301 ($M^{+\cdot}$ - C_3H_7); *m/z* 343 ($M^{+\cdot}$ - H); *m/z* 254 ($M^{+\cdot}$ -OTMS), with smaller amounts (12 % of total reaction products) of 7-cyclohexyl-1-hydroxytetralin (peak B); *m/z* 301 ($M^{+\cdot}$-H), *m/z* 212 ($M^{+\cdot}$ - OHTMS) and a third compound which was tentatively identified as the underivatised target alcohol (peak C, 20 % of total reaction products), owing to the presence of an ion at *m/z* 254 corresponding to the loss of water ($M^{+\cdot}$-H_2O) which is a characteristic fragmentation pattern for alcohols, and an ion at *m/z* 211 [($M^{+\cdot}$-H_2O)-C_3H_7]. It appears that possibly owing to the position of the hydroxyl group of the alcohol, not all of the alcohol had been derivatised. The sterically hindered nature of the hydroxy group would also account for the sharp chromatographic peak of the underivatised alcohol.

7-Cyclohexyl-1-hydroxytetralin (peak B) has been formed by reduction, a commonly occurring side reaction in Grignard reactions with hindered ketones (March, 1985). The reaction involves reduction of the carbonyl carbon to an alcohol by the Grignard reagent, which itself undergoes elimination to produce an olefin, thus;



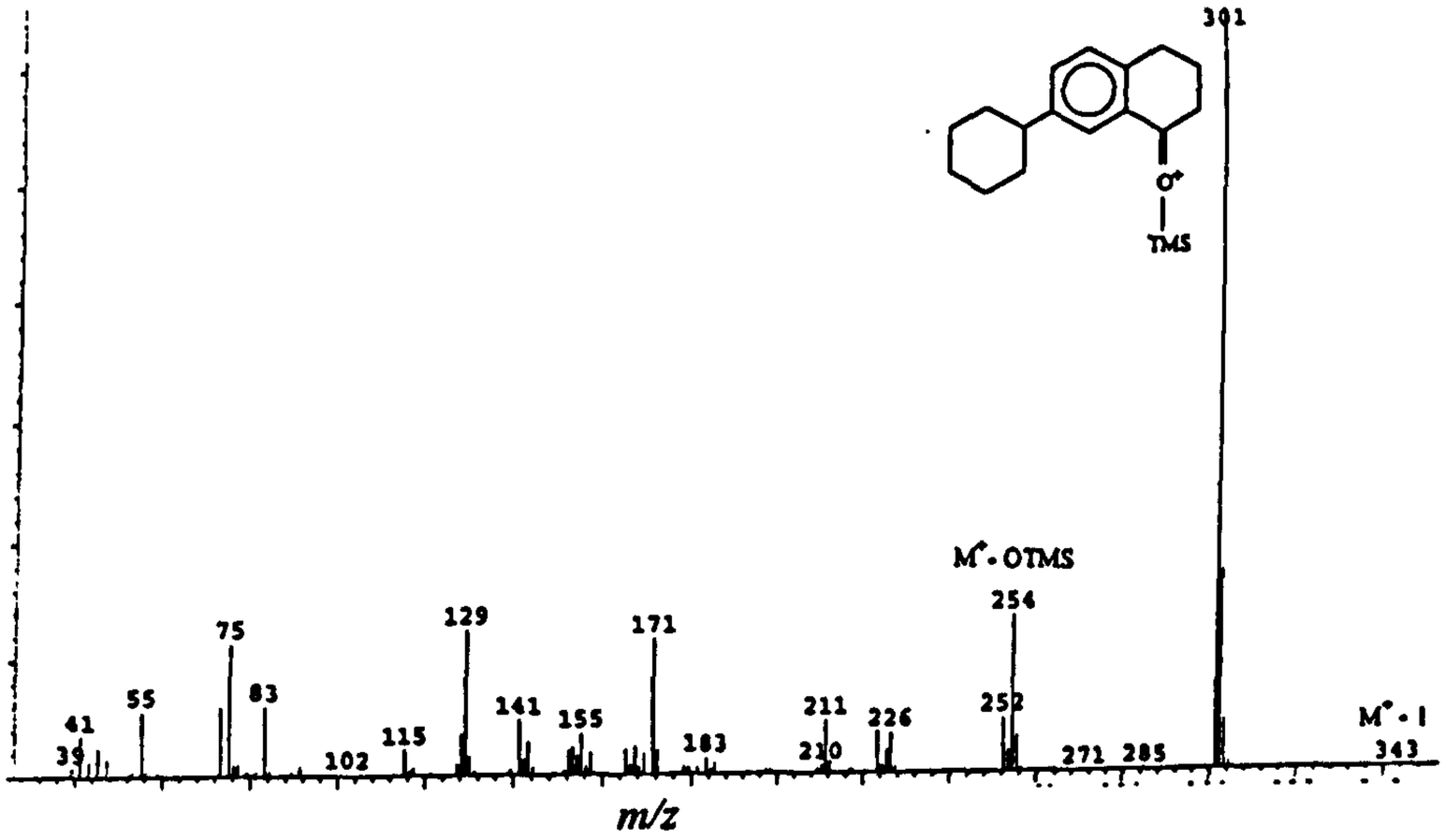
Separation of the crude reaction products by vacuum distillation is precluded, owing to the similar boiling points of each of the products (as indicated by the close elution of the three compounds by gas chromatography). Consequently, the crude reaction products were dehydrated to a mixture of alkenes without a 'clean up' stage.



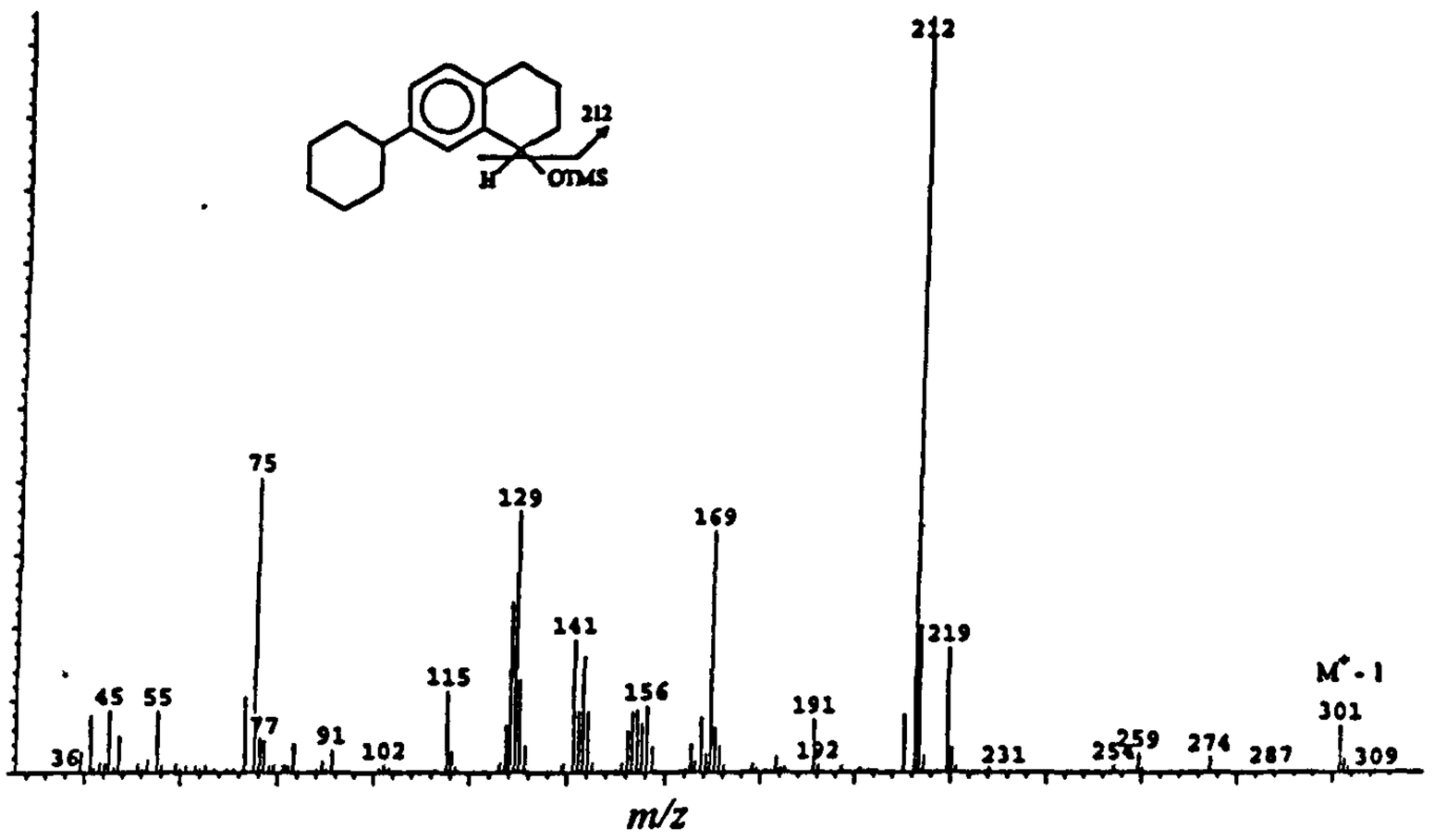
[GC details; HP-1 column, He carrier gas. Temp program 40°C -300°C @ 5° min^{-1}, hold 10 mins]

Figure 3.38 Gas chromatogram of total reaction products from Grignard reaction of 7-cyclohexyl-1-tetralone with propylmagnesium bromide (derivatised with BSTFA)

(a) Peak A



(b) Peak B



(c) Peak C

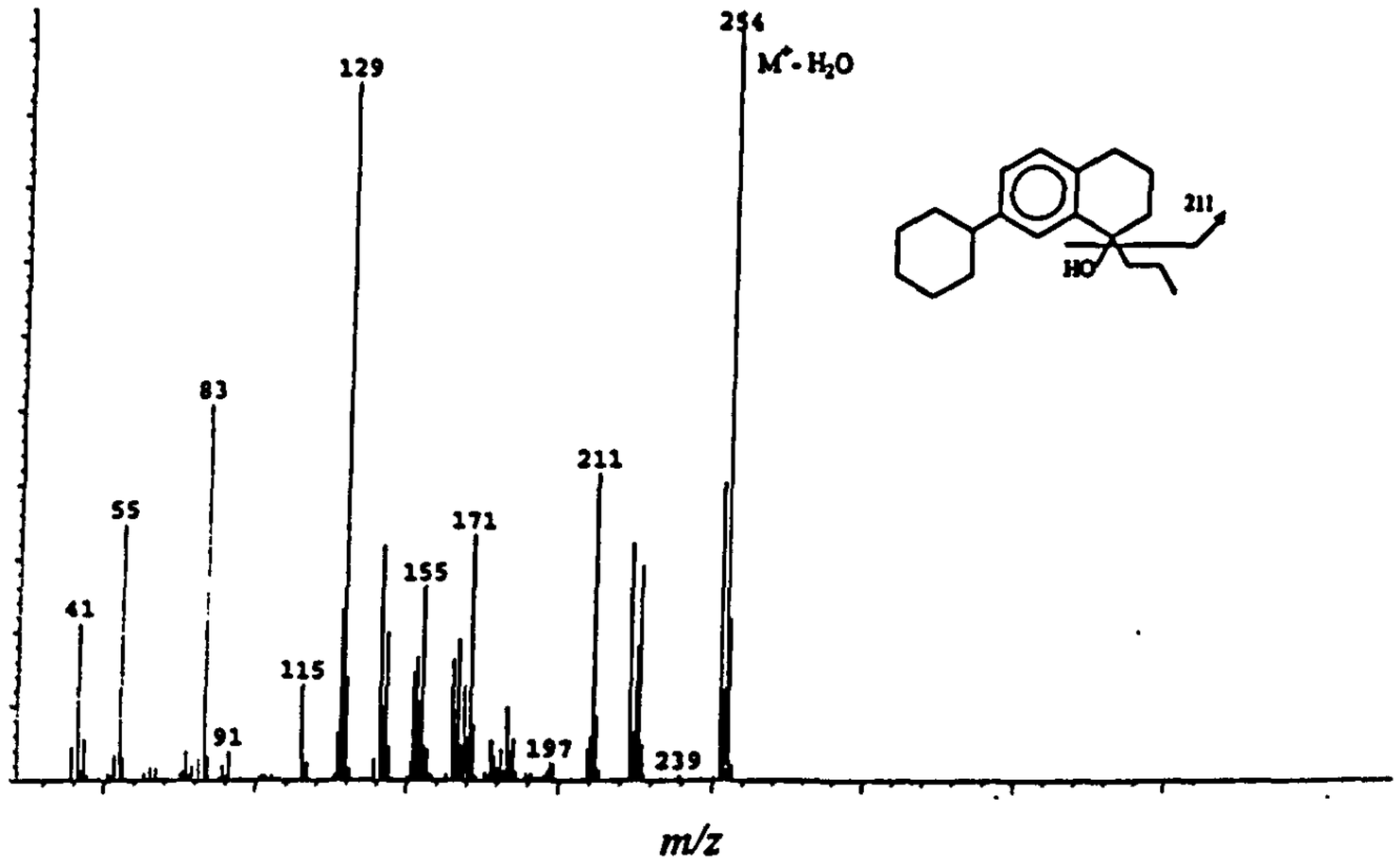
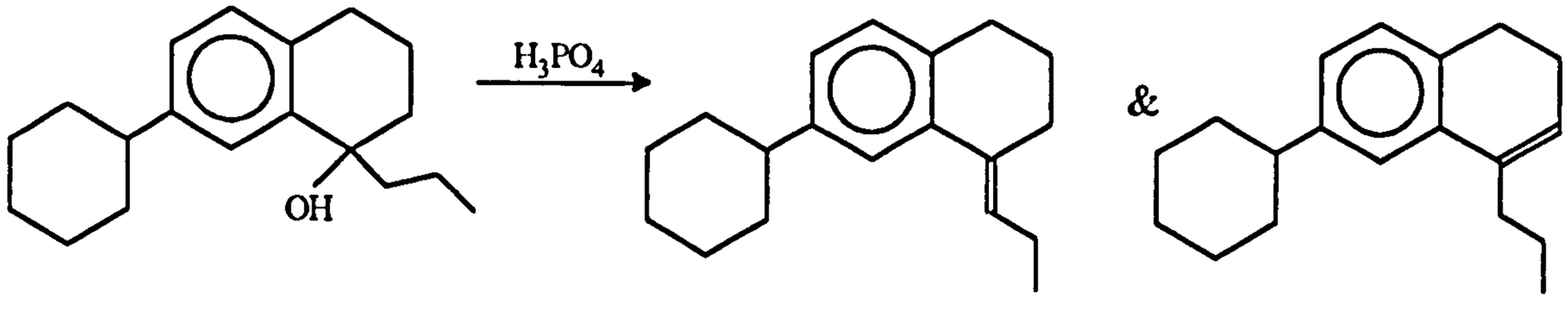


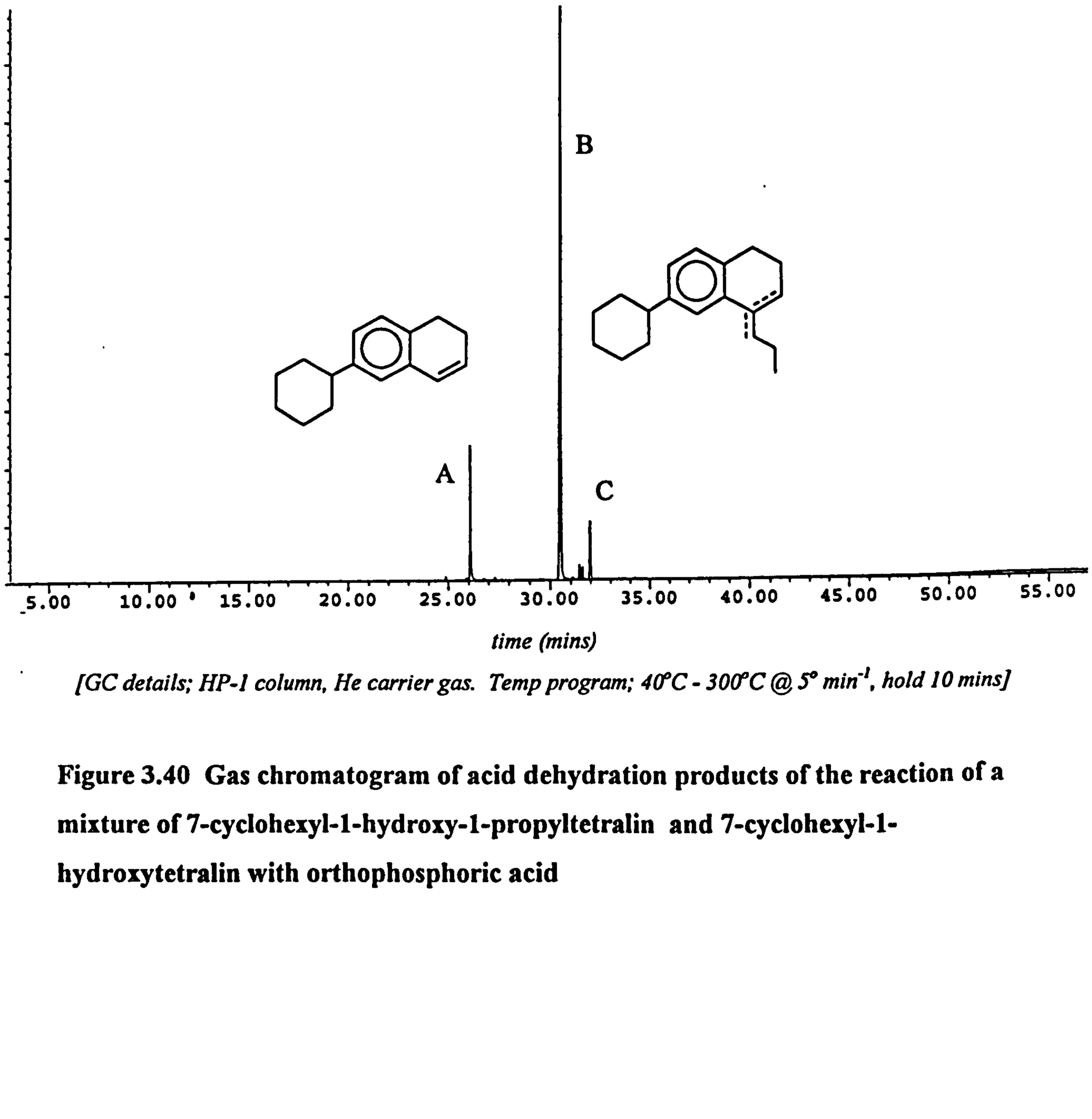
Figure 3.39 Mass spectra of products of Grignard reaction of 7-cyclohexyl-1-tetralone with propylmagnesium bromide (derivatised with BSTFA)

3.3.10 Synthesis of (E/Z)7-cyclohexyl-1-propenyltetralin (I) and 7-cyclohexyl-1-propyl-3,4-dihydronaphthalene (II)



I II

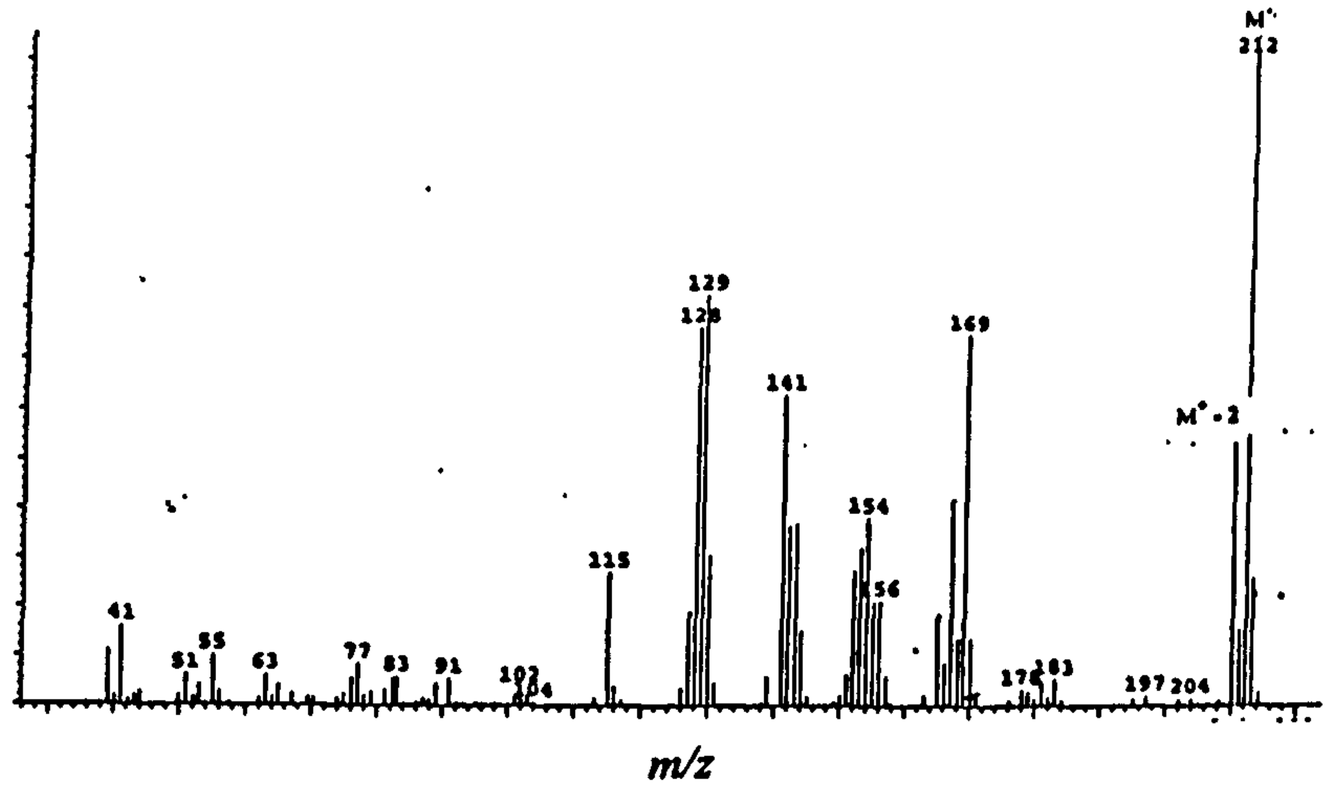
The crude alcohol mixture (Section 3.3.9) was dehydrated to an isomeric mixture of the related alkenes (the target alkenes, (E/Z) 7-cyclohexyl-1-propenyltetralin (I) and 7-cyclohexyl-1-propyl-3,4-dihydronaphthalene (II) and also unwanted 7-cyclohexyl-3,4-dihydronaphthalene *via* acid catalysed dehydration. Analysis of the crude reaction products by GC-MS identified two principal reaction products as shown in Figure 3.40. Peak A was identified by mass spectrometry (Figure 3.41) as the alkene 7-cyclohexyl-3,4-dihydronaphthalene, the dehydration product of 7-cyclohexyl-1-hydroxytetralin by the presence of a molecular ion, *m/z* 212, with diagnostic ions; *m/z* 210 ($M^{+\cdot}$ - 2); *m/z* 169, ($M^{+\cdot}$- C_3H_7); *m/z* 129 ($M^{+\cdot}$- 83). Dehydration of 7-cyclohexyl-1-hydroxy-1-propyltetralin could result in the formation of the isomers (E/Z) 7-cyclohexyl-1-propenyltetralin (I) and 7-cyclohexyl-1-propyl-3,4-dihydronaphthalene (II). Peaks B and C had virtually identical spectra and were identified as the target isomeric alkenes by the presence of a molecular ion *m/z* 254 and diagnostic ions; *m/z* 252 (base peak, $M^{+\cdot}$ - 2), *m/z* 223 presumably arising through loss of the propyl chain from the ($M^{+\cdot}$- 2) ion and *m/z* 171 ($M^{+\cdot}$-83). The isomers could not be distinguished further owing to virtually identical mass spectra. However, it is likely that 7-cyclohexyl-1-propyl-3,4-dihydronaphthalene will be the dominant product as this places the double bond in the alicyclic moiety, the more stable of the two positions. The absence of a third major



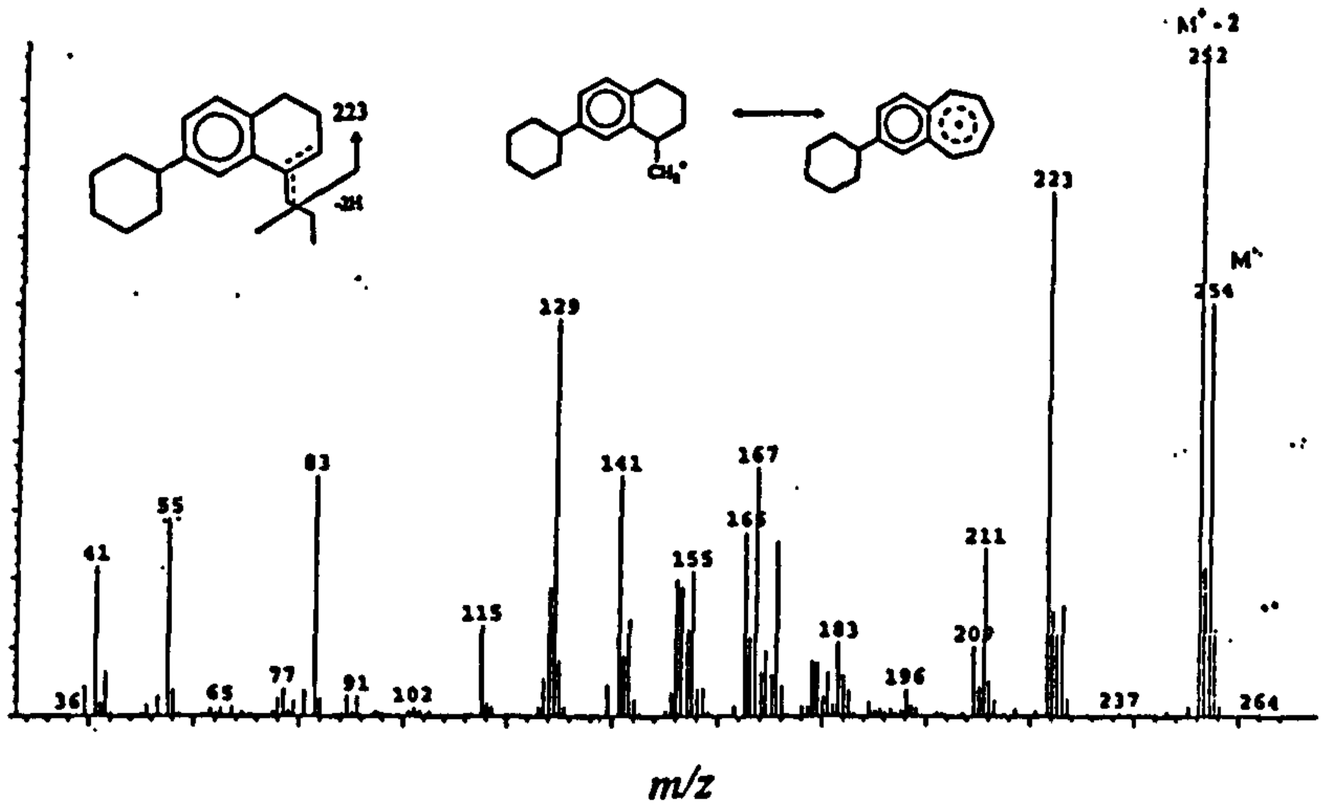
[GC details; HP-1 column, He carrier gas. Temp program; 40°C - 300°C @ 5° min^{-1}, hold 10 mins]

Figure 3.40 Gas chromatogram of acid dehydration products of the reaction of a mixture of 7-cyclohexyl-1-hydroxy-1-propyltetralin and 7-cyclohexyl-1-hydroxytetralin with orthophosphoric acid

(a) Peak A



(b) Peak B



(c) Peak C

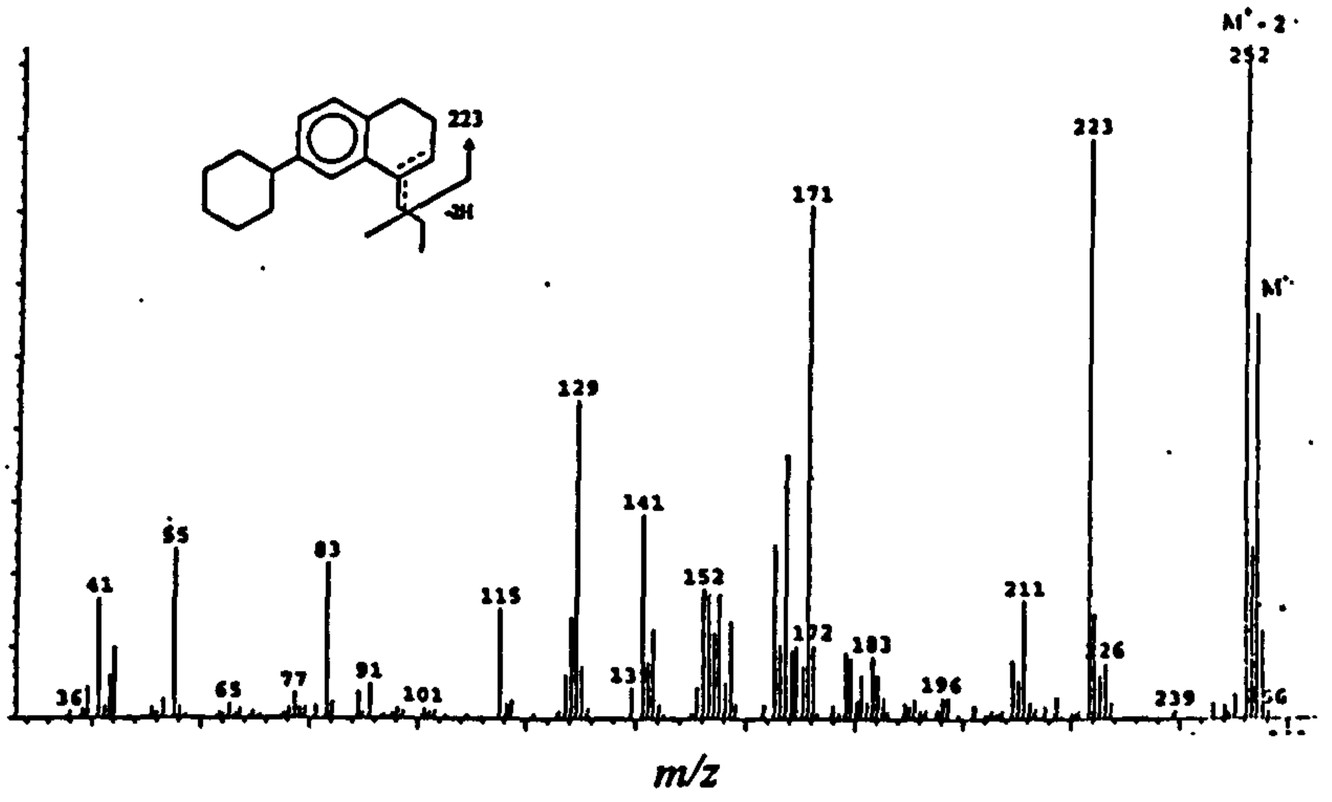
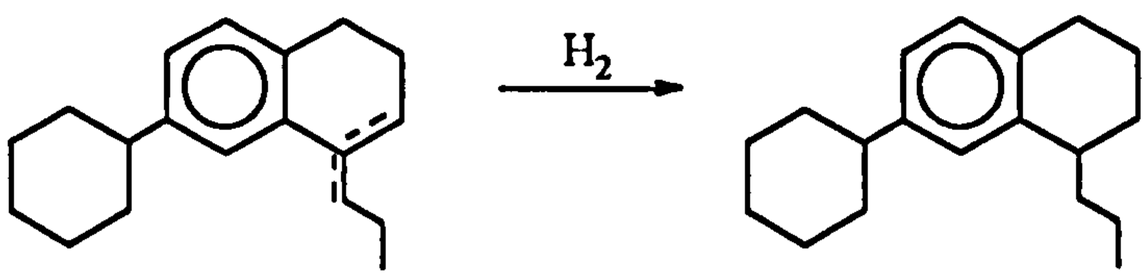


Figure 3.41 Mass spectra of acid dehydration reaction products of the reaction of a mixture of 7-cyclohexyl-1-hydroxy-1-propyltetralin and 7-cyclohexyl-1-hydroxytetralin with orthophosphoric acid

reaction product lends support to the tentative identification of peak C in the crude Grignard reaction products (Figure 3.38 and 3.39) as the underivatised alcohol 7-cyclohexyl-1-hydroxy-1-propyltetralin.

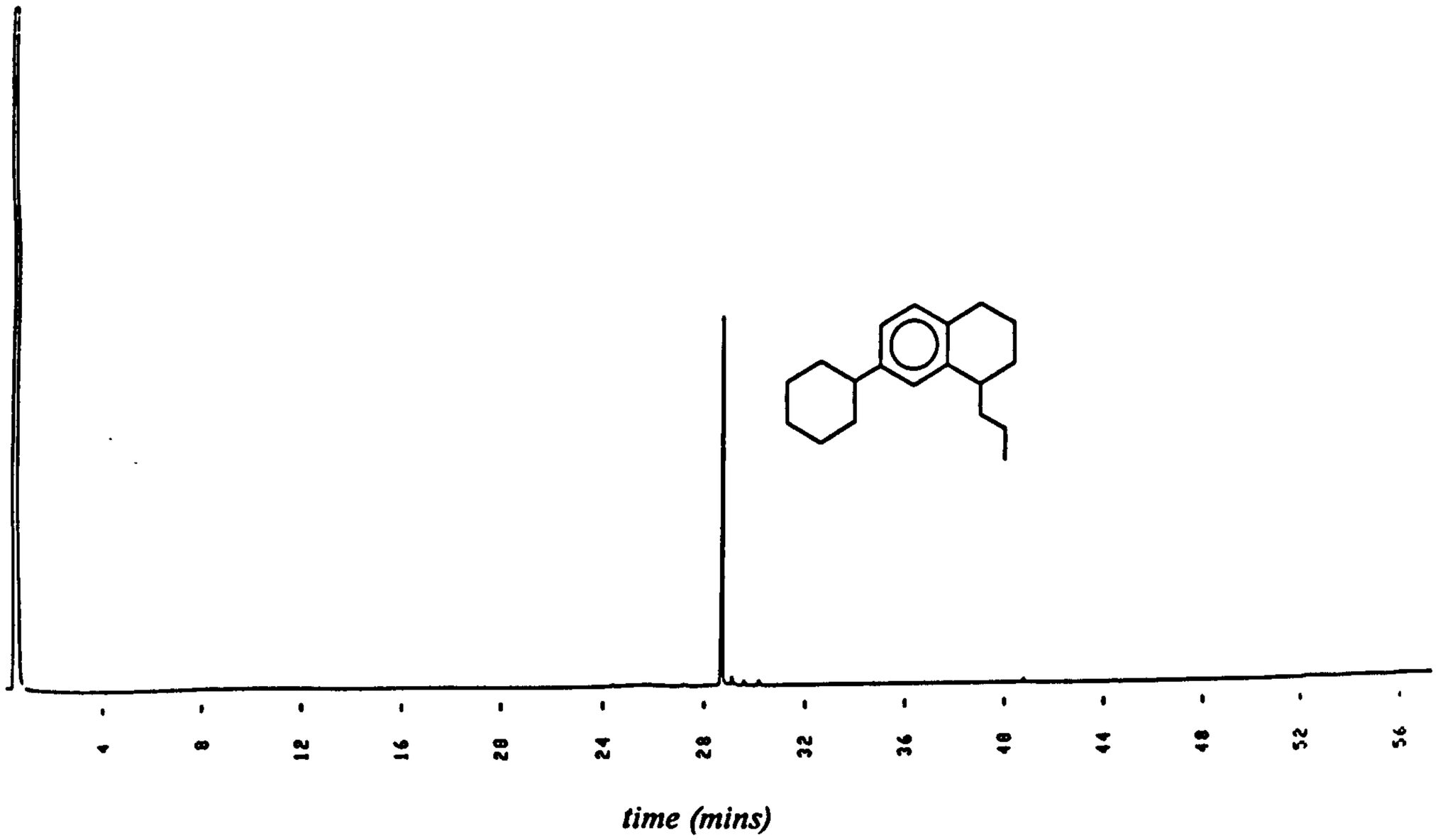
The alkene mixture was purified by column chromatography (silica column, hexane as eluent), followed by isolation of the target alkenes by Ag^+/silica TLC (R_f = 0.09 - 0.1). Purity as determined by GC was 98 % .

3.3.11 Synthesis of 7-cyclohexyl-1-propyltetralin



7-cyclohexyl-1-propyltetralin

The pure isomeric mixture of alkenes (7-cyclohexyl-1-propenyltetralin and 7-cyclohexyl-1-propyl-3,4-dihydronaphthalene) were hydrogenated to the target compound 7-cyclohexyl-1-propyltetralin by gentle bubbling of hydrogen gas through a solution of the alkenes in the presence of Adams catalyst (monohydrate). A gas chromatogram of the 7-cyclohexyl-1-propyltetralin is presented in Figure 3.42. The mass spectrum showed the molecular ion ($M^{+\cdot}$, *m/z* 256) and an ion at *m/z* 213 ($M^{+\cdot}$ - 43) corresponding to cleavage at the tertiary centre and corresponding loss of the propyl chain (Figure 3.43). Analysis of the compound by ^{13}C , DEPT and ^{1}H NMR confirmed synthesis of the target alkane. The ^{13}C NMR spectrum (Figure 3.44a) revealed the presence of 17 resonances which were assigned in conjunction with analysis *via* the DEPT sequence



[GC details; HP-1 column, He carrier gas. Temp program 40°C - 300°C @ 5° min^{-1}, hold 10 mins]

Figure 3.42 Gas chromatogram of 7-cyclohexyl-1-propyltetralin

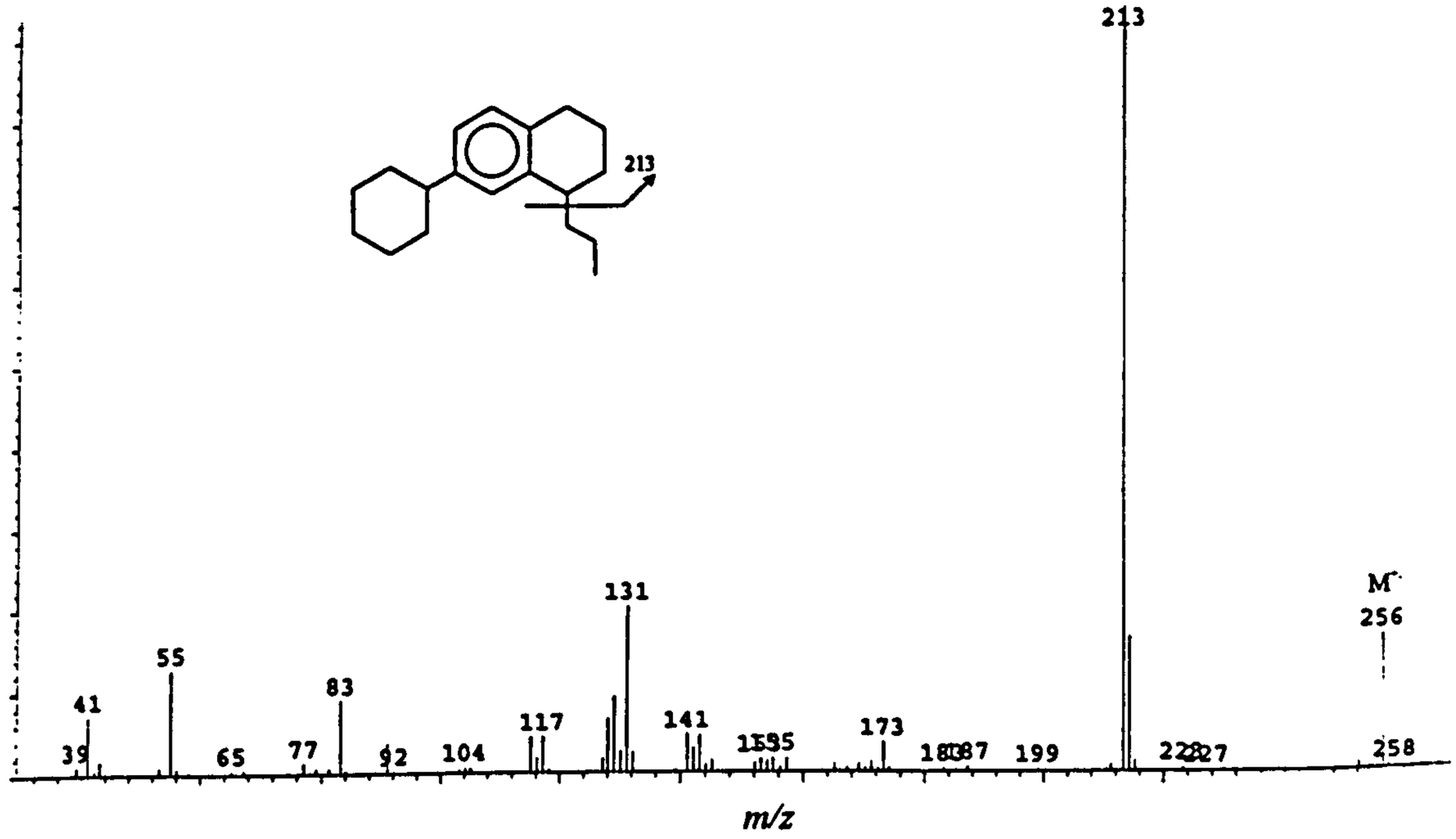
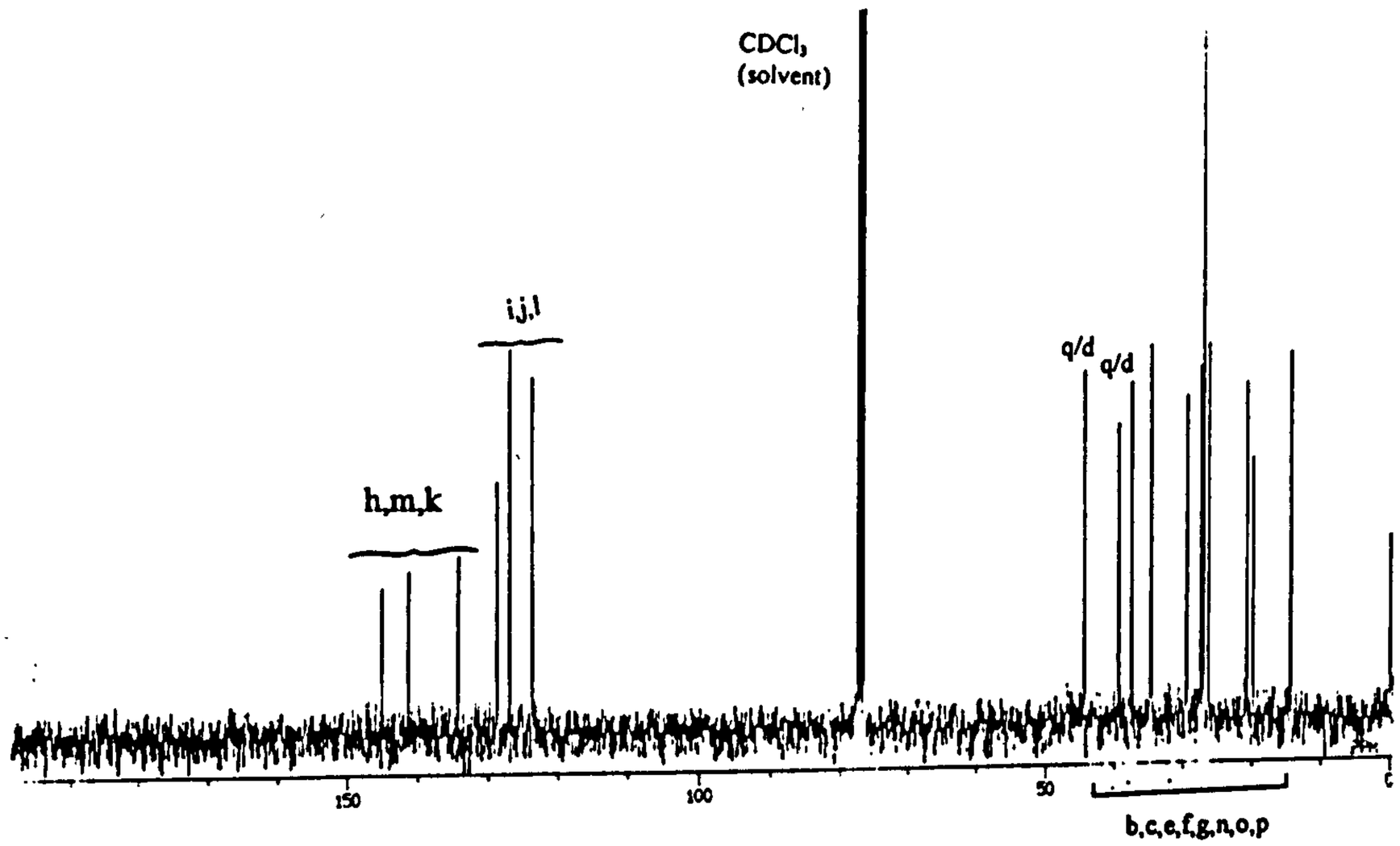
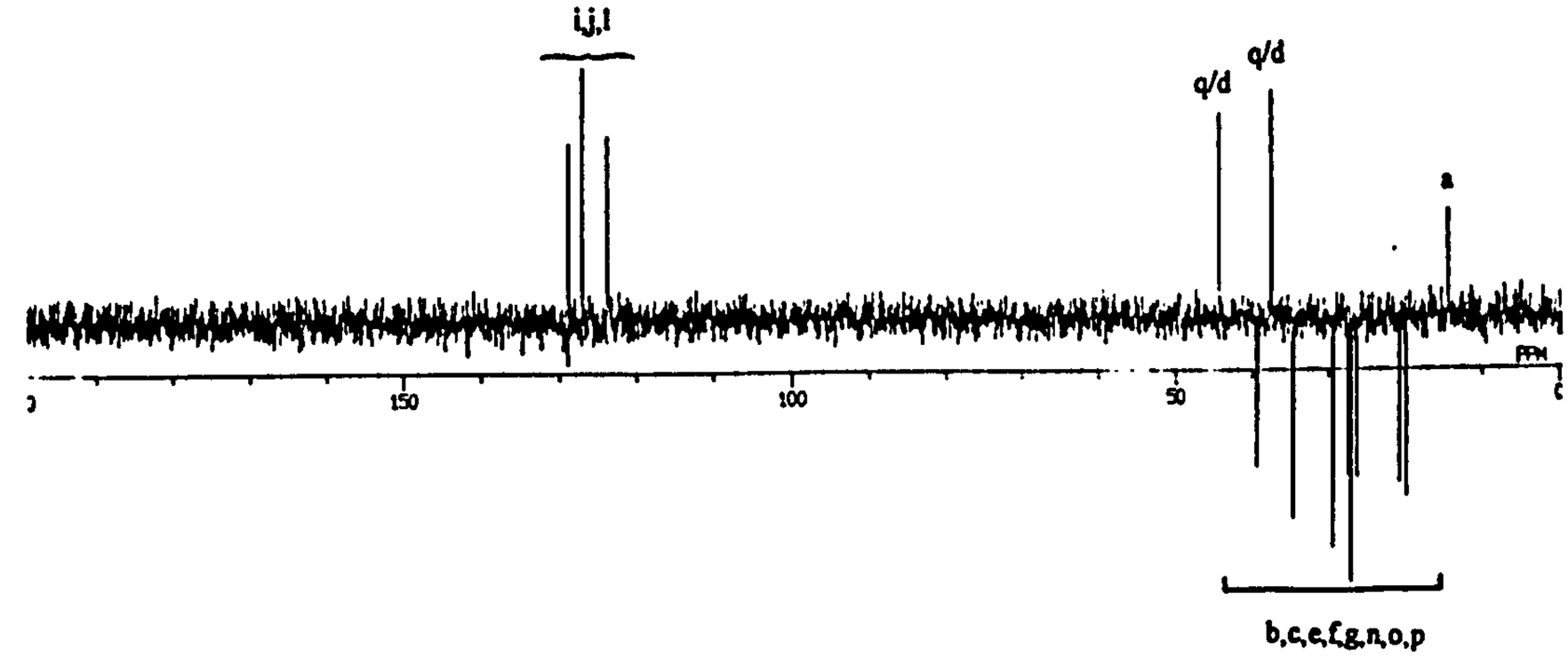


Figure 3.43 Mass spectrum of 7-cyclohexyl-1-propyltetralin

(a) ^{13}C



(b) DEPT



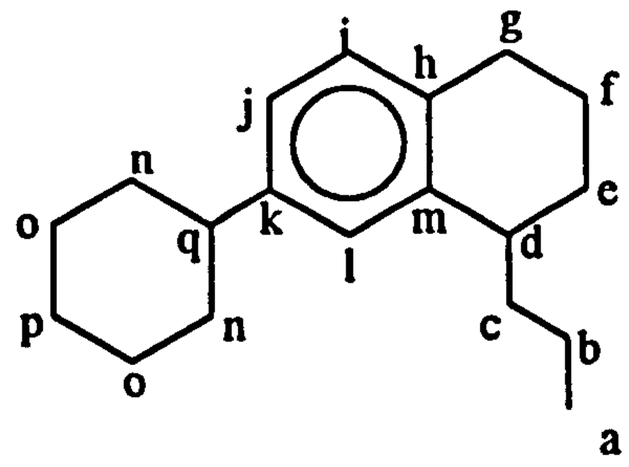
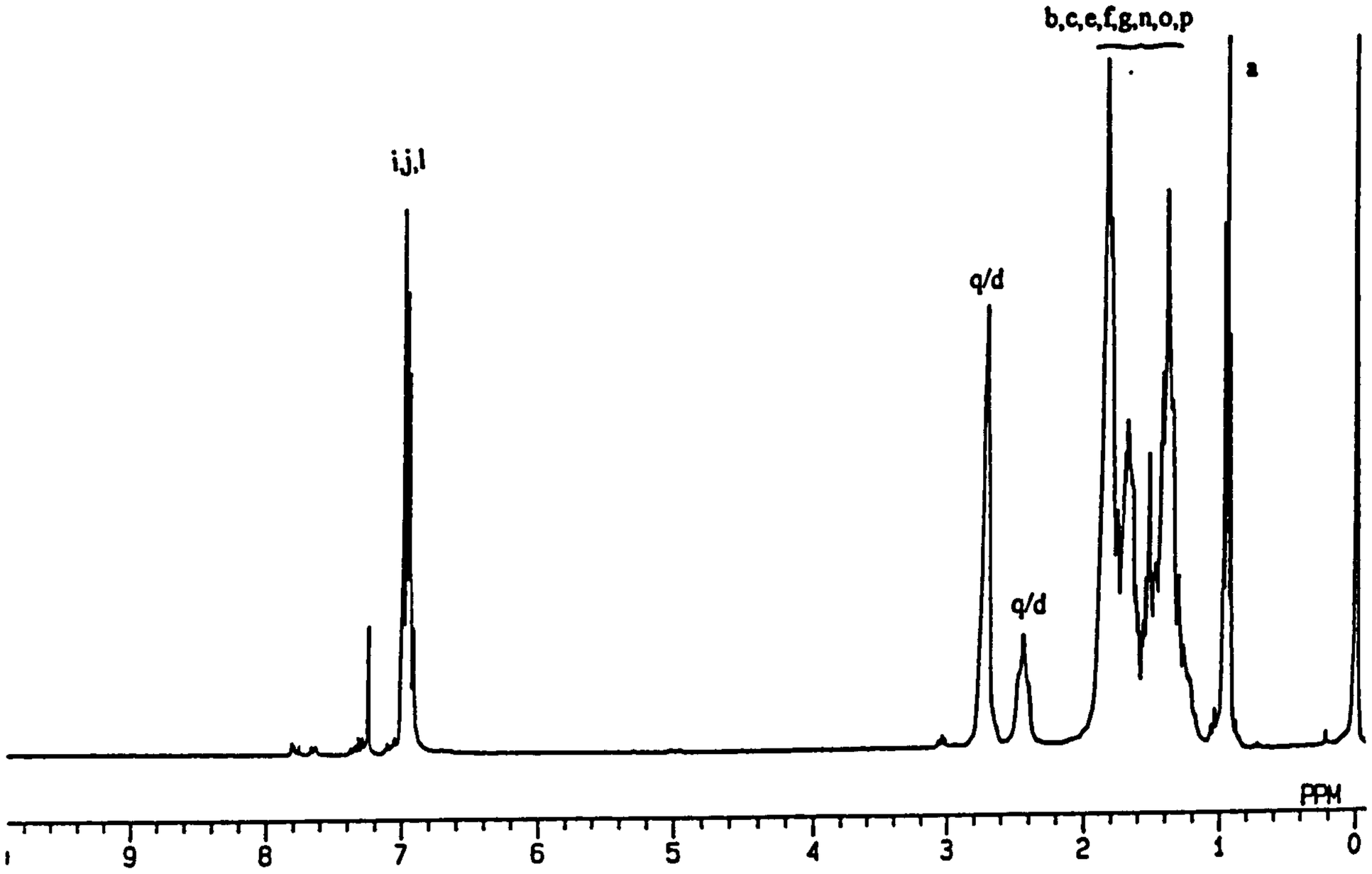


Figure 3.44 ^{13}Carbon (a) and DEPT (b) NMR spectra of 7-cyclohexyl-1-propyltetralin



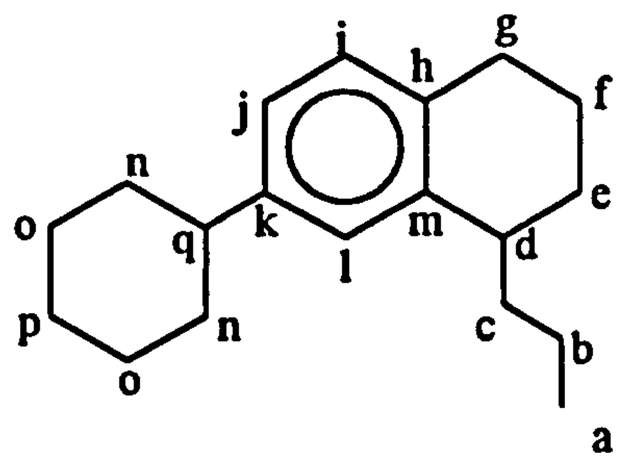


Figure 3.45 ^{1}H NMR spectrum of 7-cyclohexyl-1-propyltetralin

(Figure 3.44 b). The three quaternary carbons (h,m,k) were present at 145 ppm, 141 ppm and 134 ppm; whilst the remaining aromatic carbons (i,j,l) were evident at 128 ppm, 127 ppm and 123 ppm. Analysis *via* the DEPT sequence enabled identification of the methine carbons (d) and (q) and also the methyl carbon (a). The remaining eight methylene carbon resonances could not be unequivocally assigned to individual carbon atoms and are simply grouped together.

The ^{1}H-NMR spectrum is presented in Figure 3.45. The aromatic protons are evident at 6.9 ppm, whilst in the aliphatic region of the spectrum, the triplet at 0.9 ppm corresponds to the protons of the methyl group (a). The majority of the aliphatic protons (b,c,e,f,g,o,p,q,) are represented by the multiplet from 1.3 - 1.7 ppm. The signals at 2.7 ppm and 2.4 ppm are tentatively assigned to protons d and q respectively. These protons are all situated adjacent to the aromatic ring and this is anticipated to result in higher frequency shifts for these protons owing to the deshielding effect of the aromatic ring.

3.3.12 Conclusions

The model aromatic UCM compounds 7-cyclohexyltetralin and 7-cyclohexyl-1-propyltetralin have been successfully synthesised in sufficient quantity and purity (> 99 % and > 97 %, respectively) for future toxicological tests using the mussel *Mytilus edulis* as detailed in Chapter 5.

CHAPTER FOUR

The effect of a model aliphatic low molecular weight UCM compound (4-propyloctane) upon mussel feeding rate

4.1 Introduction

Despite the high concentrations of hydrocarbon UCMs in many sediments and aquatic organisms (*cf.* Table 1.2), the toxicological significance of UCM hydrocarbons remains largely uninvestigated. It is however, widely assumed that aliphatic UCM hydrocarbons are non-toxic owing to their relatively low aqueous solubility (*e.g.* Gilfillan *et al.*, 1977; Bobra *et al.*, 1983; Thomas *et al.*, 1995). In Chapter 2, the presence of hydrocarbon UCMs in mussels extending from a molecular weight range of approximately *n*-C_{12} upwards was demonstrated. A proportion of these low molecular weight aliphatic hydrocarbon UCMs may be of some toxicological significance and this clearly requires further investigation.

A reduction in the Scope for Growth of mussels (*Mytilus edulis*) exposed to petroleum hydrocarbons has been demonstrated to occur primarily because of a reduction in mussel feeding rate (Widdows *et al.*, 1987; *cf.* Section 1.5). The responsiveness of mussel ciliary feeding activity to a number of aliphatic and aromatic hydrocarbons has been reported previously by Donkin *et al.* (1989, 1991) who described a Quantitative Structure-Activity Relationship (QSAR) for the effects of various hydrocarbons upon mussel feeding rate. These authors demonstrated that hydrocarbons with aqueous solubilities greater than 70 $\mu g\ l^{-1}$ were toxic, and that the total body burden of toxicant required to produce a 50% reduction in mussel feeding rate was relatively constant. A 'cut-off' in toxicity was identified whereby those compounds with aqueous solubilities less than 70 $\mu g\ l^{-1}$ failed to induce a significant reduction in feeding rate (*cf.* Section 1.6) despite greater bioaccumulation into the mussels than the more soluble, toxic compounds. For the aliphatic hydrocarbons tested, this 'cut-off' in toxicity was found to occur between *n*-decane and *n*-undecane. However, no components representative of UCMs were tested.

Currently available evidence, based on spectroscopic and degradative studies reviewed in Section 1.2, suggests that a large proportion of aliphatic UCM hydrocarbons are comprised of relatively simple 'T'-branched acyclic and monocyclic alkanes. Since branched alkanes have a greater aqueous solubility than the equivalent straight chain compounds (Verscheuren, 1983; Leo, 1993) a proportion of the low molecular weight aliphatic UCM hydrocarbons may be of some toxicological significance.

The aim of the present work was to apply the mussel toxicological assay developed by Donkin *et al.* (1989, 1991) to investigate the toxicity of a model 'T-branched' low molecular weight (C_{11}) aliphatic UCM hydrocarbon, 4-propyloctane (4-PO; Figure 4.1). 4-propyloctane is predicted to have a water solubility of 297 μg l^{-1} [@ 25°C in freshwater; estimated using Syracuse Research Corporation software (SRC)] and a log K_{OW} value in the range 5.67-6.38 (estimated by SRC and MedChem software, respectively). [For comparison, *n*-decane has a log K_{OW} 5.98 and an aqueous solubility of 52 μg l^{-1} and is toxic to mussel (*Mytilus edulis*) feeding rate, whilst *n*-undecane has a log K_{OW} 6.1 and an aqueous solubility of 4 μg l^{-1} and is non-toxic (Donkin *et al.*, 1991)]. On the basis of these physico-chemical properties, 4-propyloctane would therefore be expected to exhibit a measurable effect on mussel ciliary feeding activity, according to the QSAR established by Donkin *et al.* (1989, 1991).

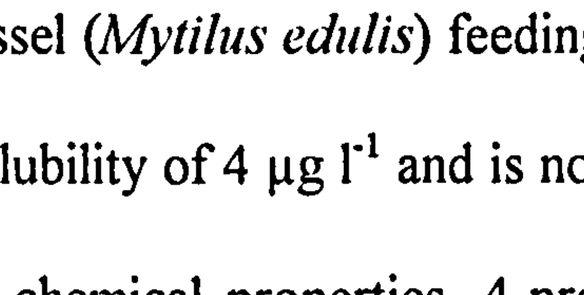


Figure 4.1. 4-propyloctane (4-PO)

4.2 Experimental details

A series of five experiments were carried out to investigate the effect of 4-PO upon mussel ciliary feeding activity. Initially, a concentration-response experiment was conducted to ascertain if exposure of *M. edulis* to 4-PO resulted in a reduction in mussel ciliary feeding activity (Experiment I). This having been demonstrated, a series of experiments investigating the effect of 4-PO upon mussel feeding rate over different exposure periods up to 120 h were then conducted (Experiments II - V). The aim of these latter experiments over longer exposure periods was to investigate the influence of bioaccumulation kinetics upon mussel feeding rate. In addition to investigating the effect of 4-PO upon mussel feeding rate, in Experiment V, a Neutral Red Retention assay (Lowe *et al.*, 1995) was conducted in parallel with the feeding rate determinations, to examine the effect of 4-PO at the sub-cellular level.

To allow the results obtained herein to be compared with those of Donkin *et al.* (1989, 1991), the toxicity of butylcyclohexane to mussel ciliary feeding activity over a 96 h exposure period was also investigated herein (Experiment VI).

4.2.1 Test materials

4-propyloctane (4-PO) was synthesised, purified and characterised as described in Section 3.2. Purity, as determined by GC, was greater than 99 %. Butylcyclohexane (BCH) was obtained from Aldrich. Purity, as determined by GC, was greater than 99 %.

4.2.2 Preparation of toxicant solutions

Toxicant solutions were prepared using seawater which had been filtered to exclude particles greater than 45 μm diameter. Test compounds were dissolved in a minimal volume of a carrier solvent (acetone), to aid their dispersion in seawater. Acetone has been shown to have no detectable effect upon mussel feeding rate at concentrations less

than 0.005 % v/v (Donkin *et al.*, 1989). Toxicant solutions were mixed for at least two hours prior to use in order to ensure thorough mixing of toxicant and seawater. 'Control' solutions were prepared by adding acetone to filtered seawater in the same manner.

4.2.3 Collection and maintenance of mussels

Mussels (*Mytilus edulis*) of between 35 mm and 40 mm shell length were collected from the intertidal zone at Exmouth, Devon. The animals were cleaned of epibionts and held in open flow tanks in recirculating seawater. The animals were allowed seven days to acclimatise to laboratory conditions prior to use in experiments. Full details are given in Section 6.5.3.

4.2.4 Exposure of mussels

In general, the procedure employed by Donkin *et al.* (1991) was followed. Modifications applied to individual experiments are described in the following sections. Mussels (shell length 40 mm ± 2 mm), were exposed to 20 l toxicant solution in a glass round bottom vessel. A control exposure vessel containing mussels exposed to 20 l of the control solution (seawater + 0.001% v/v acetone) was assembled in parallel with each 'toxicant' vessel. Mussels were fed continuously with an algal culture (*Isochrysis galbana*) by means of a peristaltic pump. Gentle water movement was maintained with a Teflon stirrer, contained within a glass dish to prevent contact with the animals.

4.2.5 Measurement of feeding rate

Mussel feeding rates were determined by measuring the rate at which algal cells were cleared from suspension in a static test system (*cf.* Coughlan, 1969). The procedure employed by Donkin *et al.* (1991) was followed. This involved transfer, following the

required exposure period, of 16 mussels into individual glass beakers, each containing 2 l of toxicant solution. Gentle water movement was maintained using a magnetic stirrer and mussels were placed such that the inhalant siphon was facing directly into the current. An additional beaker plus stirrer containing only the test solution (no mussel) was used as a control. A 30 minute acclimatisation period was allowed for the mussels to open their shells and resume feeding. A pre-determined volume of algal culture was then added to each beaker to give a concentration of 12000 - 14000 cells ml^{-1}. After a 5 minute mixing period, a 20 ml aliquot was removed from each beaker and cell numbers were counted in triplicate using a model D Coulter counter set to measure particles greater than 3 μm diameter. Further 20 ml aliquots were sampled every twenty minutes for a total period of 80 minutes. Clearance rates were calculated using the maximum decline in cell concentration over a 40 minute period (Equation 4.1). Feeding rates are expressed in litres per hour, ($l\ h^{-1}$).

$$feeding\ rate\ (l\ h^{-1}) = (\frac{v * 60}{t}) * (lnM_1 - lnM_2)$$

where; v = volume of water in beaker

t = time period of measurement

M_1 = cell count at t_0 (mean of triplicate measurements)

M_2 = cell count at t_1 (mean of triplicate measurements)

Equation 4.1 Calculation of mussel feeding rate

4.2.6 Chemical analysis of mussel tissue

Soft tissue was dissected from the mussel shells and stored at -17°C in solvent-rinsed glass jars prior to analysis. Tissues were extracted by steam distillation using the method of Donkin and Evans (1984). The internal standards 4-methylnonane and *n*-undecane

were added to the mussel homogenate immediately prior to extraction. Tissue extracts were analysed by GC. Recoveries of 4-PO and BCH were 94.1 ± 0.5% and 93.2 ± 0.5%, respectively (mean ± rsd, n=3) as determined by spike experiments.

4.2.7 Initial studies into the effect of 4-PO upon mussel feeding rate. A dose-response experiment

Experiment I: 24 h concentration-response experiment

A preliminary experiment was conducted to investigate whether a reduction in feeding rate occurred upon exposure of mussels to a solution of 4-PO. The method of Donkin *et al.* (1991) was followed. Groups of 16 mussels were exposed to a range of concentrations of 4-PO (nominal aqueous exposure concentrations 8 μg l^{-1}, 14 μg l^{-1}, 28 μg l^{-1} and 60 μg l^{-1}) for a 24 h exposure period as described in Section 4.2.4. Feeding rate measurements were conducted at the end of the 24 h exposure period (*cf.* Section 4.2.5). A control vessel was set up in parallel with each exposure concentration.

Experiment II: 72 h concentration-response experiment

A second 'preliminary' experiment was conducted by extending the time period for which mussels were exposed to 4-PO from 24 h to 72 h at aqueous concentrations of 14μg l^{-1} and 28 μg l^{-1}. Animals were returned to the exposure vessels after completion of the 24 h feeding rate measurements and the exposure time extended to 72 h. Toxicant solutions were changed every 24 h to maintain the toxicant concentrations and reduce the build up of excretory products in the exposure vessels. Mussel feeding rates were then determined after 72 h exposure.

4.2.8 Investigations into the effect of 4-PO upon mussel feeding rate over a 96 h exposure period. Relationship to bioaccumulation

Experiment III: 96 h exposure

To examine the effect of 4-PO upon mussel feeding rate over a 96 h exposure period, mussels were exposed to a solution of 4-PO (nominal aqueous exposure concentration; 23 μg l^{-1}). Mussel feeding rates and tissue concentrations of toxicant were measured every 24 h. The gill tissue was dissected out separately from the remaining soft body tissue and the concentration of 4-PO in the gill tissues, (the presumed site of toxic action for feeding rate reduction) and the remaining body tissues measured separately.

In order to measure both feeding rate and the body burden of 4-PO every 24 h, a number of modifications to the method used previously for experiments I and II were required. First, the total number of animals required for the experiment was increased from 16 to 32, to provide enough animals to allow for removal of four for tissue analysis each day, whilst still providing sufficient animals to allow accurate determination of feeding rate (16 animals required).

Mussels were exposed to a seawater solution of 4-PO (23 μg l^{-1}) in the manner described in Section 4.2.4. After 24 h, sixteen animals were removed and placed individually in beakers containing 2 l toxicant solution and their feeding rates measured in the usual manner (*cf.* Section 4.2.5). In order to maintain constancy in dosing, the animals not used for feeding rate measurements were also dosed with fresh toxicant solution during the feeding rate determination period, at a concentration equal to that received by the animals used for feeding rate measurements.

After the feeding rate measurements were completed, four animals were removed for tissue analysis and the remaining twelve mussels returned to the exposure vessel. These animals were dosed with fresh toxicant solution for a further 24 h and the procedure then repeated. A control vessel containing 32 animals was assembled and measurements

made in parallel with the toxicant vessel. For logistical reasons, the feeding rate of the control animals was only measured at the start of the exposure and subsequently every 48 h.

Experiment IV: 96 h exposure (replicate experiment)

The entire experiment was repeated to investigate if the trend in mussel feeding rate observed was a 'typical' response.

4.2.9 The effect of 4-PO on mussels at both the physiological and cellular level over 120 h exposure period: Experiment V

In addition to investigating the effect of 4-PO upon mussel feeding rate at the physiological level, an experiment was conducted in which the effect of 4-PO upon *M. edulis* was studied both at the physiological level and at the sub-cellular level, using a Neutral Red Retention assay (Lowe *et al.*, 1995). The latter was carried out in collaboration with D. Lowe, Plymouth Marine Laboratory.

Mussels were exposed to 4-PO (45 $\mu g\ l^{-1}$) over a 120 h exposure period. The experimental procedure was as for the previous experiments (III and IV), using 32 mussels in each exposure vessel. Every 24 h, sixteen animals were removed for feeding rate measurements. Once completed, four animals were retained and the remaining 12 animals returned to the exposure vessel. Blood samples were taken from each of the four animals for Neutral Red retention time measurements and the soft tissue dissected from the shells for tissue analysis. Gill tissue was dissected out separately from the remaining tissue.

4.2.10 The effect of butylcyclohexane upon mussel feeding rate over a 96 h exposure period: Experiment VI

To enable a comparison of the results obtained in the present study with those of Donkin *et al.* (1989,1991). Butylcyclohexane (BCH; 90μg l^{-1}), a compound used to establish the original QSAR describing the effect of hydrocarbons upon mussel feeding rate, was studied over a 96 h exposure period.

An identical test procedure to that described for previous experiments (III and IV) was employed whereby 32 mussels were exposed to a seawater solution of BCH (90 μg l^{-1}). Every 24 h, mussel feeding rate was measured using sixteen animals. Four animals were removed for tissue analysis and the remaining animals returned to the exposure vessel. A control vessel was set up in parallel with the exposure vessel. The concentration of BCH in the gill tissue was determined separately to the remaining soft body tissue.

4.3 Results

4.3.1 Experiment I: 24 h concentration-response experiments

The aim of Experiment I was to investigate if exposure of *M. edulis* to 4-PO resulted in a measurable reduction in mussel ciliary feeding activity. The effect of a range of concentrations of 4-PO upon mussel feeding rate was investigated (*cf.* Section 4.2.7). The results obtained from Experiment I are summarised in Table 4.1. Feeding rates for both the 'control' and 'toxicant' animals are expressed as the mean ± standard deviation (n=16). Feeding rate is also expressed as a percentage of the control value (using a 'pooled' value of all the control feeding rates). Expressing results in this manner effectively eliminates the influence of other environmental variables, and the results describe a reduction in feeding rate which is attributable to exposure to the test compound. A concentration (nominal aqueous exposure)-response curve for the effect of 4-PO upon mussel feeding rate over 24 h exposure is presented in Figure 4.2. The data in Table 4.1 and Figure 4.2 show that exposure to 4-PO resulted in a greater than 50 % reduction in mussel feeding rate at concentrations of 28 $\mu g\ l^{-1}$ and above. The present study has therefore demonstrated that the branched C_{11} compound, 4-PO is toxic to mussel ciliary feeding activity.

Nominal water conc. of 4-PO ($\mu g\ l^{-1}$)	**Feeding rate of control animals ($l\ h^{-1}$)** *mean ± sd (n=16)*	**Feeding rate of exposed animals ($l\ h^{-1}$)** *mean ± sd (n=16)*	**Mean feeding rate as a % of pooled control value**
8	2.81 ± 0.3	2.48 ± 0.5	89
14	3.02 ± 0.8	1.80 ± 0.6	65
28	2.47 ± 0.4	1.06 ± 0.4	38
60	2.81 ± 0.3	0.95 ± 0.4	34
pooled control	2.77 ± 0.6		

Table 4.1 The effect of 4-PO upon mussel feeding rate, 24 h exposure (Expt. I)

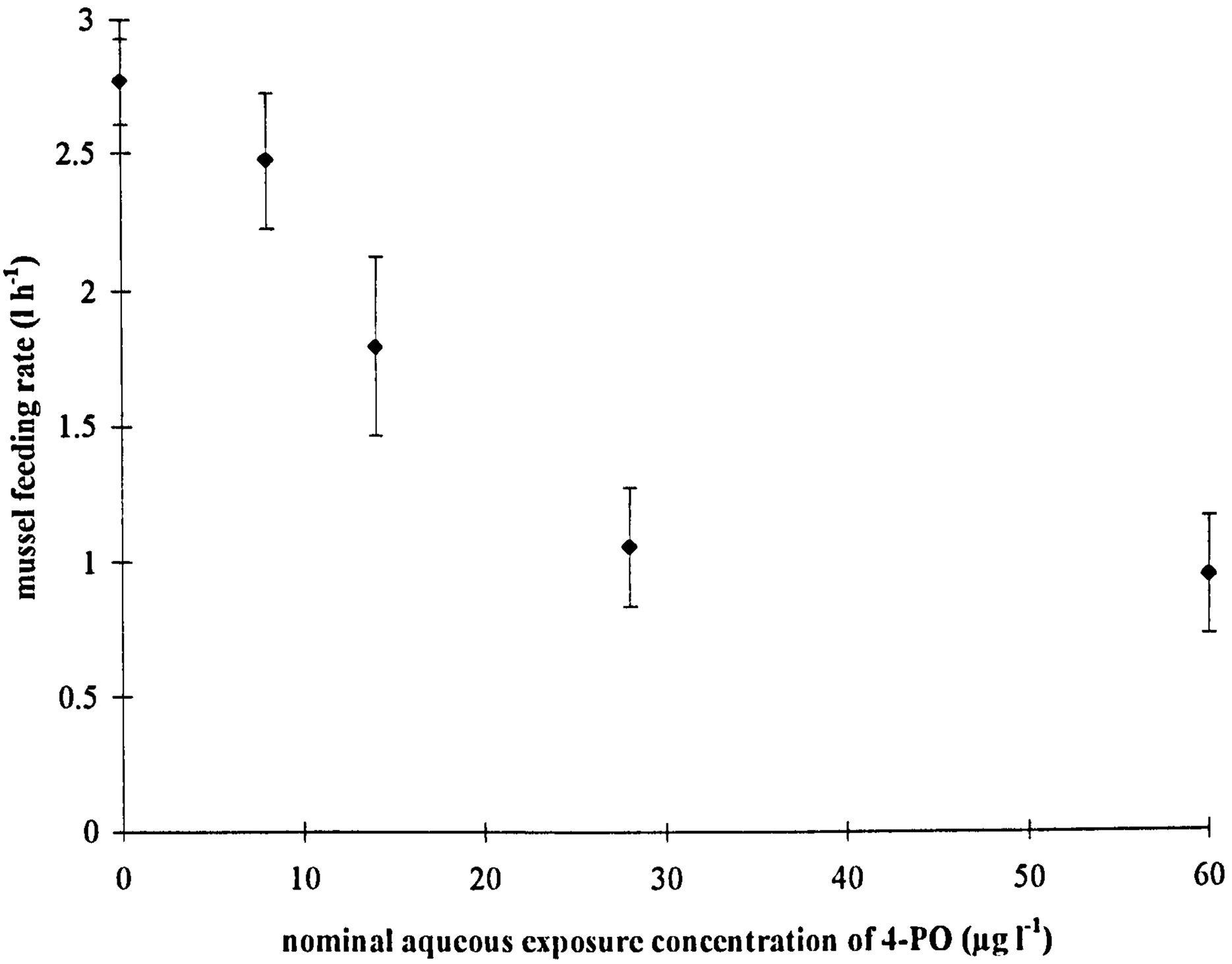


Figure 4.2 Concentration-response curve for the effect of 4-PO upon mussel feeding rate (24 h exposure, Experiment I) *mean feeding rate ± standard deviation (n=16).*

Control values pooled, n=48

4.3.2 Experiment II: 72 h concentration-response experiment

To investigate the influence of increased exposure time to 4-PO a second preliminary experiment was conducted in which the exposure time was extended from 24 h to 72 h. Narcotic effects have been demonstrated to occur at relatively constant body burdens of toxicant (*e.g.* Donkin and Widdows, 1986; McCarty, 1986; Van Hoogen and Opperhuizen, 1988; Donkin *et al.*, 1989, 1991; *cf.* Section 1.5). Variations in aqueous based expressions of toxicity are generally attributed to differences in the kinetics of uptake of the chemicals tested. In other words, prior to the attainment of a steady-state, the toxicity of slower accumulating chemicals increases with increasing exposure time

(Donkin *et al.*, 1989). The exposure time was thus increased from 24 h to 72 h, in order to allow greater accumulation of toxicant into the organisms which, it was hypothesised, would result in a larger reduction in mussel feeding rate. The results of experiment II are presented in Table 4.2 and are also illustrated in Figure 4.3. For comparison, mussel feeding rates after both 24 h and 72 h exposure to 4-PO are plotted in Figure 4.3. Interestingly, as illustrated in Figure 4.3, no further decrease in feeding rate was found after 72 h exposures, despite continued exposure to the toxicant. In fact, mussels exposed to both the 14 $\mu g\ l^{-1}$ and 28 $\mu g\ l^{-1}$ toxicant solutions showed an increase (Students t-test, P=0.05) in feeding rates after 72 h compared with 24 h exposures (Table 4.2). All raw data and summary statistics are included in Appendix E.1.

There are a number of possible explanations for the observed results. First, despite increased exposure time the animals exposed for 72 h may not have bioaccumulated any more toxicant than those exposed for 24 h. This could arise because either a steady-state had been reached between the concentration of toxicant in the organism and surrounding water, or the mussels may not have been actively filtering seawater during the 'extended' exposure period (mussels are known to close their shells and stop feeding in particularly contaminated waters). Since the same animals were used to measure the feeding rate after both 24 h and 72 h exposure measurement of the total body burden of toxicant at 24 h was precluded. Consequently, the attainment of a steady state of toxicant in the mussels could not be investigated. However, it is unlikely that equilibrium has been achieved after 24 h, as 4-PO is not anticipated to be sufficiently soluble for this (*cf.* Hawker and Connell, 1986). The valves of the animals were open and the animals were presumed to be actively filtering each day. A further possibility was that the animals became desensitised to the effects of the toxicant with increasing exposure time. In order to examine this phenomenon further, the influence of exposure time upon the toxicity of 4-PO to mussel feeding rate was investigated (Experiments III - V).

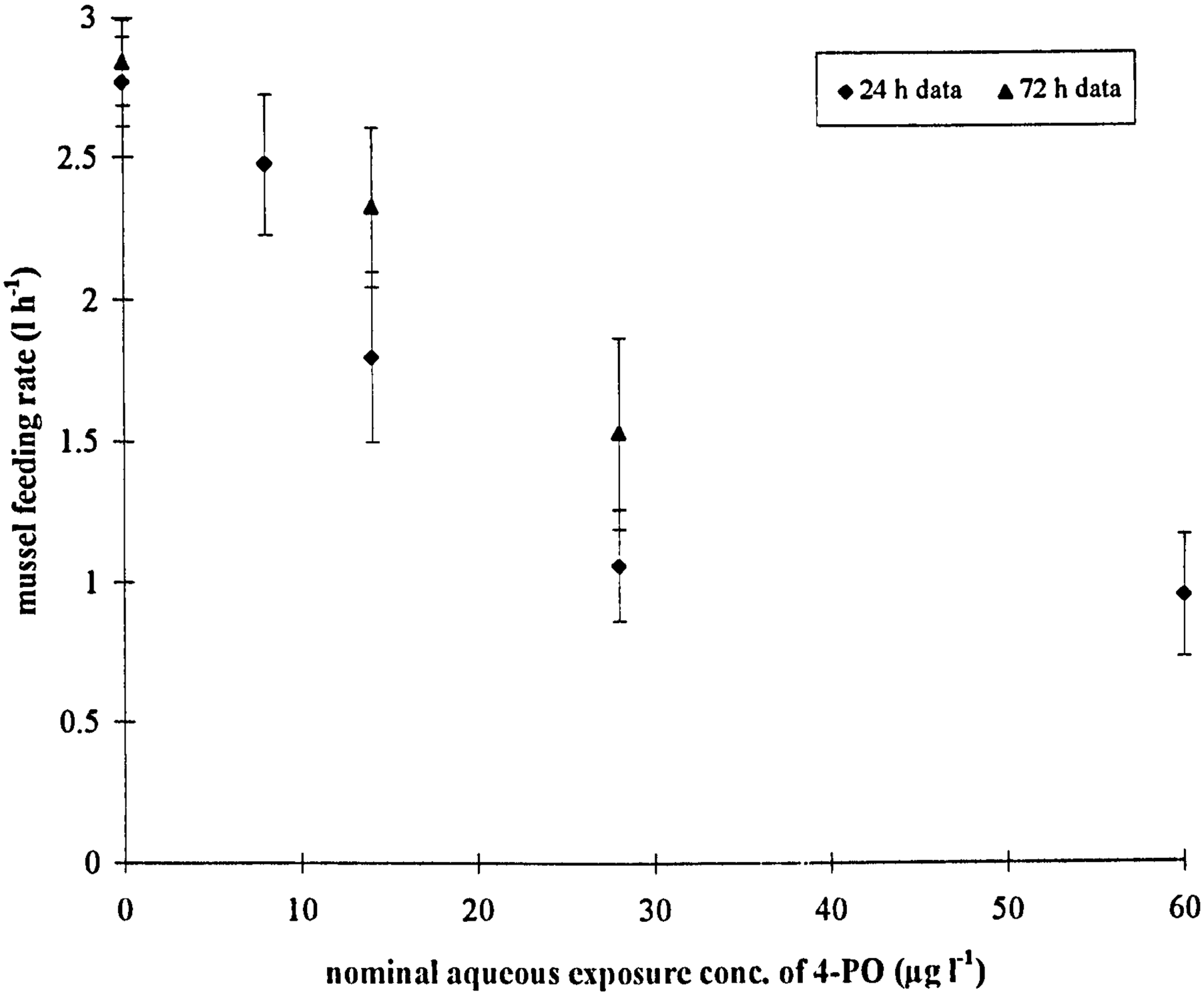


Figure 4.3 Concentration-response curve for the effect of 4-PO upon mussel feeding rate (24 h and 72 h exposure, Experiments I and II) *values plotted as mean ± s.d. (n=16)*

Nominal water conc. of 4-PO ($\mu g\ l^{-1}$)	Feeding rate of control animals ($l\ h^{-1}$) *mean ± sd (n=16)*	Feeding rate of exposed animals ($l\ h^{-1}$) *mean ± sd (n=16)*	Mean feeding rate as a % of pooled control value
14	2.83 ± 0.4	2.33 ± 0.6	82
28	2.85 ± 0.5	1.53 ± 0.7	54
pooled control	2.84 ± 0.4		

Table 4.2 The effect of 4-PO upon mussel feeding rate (72 h exposure, Experiment II)

4.3.3 Experiment III - VI

To examine the relationship between body burden of toxicant and mussel feeding rate, a series of experiments were conducted to investigate the effect of 4-PO upon mussel feeding rate over 96 h - 120 h exposure periods. The measured feeding rates for both the control and toxicant animals from each experiment are summarised in Tables 4.3 - 4.5. The variation in mussel feeding rates with time is illustrated in Figures 4.4. Values are presented as mean ± 95 % confidence intervals (n=16). All raw data and summary statistics are presented in Appendices E.2 - E.4. For logistical reasons, the feeding rate of the control animals was only measured at the start of the exposure and subsequently every 48 h. Analysis of the feeding rates of the control animals by ANOVA (P=0.05) indicated no significant differences between the values for the entire exposure period for each experiment. Consequently, for each individual experiment the control values have been pooled to provide a single value for the feeding rate. This value is used when feeding rates are expressed as a percentage of the control.

Exposure time (h)	Feeding rate control animals ($l\ h^{-1}$)	Feeding rate exposed animals ($l\ h^{-1}$)	Feeding rate expressed as a % of pooled control
0	2.59 ± 0.2	2.59 ± 0.2	100
24		2.28± 0.2	88
48	2.75 ± 0.2	1.79 ± 0.2	69
72		1.93 ± 0.2	74
96	2.42 ± 0.2	1.90 ± 0.2	73
'pooled' control	2.59 ± 0.1		

values given as mean ± 95 % confidence intervals (n=16)

Table 4.3 Feeding rate of mussels (*M. edulis*) exposed to 4-PO (23 µg l^{-1}) over a 96h exposure period. Experiment III (April 1994)

Exposure time (h)	Feeding rate control animals ($l\ h^{-1}$)	Feeding rate of exposed animals ($l\ h^{-1}$)	Feeding rate expressed as % of pooled control
0	2.53 ± 0.3	2.53 ± 0.3	100
24		2.17 ± 0.2	85
48	2.57 ± 0.2	2.20 ± 0.2	86
72		2.59 ± 0.2	102
96	2.54 ± 0.1	2.46 ± 0.3	96
'pooled' control	2.55 ± 0.1		

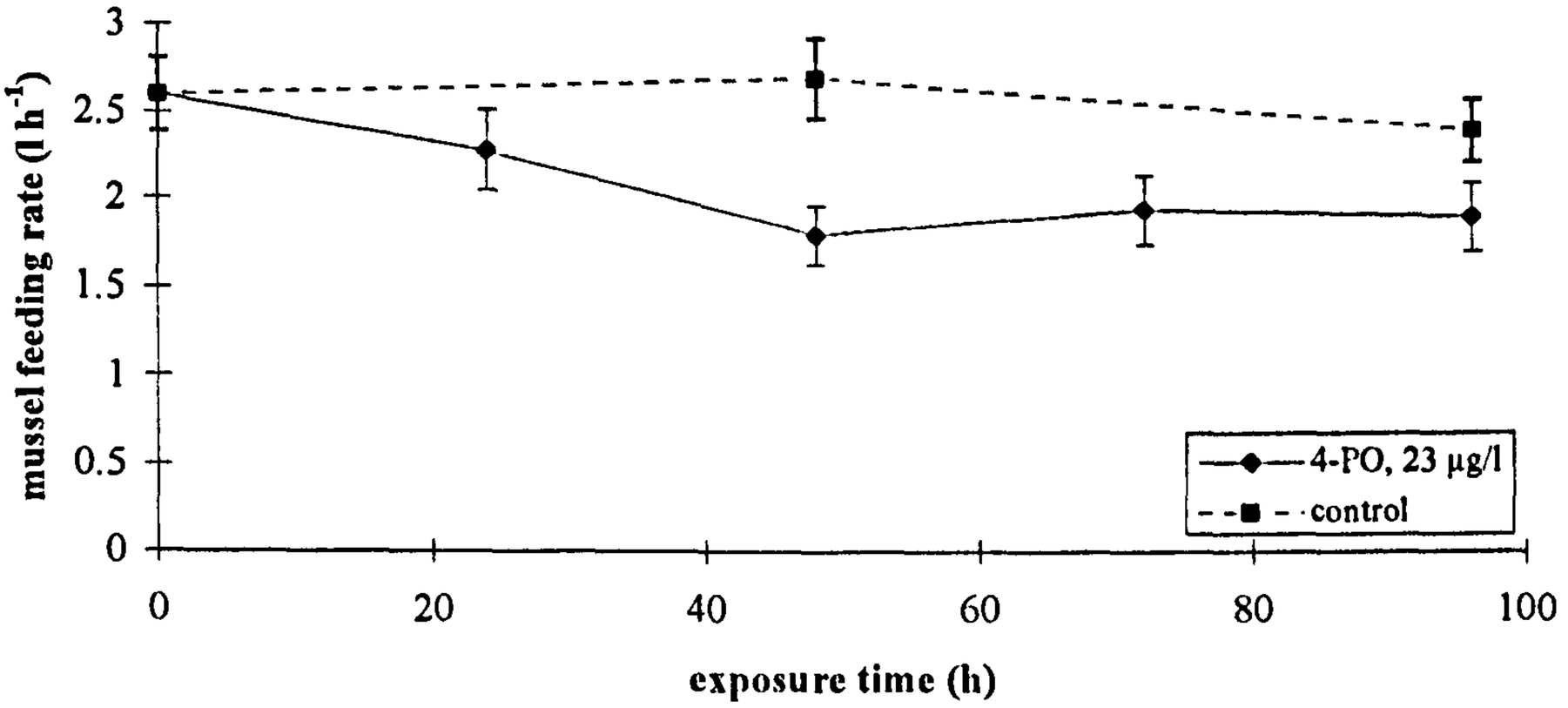
values given as mean ± 95 % confidence intervals (n=16)

Table 4.4 Feeding rate of mussels *(M. edulis)* exposed to 4-PO (23 µg l^{-1}) over a 96h exposure period. Experiment IV (November 1994)

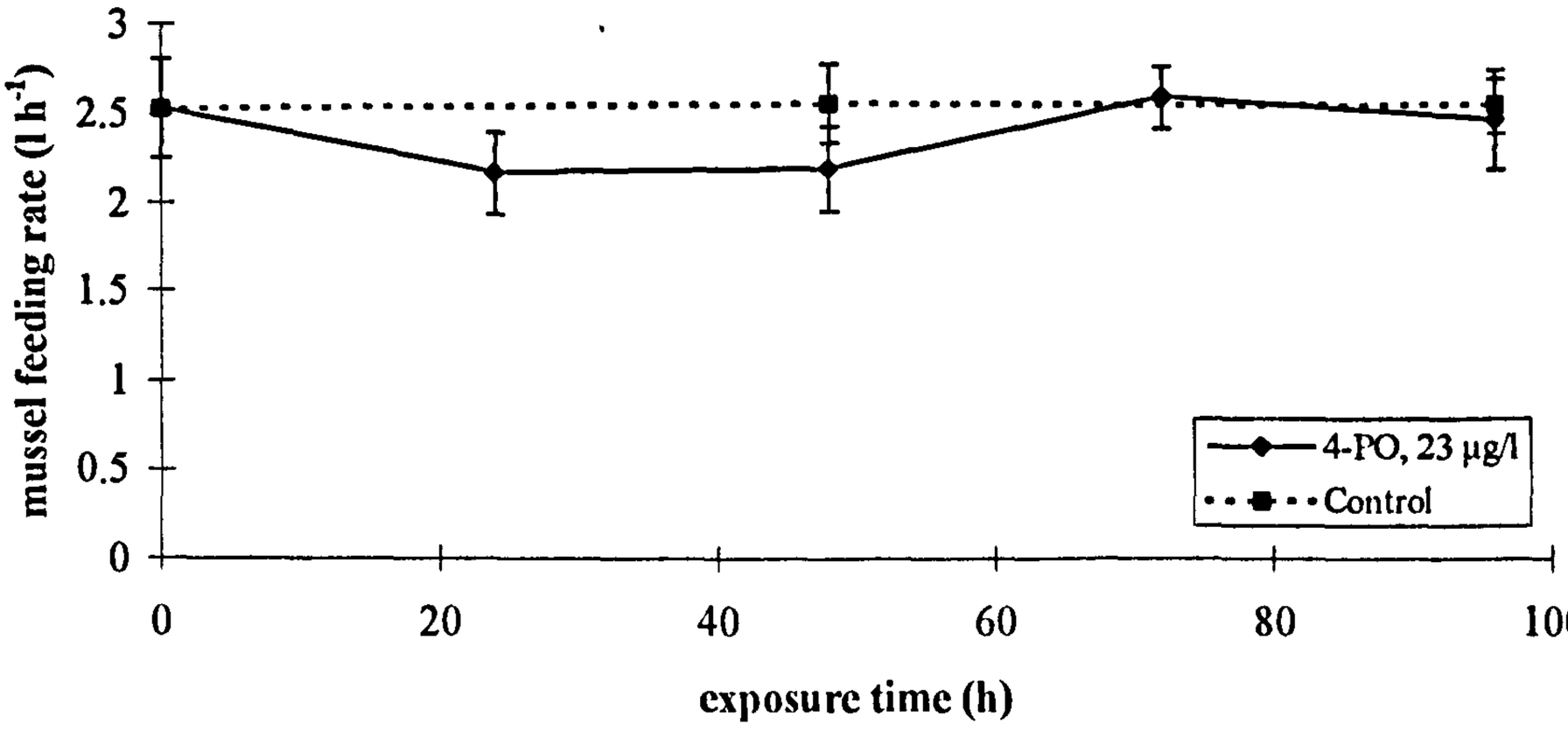
Exposure time (h)	Feeding rate control animals ($l\ h^{-1}$)	Feeding rate exposed animals ($l\ h^{-1}$)	Feeding rate expressed as a % of pooled control
0	2.56 ± 0.2	2.56 ± 0.2	100
24		1.61 ± 0.3	58
48	2.82 ± 0.2	1.70 ± 0.3	61
72		1.33 ± 0.3	48
96	2.90 ± 0.2	1.83 ± 0.4	66
120	2.83 ± 0.2	1.68 ± 0.4	61
'pooled' control	2.77 ± 0.1		

values given as mean ± 95 % confidence intervals (n=16, except 120h where n=12)

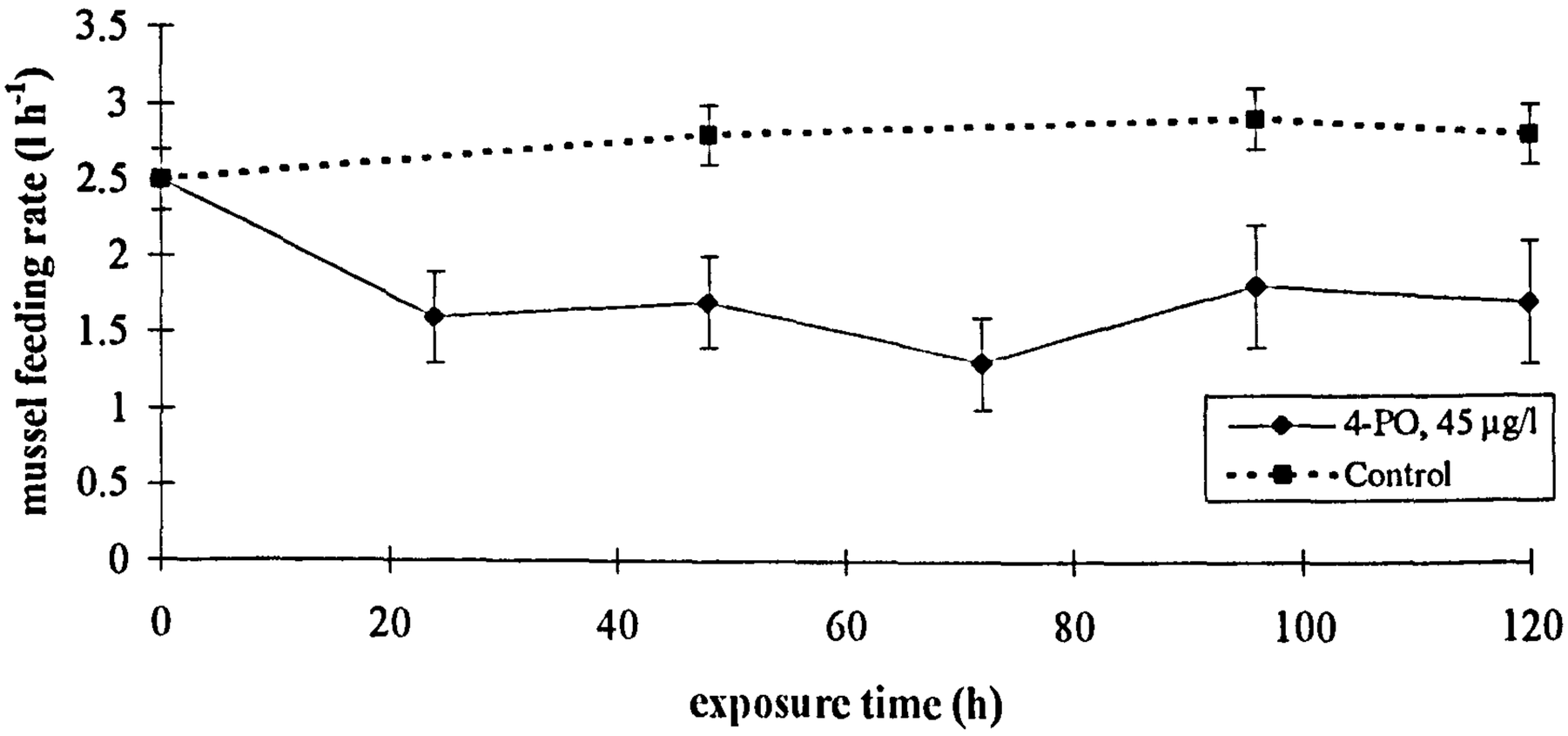
Table 4.5 Feeding rate of mussels (*M. edulis*) exposed to 4-PO (45 µg l^{-1}) over a 120h exposure period. Experiment V (July 1994)



(a) Experiment III (April 1994). 23 µg l^{-1}; 96 h exposure



(b) Experiment IV (November 1994). 23 µg l^{-1}; 96 h exposure



(c) Experiment V (July 1994). 45 µg l^{-1}; 120 h exposure

Figure 4.4 The variation in mussel feeding rate upon exposure to 4-PO over 96 h - 120 h *values expressed as mean ± 95 % confidence intervals*

Comparison (ANOVA, P=0.05) of the feeding rates of mussels in Experiment III exposed to 4-PO for the exposure period 24h - 96h indicated a statistically significant difference between the feeding rates. After 24h exposure to 4-PO there was no significant difference between the exposed and control mussels feeding rates (t-test, P=0.05). However, after 48h, mussels exposed to 4-PO have a significantly lower feeding rate than the control animals (t-test, P=0.05). Analysis of the feeding rates of the exposed animals for 48h, 72h and 96h exposure reveals no significant difference (ANOVA, P=0.05). These results suggest that the overall trend is a reduction in mussel feeding rate from 0h to 48h, after which there is no further decrease and the feeding rate remains relatively constant for the remainder of the exposure period (48h - 96h).

Analysis of the feeding rates of the exposed animals in Experiment IV showed no significant difference between mussel feeding rates over the entire exposure period from 0 h to 96 h (ANOVA, P=0.05). The lack of toxic response in Experiment IV was surprising, as a significant reduction in mussel feeding rate was observed in Experiment III after 48 h. Both experiments used identical exposure conditions.

In experiment V, mussels were dosed with a higher concentration of toxicant (*ca* x 2). A significant reduction in mussel feeding rate was observed after 24h in the exposed animals (t-test, P=0.05). There is no significant difference between the feeding rates of mussels exposed to 4-PO between 24h - 120h exposure. The overall trend in mussel feeding rate in Experiment V is therefore similar to that observed in Experiment III. In both experiments the feeding rate of mussels exposed to 4-PO has decreased initially until a point is reached at which no further reduction in mussel feeding rate is observed. The feeding rate then remains effectively constant for the remainder of the exposure period. Feeding rate has reached a steady rate in Experiment V more rapidly than in experiment III (24h *vs* 48h), presumably owing to the higher aqueous exposure concentration of toxicant employed in Experiment V.

To enable a comparison of the results obtained in the present study with those of Donkin *et al.* (1989, 1991; illustrated in Figure 1.13), a compound used to establish the original QSAR for the effect of hydrocarbons upon feeding rate was studied over a 96 h exposure period. Butylcyclohexane (BCH; 90 $\mu g\ l^{-1}$) was chosen as the test compound (Experiment VI). The feeding rates of both those mussels exposed to BCH and the control mussels are summarised in Table 4.6 and illustrated in Figure 4.5. After 24h, there is a significant reduction in the feeding rate of the animals exposed to BCH (t-test, P=0.05). From 24h to 96h there is no significant difference between the feeding rates of the exposed animals (ANOVA, P=0.05). Thus, a similar trend in feeding rate reduction is observed in the animals exposed to BCH as observed in mussels upon exposure to the 4-PO. This implies that the trend observed in mussel feeding rate over time in the present study is a common response for mussels upon exposure to all non-specific narcotic toxicants.

Exposure time (h)	**Feeding rate control animals ($l\ h^{-1}$)**	**Feeding rate exposed animals ($l\ h^{-1}$)**	**Feeding rate expressed as a % of pooled control**
0	2.96 ± 0.3	2.96 ± 0.3	100
24		1.11 ± 0.2	38
48	2.93 ± 0.3	1.38 ± 0.3	47
72		1.20 ± 0.2	41
96	2.82 ± 0.3	1.11 ± 0.3	38
'pooled' control	2.92 ± 0.2		

values given as mean ± 95 % confidence intervals (n=16)

Table 4.6 Feeding rate of mussels (*M. edulis*) exposed to butylcyclohexane (90 $\mu g\ l^{-1}$) over a 96 h exposure period (Experiment VI)

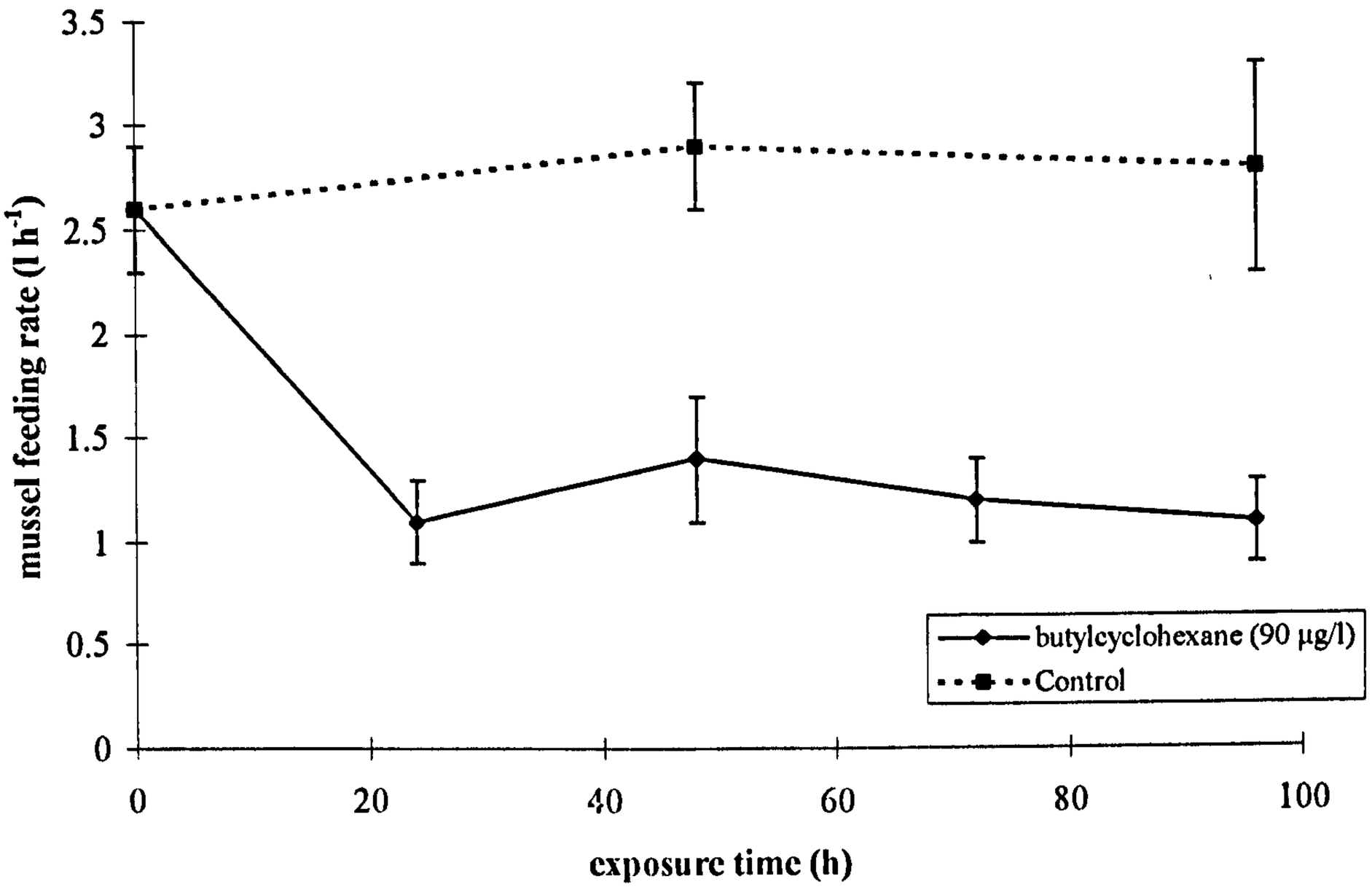


Figure 4.5 The variation in mussel feeding rate upon exposure to butylcyclohexane over a 96 h exposure period. Experiment VI

The concentrations of 4-PO measured in both the gill tissue and the total body burden of 4-PO over the duration of experiments III-V are summarised in Tables 4.7 - 4.9. Concentrations are expressed both in mg kg^{-1} and mmol kg^{-1}. Expressing the body burden of toxicant in terms of the number of moles of toxicant allows an understanding of the mechanism of toxicity at a molecular level. However, the objective of this work is ultimately to interpret the toxicological significance of a complex mixture of contaminants bioaccumulated by marine organisms, and a mass-based approach is therefore a necessity (*cf.* Donkin *et al.*, 1991). This data is represented graphically in Figures 4.6 and 4.7.

Exposure time h	**Conc. of 4-PO in gill tissue**		**Total body burden of 4-PO**		**Feeding rate as % of control**
	mg kg^{-1}	**mmol kg^{-1}**	**mg kg^{-1}**	**mmol kg**	
0	0	0	0	0	100
24	13.5	0.086	4.0	0.026	88
48	19.5	0.125	8.5	0.054	69
72	23.1	0.148	13.2	0.085	74
96	22.1	0.141	15.2	0.097	73

Table 4.7 Summary of tissue concentrations of 4-PO bioaccumulated by mussels (*M. edulis*) following 96 h exposure to 23 µg l^{-1} 4-PO. Experiment III (April 1994)

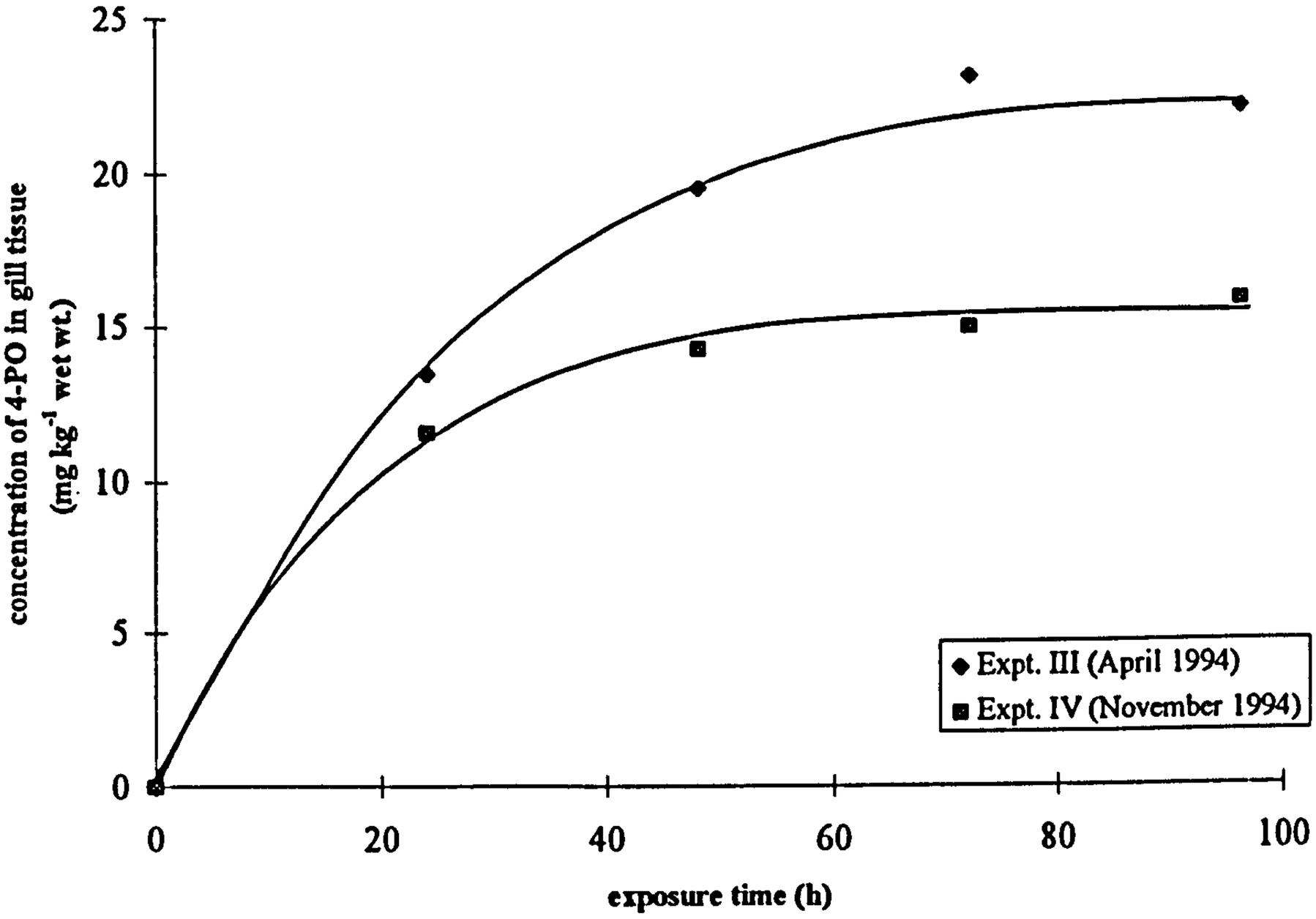
Exposure time (h)	Conc. of 4-PO in gill tissue		Total body burden of 4-PO		Feeding rate as % of control
	mg kg^{-1}	mmol kg^{-1}	mg kg^{-1}	mmol kg^{-1}	
0	0	0	0	0	100
24	11.6	0.074	1.9	0.012	85
48	14.3	0.092	4.3	0.027	86
72	15.0	0.096	5.3	0.034	102
96	15.9	0.102	7.0	0.045	96

Table 4.8 Summary of tissue concentrations of 4-PO bioaccumulated by mussels (*M. edulis*) following 96 h exposure to 23 µg l^{-1} 4-PO. Experiment IV (November 1994)

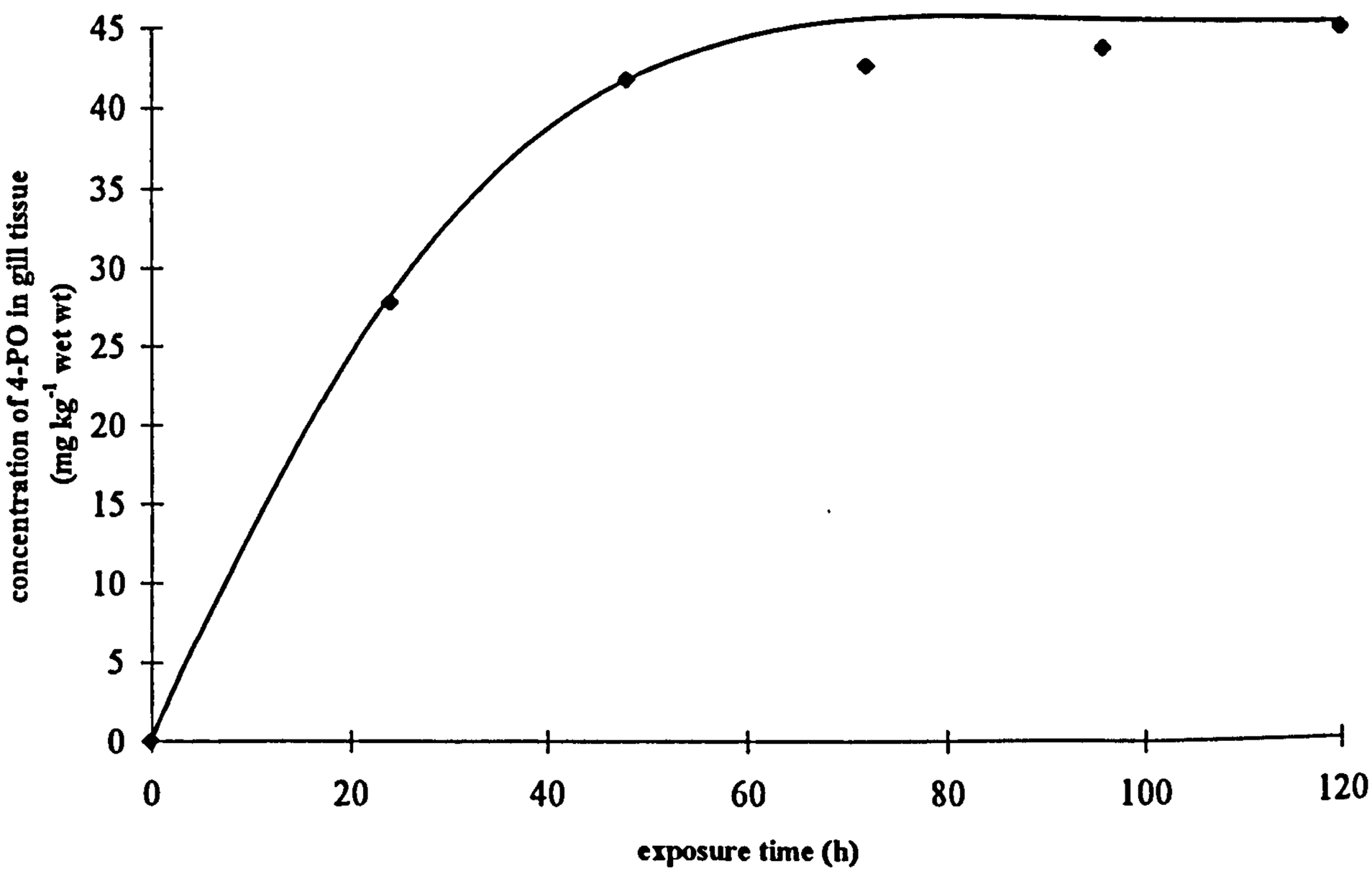
Exposure time (h)	Conc. Of 4-PO in gill tissue		Total body burden of 4-PO		Feeding rate as % of control
	mg kg^{-1}	mmol kg^{-1}	mg kg^{-1}	mmol kg^{-1}	
0	0	0	0	0	100
24	27.8 ± 0.8	0.178 ± 0.005	9.1 ± 0.2	0.058 ± 0.001	58
48	41.7 ± 1.7	0.267 ± 0.011	13.3 ± 0.6	0.085 ± 0.004	61
72	42.5 ± 2.6	0.272 ± 0.017	21.4 ± 0.9	0.137 ± 0.006	48
96	43.6 ± 2.6	0.279 ± 0.016	30.0 ± 0.5	0.192 ± 0.003	66
120	44.8 ± 1.5	0.287 ± 0.009	32.9 ± 0.4	0.211 ± 0.002	61

values as mean ± range (n=2)

Table 4.9 Summary of tissue concentrations of 4-PO bioaccumulated by mussels (*M. edulis*) following 120h exposure to 45 µg l^{-1} 4-PO. Experiment V (July 1994)

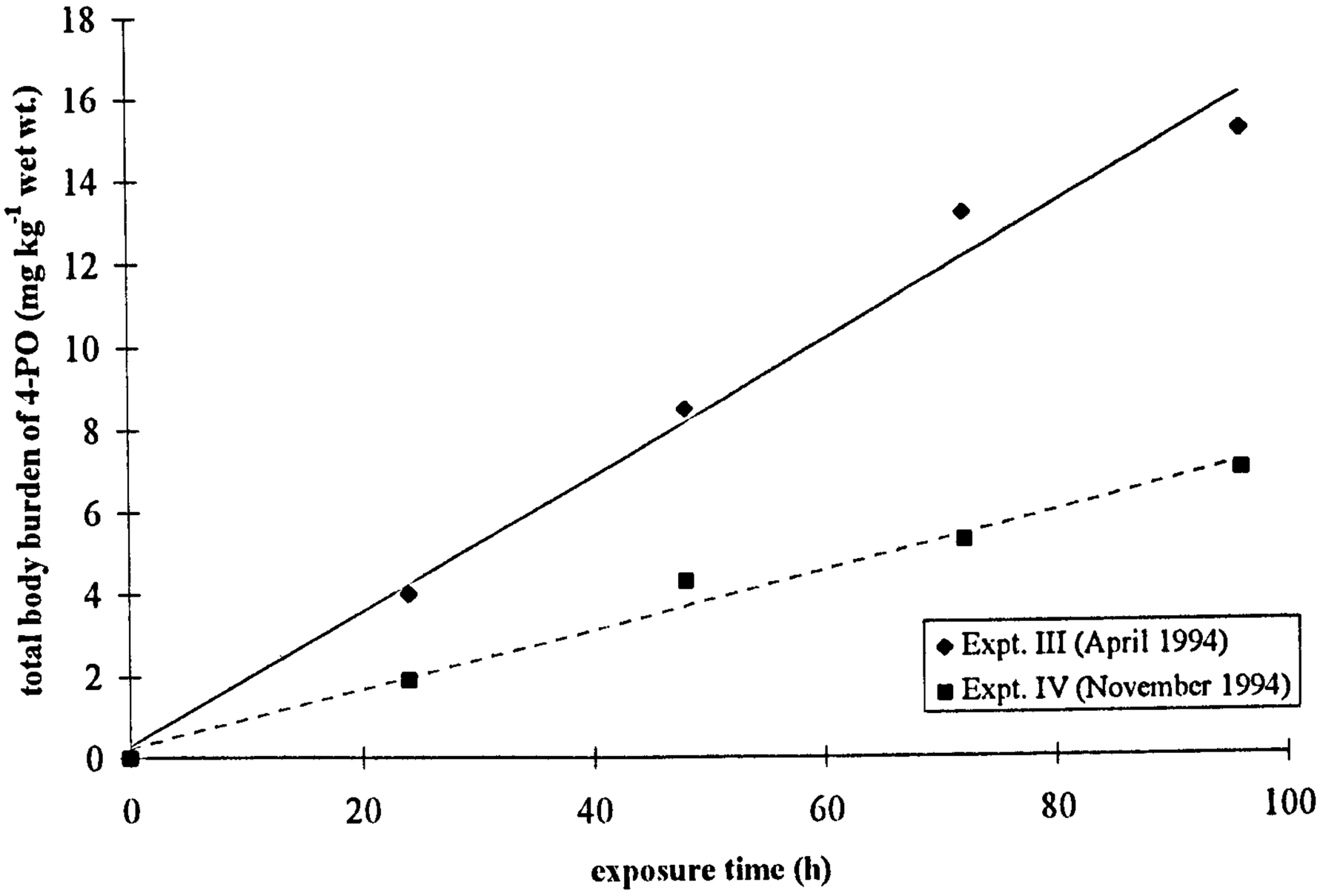


(a) Experiments III and IV. [23 µg l^{-1}; 96 h]

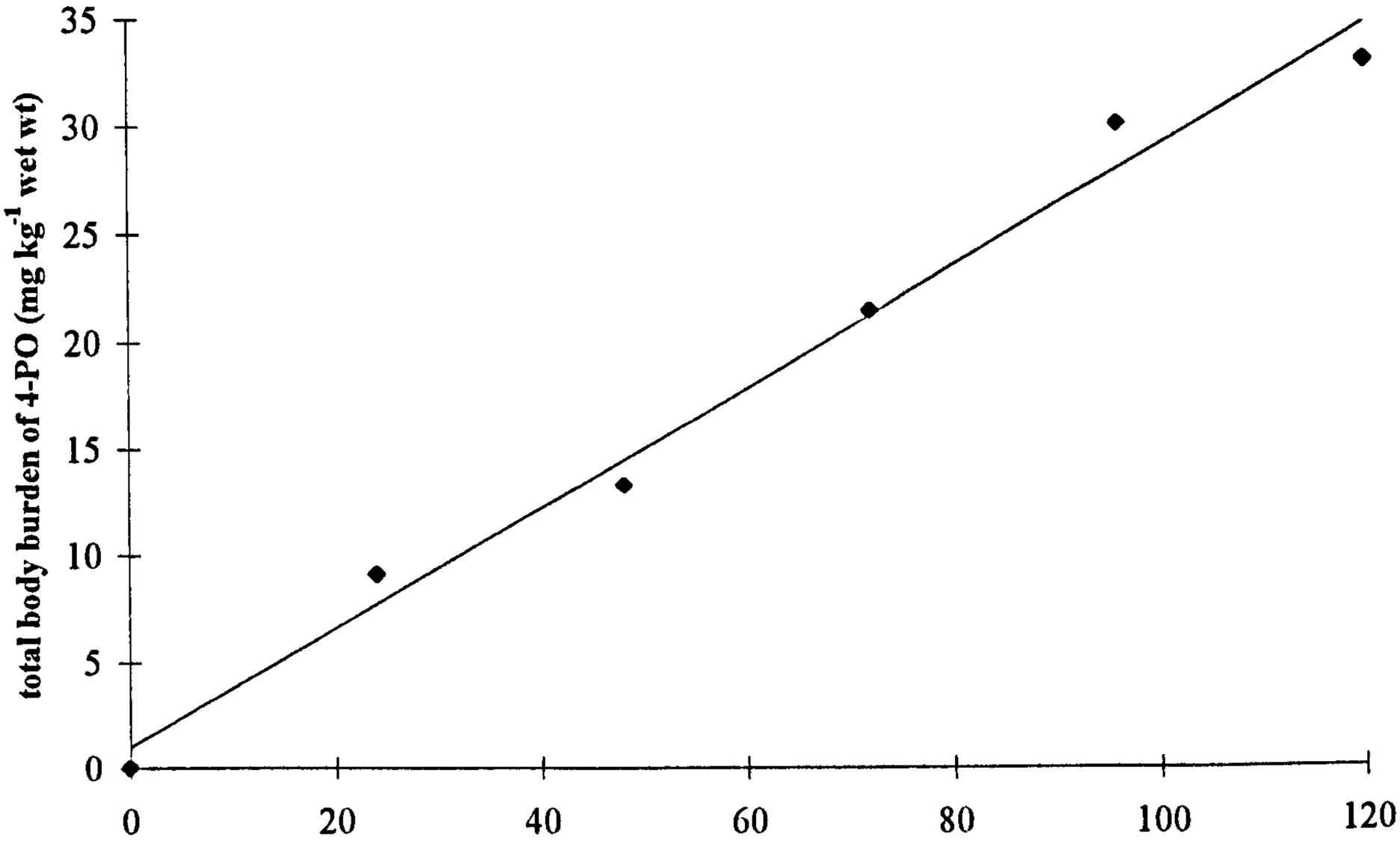


(b) Experiment V. [45 µg l^{-1}; 120 h]

Figure 4.6 Bioaccumulation of 4-PO into the gill tissue of the mussel (*M. edulis*)



(a) Experiment III and IV. [23 µg l^{-1}; 96 h]



(b) Experiment V. [45 µg l^{-1}; 120 h]

Figure 4.7 Bioaccumulation of 4-PO by the mussel (*M. edulis*)

values plotted as mean, (n=2)

As can be seen from Figures 4.6 and 4.7, a similar trend in bioaccumulation patterns is observed in each experiment. As Figure 4.6 illustrates, the concentration of 4-PO in the gills of *M. edulis* increases from 0h to approximately 40h (dependent upon experiment), after which time, the concentration of 4-PO remains relatively constant. This suggests that an equilibrium between the concentration of 4-PO in the gill tissue and surrounding seawater has been reached sometime after this point. In contrast, the total body burden of 4-PO (Figure 4.7) increases linearly for the entire exposure period, indicating equilibrium between the mussels as a whole and the surrounding seawater has not been attained in the 96h-120h exposure periods employed in the present study.

Comparing the body burden results for Experiments III and IV provides an explanation for the differences in observed feeding rates between the two experiments. Despite exposure to the same aqueous exposure concentration of 4-PO in both Experiments III and IV, as illustrated in Figure 4.6a, less toxicant was bioaccumulated into the mussel tissues in Experiment IV than Experiment III. Insufficient bioaccumulation of toxicant at the site of toxic action is the most probable explanation for the lack of a significant reduction in feeding rate of the exposed mussels in Experiment IV. Despite exposure to the same aqueous concentration of toxicant, the mussels in Experiment III have a higher tissue concentration of toxicant than those in Experiment IV. The reason why less toxicant has been bioaccumulated by the mussels in Experiment IV remains unknown. It is also interesting to note that the concentration of 4-PO in the gill tissue at steady state in the mussels from Experiment IV is lower (approximately 15 mg kg^{-1} or 0.09 mmol kg^{-1}) than the concentration of 4-PO in the gill tissue at steady state of mussels from experiment III (approximately 21 mg kg^{-1} or 0.14 mmol kg^{-1}). One difference between the two Experiments is that Experiment III was conducted in the spring (April) whilst Experiment IV was conducted in the autumn (November). A seasonal difference in the biochemical composition of the mussels in Experiments III and IV may account for the

observed differences in bioaccumulation of toxicant. Variability in measured lethal body burdens of toxicant within individual organisms in an experiment is commonly attributed to the variable lipid content of the individuals, as hydrophobic organic chemicals accumulate in the lipid phases of the organism. For example, van Wezel *et al.* (1995) examined intraspecies variation in lethal body burdens of narcotic compounds by exposing groups of Fathead Minnows (*Pimephales promelas*) to various concentrations of chlorobenzenes. The lethal body burdens reported ranged over approximately one order of magnitude. However, these authors found that about 50 % of this variability could be attributed to the variable lipid content of the individuals.

Zandee *et al.* (1980) investigated the seasonal variation in lipid composition of *Mytilus edulis* and reported that whilst there is a seasonal fluctuation in the lipid content of the whole organism, there was relatively little variation in the lipid level of the gill tissue throughout the entire year. Interestingly, the concentration of 4-PO in the gill tissue at steady state is different in each experiment (21 mg kg^{-1}, 15 mg kg^{-1} and 43 mg kg^{-1} for Experiments III, IV and V respectively). Experiment V was conducted in summer (July). Each experiment (III, IV and V) has therefore been conducted at a different time of the year. However, as discussed previously, the lipid content of the gill tissue remains relatively constant throughout the year (Zandee *et al.*, 1980). It seems unlikely therefore, that a slight fluctuation in lipid levels of the gill tissue could account for the variation in gill concentrations of 4-PO at steady state observed in the present studies. Unfortunately, as lipid determinations were not conducted in the present study, this hypothesis can neither be confirmed or excluded and may prove an interesting area of study for future work. The varying concentrations at which 4-PO reaches steady state in the gill tissue of the organism is more probably a function of the aqueous exposure concentration of toxicant, with higher aqueous exposure concentrations leading to higher bioconcentration. A concentration dependence of the bioconcentration factor (BCF) has

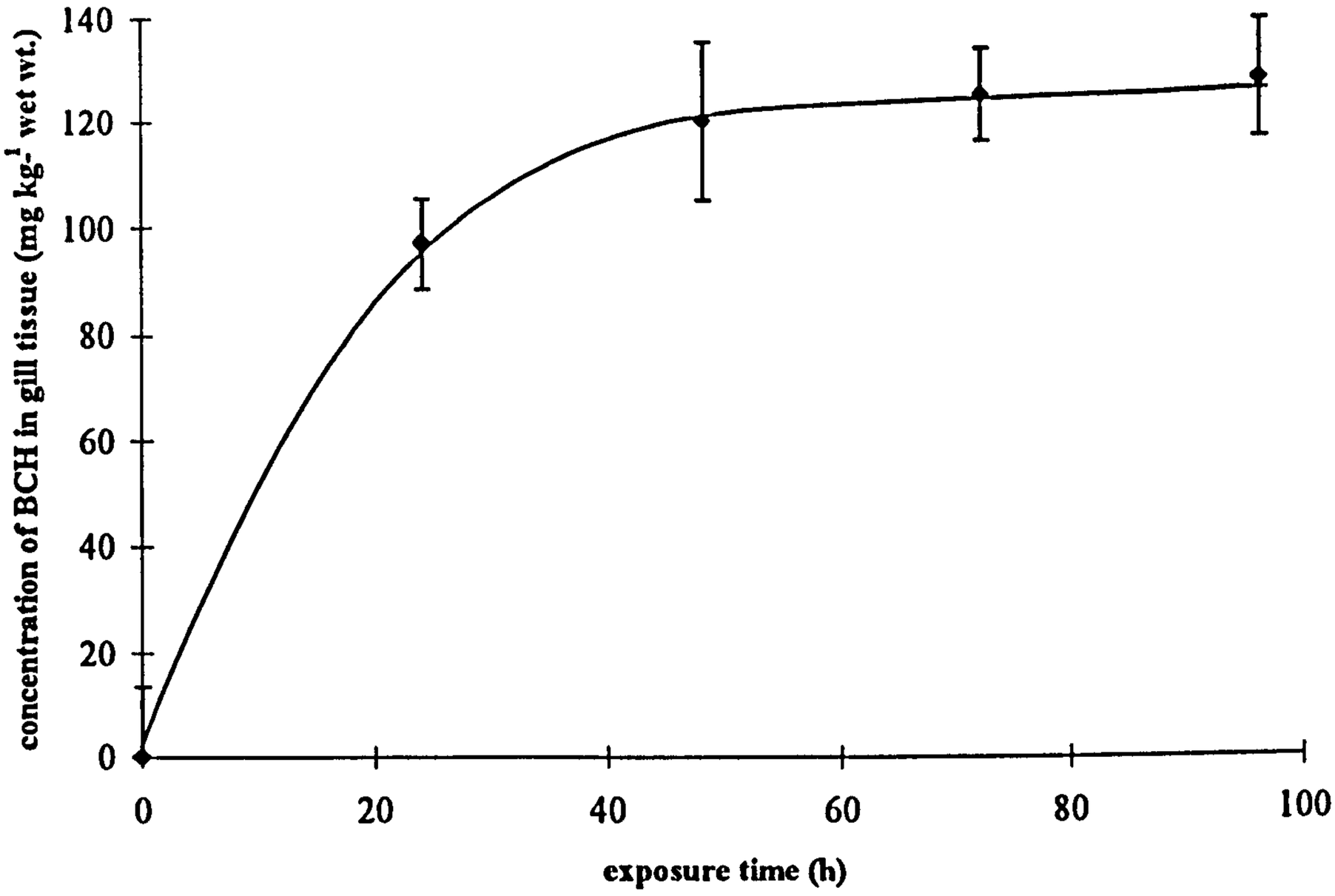
been reported by several authors (*e.g.* Oliver and Niimi, 1985; Francke, 1996) and is presumably caused by saturation of processes that control absorption, distribution or elimination (Barron, 1990).

A similar trend in bioaccumulation of BCH into the mussels was observed in experiment VI to that observed with 4-PO in experiments III-V. Measured gill concentrations and total body burdens of BCH over the 96h exposure period are summarised in Table 4.10 and illustrated in Figure 4.8.

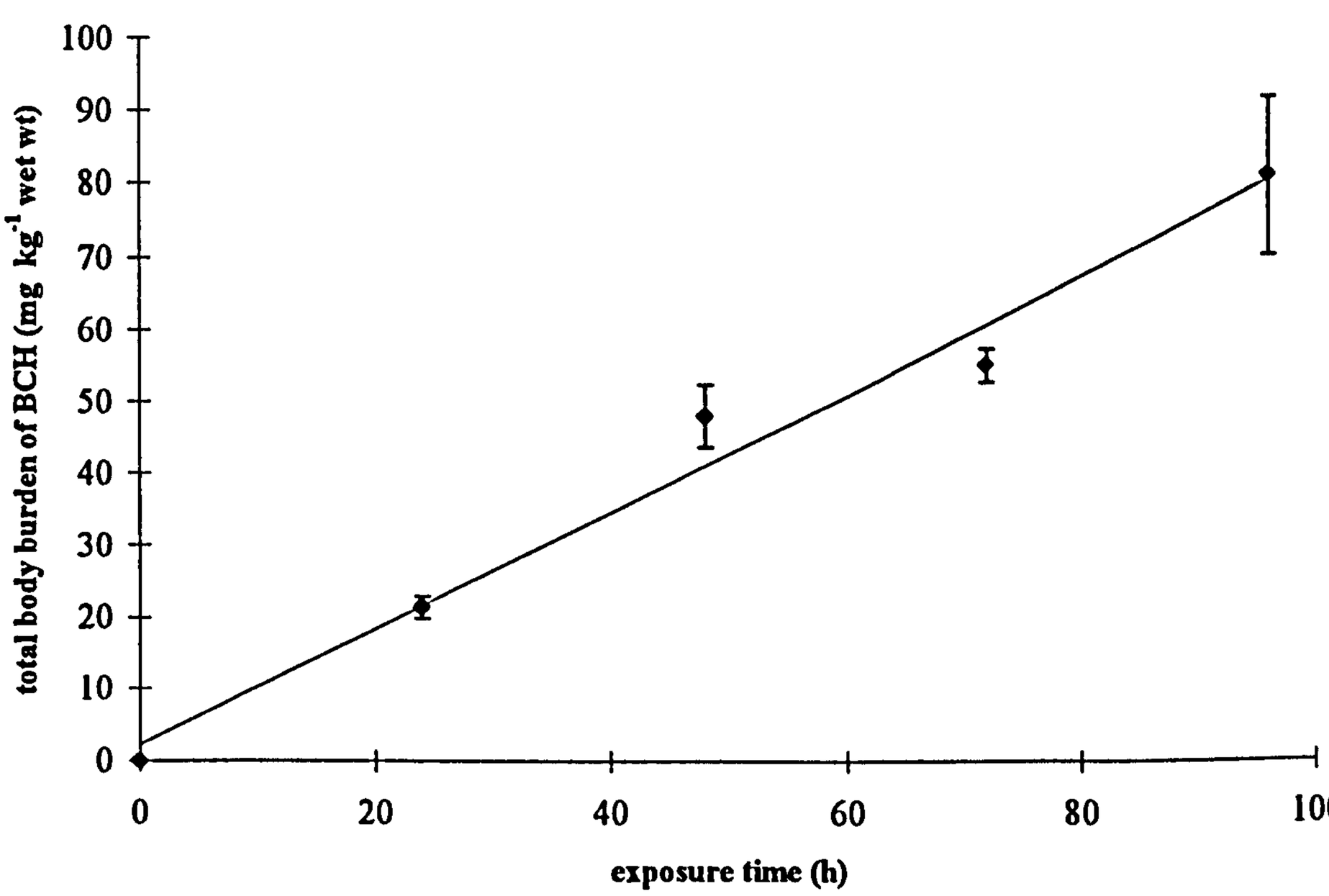
Exposure time (h)	Conc. of BCH in gill tissue		Total body burden of BCH		Feeding rate as % of pooled control
	mg kg^{-1}	mmol kg^{-1}	mg kg^{-1}	mmol kg^{-1}	
0	0	0	0	0	100
24	97.1 ± 13.4	0.694 ± 0.06	29.3 ± 1.5	0.209 ± 0.01	38
48	120.7 ± 8.4	0.862 ± 0.06	45.4 ± 4.4	0.324 ± 0.03	47
72	125.0 ± 15.0	0.893 ± 0.11	60.7 ± 2.3	0.433 ± 0.02	41
96	128.0 ± 8.7	0.914 ± 0.06	74.1 ± 10.9	0.529 ± 0.08	38

values as mean ± range (n=2)

Table 4.10 Summary of tissue concentrations of butylcyclohexane bioaccumulated by mussels *(M. edulis)* over a 96 h exposure period. Experiment VI



(a) gill *values plotted as mean ± range (n=2)*



(b) total body burden

Figure 4.8 Bioaccumulation of BCH by the mussel (*M. edulis*) over a 96h exposure period . Experiment VI *values plotted as mean ± range (n=2)*

To complement the feeding rate experiments, the effect of 4-PO at the sub-cellular level was also investigated in Experiment V. A number of studies (Lowe *et al.*, 1992, 1995; Lowe and Pipe, 1994; Moore *et al.*, 1996) have proposed that changes in cell function and sub-cellular organelles may be particularly sensitive early warning markers of contaminant-induced stress. Exposure to a wide range of contaminants including metals, PCBs and PAHs has been demonstrated to result in increased permeability and fragility of lysosomal membranes. Damage to lysosomes may be measured by the capacity of lysosomes to take up and retain Neutral Red dye over time. The 'healthy' cells take up and retain Neutral Red dye for longer periods than the damaged cells. The progress of dye uptake into the lysosomes and subsequent leakage back into the cytosol may be visualised through a microscope and quantified using time as the determinant of effect (Lowe *et al.*, 1995). By measuring the response of *M. edulis* to 4-PO at both the physiological level (feeding rate) and sub-cellular level (Neutral Red retention assay), Experiment V also provided an opportunity to compare the relative sensitivities of these two toxic end-points. The results of the Neutral Red retention assay are presented in Table 4.11. The variation in Neutral Red retention time of mussel lysosomes with exposure time is illustrated in Figure 4.9

Owing to the limitations of the experimental design, with regards to the number of animals which could be used for each exposure, it was only possible to use four animals for the Neutral Red retention assay. As a consequence of the small sample size, the 95 % confidence intervals are large, and the accuracy of the mean values is uncertain. No statistical analysis was performed on this data. However, the overall trend appears to indicate a decrease in the lysosomal retention time of Neutral Red dye with increasing exposure time.

Exposure time (h)	lysosome neutral red retention time (mins)		Retention time as a % of control
	Control	Exposed	
0	150 ± 37	150 ± 37	100
24		102 ± 39	77
48	114 ± 34	90 ± 45	79
72		60 ± 18	54
96	114 ± 34	48 ± 14	44
120	132 ± 14	78 ± 23	59

values given as mean ± 95% confidence intervals (n=4)

Table 4.11 The lysosomal retention time of Neutral Red dye by mussels (*M. edulis*) exposed to 4-PO (45 µg l^{-1}) over a 120 h exposure period. Experiment V

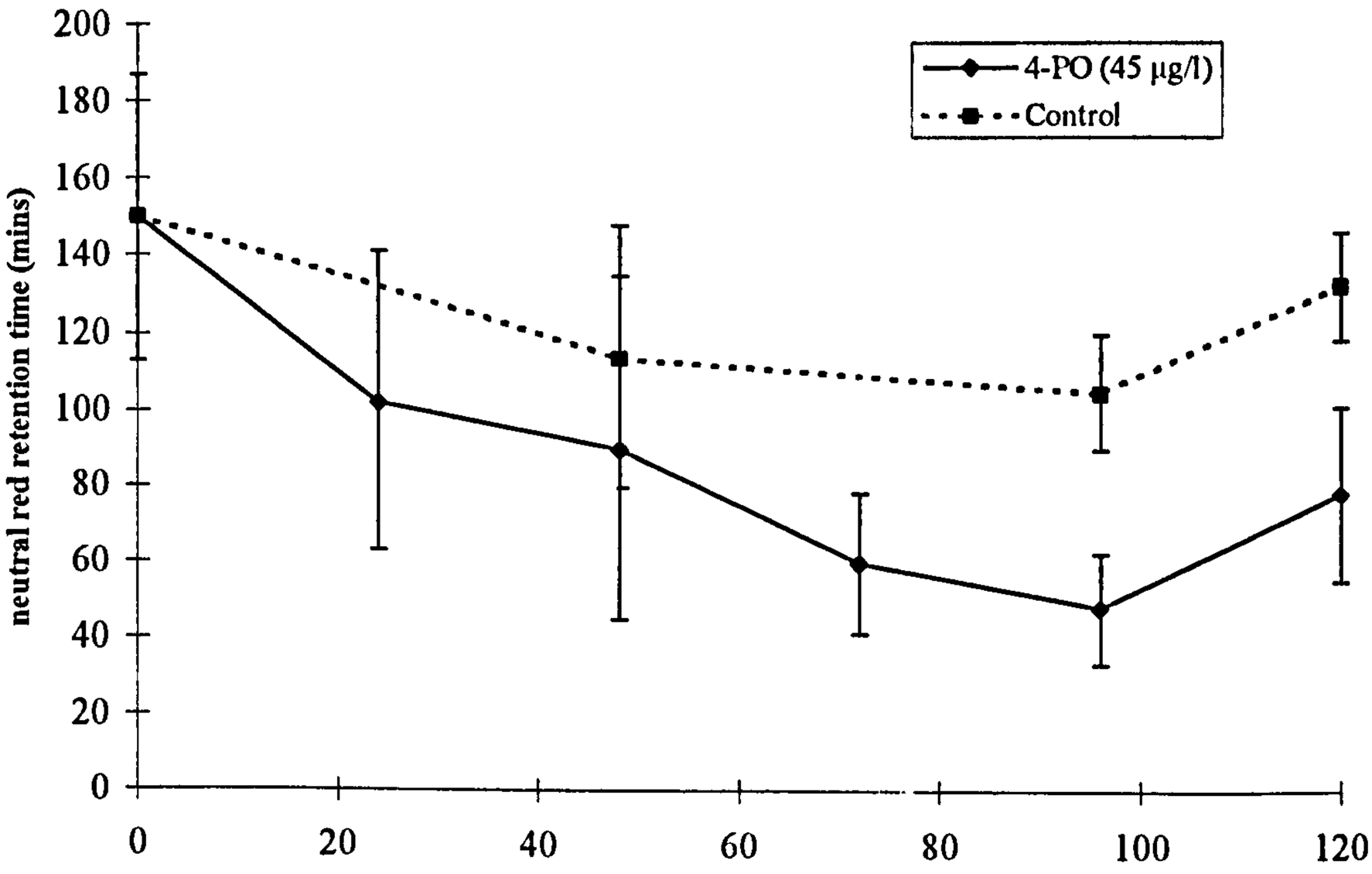


Figure 4.9 The retention time of Neutral Red dye by lysosomes of mussels (*M. edulis*) exposed to 4-PO (45 µg l^{-1}) over a 120 h exposure period. Experiment V

values plotted as mean ± 95 % confidence intervals (n=4)

4.4 Discussion

The aim of this work was to examine the effect of a low molecular weight model aliphatic UCM compound upon mussel ciliary feeding activity. Previous studies (Donkin *et al.*, 1989, 1991) have demonstrated that most test compounds with aqueous solubilities greater than 70 μg l^{-1} were toxic to mussel ciliary feeding activity when bioaccumulated to similar tissue concentrations (15-40 mg kg^{-1} or 0.1 - 0.3 mmol kg^{-1} wet weight tissue). Compounds with lower aqueous solubilities were less toxic, having little or no effect upon mussel feeding rate, despite bioaccumulation of these compounds within the mussel.

The work described herein has investigated the effects of 4-PO, a low molecular weight model aliphatic UCM hydrocarbon, upon mussel feeding rate, and demonstrated that exposure to 4-PO can indeed cause a significant reduction in mussel ciliary feeding activity. In other words, whilst the straight chain C_{11} alkane, *n*-undecane, is non-toxic (presumably owing to its low aqueous solubility; 14 μg l^{-1}), the branched C_{11} compound, 4-PO, is of sufficient aqueous solubility (estimated, 297 μg l^{-1}) to have narcotic potency and cause a reduction in mussel feeding rate. The present study has therefore demonstrated that the molecular weight range of 'narcotic' hydrocarbons is effectively extended by consideration of branched hydrocarbons, owing to their relatively higher aqueous solubilities than straight chain hydrocarbons of comparable chain length. These results suggest therefore, that, a small proportion of the low molecular weight aliphatic UCM hydrocarbons may have some toxicological significance.

The work described in Chapter 2 has demonstrated the presence of low molecular weight UCM hydrocarbons with molecular weight ranges from *n*-C_{12} upwards in mussels. It could be argued therefore, that 4-PO is not a truly representative as a model UCM compound because of its relatively low molecular weight. However, to date, there are very few studies which have specifically examined the narcotic potency of the more

hydrophobic organic compounds considered to be within the toxicity 'cut-off region'. The majority of studies tend to focus upon less hydrophobic compounds of known toxicity (typically compounds with log K_{ow} <5). The toxicity 'cut-off' therefore needs to be more clearly and accurately defined. Indeed, there is still a lack of agreement as to the best molecular descriptor (*e.g.* log K_{ow}, aqueous solubility, molar volume) with which to delineate the toxicity 'cut-off' point. There are no comparable studies which have examined either the toxicity, or physical properties such as aqueous solubility, log K_{ow} values of hydrocarbons with branched and cyclic moieties similar to those considered to constitute the aliphatic hydrocarbon UCM. Consequently, as *n*-undecane has previously been demonstrated to be non-toxic (Donkin *et al.*, 1991), in investigating the effect of a 'T'-branched compound, a C_{11} compound is clearly a logical choice with which to begin investigations into the toxicity of the aliphatic UCM. The work described in this Chapter is therefore presented as an 'initial exploratory analysis' using 4-PO as a model UCM compound.

Unfortunately, no firm conclusions can be drawn regarding the toxicity of the aliphatic UCM as the narcotic cut-off effect still needs to be defined more accurately. Indeed, it may be that a precise delineation is unattainable. The results obtained for 4-PO suggest that a proportion of the low molecular weight aliphatic UCM may be toxic and it may be of value to investigate further the effect of branched and cyclic moieties (such as those proposed as UCM components) upon the physical properties and toxicity of such hydrocarbons. A very recent study by Widdows *et al.* (1997) investigating the effects of various pollutants on the scope for growth (SFG) of mussels (*Mytilus galloprovincialis*) has reported a statistically significant negative correlation between SFG and the size of the UCM and the alkanes C_{15} to C_{30}. This is the only report to date in the literature reporting a link between the aliphatic UCM and toxic effects. These authors reported no significant correlation between SFG and the sum of 12 PAHs (> 3-ring) and attributed

this to the non-narcotic nature of PAH molecules of this size as demonstrated by Donkin *et al.* (1989, 1991). Whilst this study is encouraging, in that it suggests that a proportion of the UCM hydrocarbons may exert toxic effects on the ciliary activity of the gills (feeding rate is the principal component of SFG affected by hydrocarbons), it is unclear how these authors fractionated the total organic extracts of the mussels. Thus, a proportion of what has been classed as 'petroleum hydrocarbons' may also include smaller aromatic hydrocarbons which are known to exert a narcotic effect upon mussel ciliary feeding activity.

Comparing the results from Experiments III, IV, V and VI illustrates an overall trend in the variation of mussel feeding rate with exposure time. With the exception of experiment IV (in which no significant reduction in feeding rate was observed over the entire exposure period, possibly due to bioaccumulation of insufficient toxicant at the site of toxic action), the feeding rate has initially decreased upon exposure to 4-PO. However, within the experimental period, the feeding rate reaches a minimum at which it remains constant for the remainder of the exposure period. This is illustrated in Figure 4.4, which presents the variation in feeding rate of the mussels exposed to 4-PO over the exposure period for each individual experiment.

This trend in mussel feeding rate can be explained by examination of the gill tissue residue data. As illustrated in Figures 4.6 and 4.8a, the concentration of toxicant increases within the gill tissue initially until the concentration reaches a plateau. In other words, within the duration of the experiment, steady-state equilibrium between the concentration of toxicant in the gill tissue and the external exposure water is reached. Comparing the bioaccumulation of toxicant into the gill tissue over the exposure period, with variation in mussel feeding rate, suggests a direct relationship between the concentration of toxicant in the gill tissue and mussel feeding rate. The 'plateau' in the reduction in mussel feeding rate coincides approximately with the attainment of steady

state equilibrium in the gill tissue. In other words, once the concentration of 4-PO in the gill tissue has reached a steady state, no further reduction in mussel feeding rate is observed. A comparison of the feeding rate and gill tissue data from Experiment III illustrates this point (Figure 4.10). In contrast to the bioaccumulation pattern of toxicant into the gill tissue, the total body burden of 4-PO increases linearly throughout the entire exposure period (Figures 4.7 and 4.8b), indicating there is no clear relationship between total body burden of toxicant and mussel feeding rate over time.

A reduction in mussel feeding rate is caused by reduced pumping of the lateral cilia of the gill (Axiak and George, 1987). The cilia are under neuronal control (Paparo, 1972) and therefore the mechanism of feeding rate reduction in response to hydrocarbon exposure is consistent with a non-specific mode of narcotic action (Donkin *et al.*, 1989).

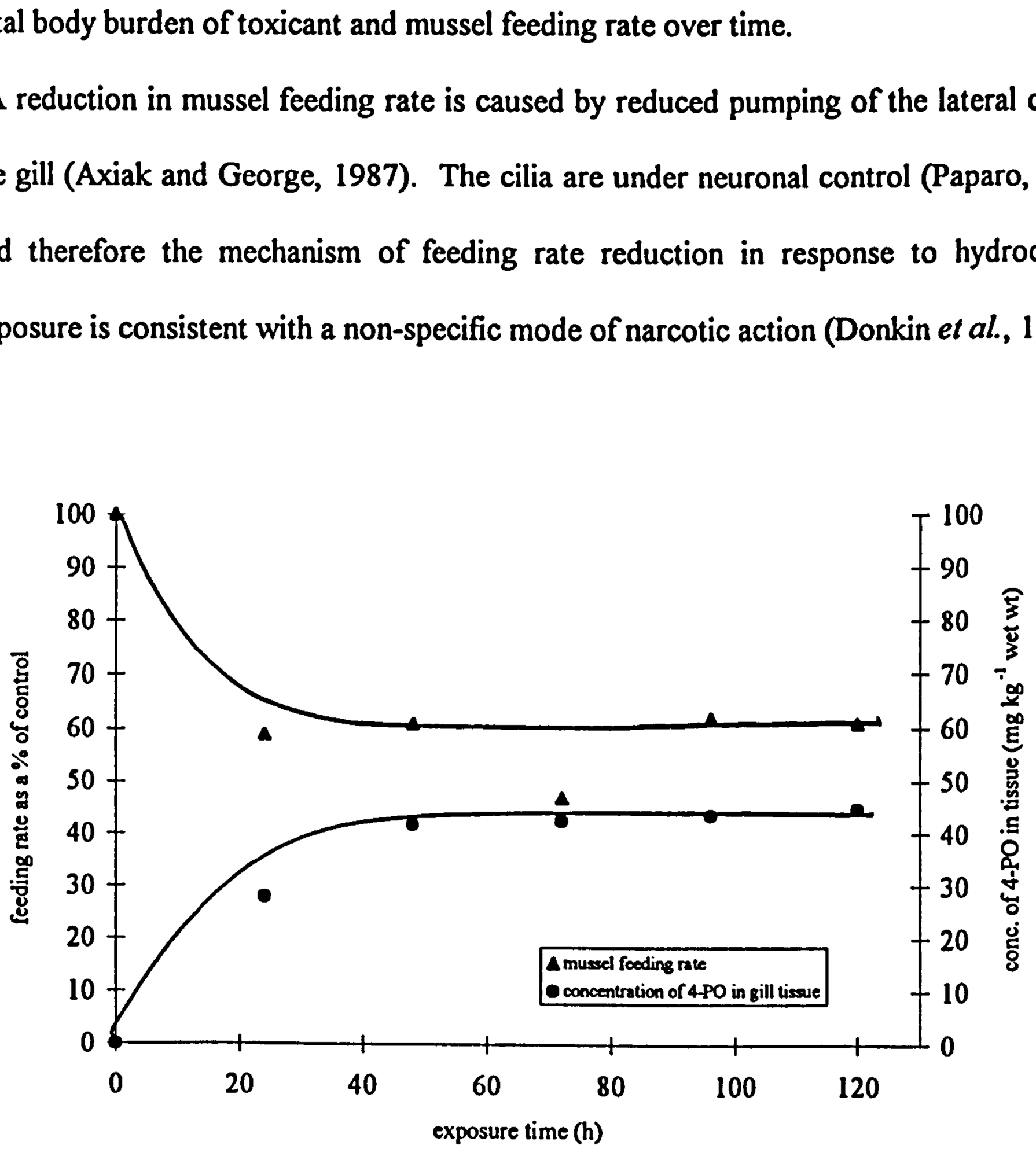


Figure 4.10 The relationship between mussel feeding rate and concentration of 4-PO in the gill tissue (data from Experiment III)

The relationship between gill tissue concentration of toxicant and mussel feeding rate demonstrated in the present study is therefore, perhaps, unsurprising.

Widdows *et al.* (1987) studied the physiological responses (feeding rate, absorption efficiency, respiration rate, ammonia excretion and nitrogen quotient) over a 60 day 'recovery period' (*i.e.* transfer to clean water) of the mussel *M. edulis*, following eight months exposure to the WAF of a diesel oil. These authors measured the concentration of aromatic hydrocarbons (2- and 3-ring) in the whole organism, and also in the gill tissue and digestive gland and noted that the loss of aromatic hydrocarbons from the gill tissue during the 'recovery period' was reflected in a comparable recovery in mussel feeding rate. The results presented herein, therefore, confirm the results of Widdows *et al.* (1987) but provide a more detailed examination of the relationship between a toxic response and the concentration of toxicant at the site of toxic action than has been demonstrated previously.

An estimate of the TEC_{50} (tissue concentration required to reduce mussel feeding rate by 50 %) was obtained by combining the data from each experiment. Donkin *et al.* (1989, 1991) obtained TEC_{50} estimates by applying the relationship; $y = \alpha + \beta \log_{10} x$, fitted by least squares linear regression to their concentration-response data. The data obtained in the present study was treated in the same manner to obtain an estimate of the gill TEC_{50}. The concentration (gill)-response data for 4-PO is presented in Figure 4.11. Summary statistics for the regression analysis are provided in Appendix E.6. The gill TEC_{50} obtained was 56 mg kg^{-1} (46 - 69 mg kg^{-1} lower and upper 95 % confidence limits; or in molar terms, TEC_{50} = 0.365 mmol kg^{-1}, 0.292 - 0.445 mmol kg^{-1})

To compare the results obtained in the present study with those of the established QSAR of Donkin *et al.* (1989) an estimate of the TEC_{50} for 4-PO in terms of the total body burden of toxicant was also obtained by the same method. The relationship

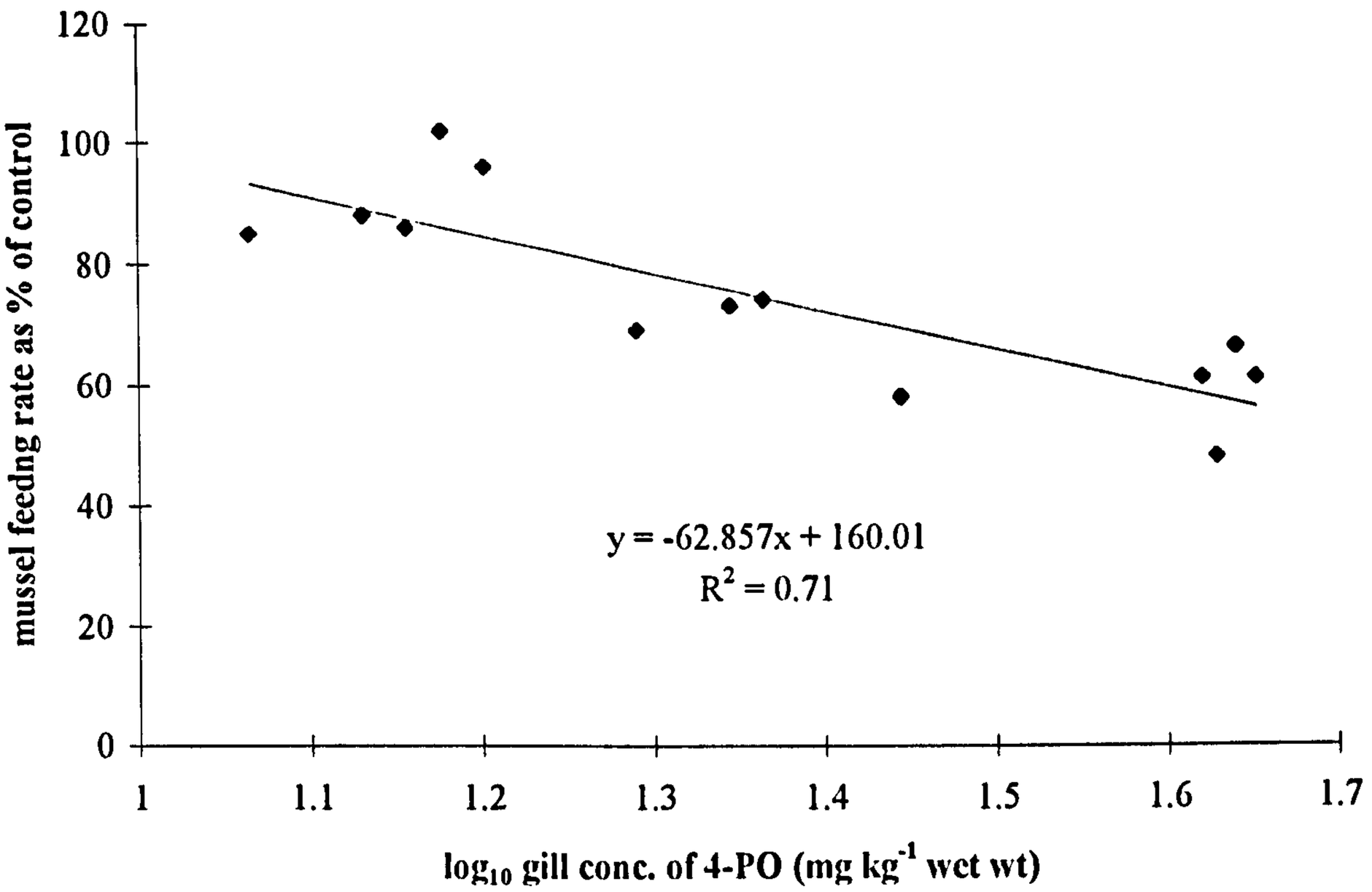


Figure 4.11 Relationship between mussel feeding rate and concentration of 4-PO in the gill tissue (combined data from Experiments III-V)

between total body burden of 4-PO and mussel feeding rate is presented in Figure 4.12. The TEC_{50} obtained was 59 mg kg^{-1} (44 - 79 mg kg^{-1} lower and upper 95 % confidence intervals; or, in molar terms, TEC_{50} 0.376 mmol kg^{-1}, 0.280 - 0.505 mmol kg^{-1}). The TEC_{50} for 4-PO calculated using the total body burden concentration of 4-PO is effectively the same as that obtained using the gill tissue concentration. The similarity of these values is most probably coincidental. Consideration of the relationship between mussel feeding rate and both the gill and total body burden concentrations of 4-PO provides an explanation for this statement. As illustrated in Figures 4.11 and 4.12, whilst there is a general decrease in mussel feeding rate with increasing body burden of toxicant, there is a much clearer relationship between the gill concentration of 4-PO and mussel feeding rate.

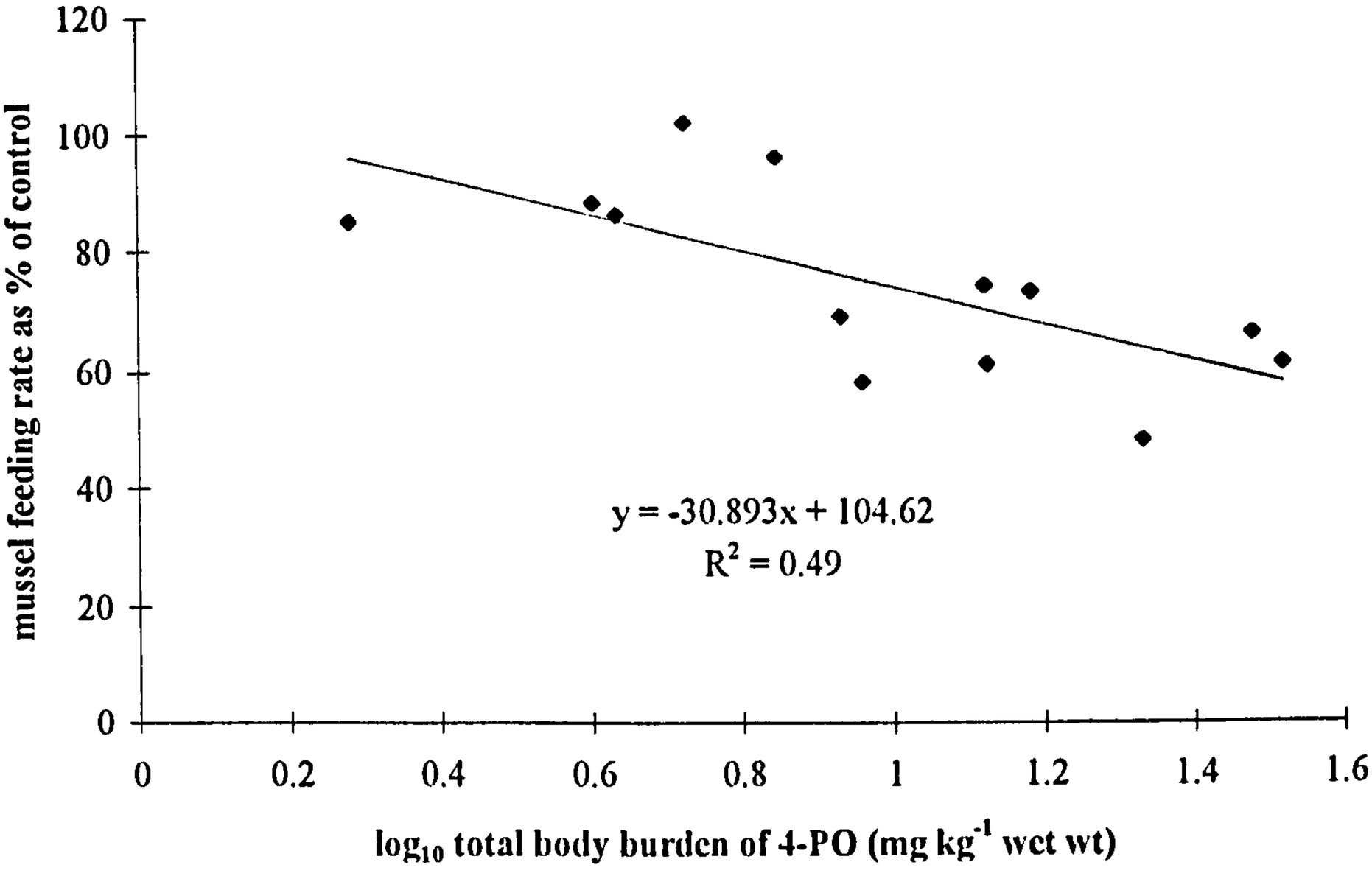


Figure 4.12 Relationship between total body burden of 4-PO and mussel feeding rate (combined data from Experiments III-V)

The gill is the major site of uptake of chemicals from the water phase by aquatic animals (Hayton and Barron, 1990). Exchange at the gills is presumed to be fundamentally a result of diffusion across the epithelium separating water and blood (Barber *et al.*, 1988) and is relatively rapid, as illustrated in the present study. In very simplistic terms, initial uptake of pollutant by the gills is followed by a slightly less rapid transfer to the circulatory fluid, followed by much slower transfer to and accumulation in, storage lipid reserves. Long term exposure results in accumulation of pollutant in lipid reserves until equilibrium or saturation of storage capacity is reached (Farrington, 1991). The continual increase of the total body burden of toxicant throughout the experimental exposure period as observed in the present study, reflects the bioaccumulation of toxicant into the storage lipids. The total body burden measured at exposure times typically greater than 48 h (*i.e.* once a steady state has been reached in the gill tissue) in the present study does

not therefore, reflect bioaccumulation at the site of toxic action (*i.e.* the gills), but will be increasingly influenced by the concentration of toxicant in the remaining body tissues, particularly the storage lipids of the organism with increasing exposure time. If toxicant response is related to the total body burden at exposure times exceeding that at which a steady state of toxicant has been reached in the gill tissue (the presumed site of toxic action), then the sensitivity of the estimate of the tissue concentration of toxicant required to induce a specific response *e.g.* a 50 % reduction in mussel feeding rate is lowered (*i.e.* a higher TEC_{50} estimate is obtained).

The ultimate aim of tissue concentration-response studies such as the work described herein is to extrapolate laboratory derived concentration-response relationships to interpretation of field residue data. For such purposes it is impracticable to relate toxicant response to gill tissue data, as it is the whole body residue which is usually measured in field studies. Consequently, in order to improve the sensitivity of the TEC_{50} estimate for 4-PO, the total body burden TEC_{50} was re-calculated using only the total body burden data from exposures up to attainment of steady state in the gill tissue (*i.e.* 24 h and 48 h data). As shown in Figure 4.13, the relationship between body burden of toxicant and mussel feeding rate is clearly improved by using only the data from exposures up to attainment of steady state in the gill tissue (24 h and 48 h data). The TEC_{50} estimate obtained was 25 mg kg^{-1} (16 - 40 mg kg^{-1} lower and upper 95 % confidence intervals; or in molar terms, TEC_{50} = 0.163 mmol kg^{-1}, 0.110 - 0.253 mmol kg^{-1}). This value is a much lower and therefore more sensitive estimate of the TEC_{50} for 4-PO than the estimate calculated using all the body burden data. Ideally the most sensitive TEC_{50} estimate is required for the purposes of interpretation of field residue data if the impact of complex tissue residues is not to be underestimated, *i.e.* laboratory derived concentration-response relationships (for feeding rate experiments) should be established over relatively short exposure periods, (*e.g.* 24 - 48 h).

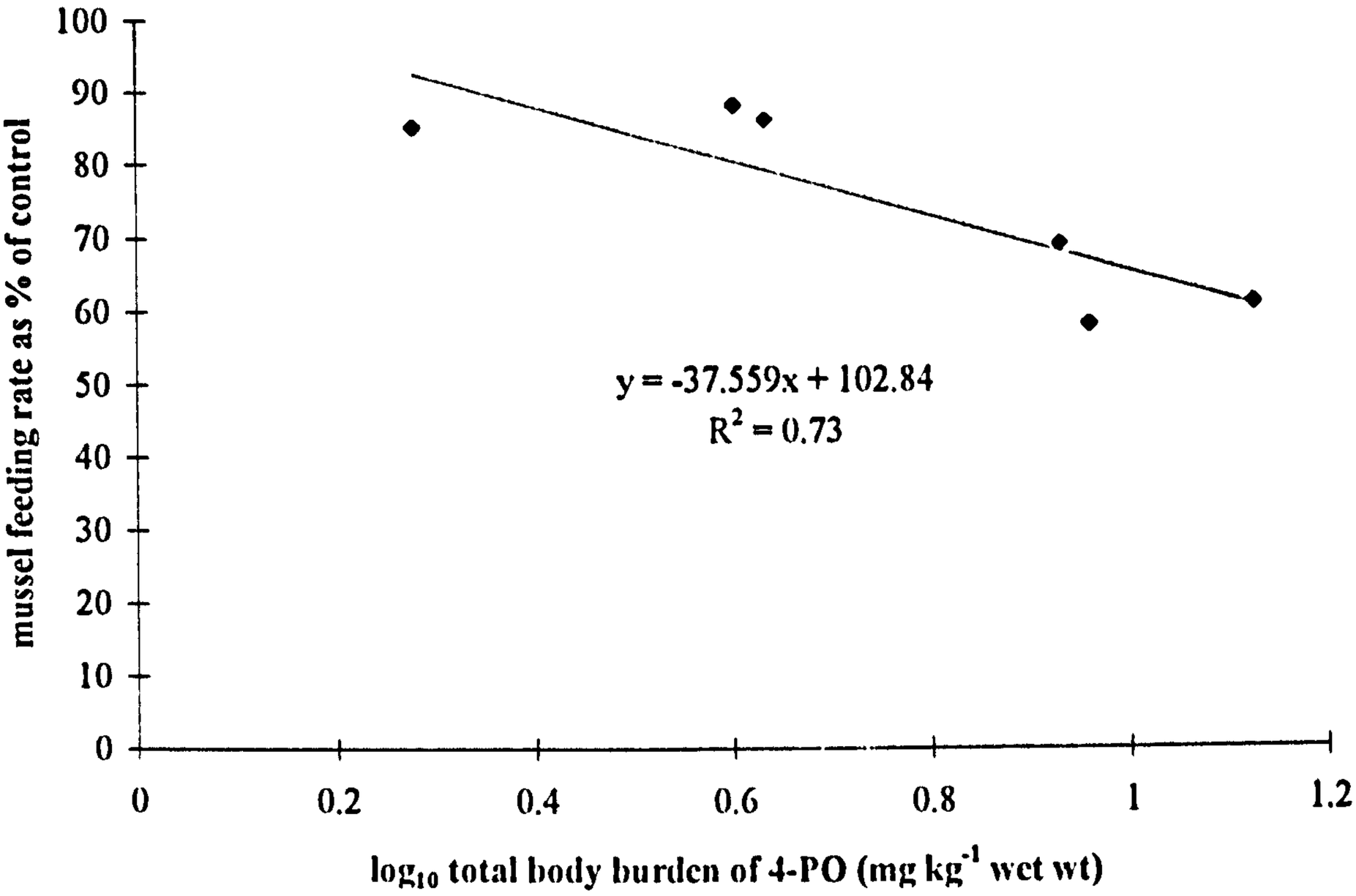


Figure 4.13 Relationship between total body burden of 4-PO and mussel feeding rate (24-48 h data only) (combined data from Experiments III-V)

The much lower TEC_{50} value obtained using only the 24 h and 48 h data illustrates the point discussed earlier that the similarity of the gill and total body burden TEC_{50} estimates is purely coincidental. In contrast to the differing TEC_{50} values calculated for total body burden of toxicant, the gill TEC_{50} value calculated using only the 24 h and 48 h data was similar to that obtained using all data up to 120 h exposure (51 mg kg^{-1}; 35 - 74 mg kg^{-1} lower and upper confidence limits; Appendix E.6.4).

As discussed previously (Section 1.5), narcotic effects are considered to occur at a relatively constant tissue concentration of toxicant, regardless of compound (for those compounds below the toxicity cut-off). A summary of the estimated physical properties and measured biological response of *M. edulis* to the test compound 4-PO is presented in Table 4.12, together with a summary of the data from some previously studied aliphatic hydrocarbons (Donkin *et al.*, 1991).

Compound	Physical properties of compound		Biological response to compound (TEC_{50})	
	Log K_{ow}[a]	Aqueous solubility[b] ($\mu g\ l^{-1}$)	mg kg^{-1}	mmol kg^{-1}
cyclooctane (C_8)	4.47	7900	32	0.29
n-octane (C_8)	4.93	682	39	0.34
n-nonane (C_9)	5.46	220	35	0.27
n-butylcyclohexane (C_{10})	5.46	590	40	0.29
1-phenylpentane (C_{11})	4.91	3850	94	0.63
4-propyloctane **(C_{11})**	**5.67 - 6.38**	**297**	**56 (gill)**	**0.36**
			59 (tbb)	**0.38**
			25(tbb, 24 & 48h only)	**0.16**
n-decane (C_{10})	5.98	52	>102	>0.72
n-undecane (C_{11})	6.51	14	>69	>0.44
1-phenylheptane (C_{13})	6.11	269	35	0.20
n-dodecane (C_{12})	7.04	4	>203	>1.19
1-phenyloctane (C_{14})	6.73	71	82	0.43
1-phenyldecane (C_{16})	7.55	5	>78	>0.36

[a] Log K_{OW} values from Donkin et al. (1991) calculated using MedChem software except for the values for 1-phenylheptane and 1-phenyloctane which were obtained by extrapolation from the data of Miller et al. (1985).
Log K_{OW} values for 4-propyloctane calculated using SRC software and MedChem respectively
[b] Aqueous solubility data from Donkin et al. (1991) originates as follows; cyclooctane, Bobra et al. (1984); n-octane and 1-phenylpentane, Miller et al. (1985); 1-phenylheptane, 1-phenyloctane and 1-phenyldecane, extrapolation of data from Miller et al. (1985); remaining n-alkanes, Coates et al. (1985); butylcyclohexane, extrapolation of data from Benville et al. (1985).
Aqueous solubility data for4-propylocatne calculated using microQSAR software
[c] TEC_{50}; test compound concentration in mussel tissue required to reduce mussel feeding rate to 50% of control value. Values shown as > are the highest concentrations tested or attained (Donkin et al. (1991)

Table 4.12 The effect of hydrocarbons on mussel feeding rate - A synthesis of the data obtained by Donkin *et al.* (1991) with those obtained in the present study

In the present study, the effect of butylcyclohexane (BCH; Experiment IV) upon mussel feeding rate over a 96 h exposure period was also investigated. The purpose of this experiment was to act as a reference compound to enable a comparison of the results reported by Donkin *et al.* (1991) with those obtained herein.

No TEC_{50} was calculated for BCH as the concentration of BCH reached a steady state in less than 24 h. Consequently, all the data obtained was over a very narrow range in concentration terms, and it was felt that this was insufficient for an accurate regression analysis. An 'estimate' of the TEC_{50} for BCH (30 mg kg^{-1} or 0.2 mmol kg^{-1}) was made by visual inspection of the data obtained (Table 4.10). This value is slightly lower than that reported by Donkin *et al.* (40 mg kg^{-1}; 1991), however, these authors typically used exposure times of 48 - 72 h. The difference between the two TEC_{50} estimates is, therefore, small.

Overall, the values obtained in the present study (25 - 59 mg kg^{-1}, 0.171 - 0.380 mmol kg^{-1} wet weight tissue) are comparable with the TEC_{50} values reported by Donkin *et al.* (1991) [15-40 mg kg^{-1}, 0.15 - 0.30 mmol kg^{-1} wet weight tissue] for those compounds with demonstrable narcotic activity. In the present study, the TEC_{50} value of 0.380 mmol kg^{-1} was obtained using exposure times up 120 h. When the TEC_{50} for 4-PO is calculated using only the 24 h and 48 h data a lower, and therefore more sensitive TEC_{50} estimate is obtained (0.163 mmol kg^{-1}). This can be explained in terms of the influence of exposure time, as previously discussed. The influence of exposure time is also evident in the results of Donkin *et al.* (1989, 1991). These workers reported TEC_{50} estimates for aromatic hydrocarbons in the range 0.10 - 0.24 mmol kg^{-1} whilst for aliphatic hydrocarbons the TEC_{50} values reported were typically in the range 0.27 - 0.43 mmol kg^{-1}. Donkin *et al.* (1991) suggested that the apparently higher toxicity of the aromatic hydrocarbons may be due, in part, to the different exposure times used in the two series of experiments (1.7 h for two/three ring aromatics, 48 h - 72 h for aliphatics). Pawlisz

and Peters (1993) tested the hypothesis of equipotency of narcotic compounds by exposing *Daphnia* to a range of narcotic compounds and measuring the lethal body burden of toxicant. These authors reported that the effective internal concentration increased with duration of exposure and ambient concentration but offered no explanation for their results. The results presented herein suggest that the reported variation in TEC_{50} values according to exposure time is a result of prolonged exposure of the organism to the toxicant such that the site of toxic action has reached a steady state, whilst bioaccumulation into discrete compartments such as storage lipids is continuing.

The relationship between mussel feeding rate and concentration of toxicant in the gill tissue demonstrated herein in the experiments with 4-PO is also apparent in Experiment VI when BCH was used as the toxicant. This shows that the relationship is true for other narcotic hydrocarbons. In addition, the overall finding that, at least for exposures up to and including 120 h, toxic response as measured by mussel ciliary feeding activity is consistent with body burden of toxicant (gill tissue concentration in this instance) implies that metabolic activation to more toxic products or sequestration processes which act as detoxifying mechanisms are not important in the gill. This finding is particularly important for the interpretation of field tissue residue data, enabling direct interpretation of the measured body burden of contaminants in field samples in toxicological terms.

Although the 'recovery' in mussel feeding rate (the increase in mussel feeding rate from 24 h to 72 h) observed in the initial dose-response experiments (Experiments I and II) remains unexplained, the time series experiments (III - VI) conducted as a result of these observations has enabled a more detailed analysis of the relationship between the measured body burden of toxicant and toxic response than has been previously reported. Whilst numerous studies have reported toxicant body burden data for toxicity tests in which lethality is the end-point and established that a relatively constant body burden of

narcotic toxicant is associated with LC_{50} estimates (typically 2-8 mmol kg^{-1}), comparison of the tissue residue data reported herein with other studies of a similar nature is limited by the sparse amount of reported tissue residue data for sub-lethal toxicity studies. A few studies (Call *et al.*, 1985; McCarty, 1986; Mortimer and Connell, 1995) have estimated the toxicant concentration in test organisms in chronic toxicity studies and reported a relatively constant body burden of approximately 0.6 mmol kg^{-1}. However differences between test species (*i.e.* differences in lipid content) and also the sub-lethal response measured will influence the critical tissue residue reported. With the exception of Donkin *et al.* (1989, 1991) there are no comparable studies reported, which have used a QSAR approach to study the sublethal responses of mussels to non-specific narcotic toxicants. The TEC_{50} values obtained in the present study are in good agreement with those reported by Donkin *et al.* (1989, 1991) and lend support to the theory of a constant body burden of toxicant to produce a given narcotic response. By examining the tissue toxicant concentration not only in terms of the total body burden, but also the concentration of toxicant at the presumed site of toxic action, the present study has provided a detailed and unique insight into the relationship between the concentration of toxicant in the mussel and the observed ciliary feeding rate. The relationship between the concentration of toxicant in the gill tissue and mussel ciliary feeding activity demonstrated in the present study demonstrates that the sensitivity of TEC_{50} estimates obtained in this manner is increased if the exposure time is kept relatively short.

4.9 Conclusions

The work described herein has investigated the effects of a model low molecular weight aliphatic UCM hydrocarbon, 4-propyloctane (4-PO) and demonstrated that exposure to 4-PO can cause a significant reduction in mussel ciliary feeding activity. The work has also demonstrated that the molecular weight range of narcotic hydrocarbons is effectively

extended by consideration of branched hydrocarbons, because of their relatively higher aqueous solubility than straight chain hydrocarbons of comparable chain length. Whilst no firm conclusions can be drawn from the present study regarding the toxicity of the aliphatic UCM, the results suggests that a small proportion of the low molecular weight aliphatic UCM may be of some toxicological significance. However, it is probable that the aromatic UCM will prove to be of greater toxicological significance than the aliphatic UCM, owing to the relatively greater solubility of aromatic hydrocarbons compared with aliphatic hydrocarbons of comparable chain length. This is investigated in Chapter 5. Further work into the effects of branched and cyclic moieties upon the solubility and narcotic potency of aliphatic hydrocarbons is required before any further conclusions can be reached. The present study has also elegantly demonstrated the relationship between the concentration of toxicant in the gill tissue, the presumed site of toxic action, and the observed reduction of mussel ciliary feeding activity upon exposure to narcotic toxicants such as 4-PO and butylcyclohexane.

CHAPTER FIVE

Concentration-response studies of the effect of two low molecular weight model aromatic UCM hydrocarbons upon mussel feeding rate

5.1 Introduction

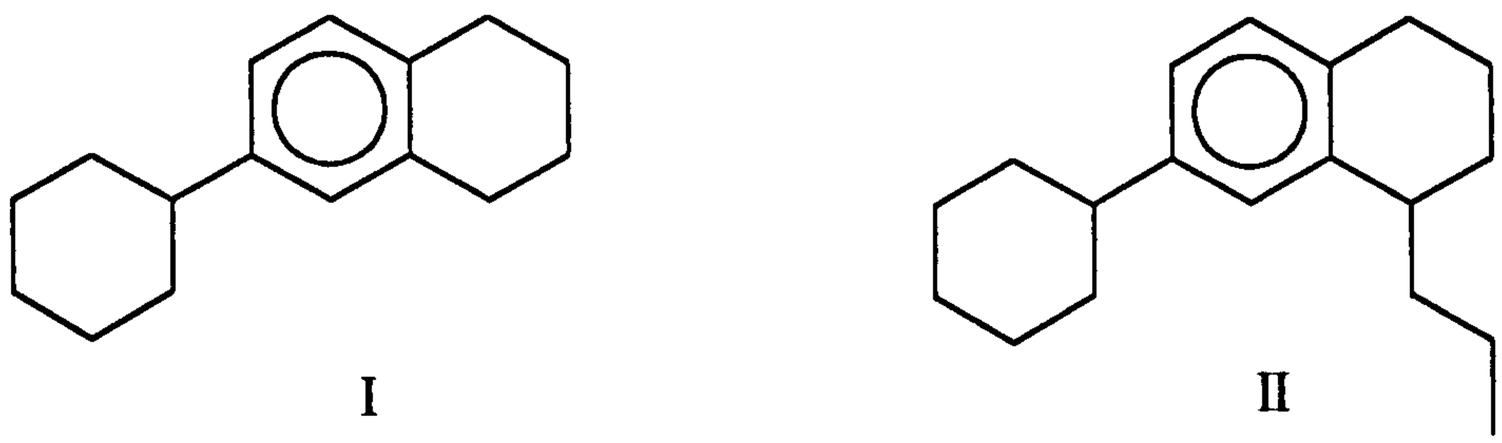
The environmental impact of aromatic hydrocarbon UCMs is currently unknown and uninvestigated. In Chapter 2 it was demonstrated that despite the paucity of reported aromatic UCM concentrations in environmental samples prior to this study, aromatic hydrocarbon UCMs may form a quantitatively significant component of the hydrocarbon body burden in petroleum hydrocarbon contaminated mussels. Aromatic hydrocarbons typically have greater aqueous solubilities than aliphatic hydrocarbons of comparable molecular weight (McAuliffe, 1966; Verscheuren, 1983) and therefore it is anticipated that the aromatic UCM may be of greater toxicological significance than the less soluble aliphatic UCM hydrocarbons. For example, Gilfillan *et al.* (1977) measured the long term effects of an oil spill on populations of the clam *Mya arenia* from different sites in Casco Bay, Maine. Two years after the spill there was no correlation of toxic response (measured in terms of carbon flux, which is effectively Scope for Growth) with the total body burden of hydrocarbons (dominated by the aliphatic UCM), but a significant reduction in carbon flux was found to correlate with elevated body burdens of low molecular weight aromatic hydrocarbons (presumably including the low molecular weight aromatic UCM). Apart from this, few reports have investigated the toxicity of this quantitatively important environmental burden.

As discussed previously (Sections 1.6 and 4.1), Donkin *et al.* (1989, 1991) established a QSAR for the effect of various hydrocarbons upon mussel feeding rate and reported a 'cut-off' in toxicity for hydrocarbons with aqueous solubilities less than 70 $\mu g\ l^{-1}$. Whilst existing knowledge on narcotic 'cut-off' effects is limited, the present study has already demonstrated that the molecular weight range of narcotic chemicals may effectively be extended by consideration of branched alkanes such as the 'T-branched' alkanes proposed as components of the aliphatic UCM (Sections 4.3-4.4). The recent

proposal of 'average' model structures for aromatic UCM hydrocarbons (Figure 1.5; Revill *et al.*, 1997; Thomas *et al.*, 1997; *cf.* Section 1.2) now enables some of the toxicological properties of aromatic UCM hydrocarbons to be studied.

Given the solubility and associated toxicity of a number of resolved low molecular weight aromatic hydrocarbons (Donkin *et al.*, 1989, 1991) it seems likely that a proportion of the lower molecular weight aromatic UCM would be of sufficient aqueous solubility to be considered as non-specific narcotic toxicants. Unfortunately, the methods commonly used to predict aqueous solubility or log K_{ow} of a particular compound are so-called 'additive-constitutive' (*cf.* Section 3.3.1) or regression methods. Given that the data used to derive both fragment contributions for chemical groups and regression equations are derived from experimentally measured values of a limited set of chemicals, the applicability of such models is not universal (Gombar and Enslein, 1996), and in the present study, the structures of the model aromatic UCM hydrocarbons proposed by Revill *et al.* (1997) and Thomas *et al.* (1997) are considerably more complex than the simple hydrocarbons used to establish and validate such predictive models. Consequently, the accuracy of any solubility or octanol-water partition coefficient (log K_{ow}) predictions obtained for the model aromatic UCM hydrocarbons by such methods is uncertain. Whilst an estimate of the physico-chemical properties of a particular compound is of use in guiding the choice of test compound (discussed previously in Section 3.3), values obtained in this manner cannot currently be used to predict the toxicity of compounds of the type proposed as 'model' aromatic UCM hydrocarbons. An alternative approach is to test the toxicity of a number of hydrocarbons with structures similar to those proposed as 'average' structural components of aromatic UCMs. This may provide an indication of the 'toxic potential' of the aromatic UCM.

The aim of the present study was to employ an approach similar to that used previously for the model aliphatic UCM hydrocarbon, 4-propyloctane (Section 4.3.1) to investigate whether two synthetic model aromatic hydrocarbons, 7-cyclohexyltetralin (7-CHT; Figure 5.1, I) and 7-cyclohexyl-1-propyltetralin (7-C-1-PT; Figure 5.1, II) are toxic to mussel ciliary feeding activity. Estimates of the log K_{OW} and aqueous solubilities of these compounds were obtained using MedChem and SRC software packages (values kindly provided by Prof. J. Dearden, John Moores University, Liverpool), and are summarised in Table 5.1.



I II

Figure 5.1 Model aromatic UCM compounds 7-cyclohexyltetralin (7-CHT, I) and 7-cyclohexyl-1-propyltetralin (7-C-1-PT, II)

Consideration of the physico-chemical properties of these two compounds in relation to the QSAR for the effect of non-specific narcotics upon mussel ciliary feeding activity (Donkin *et al.*, 1989,1991) suggests that 7-cylohexyltetralin may be toxic to mussel feeding rate, but that 7-cyclohexyl-1-propyltetralin would not be hydrophilic enough to act as a non-specific narcotic toxicant (*cf.* Section 1.6). However, values of

log K_{OW} greater than 6 may be overestimated by as much as one log unit (Brooke *et al.*, 1986; Lyman, 1990; Chessels *et al.*, 1991).

Given the trend in mussel feeding rate demonstrated previously in experiments with 4-PO (Section 4.3 - 4.6), concentration-response experiments in the present study were conducted over an exposure period of 24 h, with the aim of establishing toxicity to mussel ciliary feeding activity. The test procedure was modified slightly from that employed in the study with 4-PO (Section 4.2) in order to make the procedure simpler and faster.

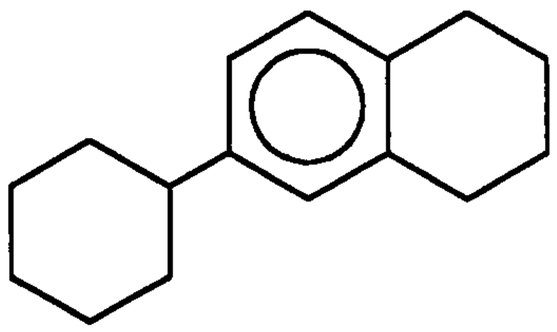
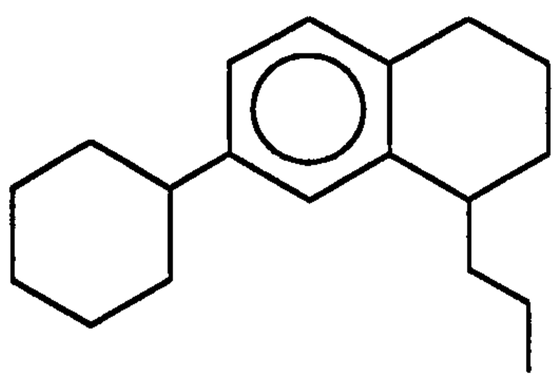
Compound	log K_{OW}			Aqueous solubility ($\mu g\ l^{-1}$)	
	estimate (this study, *cf.* Appendix D.1)	ClogP	SRC	microQSAR	SRC
	6.61	6.33	6.77	152	60
	7.73	7.91	8.17	3	2

Table 5.1 Estimates of log K_{OW} and aqueous solubility for model aromatic UCM compounds (provided by Prof. J. Dearden, Liverpool John Moores University)

5.2 Experimental details

The procedure of Donkin *et al.* (1989, 1991) employed in the present study to investigate the effect of 4-PO upon mussel feeding (Section 4.2) was modified. The initial procedure used large volumes of seawater for exposure of the animals and measurements of feeding rates. Preparation of toxicant solutions was lengthy, expensive and arduous. By using smaller animals, it was hoped that the volume of water used would be reduced and time saved. In addition, the use of smaller animals enables a steady state (in terms of total body burden of toxicant) to be reached faster than with larger animals, thereby reducing the experimental exposure period required to obtain a significant body burden of toxicant. A similar approach has recently been successfully used by Widdows *et al.* (1996).

As demonstrated in Section 4.3, mussel feeding rate is related to the concentration of toxicant in the gill tissue. Once the concentration of toxicant has reached a steady state in the gill tissue, no further decrease is observed in mussel feeding rate despite continued bioaccumulation of toxicant into the remaining body tissues. Thus, if toxicant response is related to the total body burden at exposure times exceeding that at which steady state of toxicant has been reached in the gill tissue, the sensitivity of the estimate of the tissue concentration required to induce a specific response such as a 50 % reduction in feeding rate (TEC_{50}) is lowered. Consequently, for the purposes of the work described herein, exposure was kept constant at 24 h for each compound tested. It was estimated that a steady state would have been reached in the gill tissue by this point (based on the results of experiments with 4-PO, Chapter 4).

Prior to conducting toxicity tests with the two synthetic model UCM hydrocarbons (7-CHT and 7-C-1-PT) a 'pilot' study was conducted using butylcyclohexane (BCH) as the toxicant. The toxicity of BCH has been investigated in previous feeding rate

studies (Section 4.3 this study; Donkin *et al.*, 1989, 1991). Incorporation of this experiment meant that results obtained herein could again be compared with previous studies (*cf.* Chapter 4). In addition, the use of a widely available, fairly cheap compound such as BCH enabled any flaw in the experimental protocol to be addressed without wasting valuable synthetic test compounds.

5.2.1 Test materials

7-Cyclohexyltetralin (7-CHT) and 7-cyclohexyl-1-propyltetralin (7-C-1-PT) were synthesised, purified and characterised as described in Section 3.3. GC purity was greater than 99 % and 97 %, respectively. Butylcyclohexane (BCH) was obtained from Aldrich (purity >99 %).

5.2.2 Preparation of toxicant solutions

Toxicant solutions were prepared by adding the test compound, in an acetone carrier, to filtered seawater as described in Section 4.2.2.

5.2.3 Exposure of animals

Seven mussels (*Mytilus edulis*) of shell length 12 mm ± 1mm were exposed to 1.4 l of toxicant solution in a glass beaker. For each concentration of toxicant studied, two replicate exposure vessels, each containing seven mussels were set up. For each compound studied (*i.e.* each concentration-response experiment), two control exposure vessels containing seven mussels exposed to 1.4 l of the control solution (seawater + 0.001 % acetone) were assembled. Mussels were fed continuously with an algal culture (*Isochrysis galbana*) by means of a peristaltic pump. Gentle water

movement was maintained using a Teflon stirrer bar (10 mm). The exposure period was 24 h.

5.2.4 Measurement of feeding rate

Mussel feeding rates were determined by measuring the rate at which algal cells were cleared from suspension in a static system (*cf.* Coughlan, 1969). Following a 24 h exposure period, mussels were transferred individually into glass beakers, each containing 200 ml toxicant solution. Gentle water movement was maintained using a magnetic stirrer and mussels were placed such that the inhalant siphon was facing directly into the current. An additional beaker plus stirrer containing only the test solution (no mussel) was used as a control. A 30 minute acclimatisation period was allowed for the mussels to open their shells and resume feeding. A pre-determined volume of algal culture was then added to each beaker to give a concentration of 12000 - 14000 cells ml^{-1}. After a five minute mixing period, a 20 ml aliquot was removed from each beaker and cell numbers were counted in triplicate using a model D Coulter Counter set to measure particles greater than 3 μm diameter. A further 20 ml aliquot was removed after 15 minutes and cell numbers determined using the Coulter counter. Feeding rates were calculated as described previously (Equation 4.1) using the decline in cell numbers over the 15 minute period.

5.2.5 Chemical analysis of mussel tissue

Soft tissue was dissected from the mussel shells and stored at -17°C in solvent rinsed glass vials prior to analysis. Tissues were extracted by alkaline digestion (NaOH) followed by extraction with hexane (x 3). An internal standard was spiked into the mussel tissue immediately prior to extraction. 7-CHT was used as the internal

standard for analyses of 7-C-1-PT and *vice versa*. Tissue extracts were analysed by GC-MS. Full experimental details are given in Section 6.6.6.

5.3 Results

5.3.1 Concentration-response experiment for the effect of butylcyclohexane (BCH) upon mussel feeding rate over a 24 h exposure period. A 'pilot study'

A summary of the effects of BCH upon mussel feeding rates and total body burdens of BCH obtained is presented in Table 5.2. Concentration-response curves for the effect of BCH upon mussel feeding rate over a 24 h exposure period expressed in terms of both nominal aqueous exposure concentration and total body burden of toxicant are shown in Figures 5.2 and 5.3. A reduction in mussel feeding rate upon exposure to BCH is clearly evident.

Nominal aqueous concentration ($\mu g\ l^{-1}$)	total body burden ($mg\ kg^{-1}$ wet wt)	mussel feeding rate ($l\ h^{-1}$) mean ± sd (n=7)	mussel feeding rate expressed as a % of the control feeding rate
0 (control)	0	0.43 ± 0.06 (n=14)	100
25	34.5	0.37 ± 0.05	86
25	35.9	0.31 ± 0.07	72
50	59.8	0.22 ± 0.05	51
50	57.7	0.22 ± 0.07	51
100	117.6	0.18 ± 0.04	42
100	113.1	0.11 ± 0.03	26
200	196.8	0.08 ± 0.03	19
200	197.6	0.06 ± 0.02	14

Table 5.2 The effect of butylcyclohexane (BCH) upon mussel feeding rate over a 24 h exposure period

Mussel feeding rates measured for replicate experiments at each concentration were compared using a t-test (P=0.05, two-tailed). With the exception of the feeding rates obtained at a nominal aqueous exposure concentration of 100 μg l^{-1}, there were no significant differences between replicate experiments. Consequently, for the purposes of estimating an EC_{50} value, data from replicate experiments in which there were no significant differences between the two replicates, was pooled. A summary of the pooled data is presented in Table 5.3. A TEC_{50} value for the effect of BCH upon mussel feeding rate was then obtained by applying the relationship $y = \alpha + \beta \log_{10} x$, fitted by linear least squares regression to data in Table 5.3. Figure 5.4 illustrates the concentration (tissue)-response data. A summary of the regression statistics is presented in Appendix F.1.

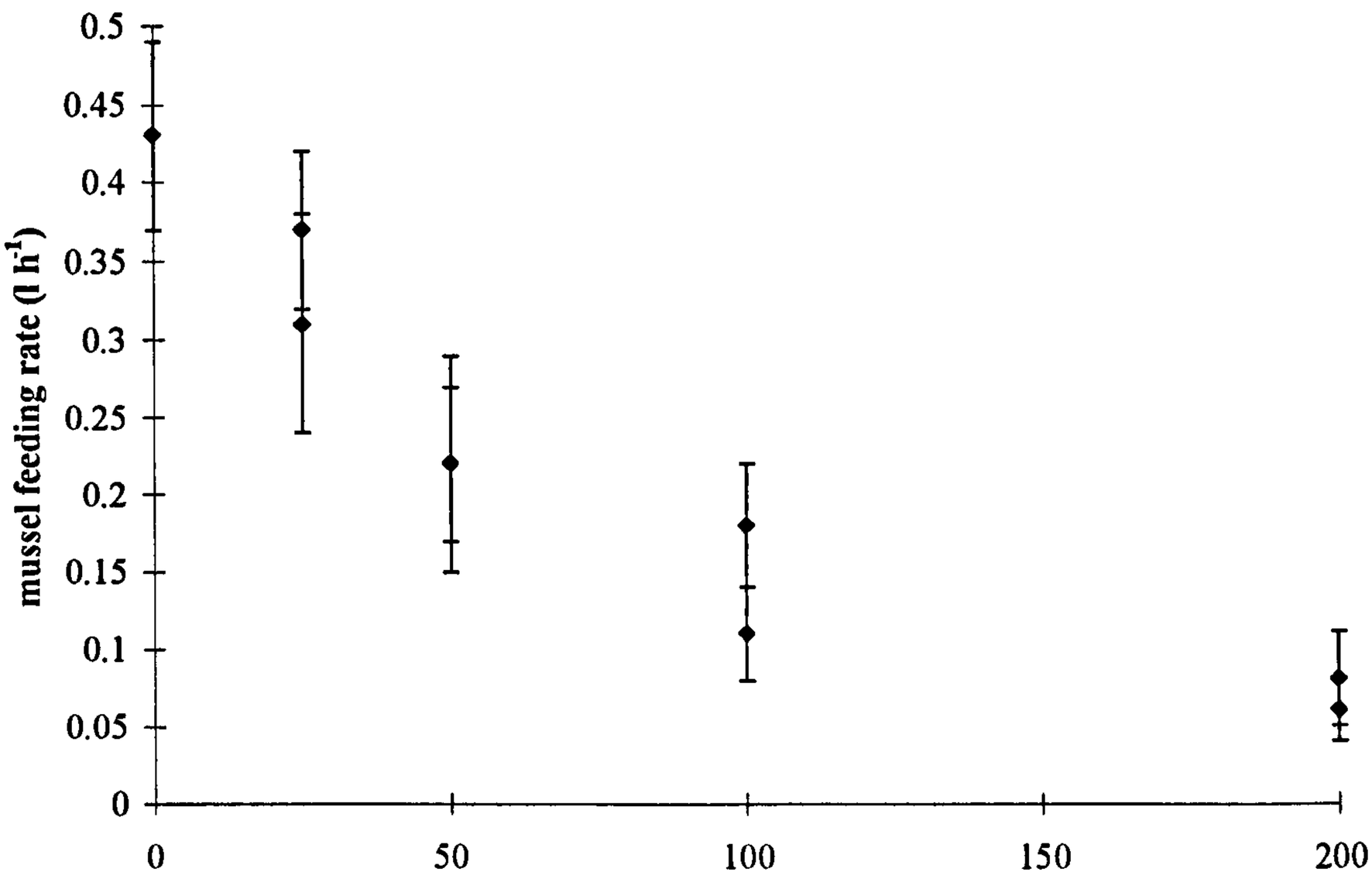


Figure 5.2 Concentration (aqueous)-response curve for the effect of butylcyclohexane (BCH) upon mussel feeding rate over a 24 h exposure period

values plotted as mean ± s.d. (n=7)

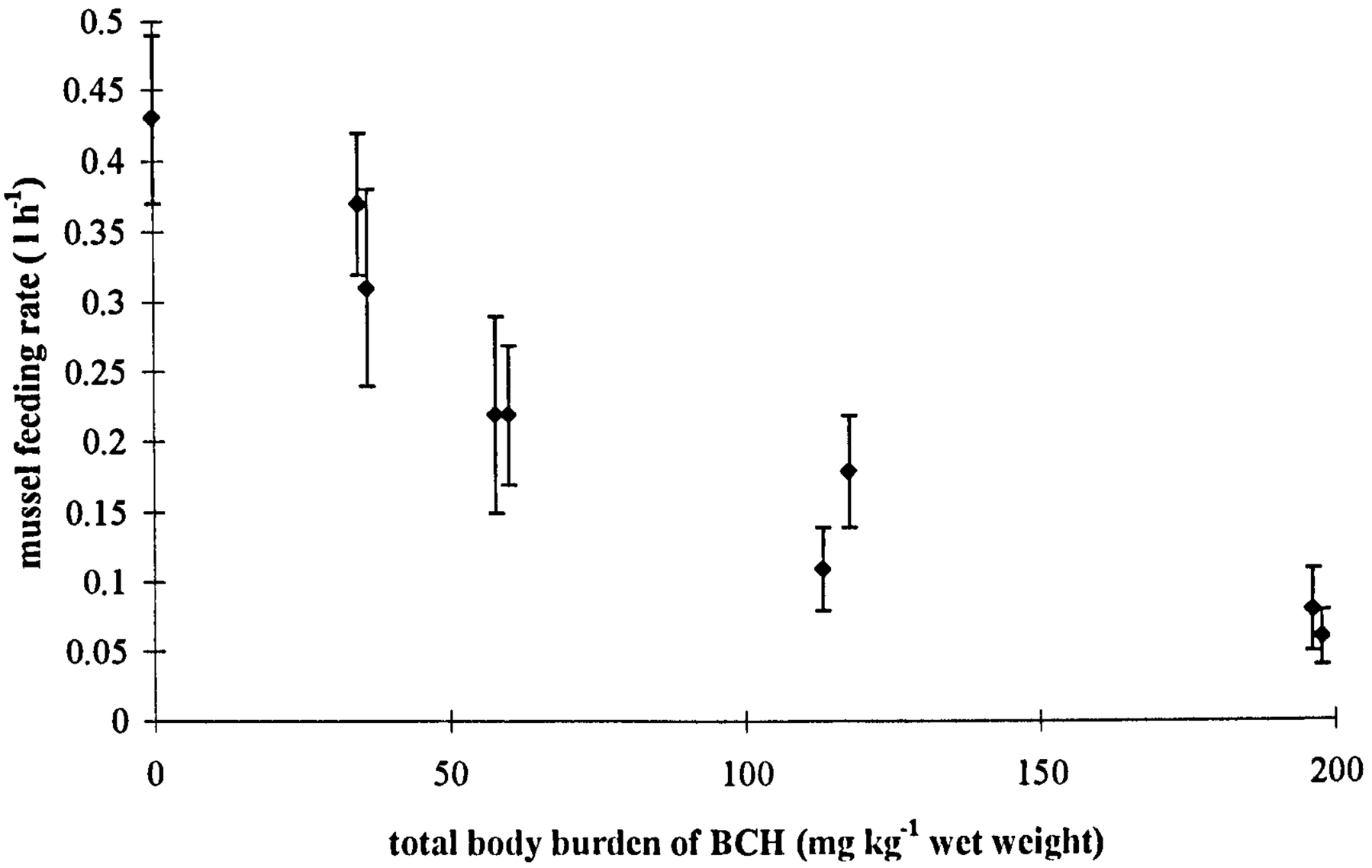


Figure 5.3 Concentration (tissue)-response curve for the effect of butylcyclohexane (BCH) upon mussel feeding rate over a 24 h exposure period

values plotted as mean ± s.d. (n=7)

Nominal aqueous concentration of BCH ($\mu g\ l^{-1}$)	total body burden of toxicant (mg kg^{-1} wet wt) (n=2)	mussel feeding rate ($l\ h^{-1}$) mean ± sd (n=14) except * where n=7	mussel feeding rate expressed as a percentage of control feeding rate
0 (control)	0	0.43 ± 0.06	100
25	35.2	0.34 ± 0.07	79
50	58.7	0.22 ± 0.06	51
100	115.3	0.18 ± 0.04*	42
100	115.3	0.11 ± 0.03*	26
200	197.2	0.07 ± 0.03	16

Table 5.3 A summary of the pooled concentration-response data for the effect of butylcyclohexane (BCH) upon mussel feeding rate

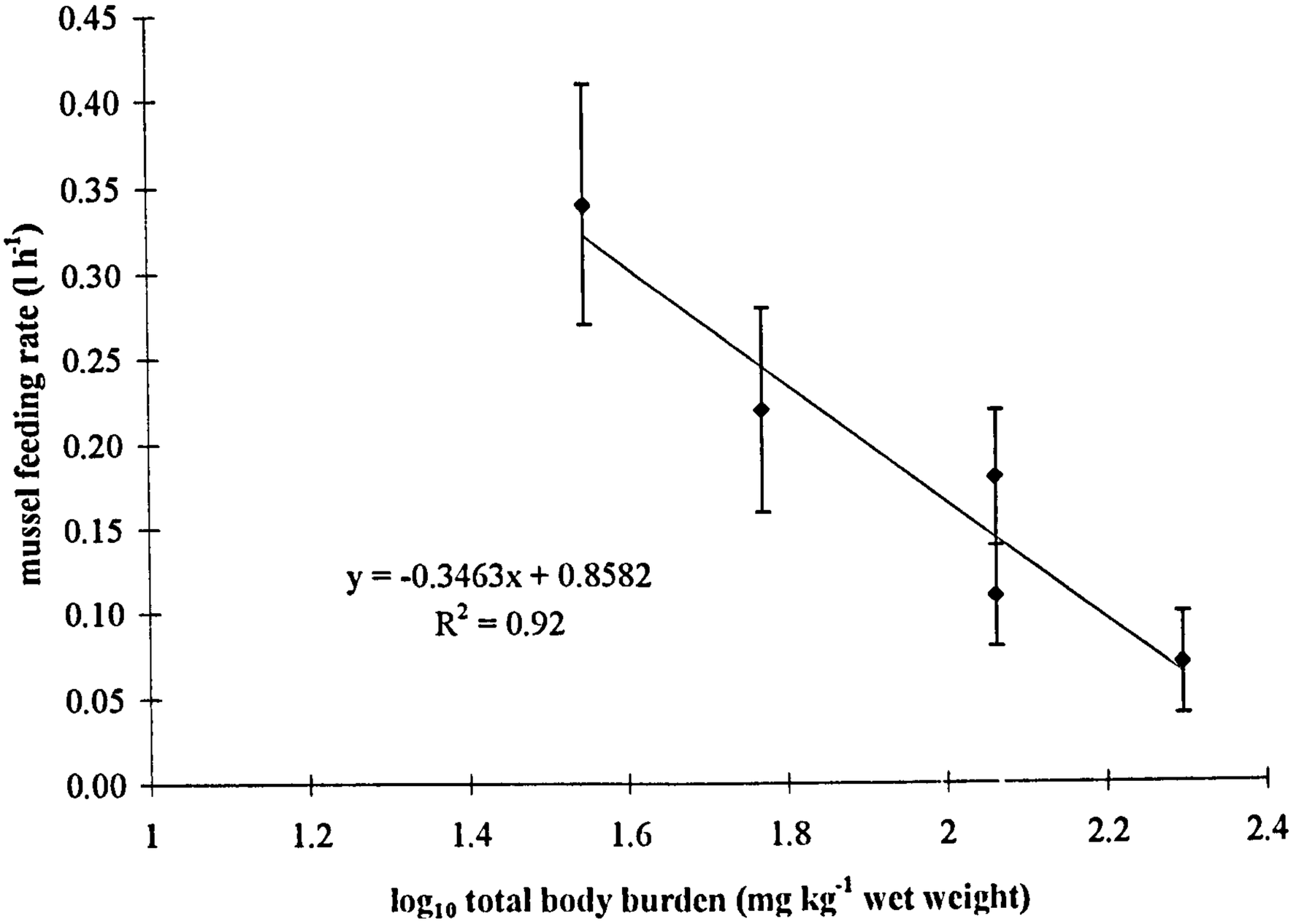


Figure 5.4 Concentration (tissue)-response curve for the effect of BCH upon mussel feeding rate over a 24 h exposure period

values plotted as mean ± s.d. (n=14 except where n=7 (See Table 5.3))

A TEC_{50} estimate of 72 mg kg^{-1} wet tissue weight (52.2 - 98.5 mg kg^{-1}; upper and lower 95 % confidence limits) was obtained. This gives a TEC_{50} value of 0.514 mmol kg^{-1} (0.373 - 0.703 mmol kg^{-1}, 95 % confidence limits). Comparison of this value with that obtained by Donkin *et al.* (1991; 40 mg kg^{-1} or 0.29 mmol kg^{-1} wet weight tissue) indicates that the TEC_{50} value obtained in the present study is slightly higher. Unfortunately, Donkin and co-workers did not cite the 95 % confidence intervals for their BCH TEC_{50} and thus the errors of this estimate are unknown. The crude TEC_{50} estimate for BCH made during studies of 4-PO in the present study (Section 4.3) was comparable with that of Donkin *et al.* (1991) at approximately 40 mg kg^{-1}. These variations in TEC_{50} values may be due to differences in the lipid content of the mussels used in each study. In addition, given the relationship between the concentration of

toxicant in the gill tissue and mussel feeding rate demonstrated in Section 4.3, it is probable that the slightly higher TEC_{50} estimate obtained in the present study occurred because the concentration of BCH in the gill tissue had reached a steady state early within the 24 h exposure period. The feeding rate of the mussels would therefore have reached a constant value, but the total body burden of BCH was still increasing, leading, as discussed previously, to a less accurate TEC_{50} estimate. The difference between the TEC_{50} estimates of Donkin *et al.* (1991) and the present study are, in any case very small. Previous studies relating total body burden of toxicant to toxic response (*e.g.* McCarty, 1986, 1987a,b; McCarty *et al.*, 1992a; Mortimer and Connell, 1995) have considered differences of less than one order of magnitude to be approximately constant. Thus the TEC_{50} value obtained by the present 'modified' method of feeding rate measurement can be considered entirely reasonable and shows that the revised method is applicable to toxicity measurements in mussels whilst at the same time representing a significant saving in time and cost. The revised method was therefore applied to the study of the two synthetic model compounds.

5.3.2 Concentration-response experiments for the effect of two model aromatic UCM compounds, 7-cyclohexyltetralin (7-CHT) and 7-cyclohexyl-1-propyltetralin (7-C-1-PT) upon mussel feeding rate over a 24 h exposure period

A summary of the effects of both aromatic UCM model compounds upon mussel feeding rate and tissue concentrations of toxicant measured in experiments with 7-CHT and 7-C-1-PT are presented in Tables 5.4 and 5.5 respectively. Concentration-

Nominal aqueous concentration ($\mu g\ l^{-1}$)	total body burden (mg kg^{-1} wet wt)	mussel feeding rate ($l\ h^{-1}$) mean ± sd (n=7)	mussel feeding rate expressed as a % of the control feeding rate
0 (control)	0	0.49 ± 0.04 (n=14)	100
12	22.9	0.30 ± 0.05	61
	25.3	0.33 ± 0.05	67
25	40.7	0.25 ± 0.06	51
	42.4	0.21 ± 0.06	43
50	79.6	0.15 ± 0.07	31
	80.5	0.18 ± 0.07	37
100	152.5	0.12 ± 0.05	24
	149.6	0.09 ± 0.04	18

Table 5.3 The effect of 7-cyclohexyltetralin (7-CHT) upon mussel feeding rate over a 24 h exposure period

Nominal aqueous concentration ($\mu g\ l^{-1}$)	total body burden (mg kg^{-1} wet wt)	mussel feeding rate ($l\ h^{-1}$) mean ± sd (n=7)	mussel feeding rate expressed as a % of the control feeding rate
0 (control)	0	0.47 ± 0.04 (n=14)	
12	23.7	0.51 ± 0.06	108
	20.6	0.48 ± 0.05	102
25	44.8	0.37 ± 0.05	79
	43.6	0.34 ± 0.04	72
50	120.9	0.27 ± 0.08	57
	129.7	0.28 ± 0.07	60
100	215.4	0.16 ± 0.11	34
	212.1	0.16 ± 0.07	34

Table 5.5 The effect of 7-cyclohexyl-1-propyltetralin (7-C-1-PT) upon mussel feeding rate over a 24 h exposure period

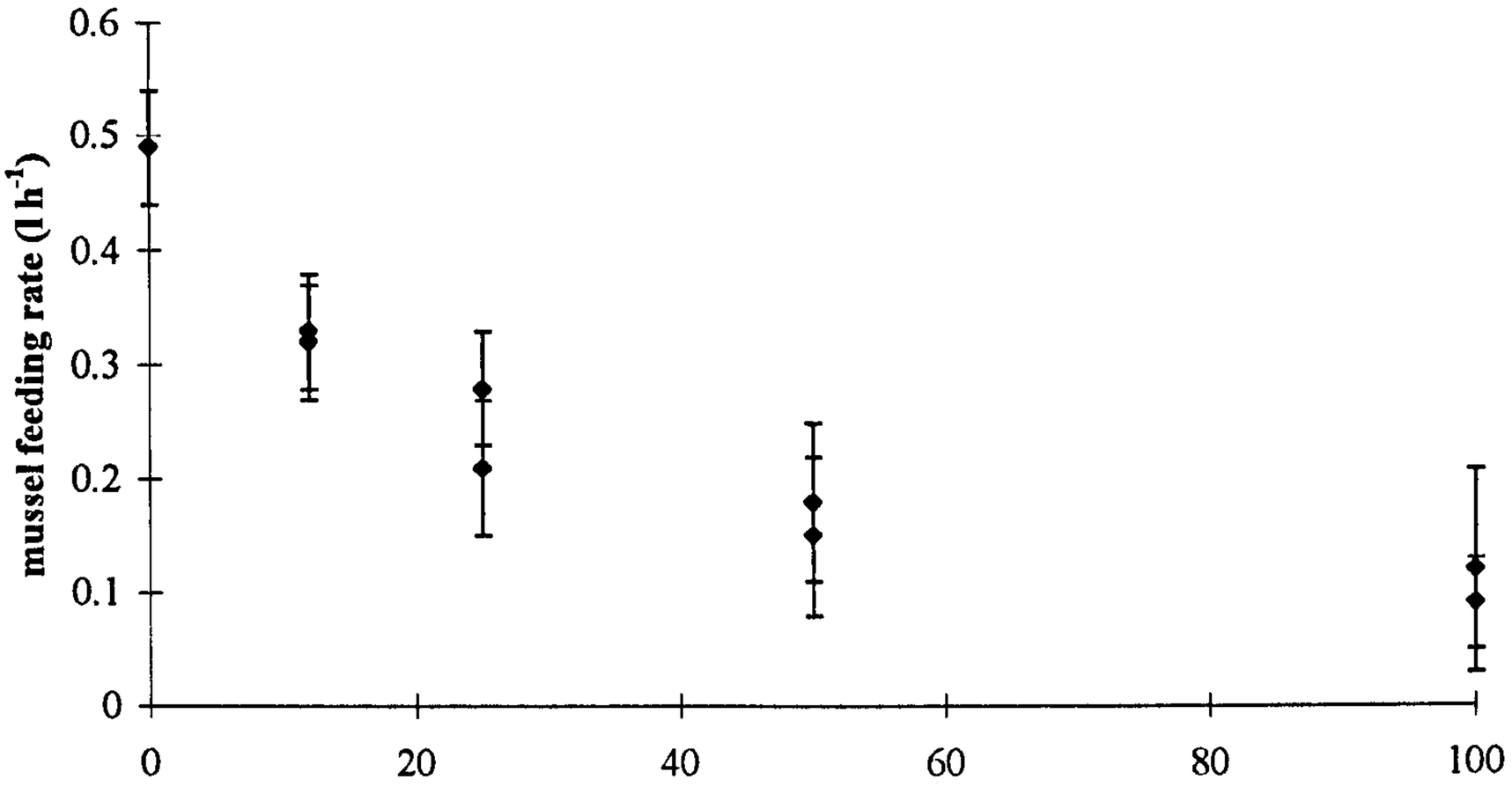


Figure 5.5 Concentration (aqueous)-response curve for the effect of 7-cyclohexyltetralin (7-CHT) upon mussel feeding rate over 24 h

(values plotted as mean ± sd, n=7)

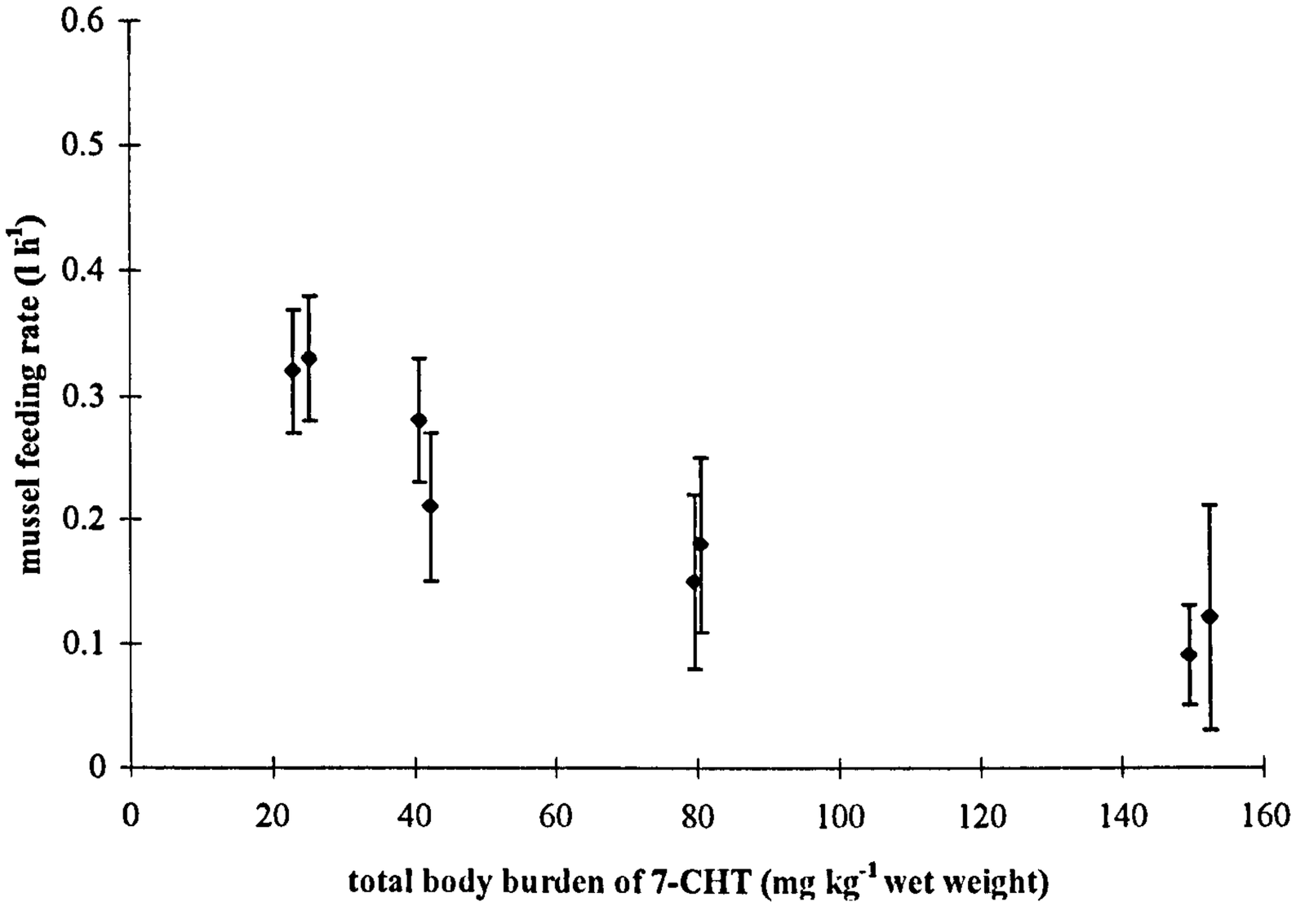


Figure 5.6 Concentration (tissue)-response curve for the effect of 7-cyclohexyltetralin (7-CHT) upon mussel feeding rate over 24 h

(values plotted as mean ± sd, n=7)

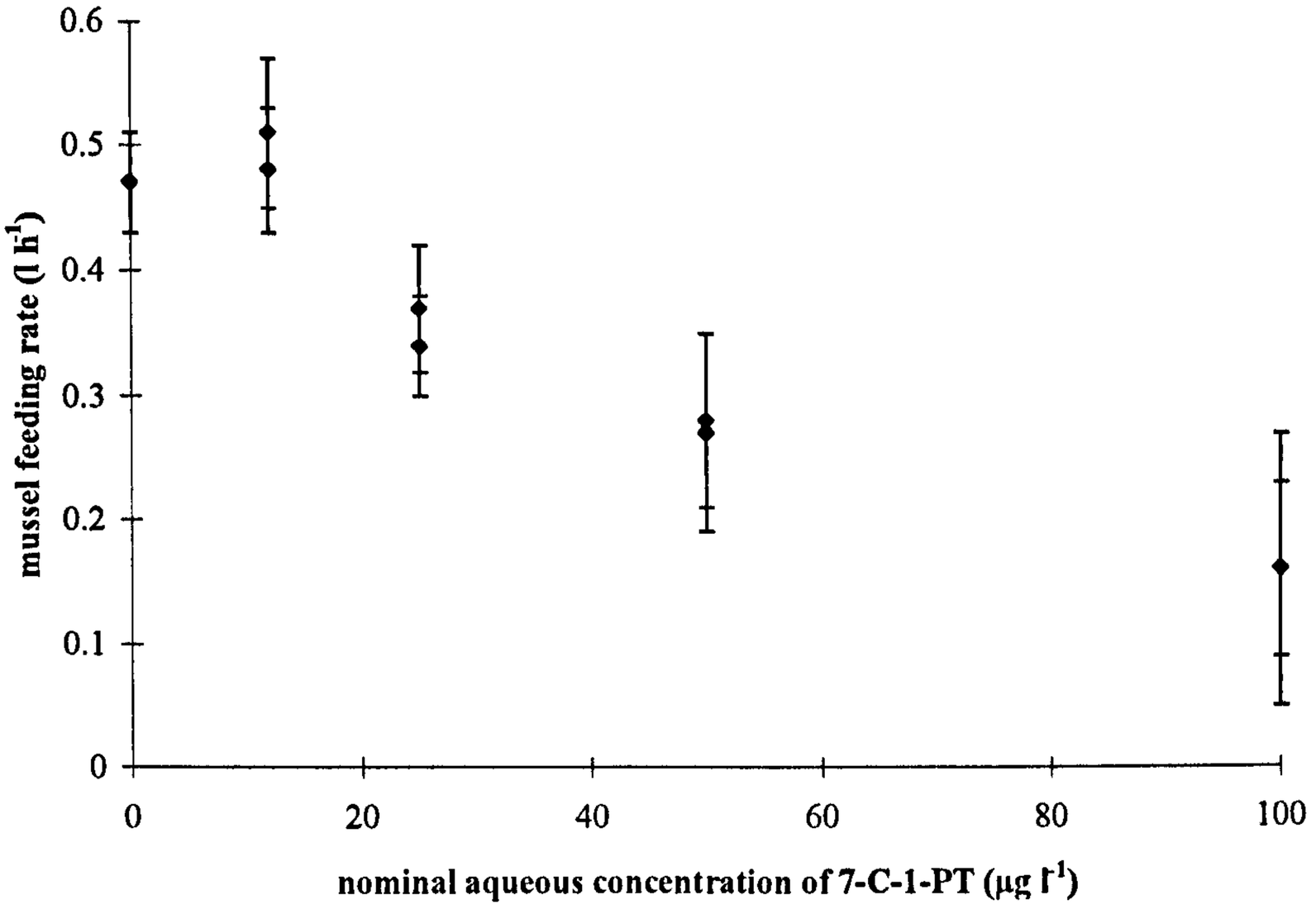


Figure 5.7 Concentration (aqueous) - response curve for the effect of 7-cyclohexyl-1-propyltetralin (7-C-1-PT) upon mussel feeding rate over a 24 h exposure period

(values plotted as mean ± sd, n=7)

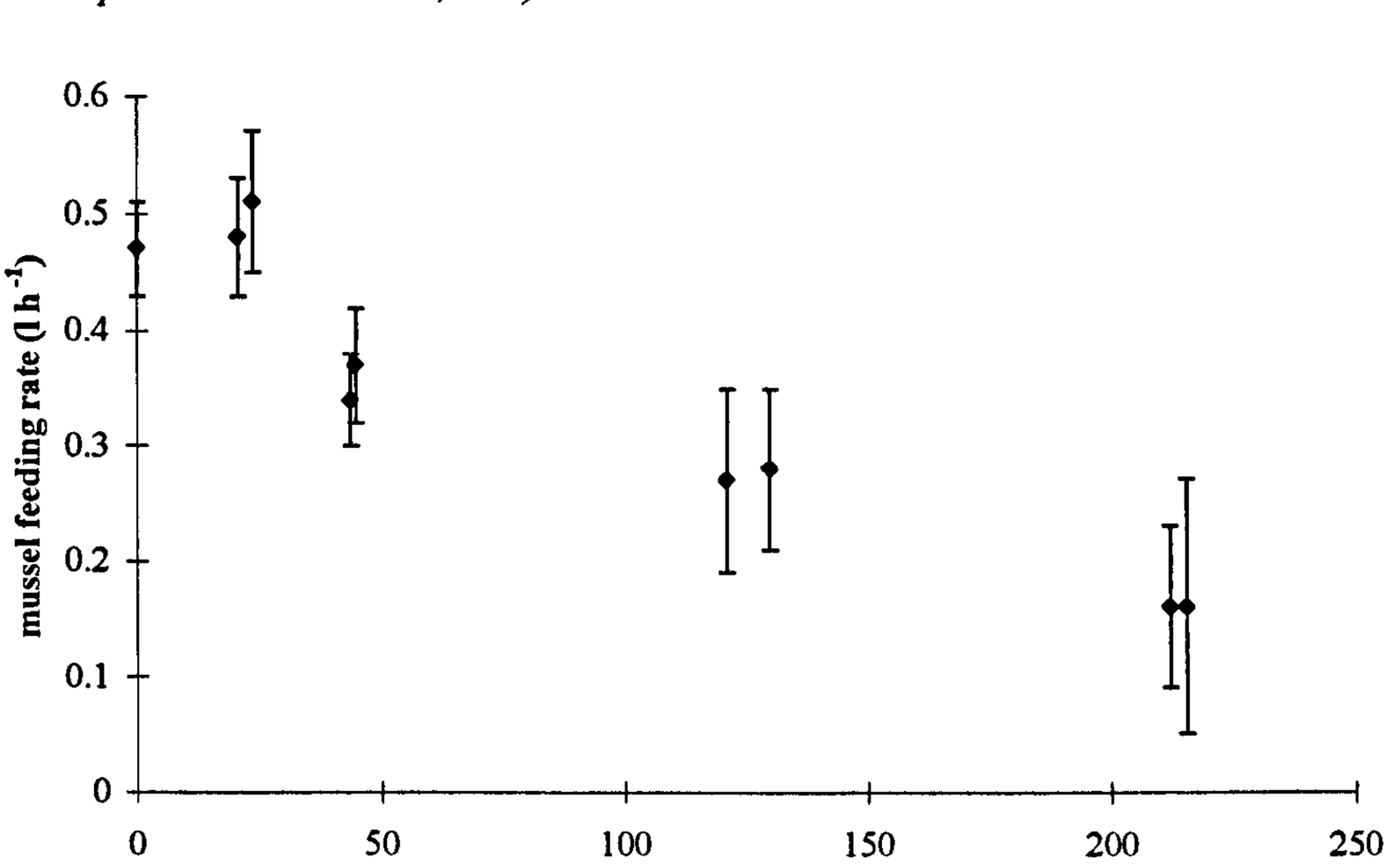


Figure 5.8 A concentration (tissue) - response curve for the effect of 7-cyclohexyl-1-propyltetralin (7-C-1-PT) upon mussel feeding rate over a 24 h exposure period *(values plotted as mean ± sd, n=7)*

response curves for the effect of both model aromatic UCM compounds upon mussel feeding rate over a 24 h exposure period (expressed in terms of both aqueous exposure concentration and total body burden of toxicant) are presented in Figures 5.5 - 5.8. Values are plotted as mean feeding rate ± standard deviation (n=7). It is apparent from these graphs that both 7-CHT and 7-C-1-PT have a narcotic effect upon mussel ciliary feeding activity.

Comparison of the feeding rates of replicate exposure concentrations within each individual experiment revealed no significant differences between the two replicates. Consequently, replicate data for each exposure concentration was pooled prior to estimation of the TEC_{50} value for each test compound. Summaries of the pooled data for both 7-CHT and 1-P-7-CHT are presented in Tables 5.6 and 5.7. TEC_{50} estimates for the effect of each model aromatic UCM compound upon mussel feeding rate were obtained in an identical manner to that described previously for BCH (Section 5.3.1) and are presented in Table 5.8. Figures 5.9 and 5.10 illustrate the concentration (tissue)-response curves used in the calculation of TEC_{50} estimates.

Nominal aqueous concentration of 7-CHT ($\mu g\ l^{-1}$)	**total body burden of toxicant (mg kg^{-1} wet wt) (mean, n=2)**	**mussel feeding rate ($l\ h^{-1}$) mean ± sd (n=14)**	**mussel feeding rate expressed as a % of the control feeding rate**
0 (control)	0	0.49 ± 0.04	100
12	24.13	0.32 ± 0.05	65
25	41.53	0.23 ± 0.06	51
50	80.08	0.17 ± 0.07	35
100	151.03	0.10 ± 0.05	20

Table 5.6 Summary of the concentration-response data for the effect of 7-cyclohexyltetralin (7-CHT) upon mussel feeding rate, 24 h exposure

nominal aqueous concentration of 7-C-1-PT ($\mu g\ l^{-1}$)	total body burden of toxicant (mg kg^{-1} wet wt) (mean, n=2)	mussel feeding rate ($l\ h^{-1}$) mean ± sd (n=14)	mussel feeding rate expressed as a % of the control feeding rate
0 (control)	0	0.47 ± 0.04	100
12	22.1	0.50 ± 0.05	106
25	44.2	0.36 ± 0.05	77
50	125.3	0.28 ± 0.07	60
100	213.8	0.16 ± 0.09	34

Table 5.7 A summary of the concentration (tissue)-response data for the effect of 7-cyclohexyl-1-propyltetralin (7-C-1-PT) upon mussel feeding rate, 24 h exposure

Test compound	TEC_{50} mg kg^{-1} (mmol kg^{-1} wet wt tissue)	Lower 95 % confidence limit	Upper 95 % confidence limit
7-cyclohexyltetralin	41.1 (0.206)	42.1mg kg^{-1} (0.196 mmol kg^{-1})	46.2 mg kg^{-1} (0.216 mmol kg^{-1})
7-cyclohexyl-1-propyltetralin	138 (0.539)	91.0 mg kg^{-1} (0.355 mmol kg^{-1})	204.2 mg kg^{-1} (0.797 mmol kg^{-1})

Table 5.8 TEC_{50} values for the effect of the model aromatic UCM hydrocarbons 7-CHT and 7-C-1-PT upon mussel ciliary feeding activity

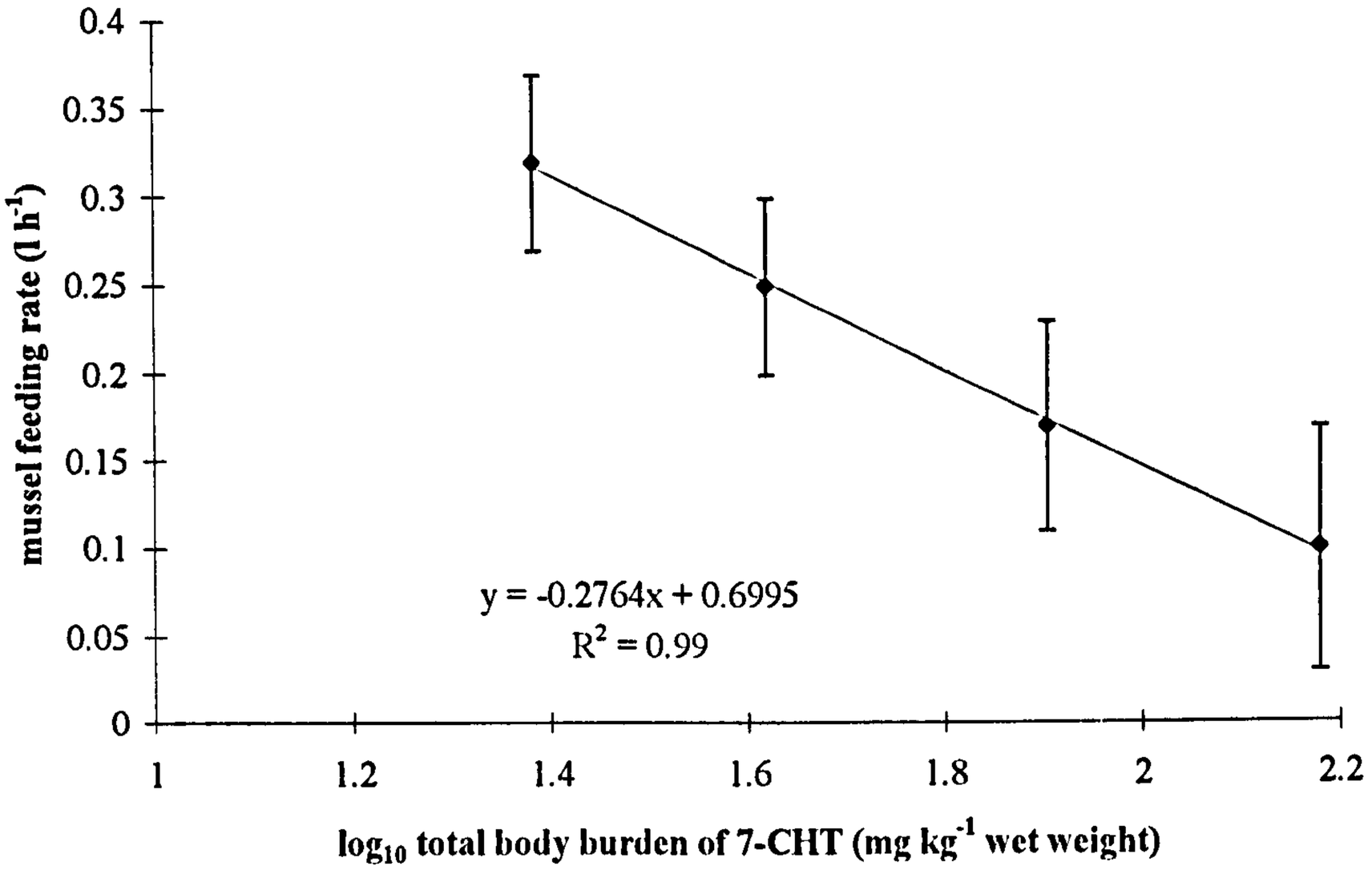


Figure 5.9 Concentration (tissue)-response curve for the effect of 7-cyclohexyltetralin (7-CHT) upon mussel feeding rate over a 24 h exposure period *(values plotted as mean ± sd, n=14)*

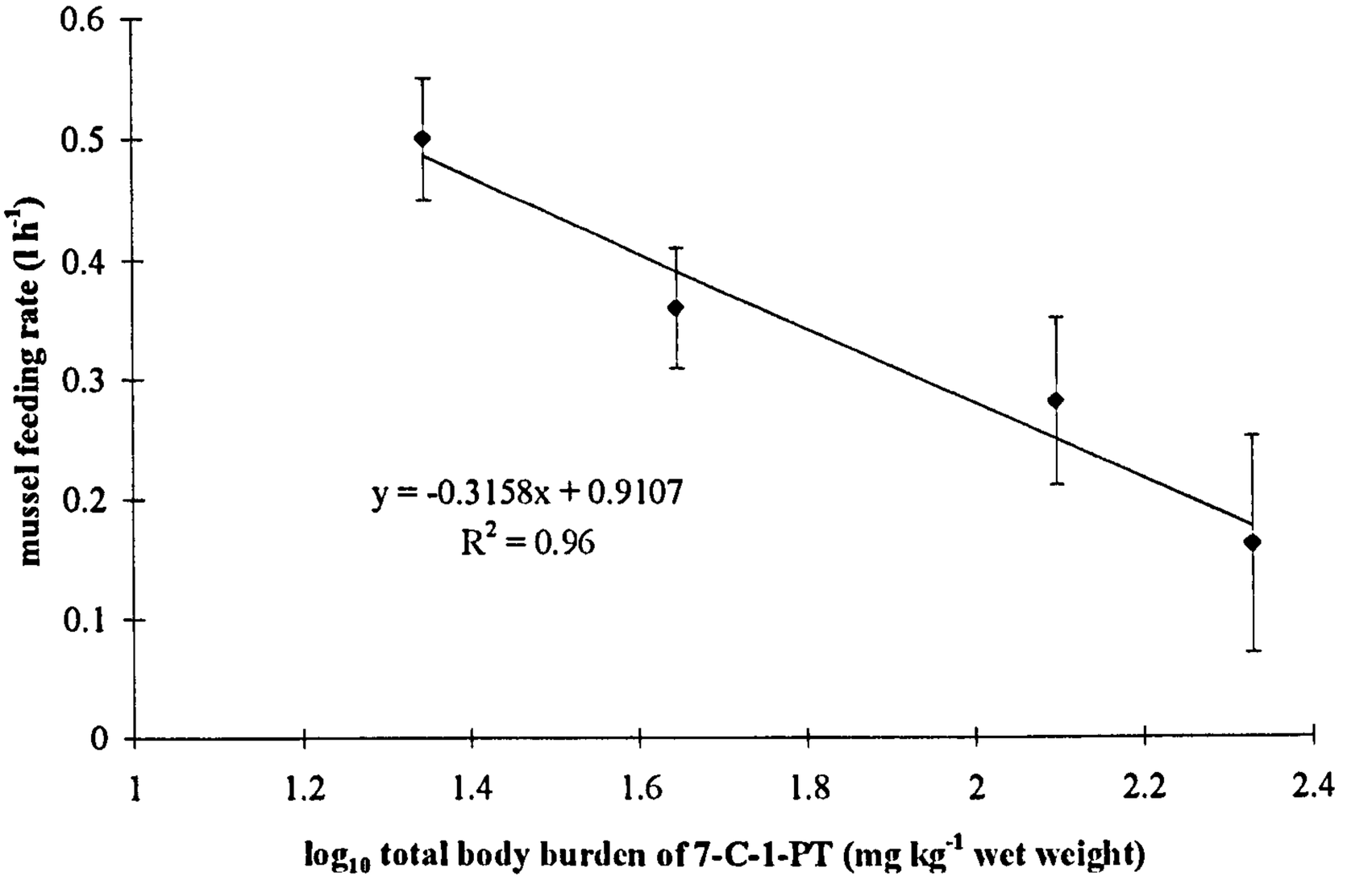


Figure 5.10 Concentration (tissue)-response curve for the effect of 7-cyclohexyl-1-propyltetralin (7-C-1-PT) upon mussel feeding rate over a 24 h exposure period *(values plotted as mean ± sd, n=14)*

5.4 Discussion

The aim of the present study was to investigate whether hydrocarbons with structures similar to those proposed as 'average' model structures of aromatic UCM hydrocarbons (Revill *et al.*, 1997; Thomas *et al.*, 1997) have a narcotic effect upon mussel ciliary feeding activity.

A further aim was to modify the established method of mussel feeding rate determination, in order to develop a simple and reproducible technique to rapidly assess if compounds are toxic or non-toxic, thus enabling further research to be directed towards those compounds of greatest toxicological interest. This was achieved by using mussels of a smaller size (12 mm, shell length) compared with the animals used in previous studies (40 mm shell length; Chapter 4, present study; Donkin *et al.*, 1989, 1991). The use of smaller animals enabled the total volume of seawater necessary for an experiment to be reduced. This effectively reduced the time, space and quantities of test chemicals required.

The study has shown that both of the model aromatic UCM hydrocarbons tested were toxic to mussel ciliary feeding activity. At nominal aqueous exposure concentrations of greater than 25 $\mu g\ l^{-1}$ and approximately 75 $\mu g\ l^{-1}$ for 7-cyclohexyltetralin and 1-propyl-7-cyclohexyltetralin respectively a reduction in mussel feeding rate of greater than 50 % was observed.

A comparison of the TEC_{50} values and physico-chemical data obtained in the present study with those reported for a number of aromatic hydrocarbons by Donkin *et al.* (1989, 1991) is presented in Table 5.9. The narcotic action of 7-cyclohexyl-1-propyltetralin upon mussel feeding rate demonstrated herein is surprising if the predicted log K_{ow} and aqueous solubility values are accurate. According to the

Compound	Physical properties of compound[3]		Biological properties of compound (TEC_{50})	
	Log K_{ow}	Solubility ($\mu g\ l^{-1}$)	mg kg^{-1} (wet wt)	mmol kg^{-1} (wet wt)
Toluene (C_7)	2.65	578600	16	0.17
Naphthalene (C_{10})	3.35	30640	31	0.24
n-Phenylpropane (C_9)	3.69	52170	27	0.23
1-Chloronaphthalene (C_{10})	4.08	22490	22	0.14
Acenaphthene (C_{12})	3.92	3930	29	0.19
Biphenyl (C_{12})	3.95	7000	16	0.10
Phenanthrene (C_{14})	4.57	1180	31	0.17
Dibenzothiophene (C_{12})	4.42	no data	14	0.08
Pyrene (C_{16})	5.18	135	> 189	>0.94
Fluoranthene (C_{16})	5.22	263	627	310
1-Phenylheptane (C_{13})	6.11	269	35	0.20
7-cyclohexyltetralin (C_{16})	**6.33[1] 6.77[2]**	**151[1] 60[2]**	**41**	**0.206**
1-Phenyloctane (C_{14})	6.73	71	82	0.43
1-Phenyldecane (C_{16})	7.55	5	>78	>0.36
1-propyl-7-cyclohexyltetralin (C_{19})	**7.91[1] 8.17[2]**	**8[1] 8[2]**	**138**	**0.539**

[1] *values calculated using MedChem software*

[2] *values calculated using Syracuse Research Corporation Software (SRC)*

[3] *All physical data used by Donkin et al. (1989) originated from Miller et al. (1985) except for (a) water solubility of 1-chloronaphthalene, from Bobra et al. (1984) and it's log K_{OW} value from Foster and Tullis (1984); (b) log K_{OW} of dibenzothiophene from Ogata et al. (1984)*

Table 5.9 The effect of hydrocarbons on the filter feeding rate of mussels (*Mytilus edulis*) - a synthesis of the data of Donkin *et al.* (1989, 1991) with that obtained in the present study

established QSAR (Donkin *et al.*, 1989, 1991) for the effect of hydrocarbons upon mussel feeding rate, a highly hydrophobic compound such as 7-cyclohexyl-1-propyltetralin (log K_{ow} 7.96-8.17, aqueous solubility, 3 $\mu g\ l^{-1}$; mean of two estimates) would be considered non-toxic because of a low aqueous solubility. As illustrated in Table 5.9, 1-phenyldecane, tested by Donkin *et al.* (1991) is of comparable hydrophobicity to 7-cyclohexyl-1-propyltetralin but exposure of *M. edulis* to 1-phenyldecane failed to produce a response. As discussed previously, errors in the estimated log K_{ow} and aqueous solubility values may be large, and the true log K_{ow} value for 7-cyclohexyl-1-propyltetralin may be much lower and the compound may have sufficient aqueous solubility to induce narcosis.

As both model aromatic UCM hydrocarbons were toxic, the present work has clearly shown that the aromatic UCM, an environmental burden previously ignored by the majority of workers, is of some toxicological significance and should prove to be an interesting area for further investigation. Clearly, an area of future work should be to measure accurately the aqueous solubilities of the two hydrocarbons tested. Interestingly, a comparison of the toxicity of aromatic hydrocarbons of similar carbon number, [*e.g.* 1-phenyldecane (C_{16}), pyrene (C_{16}) and fluoranthene (C_{16})], tested by Donkin *et al.* (1989, 1991), with those studied in the present study [7-CHT (C_{16}), 7-C-1-PT, (C_{19})] suggests that the narcotic cut-off has been effectively extended by consideration of hydrocarbons of the type tested herein. Donkin *et al.* (1989,1991) reported no measurable effect upon mussel feeding rate for either fluoranthene or 1-phenyldecane, and no consistent response for pyrene and suggested that the results obtained for fluoranthene and pyrene were anomalous (these compounds being of relatively high aqueous solubility; 263 $\mu g\ l^{-1}$ and 135 $\mu g\ l^{-1}$, respectively). The authors suggested that the apparent lack of toxicity might be due to sequestration or some

other mechanism which reduced the concentration available at the active site (Donkin *et al.*, 1991). However, the lack of narcotic action observed for 1-phenyldecane (aqueous solubility 5 $\mu g\ l^{-1}$) was considered to be indicative of a narcotic cut-off. The present study has clearly demonstrated that 7-cyclohexyltetralin (C_{16}) has a narcotic effect upon mussel feeding rate. This suggests that alicyclic moieties such as those present on 7-cyclohexyltetralin increase the solubility of the hydrocarbon sufficiently (when compared with acyclic moieties of a comparable size) to induce a narcotic effect. Indeed, the C_{19} hydrocarbon tested in this study (7-cyclohexyl-1-propyltetralin) has also been demonstrated to be of sufficient aqueous solubility to be considered a narcotic toxicant, effectively extending the molecular weight range of narcotic chemicals. Further work is now required to attempt to define the narcotic cut-off more accurately, in order to be able to predict the narcotic effects of aromatic UCM hydrocarbons bioaccumulated within marine organisms such as mussels.

5.5 Conclusions

The work described herein has demonstrated that exposure to either of the two synthetic model aromatic UCM hydrocarbons can cause a significant reduction in mussel feeding rate. To the best of the author's knowledge, this is the first study to investigate the toxicity of model aromatic UCM hydrocarbons. The aromatic UCM is a quantitatively significant, but rarely reported, environmental burden in petroleum hydrocarbon contaminated mussels (*cf.* Section 2.4). The demonstrable narcotic activity of the two hydrocarbons studied herein, suggests that the aromatic UCM is not only a quantitatively significant environmental burden, but a proportion of the low molecular weight aromatic UCM hydrocarbons may also be toxicologically significant and should not be ignored in future environmental monitoring programmes. Clearly,

further work is required to define the narcotic cut-off and determine what proportion of aromatic hydrocarbon UCMs bioaccumulated by marine mussels is toxic.

CHAPTER SIX

Experimental details

6.1 General Laboratory Procedures

All glassware was soaked in a 5% solution of Decon 90™, rinsed thoroughly with hot tap water, followed by a final rinse in distilled water and oven dried (110°C). After drying, the glassware was covered with aluminium foil. Immediately prior to use all glassware was rinsed (x 3) with clean solvent.

All solvents were HPLC or glass distilled grade (Rathburn Chemicals Ltd., Walkerburn, U.K.). Solvent purity was routinely monitored by rotary evaporation (Buchi, 40°C, 100ml), transfer to a vial, evaporation to approximately 0.5ml and analysis of an aliquot (0.5 µl) by gas chromatography (GC). Deionised water (Milli-Q grade) was further purified by extraction with DCM (x 3).

Silica gel (Aldrich, 100 mesh) and aluminium oxide (BDH, grade 1, neutral, 150 mesh) adsorbents used for chromatographic separations were Soxhlet extracted (DCM, 24 h) prior to use. Adsorbents of the required activity were prepared by activation (silica, 24 h, 120°C; aluminium oxide, 12h, 450°C), followed by cooling in a dessicator prior to de-activation by addition of water (pre-extracted Milli-Q) and mechanical shaking (3-5 h) to ensure homogenisation. Once prepared; adsorbents were stored in a dessicator and used within 24 h. Typically, aluminium oxide was deactivated to 1.5 % (w/w) and silica was deactivated to 5 % (w/w). In the instances when fully activated silica was employed, the silica was removed from the oven, cooled in a dessicator and used immediately.

Anhydrous sodium sulphate, cotton wool, anti-bumping granules and Soxhlet thimbles were Soxhlet extracted (DCM, 24 h) prior to use. Sodium hydroxide pellets were sonicated (10 mins x 3) in DCM, and hydrochloric acid was extracted with DCM (x 3).

6.2 Instrumental details

6.2.1 Gas chromatography (GC)

Instrument	Carlo Erba 5300 Mega series gas chromatograph
Column	DB-5 fused silica capillary column, 25 m x 0.32 mm i.d. (J & W inc.)
Injector	On-column
Carrier gas	Hydrogen, flow rate 2 ml min^{-1}
Oven temperature programme	40° - 300° @ 5° min^{-1}, held @ 300°C for 10 minutes

Column performance was qualitatively monitored daily by injection of an alkane mixture. All chromatograms were recorded using a Shimadzu C-R4A chromatopac integrator.

6.2.2 Gas chromatography - mass spectrometry (GC-MS)

GC details;

Instrument	Hewlett Packard MSD GC-MS
Column	HP-1 Ultra, fused silica column. 12 m x 0.2 mm i.d. (Hewlett Packard)
Injector	Auto splitless injection (250°C)
Carrier gas	Helium (40 kPa head pressure)
Oven temperature programme	40-300°C @ 5°C min^{-1}, hold @ 300°C 10 minutes

Mass spectrometer operating conditions;

Ion source temperature	250°C
Ionisation energy	70 eV
Mass range	35-600 Daltons

6.2.3 Nuclear Magnetic Resonance Spectroscopy (NMR)

^{13}C and ^{1}H-NMR spectra of samples in deuterated chloroform (trimethylsilane reference) were recorded using a Jeol EX270 MHz high resolution FT-NMR spectrometer.

6.2.4 Infra red spectroscopy (IR)

Infra-red spectra were recorded as liquid films (NaCl disks) using a Brücker IFSS spectrometer, resolution 4 cm^{-1}, 32 sample scans, 4000 - 400 wave numbers.

6.3 Determination of aliphatic and aromatic UCM hydrocarbon concentrations in mussels from U.K. coastal sites

6.3.1 Sample collection

Mussels (*Mytilus edulis*) were collected from the various sampling locations (*cf.* Section 2.4.1, Figure 2.10), wrapped in solvent rinsed aluminium foil and immediately transported back to the laboratory in Plymouth packed in dry ice. Soft tissue was then dissected from the shell over ice to minimize losses of volatile analytes, homogenised and stored in clean, solvent rinsed glass jars at -20° C until required.

6.3.2 Authentic compounds/deuterated internal standards

Authentic 5-ethyltetralin, 2-ethylnaphthalene and 1,3-diphenylhexane were provided by Dr M. Hodges, (BP Research and Engineering). Samples of *n*-phenyldecane, 4-pentylbiphenyl, phenanthrene, pyrene, acetophenone, 9-fluorenone, benzoic acid,

cyclohexanecarboxylic acid, 9-anthracenecarboxylic acid, hexanedioic acid, 1-naphthol and 9-hydroxyfluorene were purchased from Aldrich Ltd. 7-*n*-hexylnonadecane was available from previous synthetic studies in this laboratory (Gough, 1989). 4-propyloctane was synthesised in the present study (*cf.* Section 6.4). Purity of all compounds was greater than 98 % (determined by GC).

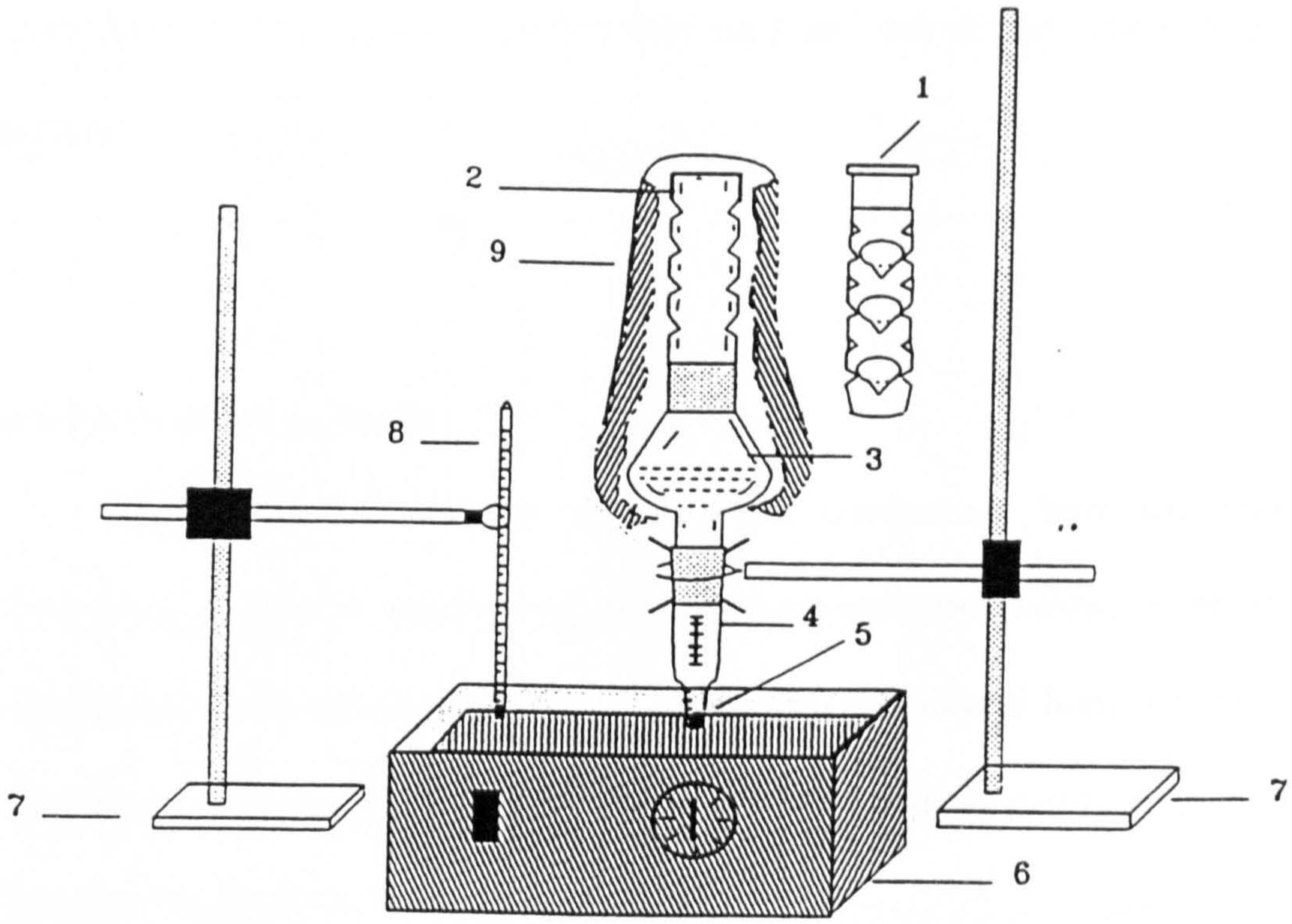
Deuterated standards (d_{10}-1-methylnaphthalene, d_{12}-tetralin and d_{10}-phenanthrene) were obtained from Aldrich Ltd. Purity of all deuterated standards was greater than 98 % (determined by GC).

6.3.3 Sample concentration

For the recovery experiments described in Section 2.3.3, authentic compounds were spiked into 250 ml DCM and concentrated to a final volume of 0.5 ml by either rotary evaporation, macro-Kuderna Danish (K.D.) or a combination of rotary evaporation and micro-K.D.

6.3.3.1 Rotary evaporation + micro-K.D. (controlled evaporation)

The sample was concentrated to a volume of *ca* 35 ml by rotary evaporation. Following rotary evaporation, the sample was transferred to the micro-K.D. apparatus (Supelco). An anti-bumping granule was added and the apparatus (with the exception of the receiving vessel) lagged in cotton wool and foil for insulation. The apparatus was then held in a water bath maintained at 52°C, with the receiving vessel fully immersed in water. Solvent volume was reduced to the required volume (typically 1 ml) and transferred quantitatively to a clean vial. The K.D. concentrator is illustrated in Figure 6.1.



1=snyder column; 2= micro condenser, 112 mm; 3= flask, 40 ml; 4=receiving vessel, 2 ml; 5= boiling chip; 6=water bath; 7=laboratory stand; 8=thermometer; 9=aluminium foil and cotton wool wrap

Figure 6.1 The micro-Kuderna Danish apparatus (Ali, 1994)

6.3.3.2 Macro-K.D.

The procedure employed was as described above in Section 6.3.3.1, but a macro-K.D. apparatus (250 ml volume capacity) was used and use of the rotary evaporator was omitted.

6.3.4 Extraction methods

To measure the recovery of the authentic compounds from the mussel tissue homogenate using the various extraction methods described below, authentic reference compounds dissolved in acetone were spiked into wet mussel homogenate. The tissue was then mixed thoroughly and left at 4°C for 4 h to allow partitioning of the analytes into the mussel tissue.

6.3.4.1 Soxhlet extraction (DCM:methanol)

Wet mussel homogenate (*ca* 40 g) was ground with anhydrous sodium sulphate, placed in a pre-extracted Soxhlet thimble and Soxhlet extracted using DCM:methanol (1:1, v/v; 24 h). The extract was then transferred to a separating funnel and extracted (x 3) with acidified pre-extracted water (pH 1, conc. HCl).

The aqueous phase was then washed (x 4) with 20 ml aliquots of DCM. The organic layers were combined and dried (18 h) over anhydrous sodium sulphate. The solution was then filtered and the solvent removed by controlled evaporation (*cf.* Section 6.3.3.1) to a volume of approximately 1 ml. The solvent system was then changed from DCM to *n*-hexane under a gentle stream of nitrogen to provide the sample in a non-polar solvent for fractionation purposes. The total organic extract (TOE) was analysed by GC.

6.3.4.2 Soxhlet extraction (DCM)

Wet mussel homogenate (*ca* 40 g) was ground with anhydrous sodium sulphate, placed in a pre-extracted Soxhlet thimble and extracted (Soxhlet, DCM, 24 h). The TOE was dried over anhydrous sodium sulphate (18 h) and the solvent removed by controlled evaporation to a final volume of approximately 1 ml, as described previously (Section 6.3.3.1). The solvent system was then changed from DCM to *n*-hexane under a gentle stream of nitrogen and the sample analysed by GC.

6.3.4.3 Two-phase extraction method

The method used was a modification of that described by Rhead *et al* (1971). Wet mussel tissue (*ca* 40 g) was acidified to pH 1 (conc. HCl) and 15 ml of a mixture of *n*-pentane:2-propanol (1:4, v/v, added). The resulting mixture was sonicated for 40 minutes. A further 120 ml of *n*-pentane and 117 ml of pre-extracted water were then added. The mixture was gently shaken for 5 minutes, followed by centrifugation at 2000 rpm for 20 minutes. The upper (*n*-pentane) layer was transferred to a stoppered conical flask and the lower aqueous layer decanted and retained. The procedure was repeated (x 2) and the *n*-pentane layers combined and dried over anhydrous sodium sulphate (18 h). The sample was concentrated to 1 ml by controlled evaporation (Section 6.3.3.1) and analysed by GC.

6.3.5 Fractionation of mussel TOE by open column chromatography

The total organic extract was fractionated into 'aliphatic', 'aromatic' and 'polar' fractions using a glass column (700 mm x 20 mm) packed with a *n*-pentane slurry of silica (60 - 100 mesh; fully activated, 20 g) under aluminium oxide (grade 1, neutral, 1.5 % deactivated, 20 g). A sample to adsorbent ratio of 1:200 (w/w) was employed. The

sample was applied to the top of the column and sequential elution of the column with solvents of increasing polarity yielded the desired fractions:

F_1 ('aliphatic'); 1.5 column volumes *n*-pentane

F_2 ('aromatic'); 2 column volumes *n*-pentane:DCM (1:1, v/v)

F_3 ('polar'); 2 column volumes DCM

F_4 ('polar'); 2 column volumes methanol

Fractions F_1 and F_2 were concentrated by controlled evaporation (Section 6.3.3.1) and analysed by GC.

6.3.6 Normal phase HPLC separation of aromatic fractions of mussel extract

A ring size separation of the 'aromatic' fraction obtained by open column chromatography (Section 6.3.5) was obtained using normal phase HPLC with a cyano/amino bonded phase according to a slight modification of the method of Killops and Readman (1985).

HPLC instrumental details were as follows;

Pumps	Merck-Hitachi L 6200A intelligent pump and L-6000 LC pump
Detector	UV/VIS spectrophotometer at 254 nm
Columns	Two 25 cm x 25 mm i.d. Partisil 5µm PAC columns (Cyano:amino (1:2) bonded phase, Whatman) connected in series. A guard column was also fitted
Mobile phase/solvent gradient	100 % *n*-hexane to 100 % DCM in 30 minutes, flow rate 1.5 ml min^{-1}

A mixture of authentic aromatic hydrocarbons (5-ethyltetralin, 2-ethylnaphthalene and phenanthrene) was examined daily to monitor fractionation reproducibility. Fractions were collected as follows;

Cut 1	0-9 mins	'mono-aromatic' hydrocarbons
2	9-13 mins	'di-aromatic' hydrocarbons

3	11-15 mins	'tri-aromatic' hydrocarbons
4	15-30 mins	> 3- ring PAHs

Each fraction was concentrated under a gentle stream of nitrogen to approximately 100µl and analysed by GC.

6.3.7 Quantification of authentic compounds in method validation

Quantification of authentic compounds for method validation was performed using either GC or GC-MS.

The percentage recovery of authentic compounds spiked into mussel tissue was calculated from external standard calibration graphs. A standard mixture containing the relevant authentic compounds (*cf.* Table 2.1) was prepared. Aliquots of this standard mixture were removed and accurately diluted to produce a concentration range covering that necessary for analysis of the 'spiked' samples. The peak areas obtained for each compound, using a Shimadzu CR4-A recording integrator, were plotted against the known concentration in the mixture to produce a calibration graph for each compound ($R^2 \geq 0.998$). The peak area of the 'spiked' authentic compound was then measured and the concentration read from the relevant calibration graph.

Analyses by GC were all performed using the same syringe, thoroughly rinsed between samples to avoid cross contamination. GC-MS analyses employed auto-injection.

6.3.8 Quantification of total unresolved and resolved hydrocarbons

Samples were analysed by GC-MS (instrumental details in Section 6.2.2) and quantification performed using the Chemstation™ (Hewlett Packard) software.

Integration parameters were as follows;

total hydrocarbons (resolved + unresolved);

threshold = -22

peak width = 0.074

area reject = 0

AREASUM on, 3.00 mins

AREASUM off, 61.5 mins

resolved peaks;

threshold = 18.9 - 20

peak width = 0.074

area reject = 0

The total resolved peaks were then subtracted from the total hydrocarbons to give a value for the area of the unresolved component. Concentrations of resolved and unresolved hydrocarbons were calculated using an average response factor, calculated from the internal standards over the appropriate molecular weight range.

6.3.9 Reproducibility of integration software

To measure the reproducibility of the integration package used, five replicate samples of Mobil Velocite oil were analysed by GC-MS and the total peak area of both the resolved and unresolved hydrocarbons measured. The results are presented in Table 6.1, with the mean and relative standard deviation (RSD) of five analyses given. RSD values are 0.7 % and 0.6 % for the resolved and unresolved hydrocarbons respectively.

Injection	area of resolved components (as % of total peak area)	area of UCM components (as % of total peak area)
1	44.2 %	55.8 %
2	44.5 %	55.6 %
3	45.0 %	55.0 %
4	44.8 %	55.2 %
5	44.3 %	55.2 %
mean	**44.6 %**	**55.4 %**
rsd	**0.7 %**	**0.6%**

Table 6.1 Precision and reproducibility of the Chemstation™ integration software used for quantification

6.4 Synthesis of model aliphatic and aromatic hydrocarbons.

6.4.1 Synthesis of 4-propyloctane (4-PO)

Synthesis of 4-propyloctane followed the scheme outlined in Figure 3.2, involving the Grignard coupling of propyl magnesium bromide to ethylpentanoate. A similar synthetic scheme was successfully employed in a previous study to synthesis a higher molecular weight 'T-branched' alkane, 7-hexylnonadecane (Gough, 1989). The purity and authenticity of all starting materials was confirmed by GC-MS and IR.

6.4.1.1 Synthesis of 4-propyloctan-4-ol

As Grignard reagents are extremely moisture sensitive, great care was taken to ensure exclusion of any moisture from the glassware and reagents. Glassware was oven dried (120°C, 18 h), assembled whilst hot and immediately placed under a stream of nitrogen. Magnesium ribbon was also oven dried prior to use. Sodium-dried diethyl ether was used and all other reagents were dried over anhydrous sodium sulphate. Whilst in use, the reaction glassware was protected from moisture with calcium chloride drying tubes.

Magnesium turnings (2.4 g, 0.1 mol) were placed in a round bottom flask with approximately 30 ml dry diethyl ether. 1-Bromopropane (10.5 ml, 0.115 mol) was dissolved in diethyl ether (20 ml, Na-dried) and approximately 5 ml of this mixture added to the magnesium turnings in the round bottom flask. The mixture was gently heated and magnetically stirred until the formation of a cloudy precipitate was observed. The remainder of the bromopropane-diethyl ether mixture was then added dropwise to the reaction mixture over a 30 minute period, with constant stirring. The reaction mixture was gently refluxed for a further 30 minutes.

Once the reaction mixture had cooled, ethylpentanoate (6.7 ml,0.045 mol) dissolved in diethyl ether was added dropwise with stirring to maintain a gently refluxing solution and

then heated gently under reflux for a further 30 minutes. When cool, the reaction mixture was poured onto ice (28 g) and saturated ammonium chloride solution (50 ml) and mixed well. Extraction with diethyl ether (3 x 20 ml) followed by rotary evaporation to remove the solvent, yielded the crude reaction products (clear yellow oil).

Crude reaction products were purified by vacuum distillation at 10 mm Hg. Pure 4-propyloctane was collected at 107°C (@ 10 mm Hg). The purified alcohol was then analysed by GC and characterised by MS and NMR (^{1}H , ^{13}C and DEPT).

GC purity: > 99 %

Yield: 44 %

MS: *m/z* 154 ($M^{+\cdot}$-H_2O, 3%); 129 ($M^{+\cdot}$ - C_3H_7, 100 %); 115 ($M^{+\cdot}$-C_4H_9, 75 %)

^{13}C-NMR: (ppm), 74.3, 41.6, 38.9, 25.6 , 23.3, 16.6, 14.6, 14.0

^{1}H-NMR: (ppm) *t* , 0.8 (9H); *m*, 1.1-1.3 (15H)

Spectral assignments are illustrated in Figures 3.4 - 3.7.

6.4.1.2 Synthesis of 4-propyloctenes

4-propyloctan-4-ol was dehydrated to an isomeric mixture of 4-propyloctenes by acid catalysed dehydration (Vogel, 1989).

4-propyloctan-4-ol (1.49 g, 0.0075 mol) was refluxed with orthophosphoric acid (1 ml, 0.017 mol) for 15 minutes. The mixture was then transferred to a separating funnel and approximately 5 ml saturated sodium chloride solution added to neutralise any residual acid. The organic reaction products were obtained by extraction with diethyl ether (3 x 2 ml), dried over anhydrous sodium sulphate and the solvent removed by rotary evaporation. The crude alkenes were purified by column chromatography on fully activated silica, and eluted with hexane. Fully activated silica was employed to dehydrate any remaining alcohol to the target alkenes. The pure alkenes were analysed by GC and fully characterised by MS.

Yield: 89 %

GC purity: > 99 %

MS; *m/z* 154 ($M^{+\cdot}$, 30%); 41,55,65

Spectral assignments are illustrated in Figure 3.9.

6.4.1.3 Synthesis of 4-propyloctane

The alkene isomeric mixture (900 mg, 0.0058 mol) was dissolved in *n*-hexane (10 ml). Adams Catalyst (20 mg, $PtO_2.H_2O$) and a few drops of glacial acetic acid were added and hydrogen gas bubbled gently through the solution for 2.5 h. The progress of the reaction was monitored by analysis of aliquots of reaction mixture by GC.

Once complete, the reaction mixture was filtered through a plug of defatted cotton wool to remove the catalyst, and the solvent removed by rotary evaporation. The crude reaction products were purified by argentation-silica column chromatography, (silica, 5% deactivated w/w + 10% w/w $AgNO_3$) and eluted with *n*-hexane. The pure alkane, 4-propyloctane was analysed by GC and characterised by MS and NMR (^1H, ^{13}C and DEPT).

GC purity: > 99 %

Yield: 92 %

MS: *m/z* 156 ($M^{+\cdot}$, 3 %); 112 ($M^{+\cdot}$- C_3H_8, 46%); 114 ($M^{+\cdot}$- C_3H_6, 10%); 57 (C_4H_9, 100 %); 43 (C_3H_7, 55%)

^{13}C-NMR: (ppm), 36.8, 36.1, 33.4, 28.9, 23.2, 19., 14.5, 14.2

^1H-NMR: (ppm) *m*, 0.8 - 0.9 (9H); *m*, 1.2 - 1.4 (15H)

Spectral assignments are illustrated in Figures 3.11 - 3.13.

6.4.2 Synthesis of the model aromatic hydrocarbons 7-cyclohexyltetralin and 7-cyclohexyl-1-propyltetralin

Synthesis of 7-cyclohexyltetralin and 7-cyclohexyl-1-propyltetralin was achieved using a modification of the Haworth synthesis (Vogel, 1989). This synthetic pathway has been extensively studied and is commonly used for the synthesis of a wide range of aromatic hydrocarbons. The synthetic route employed for both alkyltetralins is the same for the first three stages, to the cyclic ketone (7-cyclohexyl-1-tetralone). The keto group was then reduced to an alkyl group to produce 7-cyclohexyltetralin, whilst the second model compound, 7-cyclohexyl-1-propytetralin was synthesised by addition of a propyl chain to the tetralone using a Grignard reaction. The reaction scheme is illustrated in Figure 3.15.

6.4.2.1 Synthesis of 3-benzoyl(4`-cyclohexyl)propanoic acid

Nitrobenzene (40 ml), phenylcyclohexane (16.8 ml, 0.1 mol) and succinic anhydride (12 g, 0.1 mol) were placed in a round bottom flask fitted with a mechanical stirrer. The mixture was gently heated and stirred until the succinic anhydride had completely dissolved. After cooling to *ca* 50°C, aluminium chloride was added gradually to the flask with vigorous stirring, allowing each portion of aluminium chloride to dissolve before addition of the next. Addition of aluminum chloride to the reaction mixture yielded an orange solution, which darkened to a deep red colour upon addition of further aluminium chloride, with evolution of hydrogen chloride gas. Once evolution of hydrogen chloride had ceased, the reaction mixture was hydrolysed by pouring the contents of the flask onto ice (20 g) and hydrochloric acid (50 ml). Nitrobenzene was removed from the reaction mixture by steam distillation. After the initial steam distillation (3 h, 35 ml nitrobenzene collected), the flask was cooled and the crude keto-acid obtained by vacuum filtration. The crude product was then dissolved in 150 ml sodium carbonate

solution and a second steam distillation carried out to remove residual traces of nitrobenzene (1.5 h, 5 ml nitrobenzene collected). The hot alkaline solution was treated with 1 g decolourising carbon, stirred for 2-3 minutes and filtered through a heated filter funnel. The filtrate was allowed to cool (*ca* 50°C) and dilute hydrochloric acid (2:1 v/v, H_2O; conc. HCl) added slowly with vigorous stirring until effervescence ceased. The reaction mixture was left overnight to crystallise (cream coloured solid) and the crude keto-acid subsequently obtained by vacuum filtration, the solid was rinsed with dilute hydrochloric acid followed by Milli-Q water. Recrystallisation from ethanol:water (1:1, v/v) yielded pure 3-benzoyl(4`-cyclohexyl)propanoic acid (63 %) which was characterised by MS, ^{1}H and ^{13}C NMR and IR.

MS: *m/z* 332 ($M^{+\cdot}$, 4%); 317 ($M^{+\cdot}$ - CH_3, 27 %); 187 (benzylic cleavage, 100 %)

IR: 3400 cm^{-1} (ν O-H); 1709 cm^{-1}, 1679 cm^{-1} (νC=O)

^{13}C-NMR: (ppm), 197.5, 178.8, 154.0, 134.2, 128.2 , 127.1, 44.7, 34.1, 33.0, 28.1 , 26.7, 26.0

^{1}H-NMR: (ppm) *d* 7.9; *d* 7.3; *t* 3.2; *t* 2.7; *br m* 2.5; *m* 1.8 *m* 1.4

Spectral assignments are illustrated in Figures 3.18 - 3.21.

Experiment 2

The procedure described above was followed but with replacement of nitrobenzene with 1,1,2,2-tetrachloroethane. Pure keto acid was obtained in higher yield (85 %) and characterised by GC-MS, IR and NMR.

6.4.2.2 Synthesis of 4-phenyl(4'-cyclohexyl)butanoic acid

Potassium hydroxide (6 g) was dissolved in diethylene glycol (40 ml) by gentle heating and stirring. 3-Benzoyl(4'-cyclohexyl)butanoic acid (12.5 g) was added to the flask together with hydrazine hydrate (3.6 ml; 98 %). The solution was heated under reflux for 1 h. The apparatus was then re-arranged for distillation and excess hydrazine hydrate and water removed by distillation until the temperature of the reaction mixture reached 180°C. The mixture was then refluxed for a further 4 h, the temperature of the reaction mixture was maintained at approximately 250°C. After cooling, the reactants were poured into a beaker containing ice and carefully acidified whilst stirring continuously to ensure conversion of all the potassium salt to free acid. The crude acid was left to solidify (18 h), yielding a brown, waxy solid. The crude acid was then redissolved over hot water, allowed to cool slightly and extracted with diethyl ether (2 x 25 ml). The ether solution was treated with approximately 1 g decolourising carbon for five minutes, the solution filtered and the sample treated with a further 1 g decolourising carbon followed by hot filtration to yield a yellow solution. The diethyl ether was removed under vacuum to yield the acid as a brown oil which solidified overnight. The acid, (91%, yield) was characterised by GC-MS, IR and NMR.

GC purity; 98 %

MS: *m/z* 318 ($M^{+\cdot}$, 20 %); 303 ($M^{+\cdot}$ - CH_3, 27 %); 186 (benzylic cleavage,100 %); 117 ($COOSi(Me)_3$, 92 %)

IR: br 3015 cm^{-1} νOH; 1708 cm^{-1} νC=O; 2925 cm^{-1}, 2854 cm^{-1}, νC-H

^{13}C-NMR: (ppm) 180.3, 145.8,138.4,128.4, 126.8, 44.1, 34.5, 33.4, 27.1, 26.9

^{1}H-NMR: (ppm) *d* 7.1; t 2.6; *br m* 2.4; *t* 2.3; *q* 1.9; *m* 1.8; *m* 1.4

Spectral assignments are illustrated in Figures 3.24 - 3.27

6.4.2.3 Synthesis of 7-cyclohexyl-1-tetralone

Polyphosphoric acid (PPA, 26.4 g) was heated to 90°C on a hotplate. 4-cyclohexyl(4`-phenyl)butanoic acid (10.8 g), heated to 70°C was added to the PPA and stirred vigorously for 3 minutes. A further 22 g of PPA was then added to the reaction mixture and heated with stirring for 4 minutes. The reaction mixture was then cooled (*ca* 60°C) and crushed ice added with stirring to hydrolyse the mixture. A thick brown oil separated from the reaction mixture. Diethyl ether (50 ml) was added and the mixture transferred to a separating funnel. The ether layer was removed and the aqueous layer extracted with diethylether (2 x 50 ml). The organic layers were combined and washed first with 2 x 50 ml Milli-Q grade water, followed by 2 x 50 ml sodium carbonate solution and finally Milli-Q grade water until the washings were pH neutral. The organic fraction was dried over anhydrous sodium sulphate and the ether removed by rotary evaporation to yield the tetralone (88% yield). Examination of the crude products by GC revealed no further purification was necessary (> 99 % pure). 7-Cyclohexyl-1-tetralone was characterised by GC-MS and NMR.

MS; *m/z* 228 ($M^{+\cdot}$, 100 %); 200 ($M^{+\cdot}$-CO, 30 %); 185 ($M^{+\cdot}$ - CH_3O, 53 %); 172

^{13}C-NMR; (ppm) 198.6, 146.4, 141.9, 132.2, 128.7, 124.9, 44.0, 39.1, 34.2, 29.2, 26.7, 25.9, 23.2

^{1}H-NMR; (ppm) *s* 7.9; *d* 7.3; *d* 7.1; *t* 3.8; *t* 3.6; *m* 2.5; *quintet* 2.1; *m* 1.8; *m* 1.4

Spectral assignments are illustrated in Figures 3.29 - 3.32.

6.4.2.4 Synthesis of 7-cyclohexyltetralin

Potassium hydroxide (2 g) was dissolved in 30 ml diethylene glycol by gentle heating and stirring. 7-cyclohexyl-1-tetralone (3 g, 0.013 mol) and hydrazine hydrate (0.64 ml; 98 %) were then added to the flask and the solution heated under reflux for 1 h. The apparatus was re-arranged for distillation and the excess hydrazine hydrate and water

removed by distillation until the temperature reached 195°C. The mixture was then refluxed for a further 4 h at approximately 240°C. After cooling, the reactants were poured onto ice and carefully acidified to Congo Red (HCl), stirring thoroughly to ensure conversion of all the potassium salt to the free acid. The reaction products were extracted (x 3) with diethyl ether and the organic layers combined. Solvent was removed under vacuum and the crude reaction products purified by column chromatography (silica column eluting with hexane) to yield the pure alkane, 7-cyclohexyltetralin (> 99 %; 87 % yield). Confirmation of synthesis of the desired product was confirmed by MS, IR and NMR.

MS: *m/z* 214 ($M^{+\cdot}$, 100 %); 171; 145; 158; 129

^{13}C NMR; (ppm) 145.2, 136.8, 134.5, 129.0, 127.4, 124.0, 44.2, 34.6, 29.5, 26.9, 26.2, 23.3

^{1}H-NMR; (ppm) *quintet* 6.9; *d* 2.7; *br m* 2.4; *m* 1.7; *m* 1.4

Spectral assignments are illustrated in Figures 3.34 - 3.37.

6.4.2.5 Synthesis of 7-cyclohexyl-1-hydroxy-1-propyltetralin

As Grignard reactions are extremely moisture sensitive, every precaution was taken to ensure exclusion of moisture from the apparatus and reagents. The glassware was oven dried (120°C, 18 h) and assembled whilst hot. Calcium chloride guard tubes were fitted to the condenser and dropping funnel and the reaction was carried out under a blanket of nitrogen. All reagents were dried over anhydrous sodium sulphate and fresh magnesium turnings were oven dried (120°C, 18 h) and used immediately.

Magnesium turnings (2.4 g, 0.1 mol) were added to a round bottom flask containing 30 ml diethyl ether (Na-dried). 1-Bromopropane (10.5 ml, 0.115 mol) was dissolved in

diethyl ether (20 ml, Na-dried) in a dropping funnel, and 5 ml of this mixture added, with stirring to the flask. After a few minutes the solution become cloudy and the remaining bromopropane-ether mixture was added gradually over a period of 20 minutes. The mixture was gently heated under reflux for a further 30 minutes. The reaction mixture was then cooled and 7-cyclohexyl-1-tetralone (9 g, 0.04 mol), dissolved in diethyl ether (20 ml, Na-dried) added gradually with stirring. The mixture was then refluxed for 30 minutes. The mixture was cooled (ice bath) and hydrolysed by pouring onto ice (28 g) and saturated ammonium chloride solution (20 ml). The crude organic reaction products were obtained by extraction into diethyl ether (3 x 20 ml) followed by solvent removal under vacuum (Bucchi, 40°C; 8.52g). The crude reaction products were examined by GC and GC-MS. No further purification step was carried out at this stage owing to the nature of the products (*cf.* Section 3.3.9).

GC purity; 85 %

Yield: ~52 %

MS: *m/z* 343 ($M^{+\cdot}$ - H, 100 %); 301; 254

Spectral assignments are illustrated in Figure 3.39.

6.4.2.6 Synthesis of 7-cyclohexyl-1-propenyltetralin and 7-cyclohexyl-1-propyl-3,4-dihydronaphthalene

The alcohol mixture obtained from the Grignard reaction (6.95 g) was refluxed with orthophosphoric acid (10 ml) for 1.5 h. The mixture was then transferred to a separating funnel and extracted with diethyl ether (2 x 20 ml). The ether extracts were combined and washed with saturated sodium chloride solution until the aqueous washings were a neutral pH. Solvent was then removed under vacuum to yield the crude reaction products. The crude reaction products were purified by open column chromatography, using fully activated silica and eluted with *n*-hexane. Approximately

100 mg of the target alkenes were isolated from the unwanted 7-cyclohexyl-3,4-dihydronaphthalene using argentation thin layer chromatography (TLC). Ag^+ TLC plates (0.25 mm thickness) were prepared according to the method of Aitzenmuller and Goncalves (1990). Alkene mixture (20 mg) was spotted onto each plate, and the plates developed with *n*-pentane in foil covered TLC tanks. Plates were sprayed with Rhodamine 6G and visualised under UV light. The upper band (R_f 0.09 - 0.1) was carefully scraped from the plate and the target alkenes eluted from the silica with *n*-pentane.

GC purity: 98 %

MS: *m/z* 254($M^{+\cdot}$, 62 %); 252 ($M^{+\cdot}$ - 2, 100 %); 223

Spectral assignments are illustrated in Figure 3.41.

6.4.2.7 Synthesis of 7-cyclohexyl-1-propyltetralin

The pure alkene isomeric mixture (68 mg) was dissolved in approximately 10 ml *n*-hexane to which a few drops of glacial acetic acid and Adams catalyst ($PtO_2.H_2O$) were added (*ca* 50 g). Hydrogen gas was bubbled gently through the solution. An aliquot of reaction mixture was taken after 20 minutes to monitor the progress of reaction by GC. After 1.5 h a second aliquot was taken, and analysis by GC indicated that the reaction was complete. The reaction mixture was filtered through a cotton wool plug to remove catalyst and the solvent removed under a stream of nitrogen. The crude alkane mixture was examined by GC and GC-MS. To remove residual traces of alkene, the reaction products were eluted through an Ag^+ silica column with *n*-pentane. Solvent was removed under a gentle steam of nitrogen and the pure target compound, 7-cyclohexyl-1-propyltetralin (91 % yield; 97 % GC purity) characterised by MS and NMR (^{13}C, DEPT, ^1H).

MS: *m/z* 256 ($M^{+\cdot}$, 12 %); 213

^{13}C-NMR: (ppm) 145.2, 141.4, 134.4, 128.9, 127.0, 123.8, 44.3, 39.3, 37.4, 34.6, 34.5, 29.4, 27.4, 27.0, 26.2, 20.7, 19.8, 14.3,

^{1}H-NMR: (ppm) *quintet* 6.9, *br m* 2.7, *br m* 2.4 , *m* 1.3 - 1.7, *t* 0.9

Spectral assignments are illustrated in Figures 3.43 - 3.45.

6.5 Investigations into the effect of 4-propyloctane upon mussel feeding rate

Toxicity experiments, including the preparation of test solutions and exposure of mussels were conducted in a constant temperature room (15°C).

6.5.1 Test compounds

4-propyloctane was synthesised (Section 6.4). Butylcyclohexane was purchased from Aldrich Chemical Company. Purity of test compounds as monitored by GC were; 4-propyloctane > 99 %; butylcyclohexane > 99 %

The internal standards 4-methylnonane and *n*-undecane were obtained from Sigma Chemical Company.

6.5.2 Collection and maintenance of mussels (*Mytilus edulis*)

Mussels of between 35 mm and 45 mm shell length were collected from the intertidal zone at Exmouth, Devon. U.K. Mussels were cleaned of epibionts and held in open-flow polythene tanks in recirculating seawater (3m^3 volume) at 33 ‰ salinity with an artificially produced tidal regime which aerially exposed the mussels for two periods of 2.5 h each day. Mussels were fed continuously with an algal culture of *Isochrysis galbana.* The temperature of the system was maintained at 15 °C. The animals were allowed seven days to acclimate to laboratory conditions prior to use in experiments.

6.5.3 Preparation of toxicant solutions

Test compounds were dissolved in acetone which acted as a solvent carrier to aid dispersion of the test compounds in seawater. A previous study (Donkin *et al.*, 1989) has demonstrated that at the concentrations used herein (0.001 % v/v) acetone has no effect upon mussel feeding rate.

All test solutions were prepared using Eddystone Filtered Seawater (EFSW) filtered to exclude particles with diameters greater than 45 μm. Glass aspirators of 20 l capacity were filled with 20 l EFSW at 15°C and the contents magnetically stirred (35 mm Teflon coated follower) until a vortex was produced. An aliquot of the test compound dissolved in acetone was added to the aspirator using a glass syringe, discharging the solution directly into the seawater. Any emulsion formed by the test solution was held in the vortex until the material dissolved. Acetone was added to 'control' aspirators in the same way. The aspirators were stoppered and contents stirred for a minimum of 2 h before use.

6.5.4 Exposure of mussels to toxicant

The method of Donkin *et al.* (1991) was followed. Groups of mussels were held in round-bottomed reaction vessels containing 20 l test compound solution in EFSW. Gentle water movement was maintained by means of a magnetic stirrer, restrained within a glass dish to prevent contact with the mussels. The tops of the vessels were covered with perforated aluminium foil. The mussels were fed continuously with an algal culture (*Isochrysis galbana*) added by means of a peristaltic pump. 'Control' exposure vessels were assembled in an identical manner, substituting toxicant solution with 'control' solution (seawater + 0.001% v/v acetone). Test solutions in the exposure vessels were changed every 24 h.

6.5.5 Measurement of mussel feeding rate

Following the required exposure period, sixteen mussels were transferred to individual glass beakers, each containing 2 l test solution. Each beaker also contained a magnetic stirrer bar to maintain gentle water movement. Mussels were placed away from the stirrer bar and positioned such that their inhalant siphon was facing into the current. A thirty minute period was allowed for the mussels to open their valves and resume pumping. A beaker containing only test solution and a stirrer bar (no mussel) was also set up as a control.

A pre-determined volume of algal culture was then added to each beaker to give a cell concentration of 12000 - 14000 cells ml^{-1}. After a five minute mixing period, a 20 ml aliquot was removed from each beaker and cell numbers counted in triplicate using a model D Coulter Counter set to measure particles greater than 3 μm in diameter. Further 20 ml aliquots were removed, and the cell count measured every 20 minutes for an 80 minute period. Mussel feeding rates ($l\ h^{-1}$) were then calculated for each 20 minute period and every 40 minute period using Equation 6.1. The maximum feeding rate over a 40 minute period for an individual was used as the feeding rate for that animal.

$$feeding\ rate\ (l\ h^{-1}) = \left(\frac{v * 60}{t}\right) * (lnM_1 - lnM_2)$$

where; v = volume of water in beaker

t = time period of measurement

M_1 = cell count at t_0 (mean of triplicate measurements)

M_2 = cell count at t_1 (mean of triplicate measurements)

Equation 6.1 Calculation of mussel feeding rate

6.5.6 Neutral Red retention assay

The method of Lowe *et al.* (1995) was followed. Mussel valves were prised apart with a scalpel and 0.5 ml of haemolymph was withdrawn from the anterior adductor mussel into a 2.5 ml hypodermic syringe fitted with a 25 gauge needle and containing 0.5 ml physiological saline. Once the haemolympth sample was obtained, the hypodermic needle was discarded to reduce shearing forces during the subsequent expulsion of the syringe contents into a 2 ml siliconised Eppendorf tube held in ice water.

A stock solution of Neutral Red was made by dissolving 20 mg of dye (C.I. 50040) in 1 ml of dimethylsulphoxide (DMSO). A working solution was then prepared by diluting 10 µl of the stock solution with 5 ml of a mussel physiological saline.

A 50 µl aliquot of the cell suspension was dispensed onto a 26 x 76 mm microscope slide and suspended on a rack in a humidity chamber consisting of a shallow insulated vessel containing water ice (incubation temperature 10°C) for 15 minutes to allow the cells to attach. The excess solution was then carefully decanted and 40 µl of the neutral red working solution added to the area containing the attached cells. A 22 x 22 mm coverslip was then applied. After 15 minutes incubation the preparation was examined again and thereafter at 30 minute intervals to determine the time at which the dye that had been taken up by individual lysosomes (turning them red) had leached out into the cytosol. The test was terminated at 180 minutes.

6.5.7 Tissue Analysis

Soft mussel tissue was dissected from the shells over ice to minimize losses of analytes, homogenised and stored in solvent rinsed glass jars at -20°C prior to analysis. Tissue was extracted by steam distillation (Donkin and Evans, 1984). Internal standards (4-

methylnonane and *n*-undecane) were spiked into wet mussel homogenate immediately prior to extraction.

Mussel homogenate (typically 6 g) was added to a round bottom flask containing 10 ml *iso*-hexane, 10 ml sodium hydroxide (4 mol) and 400 ml solvent extracted water. A 12.5 ml capacity Dean and Stark water estimator and condenser were fitted to the flask and the aqueous homogenate heated at 80 ± 5°C for 2 h. Hydrochloric acid (50 ml, 0.68 mol) was added *via* the condenser, followed by 50 ml distilled water to reduce the pH of the homogenate to approximately 7. The heat was then increased, and the mixture distilled for a further 2 h. After cooling, the extract was transferred to a stoppered tube and stored at -20°C for several hours to freeze out any water present. The extract was then transferred to a solvent rinsed vial and analysed by GC.

Recovery of 4-PO and BCH spiked into mussel homogenate and subsequently extracted by the above steam distillation method were found to be 94.1 ± 0.05 % and 93.2 % ± 0.5 %, respectively (mean ± rsd, n=3).

6.6 Investigations into the toxicity of model aromatic UCM hydrocarbons upon mussel feeding rate

6.6.1 Test compounds

As no suitable test compounds were available commercially, two model aromatic UCM compounds were synthesised using a modification of the Haworth synthesis (Section 6.4.2). Butylcyclohexane was purchased from Aldrich Chemical Company. Purity of test compounds as monitored by GC were; Butylcyclohexane > 99 %; 7-cyclohexyltetralin > 99 %; 7-cyclohexyl-1-propyltetralin > 97 %

6.6.2 Collection and maintenance of mussels

Mussels of between 10 and 20 mm shell length were collected from Whitsand Bay, Cornwall. This is known to be a relatively uncontaminated site with respect to petroleum hydrocarbon contamination (P. Donkin, pers. comm.). Animals were cleaned and held in tanks of recirculating seawater at 15°C, as described in Section 6.5.2.

6.6.3 Preparation of toxicant solutions

Toxicant solutions were prepared by adding solutions of the toxicant in acetone to filtered seawater as described in Section 6.6.3. Control solutions were prepared using acetone only (0.001 % v/v).

6.6.4 Exposure of animals

Groups of seven mussels, shell length 12 mm ± 1 mm were exposed to 1.4 l toxicant solution in a glass beaker. Gentle water movement was maintained using a Teflon stirrer bar (10 mm) taking care to position the animals as far away from the stirrer bar as possible. The animals were fed with an algal culture (*Isochrysis galbana*). Exposure time was 24 h. A control vessel containing filtered seawater plus acetone was assembled in parallel with each set of measurements. For each exposure concentration, two separate exposure experiments were conducted.

6.6.5 Measurement of mussel feeding rate

Animals were transferred from the exposure vessel into individual 250 ml glass beakers, each containing 200 ml toxicant solution. The animals were allowed an acclimatisation period of 30 minutes to open their valves and resume pumping prior to the addition of algae. Algal culture (volume pre-determined to give a cell concentration of 12000 -

15000 cells ml^{-1}) was then added to each beaker and the water gently stirred to ensure an even distribution of algae within each beaker. An aliquot (20 ml) of water was then taken from each beaker and the cell count determined in triplicate per aliquot using a Coulter Counter set to measure particles greater than 3 μm in diameter. A further aliquot was taken after 15 minutes and the decline in cell concentration over the 15 minute period calculated using Equation 6.1.

6.6.6 Tissue analysis

Soft tissue was dissected from the shell over ice to minimize losses by volatilization, homogenised and stored in solvent rinsed vials at -20 °C prior to analysis. 7-cyclohexyltetralin was used as an internal standard for determination of 7-cyclohexyl-1-propyltetralin in mussel tissue and *vice versa*. Internal standards were spiked into mussel homogenate immediately prior to analysis. Mussel tissue from experiments with the alkyltetralins was extracted by the procedure described below. Mussel tissue from experiments with butylcyclohexane as the toxicant were extracted by steam distillation as described previously in Section 6.5.7.

Wet mussel tissue was placed in a stoppered centrifuge tube with 5 ml of sodium hydroxide (3 M) and heated in a water bath at 60°C for 20 minutes. After cooling, 10 ml *n*-hexane was added to the tube which was then stoppered and the mixture was vigorously shaken (5 minutes) followed by centrifugation (2000 rpm, 10 minutes). The *n*-hexane layer was then transferred to a stoppered conical flask and dried over anhydrous sodium sulphate. After drying, solvent was removed from the extracts by rotary evaporation until *ca* 1 ml remained, followed by transfer of the extract to a clean vial and the remaining solvent removed under a gentle stream of nitrogen. Extracts were analysed by GC-MS.

Recoveries of 7-cyclohexyltetralin and 7-cyclohexyl-1-propyltetralin from mussel tissue as determined by spike recovery experiments was 89 % ± 3 % and 92 ± 4 %, respectively.

CHAPTER SEVEN

Conclusions and suggestions for future work

7.1 Conclusions

The occurrence of unresolved complex mixtures (UCMs) of hydrocarbons in the aliphatic fractions of marine sediments and organisms is well documented and widely used as a reliable indication of petroleum hydrocarbon contamination. However, the presence of an unresolved complex mixture in the aromatic fraction is often ignored and seldom reported. The toxicological significance of both aliphatic and aromatic UCM hydrocarbons is largely unknown and uninvestigated.

The overall objectives of the current study were therefore first, to establish the quantitative significance of aromatic UCM hydrocarbons in environmental samples, and then to assess the environmental significance of both aliphatic and aromatic UCMs. More specifically, the aims were: to establish whether oil-polluted mussels contain an aromatic UCM burden, to synthesise suitable quantities of selected, pure, well characterised model aliphatic and aromatic UCM hydrocarbons for toxicological testing and, finally, to investigate the toxicity of the synthetic model UCM hydrocarbons.

A reproducible analytical method was developed to investigate the quantitative significance of aromatic UCM hydrocarbons in petroleum hydrocarbon contaminated mussels. Emphasis was placed upon good recoveries of the low molecular weight, toxicologically significant hydrocarbons, without compromising the recovery of higher molecular weight hydrocarbons which are useful compounds in environmental monitoring schemes. The use of a micro-Kuderna Danish apparatus to carefully control the final stages of sample concentration significantly increased the recovery of low molecular weight aliphatic and aromatic hydrocarbons when compared with the traditional method of rotary evaporation (t-test, P=0.05). In addition, the use of a two phase extraction method which yielded the total organic extract (TOE) in non-polar *n*-

pentane also minimized the losses of volatile low molecular weight hydrocarbons by eliminating the need for a solvent 'change over' step, prior to fractionation of the TOE.

The optimised method was used to measure the aliphatic and aromatic hydrocarbon burdens of mussels (*Mytilus edulis*) from a small number of coastal sites around the U.K. The results indicate that aromatic hydrocarbon UCMs may form a significant proportion of the total hydrocarbon body burden of mussels from petroleum hydrocarbon contaminated areas. In the samples analysed, the aromatic UCM represents approximately 20 % of the total hydrocarbon body burden and dominates the aromatic hydrocarbon fraction. Concentrations measured ranged from unobservable in relatively uncontaminated areas, up to 430 $\mu g\ g^{-1}$ dry weight tissue in the most heavily contaminated site, Whitby harbour. In contrast, naphthalene and alkylnaphthalenes, which are resolved aromatic hydrocarbons routinely measured in monitoring programmes, are present at concentrations less than 1 $\mu g\ g^{-1}$ dry weight tissue in the mussels from Whitby harbour. Aromatic UCM concentrations measured in replicate in mussels from open coastal sites (New Brighton, Cleethorpes, Teesmouth) range from 86 - 133 $\mu g\ g^{-1}$ dry weight.

Further fractionation of the aromatic UCM by normal phase HPLC indicates that the aromatic UCMs bioaccumulated by mussels consist predominantly of mono-aromatic hydrocarbons with smaller amounts of di-aromatics.

The presence of aromatic UCM hydrocarbons in marine organisms has rarely been reported before, probably because routine monitoring programmes focus upon GC resolved compounds of known toxicological significance. However, since the present study has demonstrated that aromatic UCMs are bioaccumulated by mussels in

quantitatively significant amounts, existing methodologies may have failed to provide a measure of an environmentally important burden.

In addition, aliphatic hydrocarbon UCMs were present at concentrations ranging from 3445 μg g^{-1} dry weight tissue in mussels from Whitby harbour, to approximately 7 μg g^{-1} dry weight tissue in mussels from Whitsand Bay (the 'clean' site). In mussels sampled from open coastal sites (Cleethorpes, Teesmouth and New Brighton) aliphatic UCM concentrations ranged from 231 - 573 μg g^{-1} dry weight tissue.

In order that the toxicity of UCM hydrocarbons could be investigated, a number of 'model' UCM hydrocarbons were synthesised. A low molecular weight model aliphatic UCM hydrocarbon 4-propyloctane (4-PO) was synthesised by coupling 1-bromopropane to ethylpentanoate *via* a Grignard reaction. Dehydration of the resultant tertiary alcohol followed by catalytic hydrogenation yielded the pure (> 99%) target alkane, 4-PO.

The synthetic 4-PO, which is a C_{11} alkane, had a demonstrable narcotic effect, causing a reduction in mussel ciliary feeding activity. Previous studies have suggested that a 'cut-off' in toxicity occurs for *n*-alkanes larger than *n*-decane (C_{10}) but no branched model UCM hydrocarbons were tested (Donkin *et al.*, 1991). The present work therefore demonstrates that the molecular weight range for narcotic hydrocarbons is effectively extended by consideration of branched hydrocarbons such as those of which the aliphatic UCM is believed to be composed. This is presumably because of their higher aqueous solubilities compared to straight chain hydrocarbons of comparable carbon number. This result suggests that a small proportion of aliphatic UCMs may have some toxicological significance, particularly the lower molecular weight fractions. Further work is required to establish how much of the aliphatic UCM is of toxicological importance. Two low molecular weight model aromatic

UCM hydrocarbons, 7-cyclohexyltetralin (7-CHT; purity > 99% by GC) and 7-cyclohexyl-1-propyltetralin (7-C-1-PT; purity 97 % by GC) were synthesised using a modification of the Haworth synthesis. The synthetic route for both compounds was the same for the first three stages, to the cyclic ketone (7-cyclohexyl-1-tetralone). The 'base' compound, 7-cyclohexyltetralin was then synthesised by reduction of the keto group to an alkyl group, whilst the second model compound 7-cyclohexyl-1-propyltetralin was synthesised by addition of a propyl chain to the tetralone using a Grignard reaction. Dehydration of the alcohol product followed by catalytic hydrogenation yielded the target alkyltetralin.

Both 7-cyclohexyltetralin and 7-cyclohexyl-1-propyltetralin (which are C_{16} and C_{19} hydrocarbons, respectively) were found to reduce mussel ciliary feeding activity. Previously, Donkin *et al.* (1989, 1991) have reported that a C_{16} hydrocarbon, (1-phenyldecane) had no measurable effect upon mussel feeding rate (presumably owing to its low aqueous solubility). Thus the toxicity 'cut-off' for aromatic hydrocarbons has also been extended, presumably because the model aromatic UCM hydrocarbons are of sufficient aqueous solubility to act as non-specific narcotics. To the best of the author's knowledge, this is the first study to investigate the toxicity of aromatic UCM hydrocarbons. Given the quantitative significance of the aromatic UCM, the results reported herein suggest that aromatic UCM hydrocarbons are not only a quantitatively important environmental burden, but are also of some toxicological significance.

The effective tissue concentrations required to produce a 50 % reduction in mussel feeding rate (TEC_{50}) for 4-propyloctane, 7-cyclohexyltetralin and 7-cyclohexyl-1-propyltetralin were similar and comparable with reported TEC_{50} values for the detrimental effects of other hydrocarbons upon mussel feeding rate (~ 16 - 94 mg kg^{-1}

wet weight tissue or 0.1 - 0.6 mmol kg^{-1}; Donkin *et al.*, 1989, 1991). The range (which is small given the differences in experimental exposure times in the present and published studies) is consistent with the theory that narcotic effects occur at a relatively constant body burden of toxicant.

Whilst numerous studies have reported toxicant body burden data for toxicity tests in which lethality is the end-point and established that a relatively constant body burden of narcotic toxicant is associated with LC_{50} estimates (typically 2-8 mmol kg^{-1}), comparison of the tissue residue data reported herein for sublethal toxicity studies is limited by the few studies published (Call *et al.*, 1985; McCarty *et al.*, 1986; Mortimer and Connell, 1995). Indeed, not only is there a paucity of tissue residue data for sub-lethal toxicological studies, but the majority of tissue residue data actually reported for sub-lethal endpoints is often estimated using the aqueous exposure concentration of toxicant and a calculated bioconcentration factor. Consequently, errors associated with these values may be large and comparison with data reported herein not particularly meaningful. With the exception of the reports of Donkin *et al.* (1989, 1991), no comparable studies appear to have used a QSAR approach to study the sublethal responses of mussels to non-specific narcotic toxicants and in which the body burden of toxicant has been measured.

By separately examining the concentration of toxicants in the total body and in the gill tissue (the presumed site of toxic action for a reduction in mussel ciliary feeding activity), the present study has provided a detailed and unique insight into the relationship between concentration of toxicant in the mussel and the observed mussel feeding rate. A clear relationship between the concentration of toxicant in the gill tissue and mussel feeding rate has been demonstrated. However, the relationship between mussel feeding rate and total body burden of toxicant (*cf.* Donkin *et al.*, 1989,

1991) breaks down with increasing experimental exposure time. Once a steady state for toxicant concentration has been reached in the gills of the mussel, bioaccumulation of toxicant into other discrete compartments, such as lipid-rich organs, results in an increasing body burden of toxicant but no further decrease in mussel feeding rate is observed. Thus, with increased exposure times, estimated TEC_{50} values for mussel feeding rate may be erroneously high.

One of the aims of establishing laboratory derived concentration-response relationships and the QSAR approach employed in the present study is to allow biological effects to be predicted from concentrations of contaminants measured in mussels collected in the field (Widdows and Donkin, 1992). For such purposes, the estimate of compound toxicity should be as accurate as possible. It is clear from the present study that in order for this to be achieved mussel feeding rate experiments should be kept relatively short (< 48 h).

In light of the above findings, the established method of determining mussel feeding rate determination was modified to produce a more rapid, simple and reproducible technique with which to assess the toxicity of compounds. Using butylcyclohexane as a reference, the modified procedure was shown to produce results comparable with those obtained by established procedures (Donkin *et al.*, 1991).

Overall, the research presented herein has demonstrated that aliphatic UCM and aromatic UCM hydrocarbons are quantitatively significant environmental contaminants, and that a proportion of the low molecular weight aliphatic UCM and probably a greater proportion of the aromatic UCM hydrocarbons are toxicologically significant. It is suggested that for meaningful environmental assessments of

anthropogenic hydrocarbon pollution to be made, quantification of UCM components should be included in standard methods.

7.2 Future work

Future work discussed below includes suggestions for further toxicological studies and improvements to the analytical methodology employed in the present study.

Whilst the analytical method developed for the analysis of aliphatic and aromatic hydrocarbons in mussel tissue has proved to be reproducible, the accurate quantification of polar compounds was less successful and requires further development. The importance of including polar hydrocarbon oxidation products in environmental monitoring schemes has been highlighted recently by Burns (1993a). Polar hydrocarbon oxidation products, formed by processes of photo-chemical oxidation and biological metabolism are bioaccumulated within marine organisms and in some instances are present at concentrations significant enough to contribute to the overall estimate of the toxic level of hydrocarbon contaminants (Burns *et al.*, 1990; Widdows *et al.*, 1990).

An improvement to the analytical methodology used in Chapter 2 would be the replacement of the open column chromatography 'clean-up' step with the use of solid phase extraction (SPE) to separate polar from non-polar compounds. The non-polar fraction obtained from this procedure could then be fractionated into aliphatic, mono-, di- and tri-aromatic fractions by preparative normal phase HPLC. The polar compounds could then be eluted from the SPE cartridge and analysed. This would remove the problem encountered in the present study of irreproducible recovery of polar compounds from a silica column. A potential problem in the detection and quantification of polar hydrocarbons in mussel tissue is the presence of higher

concentrations of naturally occurring polar lipids which may mask the presence of any polar compounds of interest. The inclusion of a gel filtration step (*e.g.* Sephadex) would enable separation of the higher molecular mass, naturally occurring lipids from smaller anthropogenic polar compounds of toxicological interest. Further separation and identification of the polar oxidation products could then be conducted using LC-MS-MS. No quantitative methods for the analysis of polar hydrocarbon oxidation products in mussel tissue have, as yet, been reported. However, there is currently considerable interest in the application of LC-MS-MS for the analysis of polar organic compounds in water samples (K. Thomas, pers. comm.)

The present study has demonstrated that the narcotic 'cut-off' is effectively extended by consideration of branched hydrocarbons such as the C_{11} 'T-branched' hydrocarbon, 4-propyloctane. However, to what extent the molecular weight range of narcotic hydrocarbons is extended remains unknown. Synthesis of a larger 'T-branched' alkane, *e.g.* a C_{15} hydrocarbon, followed by toxicological testing and measurement of its aqueous solubility may answer the question to a limited extent. Given that the majority of aliphatic UCM hydrocarbons are of much greater molecular size than 4-propyloctane and therefore most probably of little toxicological significance it is likely that only a very small proportion of aliphatic hydrocarbon UCMs are of any toxicological significance. Whilst concentrations of 'toxic' aliphatic UCM hydrocarbons may not be sufficient to be thought of as toxicologically significant when considered on their own they may contribute to the toxic burden as a whole. A number of studies have demonstrated that narcotic toxicants are concentration additive when present as a mixture (*e.g.* Hermens *et al.*, 1984, 1985; Deneer *et al.*, 1988; *cf.* Section 1.6). For example, Deneer *et al.* (1988) demonstrated that compounds present in very low concentrations, well below their 'no toxic-effect' levels contribute to the

joint toxicity of a mixture. To test this hypothesis a 'mini' low molecular weight UCM could be simulated by synthesising all the isomers of a branched aliphatic hydrocarbon, (for example C_{11} has 8 isomers, and there are 19 possible isomers for a C_{16} compound; C.A. Lewis, pers comm). The toxicity of the resulting 'mixture' could then be tested upon mussel feeding rate and the results compared with those obtained for single compound tests.

As discussed in Chapter 5, the accuracy of estimates of aqueous solubility or octanol-water partition coefficient obtained using established prediction methods is uncertain and errors may be large. Although there is lack of agreement as to the best molecular descriptor with which to delineate the narcotic 'cut-off', aqueous solubility appears to be a better predictor than the octanol-water partition coefficient or molar volume (*cf.* Abernethy *et al.*, 1986; Donkin *et al.*, 1989, 1991). Before any further conclusions can be made regarding what proportion of both aliphatic and aromatic hydrocarbon UCMs are toxic, it is desirable to obtain accurate aqueous solubility data for the model UCM compounds synthesised and tested. The use of the 'generator' column method (Wasik *et al.*, 1983) to measure aqueous solubility offers a number of advantages over other methods as the procedure is rapid, precise and suitable for hydrophobic compounds (Yalkowsky and Banerjee, 1992). The synthetic scheme employed in the present study for the synthesis of model aromatic UCM hydrocarbons is such that a number of hydrocarbons of differing alkyl chain length could easily be produced by synthesis of a large quantity of the 7-cyclohexyltetralone, division of the tetralone into smaller quantities and addition of a different alkyl chain to each 'batch' *via* Grignard reactions. Synthesis of a pseudo-homologous series of alkyltetralins, followed by measurement of the aqueous solubility of each compound and regression analysis of molecular size (carbon number) against aqueous solubility would produce an equation

relating the size of the molecule to its aqueous solubility. This equation could be used to predict the solubility of alkyltetralins and thus predict whether a compound will be toxic or non-toxic. Subsequent toxicological testing of the two compounds believed to bracket the narcotic cut-off point (*i.e.* the two alkyltetralins with aqueous solubilities closest to 70 $\mu g\ l^{-1}$) could then be conducted. Such an experiment would be of use in evaluating whether the existing parameters used to delineate narcotic action were correct.

A promising area of future study could be to investigate whether UCM hydrocarbons are a significant source of polar hydrocarbon oxidation products into the marine environment and, if so, whether photo-oxidation products of UCM hydrocarbons are toxic. A number of model aromatic UCM hydrocarbons could be tested, firstly to see whether they will photo-oxidise and secondly if the oxidation products produced are toxic.

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APPENDICES

COMPOUND	1	2	3	4	5	6	mean	sd	RSD
aliphatic									
4-propyloctane	76.1	64.3	72.1	58.9	43.1	48.6	**60.5**	**11.8**	**19.5**
7-hexylnonadecane	93.7	94.2	90.1	97.2	86.3	89.7	**91.9**	**3.9**	**4.2**
aromatic									
phenyldecane	72.1	62.1	70.9	74.3	68.4	62.7	**68.4**	**4.6**	**6.7**
5-ethyltetralin	78.9	73.4	68.4	71.9	50.4	65.6	**68.1**	**8.9**	**13.1**
2-ethylnaphthalene	70.1	84.1	70.9	80.1	62.3	75.4	**73.8**	**7.8**	**10.5**
1,3-diphenylhexane	83.0	79.2	69.2	93.2	75.1	77.3	**79.5**	**7.4**	**9.3**
4-pentylbiphenyl	93.8	83.1	89.3	81.8	84.7	87.5	**86.7**	**4.1**	**4.7**
phenanthrene	81.1	90.3	85.4	93.7	90.2	89.7	**88.4**	**4.1**	**4.6**
pyrene	87.4	94.2	94.3	100.2	90.1	98.4	**94.1**	**4.4**	**4.7**
polar									
acetophenone	66.8	84.0	75.2	77.1	69.4	73.1	**74.3**	**5.5**	**7.5**
9-fluorenone	93.6	72.0	90.4	98.1	86.4	89.4	**88.3**	**8.2**	**9.2**
benzoic acid	93.7	71.5	82.4	69.4	68.1	77.4	**77.1**	**8.9**	**11.6**
cyclohexanecarboxylic acid	104.6	77.0	64.2	81.3	68.1	70.4	**77.6**	**13.3**	**17.2**
9-anthracenecarboxylic acid	94.5	74.5	83.1	79.8	80.4	77.1	**81.6**	**6.4**	**7.8**
hexanedioic acid	90.5	69.7	64.1	73.2	70.1	83.2	**75.1**	**9.0**	**11.9**
1-naphthol	93.8	69.7	62.4	71.4	63.0	84.1	**74.1**	**11.4**	**15.3**
9-hydroxyfluorene	94.3	72.5	82.1	88.3	74.1	84.3	**82.6**	**7.6**	**9.2**

sd = standard deviation(n-1)
RSD relative standard deviation

RAW DATA Sample Concentration; Percentage recovery of authentic compounds obtained using Rotary evaporation method 1

COMPOUND	1	2	3	4	5	6	**mean**	**sd**	**RSD**
aliphatic									
4-propyloctane	90.7	77.3	81.4	75.2	84.9	73.7	**80.5**	**5.9**	**7.3**
7-hexylnonadecane	108.8	97.6	101.2	98.6	95.6	99.1	**100.2**	**4.6**	**4.6**
aromatic									
phenyldecane	99.81	81.9	85.4	80.3	86.4	86.4	**86.7**	**6.3**	**7.3**
5-ethyltetralin	96.5	85.7	82.3	89.7	93.0	84.3	**88.6**	**5.0**	**5.6**
2-ethylnaphthalene	100.7	88.5	85.4	80.1	86.9	95.4	**89.5**	**7.4**	**8.3**
1,3-diphenylhexane	101.0	94.2	90.7	87.7	93.2	98.9	**94.3**	**4.5**	**4.8**
4-pentylbiphenyl	101.0	90.6	95.6	97.3	94.2	94.4	**95.5**	**3.2**	**3.3**
phenanthrene	101.8	89.4	97.6	99.1	95.4	98.5	**97.0**	**3.9**	**4.0**
pyrene	103.2	90.8	96.4	98.8	96.3	98.1	**97.3**	**3.7**	**3.8**
polar									
acetophenone	105.6	91.4	90.3	96.3	92.3	95.2	**95.2**	**5.1**	**5.4**
9-fluorenone	113.3	97.1	99.2	103.1	95.1	97.6	**100.9**	**6.1**	**6.0**
benzoic acid	110.5	88.7	86.5	89.7	93.2	91.7	**93.4**	**7.9**	**8.5**
cyclohexanecarboxylic acid	100.3	89.1	87.6	89.5	95.4	91.1	**92.2**	**4.4**	**4.7**
9-anthracenecarboxylic acid	105.9	96.5	97.9	99.1	92.4	90.5	**97.1**	**5.0**	**5.1**
hexanedioic acid	111.6	80.8	84.3	90.6	87.8	98.7	**92.3**	**10.3**	**11.1**
1-naphthol	102.3	92.1	87.6	90.1	98.9	104.3	**95.9**	**6.3**	**6.6**
9-hydroxyfluorene	103.7	88.5	97.5	94.3	91.2	95.6	**95.1**	**4.8**	**5.1**

sd = standard deviation(n-1)
RSD relative standard deviation

RAW DATA Sample concentration; Percentage recovery of authentic compounds using rotary evaporation & micro-K.D. method 2

APPENDIX A.1.3
COMPARISON OF SAMPLE CONCENTRATION TESTS; Rotary evaporation *vs* rotary evaporation & micro-K.D.

4-propyloctane
P(0.01)
t-Test: Two-Sample Assuming
Equal Variances

	method 1	*method 2*
Mean	60.51666667	80.53333333
Variance	167.8576667	41.75466667
Observations	6	6
Pooled Variance	104.8061667	
Hypothesized Mean Difference	0	
df	10	
t Stat	-3.386561494	
P(T<=t) one-tail	0.00346288	
t Critical one-tail	2.7637725	
P(T<=t) two-tail	0.00692576	
t Critical two-tail	3.169261618	

phenyldecane
P(0.01)
t-Test: Two-Sample Assuming Equal Variances

	method 1	*method 2*
Mean	68.41666667	86.70333333
Variance	25.38566667	47.52922667
Observations	6	6
Pooled Variance	36.45744667	
Hypothesized Mean Difference	0	
df	10	
t Stat	-5.245683109	
P(T<=t) one-tail	0.000187857	
t Critical one-tail	2.7637725	
P(T<=t) two-tail	0.000375713	
t Critical two-tail	3.169261618	

5-ethyltetralin
P(0.01)
t-Test: Two-Sample Assuming Equal Variances

	method 1	*method 2*
Mean	68.1	88.58333333
Variance	95.76	29.91366667
Observations	6	6
Pooled Variance	62.83683333	
Hypothesized Mean Difference	0	
df	10	
t Stat	-4.475629364	
P(T<=t) one-tail	0.000593513	
t Critical one-tail	2.7637725	
P(T<=t) two-tail	0.001187026	
t Critical two-tail	3.169261618	

2-ethylnaphthalene
P(0.01)
t-Test: Two-Sample Assuming Equal Variances

	method 1	*method 2*
Mean	73.81666667	89.5
Variance	60.53766667	54.636
Observations	6	6
Pooled Variance	57.58683333	
Hypothesized Mean Difference	0	
df	10	
t Stat	-3.579623921	
P(T<=t) one-tail	0.002507418	
t Critical one-tail	2.7637725	
P(T<=t) two-tail	0.005014837	
t Critical two-tail	3.169261618	

1,3-diphenylhexane
P(0.01)
t-Test: Two-Sample Assuming Equal Variances

	method 1	*method 2*
Mean	79.5	94.28333333
Variance	66.064	24.75766667
Observations	6	6
Pooled Variance	45.41083333	
Hypothesized Mean Difference	0	
df	10	
t Stat	-3.799734578	
P(T<=t) one-tail	0.001743661	
t Critical one-tail	2.7637725	
P(T<=t) two-tail	0.003487322	
t Critical two-tail	3.169261618	

4-pentylbiphenyl
P(0.01)
t-Test: Two-Sample Assuming Equal Variances

	method 1	*method 2*
Mean	86.7	95.51666667
Variance	19.756	12.08166667
Observations	6	6
Pooled Variance	15.91883333	
Hypothesized Mean Difference	0	
df	10	
t Stat	-3.827449164	
P(T<=t) one-tail	0.001666362	
t Critical one-tail	2.7637725	
P(T<=t) two-tail	0.003332725	
t Critical two-tail	3.169261618	

phenanthrene
P(0.01)
t-Test: Two-Sample Assuming Equal Variances

	method 1	*method 2*
Mean	88.4	96.96666667
Variance	19.784	18.07466667
Observations	6	6
Pooled Variance	18.92933333	
Hypothesized Mean Difference	0	
df	10	
t Stat	-3.410396206	
P(T<=t) one-tail	0.003326927	
t Critical one-tail	2.7637725	
P(T<=t) two-tail	0.006653854	
t Critical two-tail	3.169261618	

pyrene
t-Test: Two-Sample Assuming Equal Variances
P (0.01)

	method 1	*method 2*
Mean	94.1	97.26666667
Variance	23.328	16.35066667
Observations	6	6
Pooled Variance	19.83933333	
Hypothesized Mean Difference	0	
df	10	
t Stat	-1.231400827	
P(T<=t) one-tail	0.123173169	
t Critical one-tail	1.812461505	
P(T<=t) two-tail	0.246346338	
t Critical two-tail	2.228139238	

P(0.05)
t-Test: Two-Sample Assuming Equal Variances

	method 1	*method 2*
Mean	94.1	97.26666667
Variance	23.328	16.35066667
Observations	6	6
Pooled Variance	19.83933333	
Hypothesized Mean Difference	0	
df	10	
t Stat	-1.231400827	
P(T<=t) one-tail	0.123173169	
t Critical one-tail	2.7637725	
P(T<=t) two-tail	0.246346338	
t Critical two-tail	3.169261618	

7-hexylnonadecane
P(0.01)
t-Test: Two-Sample Assuming Equal Variances

	method 1	*method 2*
Mean	91.86666667	100.15
Variance	15.21066667	21.327
Observations	6	6
Pooled Variance	18.26883333	
Hypothesized Mean Difference	0	
df	10	
t Stat	-3.35668323	
P(T<=t) one-tail	0.003641443	
t Critical one-tail	2.7637725	
P(T<=t) two-tail	0.007282887	
t Critical two-tail	3.169261618	

acetophenone
P(0.01)
t-Test: Two-Sample Assuming Equal Variances

	method 1	*method 2*
Mean	74.26666667	95.18333333
Variance	36.88666667	31.24566667
Observations	6	6
Pooled Variance	34.06616667	
Hypothesized Mean Difference	0	
df	10	
t Stat	-6.207138978	
P(T<=t) one-tail	5.0256E-05	
t Critical one-tail	2.7637725	
P(T<=t) two-tail	0.000100512	
t Critical two-tail	3.169261618	

9-fluorenone

t-Test: Two-Sample Assuming Equal Variances

P(0.05)

	method 1	*method 2*
Mean	88.31666667	100.9
Variance	79.80966667	44.092
Observations	6	6
Pooled Variance	61.95083333	
Hypothesized Mean Difference	0	
df	10	
t Stat	-2.769062462	
P(T<=t) one-tail	0.00990958	
t Critical one-tail	1.812461505	
P(T<=t) two-tail	0.01981916	
t Critical two-tail	2.228139238	

t-Test: Two-Sample Assuming Equal Variances

P(0.01)

	method 1	*method 2*
Mean	88.31666667	100.9
Variance	79.80966667	44.092
Observations	6	6
Pooled Variance	61.95083333	
Hypothesized Mean Difference	0	
df	10	
t Stat	-2.76906246	
P(T<=t) one-tail	0.00990958	
t Critical one-tail	2.7637725	
P(T<=t) two-tail	0.01981916	
t Critical two-tail	3.169261618	

benzoic acid

P (0.01)

t-Test: Two-Sample Assuming Equal Variances

	method 1	*method 2*
Mean	77.08333333	93.38333333
Variance	95.07766667	75.74566667
Observations	6	6
Pooled Variance	85.41166667	
Hypothesized Mean Difference	0	
df	10	
t Stat	-3.054848194	
P(T<=t) one-tail	0.006075396	
t Critical one-tail	2.7637725	
P(T<=t) two-tail	0.012150792	
t Critical two-tail	3.169261618	

t-Test: Two-Sample Assuming Equal Variances
(P(0.05)

	method 1	*method 2*
Mean	77.08333333	93.38333333
Variance	95.07766667	75.74566667
Observations	6	6
Pooled Variance	85.41166667	
Hypothesized Mean Difference	0	
df	10	
t Stat	-3.054848194	
P(T<=t) one-tail	0.006075396	
t Critical one-tail	1.812461505	
P(T<=t) two-tail	0.012150792	
t Critical two-tail	2.228139238	

cyclohexanecarboxylic acid

t-Test: Two-Sample Assuming Equal Variances
P (0.01)

	method 1	*method 2*
Mean	77.6	92.16666667
Variance	212.94	23.02266667
Observations	6	6
Pooled Variance	117.9813333	
Hypothesized Mean Difference	0	
df	10	
t Stat	-2.322812528	
P(T<=t) one-tail	0.021284164	
t Critical one-tail	2.7637725	
P(T<=t) two-tail	0.042568328	
t Critical two-tail	3.169261618	

t-Test: Two-Sample Assuming Equal Variances
P(0.05)

	method 1	*method 2*
Mean	77.6	92.16666667
Variance	212.94	23.02266667
Observations	6	6
Pooled Variance	117.9813333	
Hypothesized Mean Difference	0	
df	10	
t Stat	-2.322812528	
P(T<=t) one-tail	0.021284164	
t Critical one-tail	1.812461505	
P(T<=t) two-tail	0.042568328	
t Critical two-tail	2.228139238	

9-anthracenecarboxylic acid

t-Test: Two-Sample Assuming Equal Variances

P(0.01)

	method 1	*method 2*
Mean	81.56666667	97.05
Variance	48.79866667	29.615
Observations	6	6
Pooled Variance	39.20683333	
Hypothesized Mean Difference	0	
df	10	
t Stat	-4.282961867	
P(T<=t) one-tail	0.000801945	
t Critical one-tail	2.7637725	
P(T<=t) two-tail	0.00160389	
t Critical two-tail	3.169261618	

hexanedioic acid

t-Test: Two-Sample Assuming Equal Variances

P (0.05)

	method 1	*method 2*
Mean	75.13333333	92.3
Variance	96.30666667	126.568
Observations	6	6
Pooled Variance	111.4373333	
Hypothesized Mean Difference	0	
df	10	
t Stat	-2.816639375	
P(T<=t) one-tail	0.009132844	
t Critical one-tail	1.812461505	
P(T<=t) two-tail	0.018265687	
t Critical two-tail	2.228139238	

t-Test: Two-Sample Assuming Equal Variances

P(0.01)

	method 1	*method 2*
Mean	75.13333333	92.3
Variance	96.30666667	126.568
Observations	6	6
Pooled Variance	111.4373333	
Hypothesized Mean Difference	0	
df	10	
t Stat	-2.816639375	
P(T<=t) one-tail	0.009132844	
t Critical one-tail	2.7637725	
P(T<=t) two-tail	0.018265687	
t Critical two-tail	3.169261618	

1-naphthol

t-Test: Two-Sample Assuming Equal Variances

P(0.01)

	method 1	*method 2*
Mean	74.06666667	95.88333333
Variance	154.9666667	47.49766667
Observations	6	6
Pooled Variance	101.2321667	
Hypothesized Mean Difference	0	
df	10	
t Stat	-3.75569017	
P(T<=t) one-tail	0.0018743	
t Critical one-tail	2.7637725	
P(T<=t) two-tail	0.003748601	
t Critical two-tail	3.169261618	

9-hydroxyfluorene

t-Test: Two-Sample Assuming Equal Variances

P (0.05)

	method 1	*method 2*
Mean	82.6	95.13333333
Variance	69.356	27.87466667
Observations	6	6
Pooled Variance	48.61533333	
Hypothesized Mean Difference	0	
df	10	
t Stat	-3.113440594	
P(T<=t) one-tail	0.005498038	
t Critical one-tail	1.812461505	
P(T<=t) two-tail	0.010996076	
t Critical two-tail	2.228139238	

t-Test: Two-Sample Assuming Equal Variances

P (0.01)

	method 1	*method 2*
Mean	82.6	95.13333333
Variance	69.356	27.87466667
Observations	6	6
Pooled Variance	48.61533333	
Hypothesized Mean Difference	0	
df	10	
t Stat	-3.113440594	
P(T<=t) one-tail	0.005498038	
t Critical one-tail	2.7637725	
P(T<=t) two-tail	0.010996076	
t Critical two-tail	3.169261618	

COMPOUND	Replicate 1	2	3	4	5	6	mean	sd	RSD
aliphatic									
4-propyloctane (1 µ)	14.2	16.7	23.4	15.4	25.3	17.6	**18.8**	**4.1**	**22.0**
7-hexylnonadecane (1 µg)	89.7	84.3	92.3	87.6	94.3	84.3	**88.8**	**4.1**	**4.7**
aromatic									
phenyldecane (3 µg)	59.2	55.2	54.3	48.7	62.1	58.6	**56.4**	**4.3**	**7.6**
5-ethyltetralin (2 µg)	74.6	69.3	65.6	63.2	68.9	67.4	**68.2**	**3.5**	**5.2**
2-ethylnaphthalene (2 µg)	76.3	71.2	67.3	65.3	70.1	68.6	**69.8**	**3.8**	**5.4**
1,3-diphenylhexane (1 µg)	73.2	70.8	71.4	68.1	73.2	70.4	**71.2**	**1.8**	**2.5**
4-pentylbiphenyl (3 µg)	80.6	75.6	77.6	74.3	80.2	76.5	**77.5**	**2.3**	**3.0**
phenanthrene (3 µg)	82.6	80.3	84.3	79.2	85.4	81.3	**82.2**	**2.2**	**2.6**
pyrene (3 µg)	95.6	91.4	89.6	87.5	94.3	93.3	**92.0**	**2.8**	**3.0**
polar									
acetophenone (4 µg)	65.2	71.3	68.2	61.2	70.4	65.6	**67.0**	**3.4**	**5.1**
9-fluorenone (4 µg)	78.1	87.9	77.7	70.5	86.5	79.8	**80.1**	**5.8**	**7.3**
benzoic acid (2 µg)	20.4	35.4	25.5	28.3	31.2	33.4	**29.0**	**5.0**	**17.3**
cyclohexanecarboxylic acid (2 µg)	32.3	36.3	29.7	26.6	34.1	33.7	**32.1**	**3.2**	**9.9**
9-anthracenecarboxylic acid (2 µg)	54.3	62.1	58.6	50.2	60.7	62.3	**58.0**	**4.4**	**7.6**
hexanedioic acid (1µg)	n.d*	n.d	n.d	n.d	n.d	n.d	**n.d**	**n.d**	**-**
1-naphthol (2 µg)	50.2	43.7	45.6	40.3	48.7	45.0	**45.6**	**3.2**	**7.1**
9-hydroxyfluorenone (1 µg)	45.3	36.7	40.7	39.9	44.9	38.9	**41.1**	**3.1**	**7.6**

value in brackets is amount spiked into tissue
** not detected*

RAW DATA Percentage recovery of compounds spiked into mussel homogenate using DCM: MeOH soxhlet extraction

COMPOUND	Replicate 1	2	3	4	5	6	**mean**	**sd**	**RSD**
aliphatic									
4-propyloctane (1 µ)	42.3	31.2	36.7	44.1	48.2	35.6	**39.7**	**5.7**	**14.4**
7-hexylnonadecane (1 µg)	96.4	93.2	90.4	97.7	96.5	92.0	**94.4**	**2.9**	**3.1**
aromatic									
phenyldecane (3 µg)	51.7	56.4	55.4	67.3	64.2	58.0	**58.8**	**5.3**	**9.0**
5-ethyltetralin (2 µg)	70.3	65.3	68.4	69.4	68.5	70.1	**68.7**	**1.7**	**2.4**
2-ethylnaphthalene (2 µg)	72.3	67.7	70.4	68.0	66.4	72.3	**69.5**	**2.5**	**3.6**
1,3-diphenylhexane (1 µg)	84.5	78.9	77.4	79.8	83.2	77.7	**80.3**	**2.7**	**3.4**
4-pentylbiphenyl (3 µg)	88.4	79.3	81.3	79.1	85.6	83.4	**82.9**	**3.4**	**4.1**
phenanthrene (3 µg)	84.6	80.0	84.3	82.9	87.6	84.1	**83.9**	**2.3**	**2.7**
pyrene (3 µg)	97.8	83.2	89.2	93.2	95.6	89.7	**91.5**	**4.8**	**5.2**
polar									
acetophenone (4 µg)	72.0	65.4	68.4	73.4	69.1	70.4	**69.8**	**2.6**	**3.7**
9-fluorenone (4 µg)	81.6	73.2	85.6	78.1	86.2	77.4	**80.4**	**4.6**	**5.8**
benzoic acid (2 µg)	79.2	71.0	63.1	72.3	68.4	69.7	**70.6**	**4.8**	**6.8**
cyclohexanecarboxylic acid (2 µg)	79.6	72.3	73.2	68.4	63.1	68.7	**70.9**	**5.1**	**7.2**
9-anthracenecarboxylic acid (2 µg)	75.4	72.1	68.1	79.6	74	77.9	**74.5**	**3.8**	**5.1**
hexanedioic acid (1µg)	n.d*	n.d	n.d	n.d	n.d	n.d	**n.d**	**n.d**	**-**
1-naphthol (2 µg)	74.5	68.7	71.2	70.8	68.5	75.3	**71.5**	**2.6**	**3.6**
9-hydroxyfluorenone (1 µg)	67.5	61.2	78.3	68.4	71.7	65	**68.7**	**5.4**	**7.8**

value in brackets is amount spiked into tissue
** not detected*

RAW DATA Percentage recovery of compounds spiked into mussel homogenate using DCM soxhlet extraction

COMPOUND	Replicate 1	2	3	4	5	6	mean	sd	RSD
aliphatic									
4-propyloctane (1 µ)	52.8	63.4	60.6	57.3	55.4	56.8	**57.7**	**3.4**	**6.0**
7-hexylnonadecane (1 µg)	90.4	92.5	96.7	93.4	92.2	97.8	**93.8**	**2.8**	**3.0**
aromatic									
phenyldecane (3 µg)	67.8	70.8	74.3	68.9	72.3	71.2	**70.9**	**2.1**	**3.0**
5-ethyltetralin (2 µg)	74.5	77.4	73.4	75.6	72.1	73.9	**74.5**	**1.7**	**2.3**
2-ethylnaphthalene (2 µg)	75.2	74.1	76.4	77.8	72.5	73.9	**75.0**	**1.9**	**2.5**
1,3-diphenylhexane (1 µg)	86.5	91.2	89.2	90.3	86.5	87.7	**88.6**	**1.8**	**2.0**
4-pentylbiphenyl (3 µg)	86.7	89.2	87.7	85.4	90.2	85.6	**87.5**	**1.8**	**2.0**
phenanthrene (3 µg)	88.0	93.4	90.2	93.1	89.6	88.4	**90.5**	**2.1**	**2.3**
pyrene (3 µg)	87.3	94.0	95.6	97.3	92.1	98.4	**94.1**	**3.7**	**3.9**
polar									
acetophenone (4 µg)	60.4	65.5	60.2	63.4	55.3	70.1	**62.5**	**4.6**	**7.4**
9-fluorenone (4 µg)	99.4	90.9	94.3	97.2	91.1	96.3	**94.9**	**3.1**	**3.3**
benzoic acid (2 µg)	77.8	69.0	64.0	71.5	70.8	68.5	**70.3**	**4.1**	**5.9**
cyclohexanecarboxylic acid (2 µg)	64.8	60.4	67.3	78.2	61.1	69.2	**66.8**	**6.0**	**8.9**
9-anthracenecarboxylic acid (2 µg)	72.2	61.4	75.4	73.2	77.1	70.5	**71.6**	**5.0**	**7.0**
hexanedioic acid (1µg)	n.d*	n.d	n.d	n.d	n.d	n.d	**n.d**	**n.d**	**-**
1-naphthol (2 µg)	57.7	67.7	69.8	65.4	74.3	71.2	**67.7**	**5.3**	**7.8**
9-hydroxyfluorenone (1 µg)	56.2	65.3	74.3	77.2	68.4	69.1	**68.4**	**6.7**	**9.8**

value in brackets is amount of compound spiked into tissue

- *not detected*

RAW DATA Percentage recovery of compounds spiked into mussel homogenate using the two-phase extraction method

APPENDIX A.2.4
COMPARISON OF SAMPLE EXTRACTION TECHNIQUES; DCM Soxhlet extraction *vs* two-phase extraction method

4-propyloctane

t-Test: Two-Sample Assuming Equal Variances
P(0.05)

	Soxhlet	*2-phase*
Mean	39.68333333	57.71666667
Variance	39.28566667	14.23366667
Observations	6	6
Pooled Variance	26.75966667	
Hypothesized Mean Difference	0	
df	10	
t Stat	-6.038044203	
P(T<=t) one-tail	6.2793E-05	
t Critical one-tail	2.7637725	
P(T<=t) two-tail	0.000125586	
t Critical two-tail	3.169261618	

phenyldecane

t-Test: Two-Sample Assuming Equal Variances
P(0.05)

	Soxhlet	*2-phase*
Mean	58.83333333	70.88333333
Variance	33.95466667	5.445666667
Observations	6	6
Pooled Variance	19.70016667	
Hypothesized Mean Difference	0	
df	10	
t Stat	-4.702325889	
P(T<=t) one-tail	0.000419301	
t Critical one-tail	1.812461505	
P(T<=t) two-tail	0.000838602	
t Critical two-tail	2.228139238	

5-ethyltetralin

t-Test: Two-Sample Assuming Equal Variances
P (0.05)

	Soxhlet	*2-phase*
Mean	68.66666667	74.48333333
Variance	3.338666667	3.389666667
Observations	6	6
Pooled Variance	3.364166667	
Hypothesized Mean Difference	0	
df	10	
t Stat	-5.49282866	
P(T<=t) one-tail	0.000132218	
t Critical one-tail	1.812461505	
P(T<=t) two-tail	0.000264436	
t Critical two-tail	2.228139238	

2-ethylnaphthalene

t-Test: Two-Sample Assuming Equal Variances

P (0.05)

	Soxhlet	*2-phase*
Mean	69.51666667	74.98333333
Variance	6.317666667	3.621666667
Observations	6	6
Pooled Variance	4.969666667	
Hypothesized Mean Difference	0	
df	10	
t Stat	-4.247365066	
P(T<=t) one-tail	0.000848278	
t Critical one-tail	1.812461505	
P(T<=t) two-tail	0.001696555	
t Critical two-tail	2.228139238	

1,3-diphenylhexane

t-Test: Two-Sample Assuming Equal Variances

P (0.05)

	Soxhlet	*2-phase*
Mean	80.25	88.566667
Variance	8.683	3.9266667
Observations	6	6
Pooled Variance	6.304833333	
Hypothesized Mean Difference	0	
df	10	
t Stat	-5.73684502	
P(T<=t) one-tail	9.42658E-05	
t Critical one-tail	1.812461505	
P(T<=t) two-tail	0.000188532	
t Critical two-tail	2.228139238	

4-pentylbiphenyl

t-Test: Two-Sample Assuming Equal Variances

P (0.05)

	Soxhlet	*2-phase*
Mean	82.85	87.466667
Variance	13.547	3.7746667
Observations	6	6
Pooled Variance	8.660833333	
Hypothesized Mean Difference	0	
df	10	
t Stat	-2.71712304	
P(T<=t) one-tail	0.010833531	
t Critical one-tail	1.812461505	
P(T<=t) two-tail	0.021667063	
t Critical two-tail	2.228139238	

phenanthrene

t-Test: Two-Sample Assuming Equal Variances

P (0.05)

	Soxhlet	*2-phase*
Mean	83.91666667	90.45
Variance	6.117666667	5.343
Observations	6	6
Pooled Variance	5.730333333	
Hypothesized Mean Difference	0	
df	10	
t Stat	-4.72721659	
P(T<=t) one-tail	0.000403785	
t Critical one-tail	1.812461505	
P(T<=t) two-tail	0.000807571	
t Critical two-tail	2.228139238	

pyrene

t-Test: Two-Sample Assuming Equal Variances

P (0.05)

	Soxhlet	*2-phase*
Mean	91.45	94.116667
Variance	27.359	16.245667
Observations	6	6
Pooled Variance	21.80233333	
Hypothesized Mean Difference	0	
df	10	
t Stat	-0.9891858	
P(T<=t) one-tail	0.172951226	
t Critical one-tail	1.812461505	
P(T<=t) two-tail	0.345902452	
t Critical two-tail	2.228139238	

7-hexylnonadecane

t-Test: Two-Sample Assuming Equal Variances

P(0.05)

	Soxhlet	*2-phase*
Mean	94.36666667	93.83333333
Variance	8.498666667	8.074666667
Observations	6	6
Pooled Variance	8.286666667	
Hypothesized Mean Difference	0	
df	10	
t Stat	0.320899781	
P(T<=t) one-tail	0.377446054	
t Critical one-tail	2.7637725	
P(T<=t) two-tail	0.754892108	
t Critical two-tail	3.169261618	

acetophenone

t-Test: Two-Sample Assuming Equal Variances

P (0.05)

	Soxhlet	*2-phase*
Mean	69.78333333	62.48333333
Variance	7.993666667	25.82166667
Observations	6	6
Pooled Variance	16.90766667	
Hypothesized Mean Difference	0	
df	10	
t Stat	3.074975422	
P(T<=t) one-tail	0.005870401	
t Critical one-tail	1.812461505	
P(T<=t) two-tail	0.011740801	
t Critical two-tail	2.228139238	

9-fluorenone

t-Test: Two-Sample Assuming Equal Variances

P (0.05)

	Soxhlet	*2-phase*
Mean	80.35	94.86666667
Variance	25.647	11.65866667
Observations	6	6
Pooled Variance	18.65283333	
Hypothesized Mean Difference	0	
df	10	
t Stat	-5.821771117	
P(T<=t) one-tail	8.39573E-05	
t Critical one-tail	1.812461505	
P(T<=t) two-tail	0.000167915	
t Critical two-tail	2.228139238	

benzoic acid

P (0.05)

t-Test: Two-Sample Assuming Equal Variances

	Soxhlet	*2-phase*
Mean	70.61666667	70.26666667
Variance	27.78166667	20.51066667
Observations	6	6
Pooled Variance	24.14616667	
Hypothesized Mean Difference	0	
df	10	
t Stat	0.123368582	
P(T<=t) one-tail	0.452129799	
t Critical one-tail	1.812461505	
P(T<=t) two-tail	0.904259597	
t Critical two-tail	2.228139238	

cyclohexanecarboxylic acid

t-Test: Two-Sample Assuming Equal Variances

P (0.05)

	Soxhlet	*2-phase*
Mean	70.88333333	66.83333333
Variance	30.97366667	42.68266667
Observations	6	6
Pooled Variance	36.82816667	
Hypothesized Mean Difference	0	
df	10	
t Stat	1.155914205	
P(T<=t) one-tail	0.137293051	
t Critical one-tail	1.812461505	
P(T<=t) two-tail	0.274586102	
t Critical two-tail	2.228139238	

9-anthracenecarboxylic acid

t-Test: Two-Sample Assuming Equal Variances

P (0.05)

	Soxhlet	*2-phase*
Mean	74.51666667	71.63333333
Variance	17.06966667	30.57066667
Observations	6	6
Pooled Variance	23.82016667	
Hypothesized Mean Difference	0	
df	10	
t Stat	1.023253131	
P(T<=t) one-tail	0.165152115	
t Critical one-tail	1.812461505	
P(T<=t) two-tail	0.33030423	
t Critical two-tail	2.228139238	

1-naphthol

t-Test: Two-Sample Assuming Equal Variances

P (0.05)

	Soxhlet	*2-phase*
Mean	71.5	67.68333333
Variance	8.172	33.10166667
Observations	6	6
Pooled Variance	20.63683333	
Hypothesized Mean Difference	0	
df	10	
t Stat	1.455202161	
P(T<=t) one-tail	0.088137534	
t Critical one-tail	1.812461505	
P(T<=t) two-tail	0.176275068	
t Critical two-tail	2.228139238	

9-hydroxyfluorene
t-Test: Two-Sample Assuming Equal Variances
P (0.05)

	Soxhlet	*2-phase*
Mean	68.68333333	68.36666667
Variance	34.52566667	54.62666667
Observations	6	6
Pooled Variance	44.57616667	
Hypothesized Mean Difference	0	
df	10	
t Stat	0.082150766	
P(T<=t) one-tail	0.468073946	
t Critical one-tail	1.812461505	
P(T<=t) two-tail	0.936147892	
t Critical two-tail	2.228139238	

Sample Site		Aliphatic		Aromatic		
		total resolved ($\mu g\ g^{-1}$ wet wt)	total unresolved ($\mu g\ g^{-1}$ wet wt)	total resolved ($\mu g\ g^{-1}$ wet wt)	total unresolved ($\mu g\ g^{-1}$ wet wt)	total hydrocarbons ($\mu g\ g^{-1}$ wet wt)
New Brighton (Mersey)	(i)	3	176	4	27	210
	(ii)	1	97	7	36	141
Cleethorpes	(i)	4	60	3	15	82
	(ii)	3	25	3	20	51
Teesmouth	(i)	2	44	4	19	69
	(ii)	3	64	4	22	93
Whitby Harbour	(i)	27	857	15	87	986
	(ii)	29	779	13	118	939
Whitsand Bay	(i)	3	3	1	n.o	7
	(ii)	2	1	1	n.o	4

n.o; not observed

total hydrocarbons are defined as total (resolved + unresolved) aromatic and aliphatic hydrocarbons

HYDROCARBON CONCENTRATIONS IN MUSSELS FROM SELECTED U.K. COASTAL SITES, EXPRESSED ON A WET TISSUE WEIGHT BASIS

Sample Site		Aliphatic		Aromatic		
		total resolved ($\mu g\ mg^{-1}$ lipid)	total unresolved ($\mu g\ mg^{-1}$ lipid)	total resolved ($\mu g\ mg^{-1}$ lipid)	total unresolved ($\mu g\ mg^{-1}$ lipid)	total hydrocarbons ($\mu g\ mg^{-1}$ lipid)
New Brighton (Mersey)	(i)	0.7	40.2	1.0	6.2	48.1
	(ii)	0.2	22.2	1.7	8.3	32.4
Cleethorpes	(i)	1.5	24.6	1.4	6.3	33.8
	(ii)	1.3	10.4	1.1	8.3	21.1
Teesmouth	(i)	0.7	16.3	1.6	7.2	25.8
	(ii)	1.0	23.9	1.6	8.2	34.7
Whitby Harbour	(i)	9.3	294.4	5.2	29.8	338.7
	(ii)	10.0	267.5	4.6	40.4	332.5
Whitsand Bay	(i)	1.4	1.3	0.3	n.o	3
	(ii)	1.0	0.7	0.6	n.o	2.3

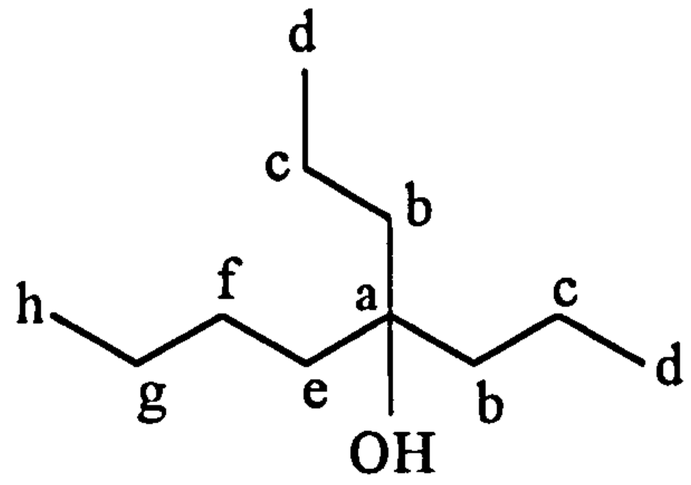
n.o; not observed

total hydrocarbons are defined as total (resolved + unresolved) aromatic and aliphatic hydrocarbons

HYDROCARBON CONCENTRATIONS IN MUSSELS FROM SELECTED U.K. COASTAL SITES, EXPRESSED ON A LIPID WEIGHT BASIS

APPENDIX C.1

^{13}C-NMR spectral assignments for 4-propyloctan-4-ol

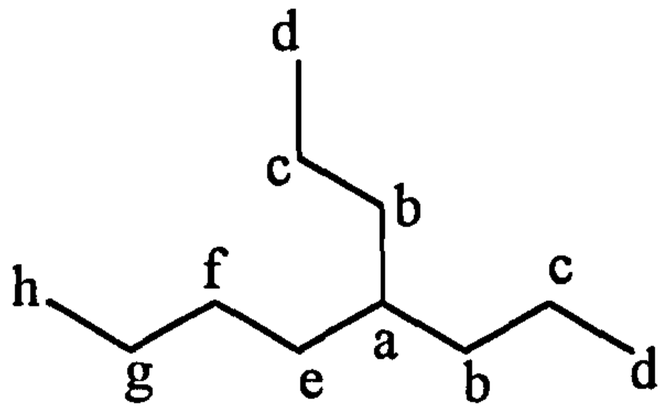


Carbon	multiplicity	chemical shift (ppm)
a	C	74.3
b	CH_2	41.6
c	CH_2	16.6
d	CH_3	14.6
e	CH_2	38.9
f	CH_2	25.6
g	CH_2	23.3
h	CH_3	14.0

refer to Figure 3.6 for spectra (^{13}C and DEPT)

APPENDIX C.2

^{13}C-NMR spectral assignments for 4-propyloctane



Carbon	multiplicity	chemical shift (ppm)
a	CH	36.8
b	CH_2	36.1
c	CH_2	19.0
d	CH_3	14.2
e	CH_2	33.4
f	CH_2	28.9
g	CH_2	23.2
h	CH_3	14.5

refer to Figure 3.12 for spectra (^{13}C and DEPT)

APPENDIX D.1

Estimation of octanol-water partition coefficients (log K_{OW}) using Leo's fragment constant method (from Lyman, 1990).

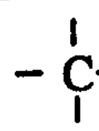
This approach uses empirically derived atomic or group fragment constants (f) and structural factors (F). All calculations are carried out in terms of log K_{OW}:

$$\log K_{OW} = \text{sum of fragments (f)} + \text{factors (F)}$$

(i) Fragment constants (f values)

Fragment constants (f values) used in the calculations described herein are listed in Table 1 and taken from Lyman (1990). A fragment has different f values, depending on the type of structure (*e.g.* aliphatic or aromatic) it is bonded to.

A fragment is defined as an atom, or string of atoms, whose exterior bonds are to isolating carbon atoms. (An isolating carbon is one that has either four single bonds, at least two of which are to non-hetero atoms, or is multiply bonded to other carbon atoms.

fragment	f	f^{Φ}
	0.20	0.20
CH_3	0.89	0.89
-H	0.23	0.23
<u>CH</u>		0.35_5
<u>C</u>		0.13

$^{\Phi}$denotes attachment to aromatic ring

underlining any symbol associated with a fragment constant means the fragment is present in a ring

Table 1. Fragment values used in log K_{OW} calculations (from Lyman, 1990)

(ii) Factors (F values)

F_b = bond factor

A bond factor of -0.12 for chains and -0.09 for non-aromatic rings is taken (n-1) times, where n is the number of bonds in the molecule, with the following provisions:

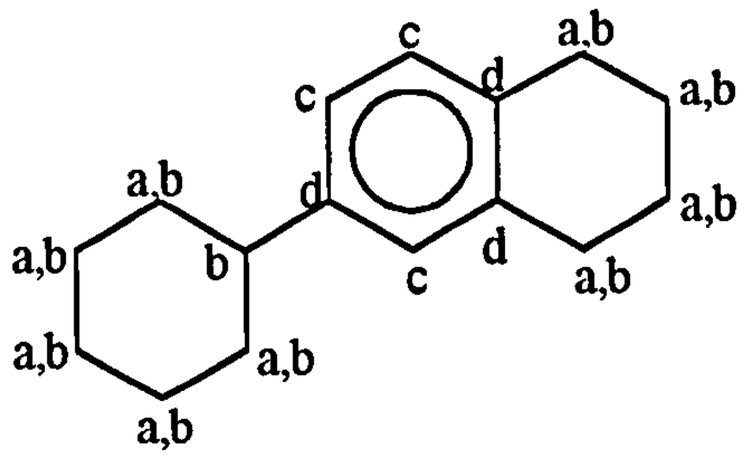
- Do not count between hydrogen and any other atom
- In ring chain combinations, consider that the ring stops the count.
- Double and triple bonds are considered equivalent to single bonds for the calculation of F_b.

Branching factors

The length of the branching must be just one or two carbon atoms; or, two or more of the branches must contain hydrophilic groups. If the branching is more than two carbons long, use the factor F_{bYN} (-0.20) , which is taken (n-1) times, as described above for branching factors.

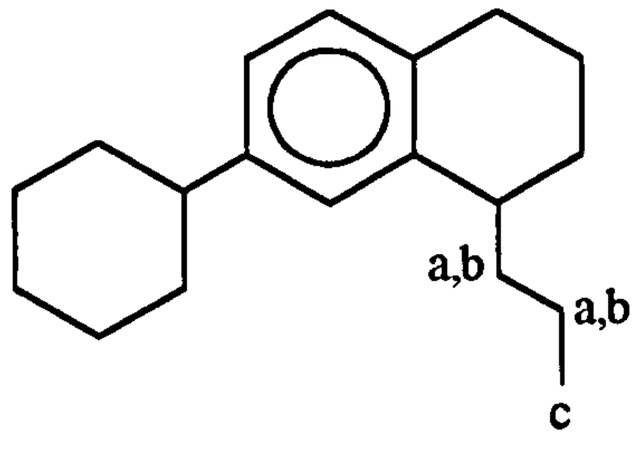
Calculation of the log K_{ow} of 7-cyclohexyltetralin and 7-cyclohexyl-1-propyl-tetralin using Leo's fragments method (Leo and Hansch, 1979) was carried out following the guidelines described in Lyman (1990) and summarised above. Fragments have been labelled a,b *etc.* in order to clarify the fragmentation of the molecule for the reader.

(i) 7-cyclohexyltetralin



(a)	$9 \times f_C = 9 \times (0.20)$	$= 1.8$
+ (b)	$19 \times f_H = 19 \times (0.23)$	$= 4.37$
+ (c)	$3 \times f^{\phi}_{\underline{CH}} = 3 \times (0.355)$	$= 1.065$
+ (d)	$3 \times f^{\phi}_{\underline{C}} = 3 \times (0.13)$	$= 0.39$
+	$(11\text{-}1) \times F_b = 10 \times (-0.09)$ (correction for cyclisation)	$= -0.9$
+	$1 \times F_b = 1 \times (-0.12)$	$= -0.12$
	$\log K_{OW}$	**$= 6.61$**

(ii) 7-cyclohexyl-1-propyl-tetralin



$\log K_{OW}$ of 'base compound'	(- 1H)	$= 6.61\text{-}0.23$
+ (a)	$2 \times f_C = 2 \times (0.20)$	$= 0.40$
+ (b)	$4 \times f_H = 4 \times (0.23)$	$= 0.46$
+ (c)	$1 \times f_{CH3}$	$= 0.89$
+	$(3\text{-}1) \times F_{bYN} = 2 \times (-0.20)$	$= -0.40$
	$\log K_{OW}$	**$= 7.73$**

animal	8 µg/l		14µg/l (24h)		14 µg/l (72 h)		28 µg/l (24 h)		28 µg/l (72h)		60 µg/l	
	control	exposed	control	exposed	control	exposed	control	exposed	control	exposed	control	exposed
1	2.86	2.31	2.83	1.75	2.78	1.91	2.09	1.07	2.30	0.92	2.86	0.92
2	3.05	2.78	3.26	0.96	2.88	1.71	2.55	0.69	1.70	1.56	3.05	1.00
3	2.94	2.82	3.23	1.20	2.95	1.79	1.80	1.02	2.68	1.02	2.94	0.00
4	3.12	2.28	1.56	1.56	1.79	2.43	2.54	1.30	3.12	1.45	3.12	0.86
5	2.30	1.75	2.84	2.93	2.48	3.33	3.03	1.02	2.94	1.13	2.30	0.46
6	2.53	1.72	4.10	0.77	2.82	1.85	2.31	1.60	3.43	2.92	2.53	1.43
7	2.96	2.66	1.43	1.22	2.81	1.37	2.67	0.78	2.56	0.67	2.96	1.32
8	2.93	2.22	3.50	2.47	3.03	2.80	1.59	0.94	3.64	1.45	2.93	1.63
9	2.91	1.62	3.15	1.98	2.81	1.56	3.26	0.90	2.40	1.37	2.91	0.91
10	2.98	2.72	3.13	2.07	3.03	2.26	2.29	1.94	3.14	1.66	2.98	1.13
11	2.41	3.07	4.34	1.83	3.61	3.02	2.10	0.77	3.11	1.09	2.41	1.37
12	3.19	2.80	2.48	2.35	2.85	2.78	2.63	0.46	3.20	3.39	3.19	1.14
13	2.68	2.58	3.16	1.51	2.49	2.54	3.13	1.53	3.00	1.42	2.68	0.91
14	2.11	3.25	2.66	1.70	3.09	2.10	2.59	1.10	3.12	1.26	2.11	0.71
15	3.34	2.43	3.38	2.79	3.13	3.07	2.64	0.45	2.57	1.33	3.34	0.39
16	2.64	2.66	3.25	1.73	2.77	2.69	2.32	1.35	2.76	1.90	2.64	1.03
mean	2.81	2.48	3.02	1.80	2.83	2.33	2.47	1.06	2.85	1.53	2.81	0.95
n	16.00	16.00	16.00	16.00	16.00	16.00	16.00	16.00	16.00	16.00	16.00	16.00
sd	0.34	0.47	0.76	0.62	0.38	0.59	0.45	0.41	0.48	0.70	0.34	0.42
se	0.08	0.12	0.19	0.15	0.10	0.15	0.11	0.10	0.12	0.18	0.08	0.10

RAW DATA; DOSE-RESPONSE EXPERIMENTS I AND II

APPENDIX E.1.1

Comparison of 24 h and 72 h data, 14 µg l^{-1}

t-Test: Two-Sample Assuming Equal Variances
P (0.05)

	24 h	*72 h*
Mean	1.80125	2.325625
Variance	0.382438333	0.352372917
Observations	16	16
Pooled Variance	0.367405625	
Hypothesized Mean Difference	0	
df	30	
t Stat	-2.446887887	
P(T<=t) one-tail	0.010241853	
t Critical one-tail	1.697260359	
P(T<=t) two-tail	0.020483707	
t Critical two-tail	2.042270353	

Comparison of 24 h and 72 h data, 28 µg l^{-1}

t-Test: Two-Sample Assuming Equal Variances
P (0.05)

	24 h	*72 h*
Mean	1.53375	1.0575
Variance	0.495665	0.166993333
Observations	16	16
Pooled Variance	0.331329167	
Hypothesized Mean Difference	0	
df	30	
t Stat	2.340184767	
P(T<=t) one-tail	0.013058863	
t Critical one-tail	1.697260359	
P(T<=t) two-tail	0.026117726	
t Critical two-tail	2.042270353	

APPENDIX E.2

RAW DATA;
EXPERIMENT III
4-PO, 96 h (i) exposure: 23 µg/l

animal	0h	24h	48hC	48h	72h	96hC	96h
1	3.41	2.20	1.93	2.10	2.03	2.44	2.45
2	2.44	3.01	3.26	1.40	2.66	2.81	1.72
3	2.72	2.97	2.44	1.63	1.62	2.37	2.39
4	3.00	1.58	3.01	1.70	2.29	1.91	2.19
5	2.70	1.87	2.73	2.24	2.19	2.81	1.21
6	2.58	2.37	2.62	2.16	1.86	2.89	1.72
7	2.87	2.05	2.18	1.29	1.67	2.02	2.26
8	1.79	2.60	3.30	1.39	2.20	2.28	1.76
9	1.79	1.78	2.11	1.50	1.78	2.59	2.54
10	2.64	2.70	3.50	1.98	1.49	3.03	2.08
11	2.98	1.80	3.28	1.85	1.99	1.81	2.11
12	2.90	2.75	2.97	2.27	1.86	2.47	1.66
13	2.21	2.58	2.67	1.71	2.18	2.14	1.29
14	2.65	2.33	2.98	2.05	1.07	2.20	1.38
15	2.45	2.19	2.78	1.27	1.60	2.68	1.62
16	2.41	1.65	2.27	2.08	2.47	2.29	2.03
mean	2.59	2.28	2.75	1.79	1.93	2.42	1.90
n	16	16	16	16	16	16	16
sd	0.42	0.46	0.47	0.35	0.40	0.36	0.42
se	0.11	0.11	0.12	0.09	0.10	0.09	0.10
2 se	0.21	0.23	0.23	0.17	0.20	0.18	0.21
95% CI	0.21	0.23	0.23	0.17	0.20	0.18	0.20

E.2.1 COMPARISON OF MUSSEL FEEDING RATES OF CONTROL ANIMALS OVER THE EXPOSURE PERIOD; EXPERIMENT III

96 h (i) - CONTROL values

animal	0h	48hC	96hC
1	3.41	1.93	2.44
2	2.44	3.26	2.81
3	2.72	2.44	2.37
4	3.00	3.01	1.91
5	2.70	2.73	2.81
6	2.58	2.62	2.89
7	2.87	2.18	2.02
8	1.79	3.30	2.28
9	1.79	2.11	2.59
10	2.64	3.50	3.03
11	2.98	3.28	1.81
12	2.90	2.97	2.47
13	2.21	2.67	2.14
14	2.65	2.98	2.20
15	2.45	2.78	2.68
16	2.41	2.27	2.29

Anova: Single Factor

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
Column 1	16	41.54	2.59625	0.180038
Column 2	16	44.03	2.751875	0.22219
Column 3	16	38.74	2.42125	0.130305

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	0.875504	2	0.437752	2.466056	0.096324	3.20432
Within Groups	7.987994	45	0.177511			
Total	8.863498	47				

E.2.2 COMPARISON OF MUSSEL FEEDING RATES OF EXPOSED ANIMALS OVER THE 96 H EXPOSURE PERIOD; EXPERIMENT III

96 h (i) TOXICANT values

animal	0h	24h	48h	72h	96h
1	3.41	2.20	2.10	2.03	2.45
2	2.44	3.01	1.40	2.66	1.72
3	2.72	2.97	1.63	1.62	2.39
4	3.00	1.58	1.70	2.29	2.19
5	2.70	1.87	2.24	2.19	1.21
6	2.58	2.37	2.16	1.86	1.72
7	2.87	2.05	1.29	1.67	2.26
8	1.79	2.60	1.39	2.20	1.76
9	1.79	1.78	1.50	1.78	2.54
10	2.64	2.70	1.98	1.49	2.08
11	2.98	1.80	1.85	1.99	2.11
12	2.90	2.75	2.27	1.86	1.66
13	2.21	2.58	1.71	2.18	1.29
14	2.65	2.33	2.05	1.07	1.38
15	2.45	2.19	1.27	1.60	1.62
16	2.41	1.65	2.08	2.47	2.03

Anova: Single Factor

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
Column 1	16	41.54	2.59625	0.180038
Column 2	16	36.43	2.276875	0.21413
Column 3	16	28.62	1.78875	0.120932
Column 4	16	30.96	1.935	0.16056
Column 5	16	30.41	1.900625	0.17322

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	7.062393	4	1.765598	10.39958	9.37E-07	2.493692
Within Groups	12.73319	75	0.169776			
Total	19.79558	79				

E.2.3 COMPARISON OF MUSSEL FEEDING RATES (EXPOSED ANIMALS) FROM 24 H EXPOSURE TO 96 H EXPOSURE; EXPERIMENT III

96 h (i) TOXICANT values

animal	24h	48h	72h	96h
1	2.20	2.10	2.03	2.45
2	3.01	1.40	2.66	1.72
3	2.97	1.63	1.62	2.39
4	1.58	1.70	2.29	2.19
5	1.87	2.24	2.19	1.21
6	2.37	2.16	1.86	1.72
7	2.05	1.29	1.67	2.26
8	2.60	1.39	2.20	1.76
9	1.78	1.50	1.78	2.54
10	2.70	1.98	1.49	2.08
11	1.80	1.85	1.99	2.11
12	2.75	2.27	1.86	1.66
13	2.58	1.71	2.18	1.29
14	2.33	2.05	1.07	1.38
15	2.19	1.27	1.60	1.62
16	1.65	2.08	2.47	2.03

Anova: Single Factor

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
Column 1	16	36.43	2.276875	0.21413
Column 2	16	28.62	1.78875	0.120932
Column 3	16	30.96	1.935	0.16056
Column 4	16	30.41	1.900625	0.17322

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	2.127181	3	0.70906	4.240533	0.008758	2.758078
Within Groups	10.03261	60	0.16721			
Total	12.15979	63				

E.2.4 COMPARISON OF MUSSEL FEEDING RATES (EXPOSED ANIMALS) OVER 48 H - 72 H EXPOSURE PERIOD; EXPERIMENT III

Anova: Single
Factor

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
Column 1	16	28.62	1.78875	0.120932
Column 2	16	30.96	1.935	0.16056
Column 3	16	30.41	1.900625	0.17322

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	0.187129	2	0.093565	0.617301	0.543903	3.20432
Within Groups	6.820669	45	0.15157			
Total	7.007798	47				

E.2.5 COMPARSION OF MUSSEL FEEDING RATES (EXPOSED ANIMALS) AT 0H AND 24 H; EXPERIMENT III

t-Test: Two-Sample Assuming Equal
Variances

	Variable 1	*Variable 2*
Mean	2.59625	2.276875
Variance	0.180038333	0.214129583
Observations	16	16
Pooled Variance	0.197083958	
Hypothesized Mean Difference	0	
df	30	
t Stat	2.034793178	
P(T<=t) one-tail	0.02539616	
t Critical one-tail	1.697260359	
P(T<=t) two-tail	0.050792319	
t Critical two-tail	2.042270353	

E.2.6 COMPARISON OF CONTROL FEEDING RATE AND EXPOSED FEEDING RATE AT 48 H EXPOSURE; EXPERIMENT III

t-Test: Two-Sample Assuming Equal Variances

	Variable 1	*Variable 2*
Mean	2.751875	1.78875
Variance	0.222189583	0.120931667
Observations	16	16
Pooled Variance	0.171560625	
Hypothesized Mean Difference	0	
df	30	
t Stat	6.576863672	
P(T<=t) one-tail	1.40735E-07	
t Critical one-tail	1.697260359	
P(T<=t) two-tail	2.81469E-07	
t Critical two-tail	2.042270353	

APPENDIX E.3

RAW DATA; EXPERIMENT IV

4-PO, 96 h (ii) exposure: 23 µg/l

animal	0h	24h	48hC	48h	72h	96hC	96h
1	2.81	2.11	2.15	2.14	2.24	2.79	2.14
2	2.28	1.77	3.30	1.77	2.39	2.71	3.06
3	2.64	2.77	2.17	2.68	2.99	2.43	3.45
4	2.26	2.48	2.75	2.48	2.87	2.82	2.70
5	0.91	1.59	3.40	1.59	2.50	2.25	2.02
6	2.79	2.55	2.01	2.55	2.64	1.90	2.12
7	2.64	2.45	2.79	2.58	2.08	2.53	2.84
8	1.88	1.16	2.81	1.16	1.79	3.01	2.73
9	2.24	1.91	2.13	1.90	2.86	2.13	2.91
10	3.13	2.30	2.24	2.31	2.90	2.70	2.32
11	2.87	2.57	2.87	2.76	2.74	2.29	1.44
12	2.46	2.85	2.45	2.85	3.07	2.65	1.31
13	2.97	1.53	3.01	1.53	2.49	2.24	2.40
14	3.28	2.36	2.82	2.36	2.68	2.82	2.44
15	2.96	2.33	2.24	2.46	2.72	2.87	2.69
16	2.31	2.07	2.02	2.07	2.56	2.45	2.75
mean	2.53	2.17	2.57	2.20	2.59	2.54	2.46
n	16	16	16	16	16	16	16
sd	0.57	0.47	0.45	0.49	0.34	0.31	0.56
se	0.14	0.12	0.11	0.12	0.08	0.08	0.14
2 se	0.28	0.24	0.23	0.24	0.17	0.15	0.28
95% CI	0.28	0.23	0.22	0.24	0.17	0.15	0.27

E.3.1 COMPARISON OF MUSSEL FEEDING RATES OF CONTROL ANIMALS OVER THE EXPOSURE PERIOD; EXPERIMENT IV

96 h (ii) CONTROL values

animal	0h	48hC	96hC
1	2.81	2.15	2.79
2	2.28	3.30	2.71
3	2.64	2.17	2.43
4	2.26	2.75	2.82
5	0.91	3.40	2.25
6	2.79	2.01	1.90
7	2.64	2.79	2.53
8	1.88	2.81	3.01
9	2.24	2.13	2.13
10	3.13	2.24	2.70
11	2.87	2.87	2.29
12	2.46	2.45	2.65
13	2.97	3.01	2.24
14	3.28	2.82	2.82
15	2.96	2.24	2.87
16	2.31	2.02	2.45

Anova: Single Factor

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
Column 1	16	40.43	2.526875	0.32709
Column 2	16	41.16	2.5725	0.205633
Column 3	16	40.59	2.536875	0.096676

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	0.018404	2	0.009202	0.043861	0.957128	3.20432
Within Groups	9.440987	45	0.2098			
Total	9.459392	47				

A.3.2 COMPARISON OF MUSSEL FEEDING RATES (EXPOSED ANIMALS) FROM 0 H EXPOSURE TO 96 H EXPOSURE; EXPERIMENT IV

96 h (ii) TOXICANT values

animal	0h	24h	48h	72h	96h
1	2.81	2.11	2.14	2.24	2.14
2	2.28	1.77	1.77	2.39	3.06
3	2.64	2.77	2.68	2.99	3.45
4	2.26	2.48	2.48	2.87	2.70
5	0.91	1.59	1.59	2.50	2.02
6	2.79	2.55	2.55	2.64	2.12
7	2.64	2.45	2.58	2.08	2.84
8	1.88	1.16	1.16	1.79	2.73
9	2.24	1.91	1.90	2.86	2.91
10	3.13	2.30	2.31	2.90	2.32
11	2.87	2.57	2.76	2.74	1.44
12	2.46	2.85	2.85	3.07	1.31
13	2.97	1.53	1.53	2.49	2.40
14	3.28	2.36	2.36	2.68	2.44
15	2.96	2.33	2.46	2.72	2.69
16	2.31	2.07	2.07	2.56	2.75

Anova: Single Factor

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
Column 1	16	40.43	2.526875	0.32709
Column 2	16	34.8	2.175	0.225787
Column 3	16	35.19	2.199375	0.241006
Column 4	16	41.52	2.595	0.117933
Column 5	16	39.32	2.4575	0.316833

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	2.366017	4	0.591504	2.407133	0.056812	2.493692
Within Groups	18.42974	75	0.24573			
Total	20.79575	79				

animal	0h	24h	48hC	48h	72h	96hC	96h	120 hC	120h
1	3.15	1.20	2.15	1.27	0.93	2.63	1.34	2.71	2.00
2	2.67	1.70	2.87	1.73	0.72	3.20	0.02	2.93	1.67
3	2.40	1.89	2.74	2.13	2.44	3.40	0.00	3.48	2.26
4	3.03	2.32	2.90	1.69	0.81	3.15	2.18	3.03	2.74
5	2.86	2.08	2.84	0.95	1.52	2.81	2.83	3.02	1.31
6	2.64	2.45	2.76	2.48	0.83	2.77	1.62	2.85	2.79
7	2.56	1.70	2.77	0.16	2.45	2.96	1.80	3.17	0.31
8	2.55	2.00	3.23	2.94	1.12	3.67	2.62	2.92	1.27
9	2.64	1.54	2.37	1.89	1.54	3.21	2.65	2.82	0.67
10	2.96	1.56	3.02	1.68	0.80	2.46	1.80	2.47	1.32
11	2.10	0.30	3.36	1.72	0.69	2.56	2.53	2.52	2.25
12	2.34	1.26	3.05	2.17	1.42	3.10	2.77	2.09	1.60
13	2.36	0.93	3.09	1.92	2.15	1.68	2.15		
14	2.12	2.07	3.06	1.33	0.82	3.23	1.88		
15	2.40	1.91	2.10	2.02	1.07	2.87	1.75		
16	2.11	0.88	2.83	1.06	1.91	2.68	1.39		
mean	2.56	1.61	2.82	1.70	1.33	2.90	1.83	2.83	1.68
n	16	16	16	16	16	16	16	12	12
sd	0.33	0.58	0.35	0.65	0.62	0.46	0.86	0.36	0.77
se	0.08	0.14	0.09	0.16	0.15	0.12	0.21	0.09	0.19
2 se	0.16	0.29	0.18	0.33	0.31	0.23	0.43	0.18	0.38
95% CI	0.16	0.28	0.17	0.32	0.30	0.23	0.42	0.18	0.38

RAW DATA EXPERIMENT V 4-PO, 120 h exposure (45µg l^{-1})

E.4.1 COMPARISON OF MUSSEL FEEDING RATES IN THE CONTROL ANIMALS OVER 120 H EXPOSURE PERIOD; EXPERIMENT V

4-PO, 120 h 48 µg/l - CONTROL

animal	0h	48 h C	96 h C	120 h C
1	3.15	2.15	2.63	2.71
2	2.67	2.87	3.20	2.93
3	2.40	2.74	3.40	3.48
4	3.03	2.90	3.15	3.03
5	2.86	2.84	2.81	3.02
6	2.64	2.76	2.77	2.85
7	2.56	2.77	2.96	3.17
8	2.55	3.23	3.67	2.92
9	2.64	2.37	3.21	2.82
10	2.96	3.02	2.46	2.47
11	2.10	3.36	2.56	2.52
12	2.34	3.05	3.10	2.09
13	2.36	3.09	1.68	
14	2.12	3.06	3.23	
15	2.40	2.10	2.87	
16	2.11	2.83	2.68	

Anova: Single Factor CONTROL

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
Column 1	16	40.89	2.555625	0.106066
Column 2	16	45.14	2.82125	0.124985
Column 3	16	46.38	2.89875	0.212585
Column 4	12	34.01	2.834167	0.129117

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	1.091158	3	0.363719	2.522439	0.066966	2.769433
Within Groups	8.074835	56	0.144193			
Total	9.165993	59				

E.4.2 COMPARISON OF MUSSEL FEEDING RATES OF EXPOSED ANIMALS OVER THE 120 H EXPOSURE PERIOD; EXPERIMENT V

4-PO, 120 h, 45 µg/l - TOXICANT

animal	0h	24h	48h	72h	96h	120h
1	3.15	1.20	1.27	0.93	1.34	2.00
2	2.67	1.70	1.73	0.72	0.02	1.67
3	2.40	1.89	2.13	2.44	0.00	2.26
4	3.03	2.32	1.69	0.81	2.18	2.74
5	2.86	2.08	0.95	1.52	2.83	1.31
6	2.64	2.45	2.48	0.83	1.62	2.79
7	2.56	1.70	0.16	2.45	1.80	0.31
8	2.55	2.00	2.94	1.12	2.62	1.27
9	2.64	1.54	1.89	1.54	2.65	0.67
10	2.96	1.56	1.68	0.80	1.80	1.32
11	2.10	0.30	1.72	0.69	2.53	2.25
12	2.34	1.26	2.17	1.42	2.77	1.60
13	2.36	0.93	1.92	2.15	2.15	
14	2.12	2.07	1.33	0.82	1.88	
15	2.40	1.91	2.02	1.07	1.75	
16	2.11	0.88	1.06	1.91	1.39	

Anova: Single Factor

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
Column 1	16	40.89	2.555625	0.106066
Column 2	16	25.79	1.611875	0.33255
Column 3	16	27.14	1.69625	0.427052
Column 4	16	21.22	1.32625	0.379878
Column 5	16	29.33	1.833125	0.73389
Column 6	12	20.19	1.6825	0.587039

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	13.63649	5	2.727297	6.488364	3.66E-05	2.320526
Within Groups	36.14896	86	0.420337			
Total	49.78544	91				

E.4.3 COMPARISON OF MUSSEL FEEDING RATES (EXPOSED ANIMALS) FROM 24 H - 120 H EXPOSURE TIME; EXPERIMENT V

Anova: Single Factor

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
Column 1	16	25.79	1.611875	0.33255
Column 2	16	27.14	1.69625	0.427052
Column 3	16	21.22	1.32625	0.379878
Column 4	16	29.33	1.833125	0.73389
Column 5	12	20.19	1.6825	0.587039

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	2.244357	4	0.561089	1.152769	0.339043	2.500762
Within Groups	34.55796	71	0.486732			
Total	36.80232	75				

E.4.4 COMPARISON OF CONTROL FEEDING RATE AND EXPOSED FEEDING RATE AT 48 H EXPOSURE TIME; EXPERIMENT V

t-Test: Two-Sample Assuming Equal Variances

	Variable 1	*Variable 2*
Mean	2.82125	1.69625
Variance	0.124985	0.427051667
Observations	16	16
Pooled Variance	0.276018333	
Hypothesized Mean Difference	0	
df	30	
t Stat	6.056595246	
P(T<=t) one-tail	5.95196E-07	
t Critical one-tail	1.697260359	
P(T<=t) two-tail	1.19039E-06	
t Critical two-tail	2.042270353	

APPENDIX E.5

RAW DATA; EXPERIMENT VI

BCH, 96 h exposure; 90 µg l^{-1}

animal	0h	24h	48hC	48h	72h	96 h C	96h
1	2.64	0.36		1.73	1.73	2.07	1.23
2	3.50	1.70	3.55	0.70	0.85	3.49	0.69
3	3.46	0.21	4.03	2.73	0.92	1.65	1.13
4	2.68	1.33	2.62	1.50	0.59	2.32	1.58
5	2.11	0.44	2.65	1.34	1.28	3.26	0.57
6	3.28	1.45	2.72	0.52	1.85	3.27	1.07
7	2.17	1.65	3.05	2.17	1.74	2.30	0.47
8	2.31	0.99	2.41	1.68	1.59	4.02	0.88
9	3.22	1.67	4.18	1.07	1.63	3.36	1.67
10	2.98	1.33	2.71	1.06	0.67	1.40	0.50
11	2.74	1.34	2.67	0.43	0.49	3.20	1.83
12	4.34	0.51	1.66	1.66	1.38	3.01	0.61
13	3.42	1.45	2.74	1.85	1.46	2.87	1.03
14	2.80	1.28	2.30	1.48	0.93	2.64	0.97
15	2.69	1.24	3.15	1.29	0.84	3.01	2.37
16	2.94	0.73	3.47	0.84	1.25	3.22	
mean	2.96	1.11	2.93	1.38	1.20	2.82	1.11
n	16.00	16.00	16.00	16.00	16.00	16.00	16.00
sd	0.57	0.50	0.66	0.61	0.45	0.71	0.55
se	0.14	0.12	0.17	0.15	0.11	0.18	0.14
2 se	0.29	0.25	0.33	0.31	0.22	0.35	0.28
95% CI	0.28	0.24	0.33	0.30	0.22	0.35	0.27

E.5.1 COMPARISON OF MUSSEL FEEDING RATES OF CONTROL ANIMALS OVER 96 H EXPOSURE PERIOD; EXPERIMENT VI

BCH (90 μg l^{-1}); Control animals

animal	0h	48h C	96 h C
1	2.64	-	2.07
2	3.50	3.55	3.49
3	3.46	4.03	1.65
4	2.68	2.62	2.32
5	2.11	2.65	3.26
6	3.28	2.72	3.27
7	2.17	3.05	2.30
8	2.31	2.41	4.02
9	3.22	4.18	3.36
10	2.98	2.71	1.40
11	2.74	2.67	3.20
12	4.34	1.66	3.01
13	3.42	2.74	2.87
14	2.8	2.30	2.64
15	2.69	3.15	3.01
16	2.94	3.47	3.22

Anova: Single Factor

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
Column 1	16	47.28	2.955	0.32832
Column 2	15	43.91	2.927333	0.44015
Column 3	16	45.09	2.818125	0.497083

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	0.166854	2	0.083427	0.19796	0.821129	3.20928
Within Groups	18.54314	44	0.421435			
Total	18.70999	46				

E.5.2 COMPSRISON OF MUSSEL FEEDING RATES OF EXPOSED ANIMALS OVER 96 H EXPOSURE PERIOD; EXPERIMENT VI

BCH (90 $\mu g\ l^{-1}$); Exposed animals

animal	0h	24h	48h	72h	96h
1	2.64	0.36	1.73	1.73	1.23
2	3.50	1.70	0.70	0.85	0.69
3	3.46	0.21	2.73	0.92	1.13
4	2.68	1.33	1.50	0.59	1.58
5	2.11	0.44	1.34	1.28	0.57
6	3.28	1.45	0.52	1.85	1.07
7	2.17	1.65	2.17	1.74	0.47
8	2.31	0.99	1.68	1.59	0.88
9	3.22	1.67	1.07	1.63	1.67
10	2.98	1.33	1.06	0.67	0.50
11	2.74	1.34	0.43	0.49	1.83
12	4.34	0.51	1.66	1.38	0.61
13	3.42	1.45	1.85	1.46	1.03
14	2.80	1.28	1.48	0.93	0.97
15	2.69	1.24	1.29	0.84	2.37
16	2.94	0.73	0.84	1.25	

Anova: Single Factor

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
Column 1	16	47.28	2.955	0.32832
Column 2	16	17.68	1.105	0.247987
Column 3	16	22.05	1.378125	0.37247
Column 4	16	19.2	1.2	0.200093
Column 5	15	16.6	1.106667	0.302524

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	40.13198	4	10.033	34.58304	2.9E-16	2.495391
Within Groups	21.46838	74	0.290113			
Total	61.60036	78				

E.5.3 COMPARISON OF MUSSEL FEEDING RATES AT 0H AND 24H EXPOSURE; EXPERIMENT VI

t-Test: Paired Two Sample for Means

	Variable 1	*Variable 2*
Mean	2.955	1.105
Variance	0.32832	0.247986667
Observations	16	16
Pearson Correlation	-0.05855001	
Hypothesized Mean Difference	0	
df	15	
t Stat	9.476904392	
P(T<=t) one-tail	5.04199E-08	
t Critical one-tail	1.753051038	
P(T<=t) two-tail	1.0084E-07	
t Critical two-tail	2.131450856	

E.5.4 COMPARISON OF MUSSEL FEEDING RATES (EXPOSED) FROM 24H TO 96 H EXPOSURE; EXPERIMENT VI

Anova: Single Factor

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
Column 1	16	17.68	1.105	0.247987
Column 2	16	22.05	1.37812	0.37247
Column 3	16	19.2	1.2	0.200093
Column 4	15	16.6	1.106667	0.302524

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	0.78264	3	0.26088	0.93039	0.43179	2.76077
Within Groups	16.5435	59	0.2804			
Total	17.3262	62				

SUMMARY
OUTPUT

Regression Statistics	
Multiple R	0.843385124
R Square	0.711298468
Adjusted R Square	0.685052874
Standard Error	9.00123142
Observations	13

ANOVA

	df	*SS*	*MS*	*F*	*Significance F*
Regression	1	2195.833085	2195.833085	27.10163359	0.000291791
Residual	11	891.2438379	81.02216709		
Total	12	3087.076923			

	Coefficients	*Standard Error*	*t Stat*	*P-value*	*Lower 95%*	*Upper 95%*	*Lower 95.0%*	*Upper 95.0%*
Intercept	160.0103127	16.63612989	9.618241366	1.08925E-06	123.3944191	196.6262062	123.3944191	196.6262062
X Variable 1	-62.85700692	12.0741332	-5.205922934	0.000291791	-89.43200835	-36.28200549	-89.43200835	-36.28200549

Regression statistics summary for TEC_{50}, (4-PO) using gill concentration

SUMMARY
OUTPUT

Regression Statistics	
Multiple R	0.699107063
R Square	0.488750686
Adjusted R Square	0.442273475
Standard Error	11.97826046
Observations	13

ANOVA

	df	*SS*	*MS*	*F*	*Significance F*
Regression	1	1508.810963	1508.810963	10.51592127	0.007834499
Residual	11	1578.26596	143.4787237		
Total	12	3087.076923			

	Coefficients	*Standard Error*	*t Stat*	*P-value*	*Lower 95%*	*Upper 95%*	*Lower 95.0%*	*Upper 95.0%*
Intercept	104.6196811	9.897868006	10.56992081	4.23743E-07	82.83460945	126.4047527	82.83460945	126.4047527
X Variable 1	-30.89304873	9.526581922	-3.242826124	0.007834499	-51.86092477	-9.925172692	-51.86092477	-9.925172692

Regression statistics summary for the estimation of TEC_{50}, (4-PO, total body burden)

SUMMARY
OUTPUT

Regression Statistics	
Multiple R	0.856605871
R Square	0.733773618
Adjusted R Square	0.667217023
Standard Error	7.780309992
Observations	6

ANOVA

	df	*SS*	*MS*	*F*	*Significance F*
Regression	1	667.3671057	667.3671057	11.02480698	0.029368588
Residual	4	242.1328943	60.53322357		
Total	5	909.5			

	Coefficients	*Standard Error*	*t Stat*	*P-value*	*Lower 95%*	*Upper 95%*	*Lower 95.0%*	*Upper 95.0%*
Intercept	102.8359251	9.105919975	11.29330429	0.000350348	77.55378583	128.1180644	77.55378583	128.1180644
X Variable 1	-37.55926654	11.31179707	-3.320362477	0.029368588	-68.96591519	-6.152617882	-68.96591519	-6.152617882

Regression statistics summary for the TEC_{50} (4-PO) calculated using only 24 h and 48 h total body burden data

SUMMARY
OUTPUT

Regression Statistics	
Multiple R	0.909689171
R Square	0.827534387
Adjusted R Square	0.784417984
Standard Error	6.262137708
Observations	6

ANOVA

	df	*SS*	*MS*	*F*	*Significance F*
Regression	1	752.6425253	752.6425253	19.19302926	0.011865779
Residual	4	156.8574747	39.21436867		
Total	5	909.5			

	Coefficients	*Standard Error*	*t Stat*	*P-value*	*Lower 95%*	*Upper 95%*	*Lower 95.0%*	*Upper 95.0%*
Intercept	148.436931	17.06931517	8.69612691	0.000962719	101.0448163	195.8290457	101.0448163	195.8290457
X Variable 1	-57.58071404	13.1433261	-4.380984965	0.011865779	-94.07251305	-21.08891503	-94.07251305	-21.08891503

Regression statistics summary for the estimation of TEC_{50} (4-PO, gill tissue, 24 h and 48 h)

APPENDIX F.1
CONCENTRATION-RESPONSE DATA FOR THE EFFECT OF BUTYLCYCLOHEXANE UPON MUSSEL FEEDING RATE
RAW DATA

animal	25 µg/l	50 µg/l	100 µg/l	200 µg/l	control
1	0.46	0.23	0.15	0.08	0.47
2	0.38	0.26	0.20	0.10	0.50
3	0.35	0.24	0.24	0.08	0.34
4	0.39	0.16	0.17	0.07	0.37
5	0.39	0.14	0.20	0.03	0.45
6	0.31	0.24	0.16	0.10	0.41
7	0.32	0.26	0.14	0.10	0.41
1	0.34	0.21	0.15	0.06	0.36
2	0.30	0.32	0.12	0.08	0.47
3	0.40	0.20	0.09	0.04	0.42
4	0.37	0.16	0.07	0.06	0.53
5	0.21	0.11	0.12	0.03	0.44
6	0.30	0.25	0.12	0.04	0.40
7	0.25	0.28	0.10	0.10	0.39
mean[1]	0.34	0.22	0.15	0.07	0.43
sd[1]	0.07	0.06	0.05	0.03	0.06

F.1.1 COMPARISON OF REPLICATE EXPOSURE CONCENTRATION DATA

25 µg l^{-1} butylcyclohexane
t-Test: Two-Sample Assuming Equal Variances

	Variable 1	*Variable 2*
Mean	0.371428571	0.31
Variance	0.002580952	0.0044
Observations	7	7
Pooled Variance	0.003490476	
Hypothesized Mean Difference	0	
df	12	
t Stat	1.945190311	
P(T<=t) one-tail	0.037779107	
t Critical one-tail	1.782286745	
P(T<=t) two-tail	0.075558214	
t Critical two-tail	2.178812792	

[1] Mean and standard deviation of combined data (mean and standard deviation of individual data sets are given in Table 5.2)

50 µg l^{-1} butylcyclohexane
t-Test: Two-Sample Assuming Equal Variances

	Variable 1	*Variable 2*
Mean	0.218571429	0.218571429
Variance	0.002347619	0.005114286
Observations	7	7
Pooled Variance	0.003730952	
Hypothesized Mean Difference	0	
df	12	
t Stat	0	
P(T<=t) one-tail	0.5	
t Critical one-tail	1.782286745	
P(T<=t) two-tail	1	
t Critical two-tail	2.178812792	

100 µg l^{-1} butylcyclohexane
t-Test: Two-Sample Assuming Equal Variances

	Variable 1	*Variable 2*
Mean	0.18	0.11
Variance	0.001233333	0.000666667
Observations	7	7
Pooled Variance	0.00095	
Hypothesized Mean Difference	0	
df	12	
t Stat	4.248838851	
P(T<=t) one-tail	0.000564813	
t Critical one-tail	1.782286745	
P(T<=t) two-tail	0.001129627	
t Critical two-tail	2.178812792	

200 µg l^{-1} butylcyclohexane

t-Test: Two-Sample Assuming Equal Variances

	Variable 1	*Variable 2*
Mean	0.08	0.058571429
Variance	0.000633333	0.000614286
Observations	7	7
Pooled Variance	0.00062381	
Hypothesized Mean Difference	0	
df	12	
t Stat	1.605096844	
P(T<=t) one-tail	0.067225678	
t Critical one-tail	1.782286745	
P(T<=t) two-tail	0.134451356	
t Critical two-tail	2.178812792	

control, butylcyclohexane

t-Test: Two-Sample Assuming Equal Variances

	Variable 1	*Variable 2*
Mean	0.421387457	0.4308453
Variance	0.00316395	0.0034196
Observations	7	7
Pooled Variance	0.003291755	
Hypothesized Mean Difference	0	
df	12	
t Stat	-0.30839762	
P(T<=t) one-tail	0.381533886	
t Critical one-tail	1.782286745	
P(T<=t) two-tail	0.763067773	
t Critical two-tail	2.178812792	

SUMMARY
OUTPUT

Regression Statistics	
Multiple R	0.959995273
R Square	0.921590925
Adjusted R Square	0.895454566
Standard Error	0.033957858
Observations	5

ANOVA

	df	*SS*	*MS*	*F*	*Significance F*
Regression	1	0.040660592	0.040660592	35.26087713	0.009547232
Residual	3	0.003459408	0.001153136		
Total	4	0.04412			

	Coefficients	*Standard Error*	*t Stat*	*P-value*	*Lower 95%*	*Upper 95%*	*Lower 95.0%*	*Upper 95.0%*
Intercept	0.858238692	0.114555842	7.491880631	0.004926308	0.493670535	1.222806849	0.493670535	1.222806849
X Variable 1	-0.346295727	0.058317726	-5.938086993	0.009547232	-0.531888932	-0.160702523	-0.531888932	-0.160702523

SUMMARY STATISTICS FROM REGRESSION ANALYSIS OF BUTYLCYCLOHEXANE DATA

APPENDX F.2
CONCENTRATION-RESPONSE DATA FOR THE EFFECT OF 7-CYCLOHEXYLTETRALIN UPON MUSSEL FEEDING RATE
RAW DATA

animal	12 µg l^{-1}	25 µg l^{-1}	50 µg l^{-1}	100 µg l^{-1}	control
1	0.42	0.30	0.12	0.12	0.47
2	0.30	0.20	0.18	0.14	0.44
3	0.27	0.17	0.16	0.19	0.47
4	0.35	0.24	0.06	0.04	0.49
5	0.32	0.23	0.13	0.11	0.50
6	0.29	0.30	0.14	0.07	0.46
7	0.34	0.33	0.28	0.13	0.45
1	0.30	0.17	0.15	0.01	0.57
2	0.31	0.21	0.16	0.12	0.49
3	0.30	0.23	0.18	0.07	0.51
4	0.36	0.17	0.06	0.08	0.53
5	0.21	0.34	0.29	0.15	0.50
6	0.28	0.19	0.22	0.10	0.46
7	0.36	0.19	0.20	0.08	0.46
mean[2]	0.32	0.23	0.17	0.10	0.49
sd[2]	0.05	0.06	0.07	0.05	0.04

APPENDIX F.2.1
COMPARISON OF REPLICATE EXPOSURE DATA
12 µg l^{-1} 7-cyclohexyltetralin

t-Test: Two-Sample Assuming Equal Variances

	Variable 1	*Variable 2*
Mean	0.327142857	0.303969714
Variance	0.002457143	0.002572151
Observations	7	7
Pooled Variance	0.002514647	
Hypothesized Mean Difference	0	
df	12	
t Stat	0.864530758	
P(T<=t) one-tail	0.202123684	
t Critical one-tail	1.782286745	
P(T<=t) two-tail	0.404247368	
t Critical two-tail	2.178812792	

[2] mean and standard deviation of combined data sets (mean and standard deviation of individual data sets are given in Table 5.4)

25 µg l^{-1} 7-cyclohexyltetralin

t-Test: Two-Sample Assuming Equal Variances

	Variable 1	*Variable 2*
Mean	0.252238714	0.214228714
Variance	0.003384399	0.003530353
Observations	7	7
Pooled Variance	0.003457376	
Hypothesized Mean Difference	0	
df	12	
t Stat	1.20936832	
P(T<=t) one-tail	0.124904542	
t Critical one-tail	1.782286745	
P(T<=t) two-tail	0.249809084	
t Critical two-tail	2.178812792	

50 µg l^{-1} 7-cyclohexyltetralin

t-Test: Two-Sample Assuming Equal Variances

	Variable 1	*Variable 2*
Mean	0.152973	0.181029143
Variance	0.004512835	0.0047877
Observations	7	7
Pooled Variance	0.004650267	
Hypothesized Mean Difference	0	
df	12	
t Stat	-0.76970273	
P(T<=t) one-tail	0.228180509	
t Critical one-tail	1.782286745	
P(T<=t) two-tail	0.456361018	
t Critical two-tail	2.178812792	

100 µg l^{-1} 7-cyclohexyltetralin
t-Test: Two-Sample Assuming Equal Variances

	Variable 1	*Variable 2*
Mean	0.115445429	0.0868566
Variance	0.002382709	0.001914771
Observations	7	7
Pooled Variance	0.00214874	
Hypothesized Mean Difference	0	
df	12	
t Stat	1.153820994	
P(T<=t) one-tail	0.135518346	
t Critical one-tail	1.782286745	
P(T<=t) two-tail	0.271036691	
t Critical two-tail	2.178812792	

control, 7-cyclohexyltetralin

t-Test: Two-Sample Assuming Equal Variances

	Variable 1	*Variable 2*
Mean	0.468571429	0.502857143
Variance	0.000447619	0.00152381
Observations	7	7
Pooled Variance	0.000985714	
Hypothesized Mean Difference	0	
df	12	
t Stat	-2.043015674	
P(T<=t) one-tail	0.031825696	
t Critical one-tail	1.782286745	
P(T<=t) two-tail	0.063651392	
t Critical two-tail	2.178812792	

SUMMARY OUTPUT

Regression Statistics	
Multiple R	0.999436629
R Square	0.998873576
Adjusted R Square	0.998310364
Standard Error	0.00392836
Observations	4

ANOVA

	df	*SS*	*MS*	*F*	*Significance F*
Regression	1	0.027369136	0.027369136	1773.530317	0.000563371
Residual	2	3.0864E-05	1.5432E-05		
Total	3	0.0274			

	Coefficients	*Standard Error*	*t Stat*	*P-value*	*Lower 95%*	*Upper 95%*	*Lower 95.0%*
Intercept	0.699459894	0.011787257	59.34034656	0.000283867	0.648743387	0.750176402	0.648743387
X Variable 1	-0.276394123	0.006563107	-42.11330332	0.000563371	-0.304632914	-0.248155332	-0.304632914

Upper 95.0%
0.750176402
-0.248155332

SUMMARY STATISTICS FROM REGRESSION ANALYSIS OF 7-CYCLOHEXYLTETRALIN DATA

APPENDIX F.3
CONCENTRATION-RESPONSE DATA FOR THE EFFECT OF 7-CYCLOHEXYL-1-PROPYLTETRALIN UPON MUSSEL FEEDNG RATE

RAW DATA

animal	12 µg/l	25 µg/l	50 µg/l	100 µg/l	controls
1	0.59	0.36	0.23	0.07	0.43
2	0.42	0.33	0.31	0.22	0.41
3	0.50	0.40	0.34	0.06	0.49
4	0.55	0.46	0.27	0.15	0.42
5	0.51	0.35	0.12	0.35	0.45
6	0.52	0.38	0.29	0.21	0.50
7	0.46	0.32	0.36	0.06	0.45
1	0.52	0.27	0.21	0.19	0.47
2	0.47	0.31	0.34	0.09	0.55
3	0.45	0.37	0.17	0.29	0.57
4	0.45	0.34	0.32	0.08	0.48
5	0.42	0.39	0.31	0.12	0.48
6	0.52	0.35	0.29	0.16	0.44
7	0.56	0.34	0.35	0.20	0.46
mean[3]	0.50	0.36	0.28	0.16	0.47
sd[3]	0.05	0.05	0.07	0.09	0.04

APPENDIX F.3.1
COMPARISON OF DATA FROM REPLICATE EXPOURE CONCENTRATIONS

12 µg l^{-1} 7-cyclohexyl-1-propyltetralin
t-Test: Two-Sample Assuming Equal Variances

	Variable 1	*Variable 2*
Mean	0.507142857	0.484285714
Variance	0.00312381	0.002495238
Observations	7	7
Pooled Variance	0.002809524	
Hypothesized Mean Difference	0	
df	12	
t Stat	0.806751175	
P(T<=t) one-tail	0.217754043	
t Critical one-tail	1.782286745	
P(T<=t) two-tail	0.435508086	
t Critical two-tail	2.178812792	

[3] Mean and standard deviation given are for the combined data sets (mean and standard deviation for individual data sets are given in Table 5.5)

25 µg l^{-1} 7-cyclohexyl-1-propyltetralin
t-Test: Two-Sample Assuming Equal Variances

	Variable 1	*Variable 2*
Mean	0.371428571	0.338571429
Variance	0.002280952	0.001547619
Observations	7	7
Pooled Variance	0.001914286	
Hypothesized Mean Difference	0	
df	12	
t Stat	1.40494861	
P(T<=t) one-tail	0.092695599	
t Critical one-tail	1.782286745	
P(T<=t) two-tail	0.185391199	
t Critical two-tail	2.178812792	

50 µg l^{-1} 7-cyclohexyl-1-propyltetralin
t-Test: Two-Sample Assuming Equal Variances

	Variable 1	*Variable 2*
Mean	0.274285714	0.284285714
Variance	0.006495238	0.004661905
Observations	7	7
Pooled Variance	0.005578571	
Hypothesized Mean Difference	0	
df	12	
t Stat	-0.250479693	
P(T<=t) one-tail	0.403226756	
t Critical one-tail	1.782286745	
P(T<=t) two-tail	0.806453513	
t Critical two-tail	2.178812792	

100 µg l^{-1} 7-cyclohexyl-1-propyltetralin
t-Test: Two-Sample Assuming Equal Variances

	Variable 1	*Variable 2*
Mean	0.16	0.161428571
Variance	0.011733333	0.005380952
Observations	7	7
Pooled Variance	0.008557143	
Hypothesized Mean Difference	0	
df	12	
t Stat	-0.0288916	
P(T<=t) one-tail	0.488712993	
t Critical one-tail	1.782286745	
P(T<=t) two-tail	0.977425986	
t Critical two-tail	2.178812792	

control, 7-cyclohexyl-1-propyltetralin
t-Test: Two-Sample Assuming Equal Variances

	Variable 1	*Variable 2*
Mean	0.45	0.492334286
Variance	0.001166667	0.002231609
Observations	7	7
Pooled Variance	0.001699138	
Hypothesized Mean Difference	0	
df	12	
t Stat	-1.921374225	
P(T<=t) one-tail	0.039376258	
t Critical one-tail	1.782286745	
P(T<=t) two-tail	0.078752516	
t Critical two-tail	2.178812792	

SUMMARY
OUTPUT

Regression Statistics	
Multiple R	0.980143446
R Square	0.960681176
Adjusted R Square	0.941021763
Standard Error	0.034658189
Observations	4

ANOVA

	df	*SS*	*MS*	*F*	*Significance F*
Regression	1	0.05869762	0.05869762	48.86622059	0.019856554
Residual	2	0.00240238	0.00120119		
Total	3	0.0611			

	Coefficients	*Standard Error*	*t Stat*	*P-value*	*Lower 95%*	*Upper 95%*	*Lower 95.0%*	*Upper 95.0%*
Intercept	0.910703538	0.085559666	10.64407543	0.008711245	0.542569751	1.278837324	0.542569751	1.278837324
X Variable 1	-0.315808065	0.045177151	-6.990437797	0.019856554	-0.510189793	-0.121426337	-0.510189793	-0.121426337

SUMMARY STATISTICS FROM REGRESSION ANALYSIS OF 7-CYCLOHEXYL-1-PROPYLTETRALIN DATA

The errors of the EC_{50} estimates for all toxicants tested were calculated using the following formula (Miller and Miller, 1994);

$$s_{x0} = \frac{s_{y/x}}{b}\left\{1 + \frac{1}{n} + \frac{(y_0 - \bar{y})^2}{b^2 \sum (x_i - \bar{x})^2}\right\}^{1/2}$$

where y_0 is the experimental value of y from the concentration of x_0 has been determined.

S_{x0} is the estimated standard deviation required

$S_{y/x}$ is the standard deviation of the y residuals

b is the gradient of the regression line

95% confidence intervals were then calculated from the standard deviation (s_{x0}) in the usual manner (*i.e.* $95\ \%\ C.I. = t * s_{x0} / \sqrt{n}$)