University of Plymouth

PEARL **https://pearl.plymouth.ac.uk**

04 University of Plymouth Research Theses 01 Research Theses Main Collection

2002

Unresolved complex mixtures of aromatic hydrocarbons in the marine environment : toxicity, solubility and photodegradation studies

Smith, Emma Louise

http://hdl.handle.net/10026.1/464

http://dx.doi.org/10.24382/4518
University of Plymouth

All content in PEARL is protected by copyright law. Author manuscripts are made available in accordance with publisher policies. Please cite only the published version using the details provided on the item record or document. In the absence of an open licence (e.g. Creative Commons), permissions for further reuse of content should be sought from the publisher or author.

UNRESOLVED COMPLEX MIXTURES OF AROMATIC HYDROCARBONS IN THE MARINE ENVIRONMENT: TOXICITY, SOLUBILITY AND PHOTODEGRADATION STUDIES

by

EMMA LOUISE SMITH

A thesis submitted to the University of Plymouth

in partial fulfillment for the degree of

DOCTOR OF PHILOSOPHY

Department of Environmental Sciences
Faculty of Science
University of Plymouth

In collaboration with

Plymouth Marine Laboratory

March 2002

BEST COPY

AVAILABLE

This copy of the thesis has been supplied on condition that anyone who consults it is understood to recognise that its copyright rests with its author and that no quotation and no information derived from it may be published without the authors prior consent.

Signed .....

Dated .....

UNRESOLVED COMPLEX MIXTURES OF AROMATIC HYDROCARBONS IN THE MARINE ENVIRONMENT: TOXICITY, SOLUBILITY AND PHOTODEGRADATION STUDIES

by

Emma Louise Smith

ABSTRACT

Unresolved complex mixtures (UCMs) of aromatic hydrocarbons have been found in a wide range of environmental matrices at high concentrations. However, limited consideration has been given to the potential detrimental effects of the accumulation of these compounds in the marine environment. In particular, there is a need to evaluate these compounds in the light of recent evidence that points to the long term effects of oil in the marine environment. The overall aim of this work was therefore to investigate unresolved complex mixtures of aromatic hydrocarbons in the marine environment with particular emphasis on assessment of toxicity, aqueous solubility and photodegradation behaviour.

A previously established link between the reduced Scope for Growth (SFG) of mussels with the concentration of 2-3 ringed aromatic hydrocarbons in mussels from petroleum impacted sites was found also to correlate well with concentrations of aromatic hydrocarbon UCMs in mussel tissues found at the same sites. This suggests that aromatic hydrocarbon UCMs or components within may be responsible for the observed effects.

To determine whether an aromatic hydrocarbon UCM was capable of eliciting a toxic response in mussels, a monoaromatic UCM was isolated from a Gullfaks (North Sea) produced crude oil. At the highest nominal aqueous concentration tested, mussel feeding rate was reduced by ~40 % in the 24 hour exposure period, during which the mussels accumulated a body burden similar to the body burden of monoaromatic UCM hydrocarbons in wild mussel populations, previously shown to exhibit reduced SFG.

Recent studies of hydrocarbon UCM composition using chemical degradation methods have led to the proposition of alkylcyclohexyltetralins as 'average' structures for some monoaromatic UCM hydrocarbons. The aromatic hydrocarbon 7-cyclohexyl-1-methyltetralin was synthesised herein in good yield and purity. This compound reduced mussel feeding rate by 50% during the 24-hour exposure period.

The aqueous solubility of a compound is an important physicochemical parameter that influences behaviour within the marine environment and is thought to be a limiting factor in the onset of measurable toxicological response. The aqueous solubilities of three 'model' aromatic UCM hydrocarbons were determined in distilled water at 25°C using a generator column set-up. The effects of salinity and temperature on aqueous solubility were also investigated. An aqueous solution comprising an aromatic UCM was also generated.

Another influence on the fate of aromatic hydrocarbons in the environment is the action of sunlight (phototransformation). Therefore aqueous solutions of three model aromatic UCM hydrocarbons were exposed to light simulated under environmental conditions. The half-lives of these compounds suggested that if these compounds are indeed representative of the aromatic UCM phototransformation may influence its fate in the marine environment.

The results of this study have furthered knowledge on the environmental behaviour of unresolved aromatic hydrocarbons, and suggest that these compounds should be considered in the long term impacts of oil in the environment and also warrant further study.

Parts of this work have been published and the author (Smith, E.L.) was awarded the Procter and Gamble Eurorcar prize at the 3rd World Congress of the Society of Environmental Toxicology and Chemistry, 21-25 May 2000, Brighton, for the best presentation by a young scientist.

CONTENTS

ABSTRACT		i
CONTENTS		ii
LIST OF FIGURES		vi
LIST OF TABLES		xi
LIST OF COMMON ABBREVIATIONS		xiii
AUTHOR'S DECLARATION		xiv
ACKNOWLEDGEMENTS		xv
1.0	**Introduction**	1
1.1	Unresolved complex mixtures of hydrocarbons	2
1.2	Compositional studies of aromatic hydrocarbon UCMs	6
1.3	Environmental occurrences of aromatic UCM hydrocarbons	11
1.4	Circumstantial field evidence for the environmental impact of aromatic unresolved hydrocarbons	16
1.5	Responses of marine organisms to petroleum hydrocarbons	28
1.6	The bioavailability of UCM hydrocarbons to organisms in the marine environment	34
1.7	Aims of the present study	38
2.0	**Isolation and toxicological assessment of a monoaromatic hydrocarbon UCM**	41
2.1	Introduction	42
2.2	Aims	43
2.3	Isolation of a monoaromatic UCM	44
2.4	Concentration-response studies of the effect of an isolated monoaromatic UCM upon mussel feeding rate	49
2.4.1	Experimental approach	49
2.5	Results	51
2.6	Discussion	58
2.7	Summary	64
3.0	**Synthesis of a model monoaromatic UCM hydrocarbon**	66
3.1	Introduction	67
3.2	Aims	69
3.3	Synthetic strategy	69
3.4	Synthesis of 3-benzoyl(4'-cyclohexyl)propanoic acid	72
3.4.1	Preparation	72
3.4.2	Characterisation	72
3.5	Synthesis of 4-phenyl(4'-cyclohexyl)butanoic acid	78
3.5.1	Preparation	78
3.5.2	Characterisation	78
3.6	Synthesis of 7-cyclohexyl-1-tetralone	84
3.6.1	Preparation	84
3.6.2	Characterisation	84
3.7	Synthesis of 7-cyclohexyl-1-hydroxy-1-methyltetralin	90
3.7.1	Preparation	90
3.7.2	Characterisation	90
3.8	Synthesis of an isomeric mixture of 7-cyclohexyl-1-methyl-3,4-	96

	dihydronaphthalene (Va) and 7-cyclohexyl-1-methenyltetralin (Vb)	
3.8.1	Preparation	96
3.8.2	Characterisation	96
3.9	Synthesis of 7-cycloheyxl-1-methyltetralin	101
3.9.1	Preparation	101
3.9.2	Characterisation	101
3.10	Isolation of 6-cyclohexyltetralin and 7-cyclohexyl-1-propyltetralin	106
3.11	Summary	109
4.0	**Toxicological assessment of a model monoaromatic UCM hydrocarbon**	111
4.1	Introduction	112
4.2	Aims	115
4.3	Experimental approach	116
4.4	Results	117
4.5	Discussion	133
4.6	Summary	141
5.0	**Aqueous solubility studies of model monoaromatic UCM compounds and an isolated monoaromatic UCM**	144
5.1	Introduction	145
5.1.1	Methods for the determination of aqueous solubility	148
5.1.1.1	Estimation methods	148
5.1.1.2	Direct determination	150
5.2	Aims	151
5.3	Experimental approach	151
5.4	Results	154
5.4.1	Validation of experimental set-up by determination of aqueous solubility of anthracene	154
5.4.2	Determination of the aqueous solubility of model aromatic UCM compounds	156
5.4.3	Generation of an aqueous solution of an isolated monoaromatic UCM	162
5.5	Discussion	164
5.6	Summary	171
6.0	**Photodegradation studies of model monoaromatic UCM compounds**	173
6.1	Introduction	174
6.2	Aims	177
6.3	Experimental approach	177
6.4	Results	181
6.4.1	Experiment 1. Photodegradation of 6-cyclohexyltetralin	182
6.4.2	Experiment 2. Photodegradation of 7-cyclohexyl-1-methyltetralin	185
6.4.3	Experiment 3. Photodegradation of 7-cyclohexyl-1-propyltetralin	188
6.4.4	Comparison of results from the photodegradation of model monoaromatic UCM compounds	191
6.5	Discussion	194
6.6	Summary	200

7.0	**Experimental details**	202
7.1	General laboratory procedures	203
7.2	Instrumental details	204
7.2.1	Gas chromatography (GC)	204
7.2.2	Gas chromatography-mass spectrometry (GCMS)	204
7.2.3	Nuclear magnetic resonance spectroscopy (NMR)	205
7.2.4	Infrared spectroscopy (IR)	205
7.3	Determination of aromatic UCM hydrocarbon concentrations in mussels from Whitby 1990	206
7.3.1	Sample collection	206
7.3.2	Two phase extraction method	206
7.3.3	Fractionation of mussel tissue by open column chromatography	206
7.3.4	Quantification of total unresolved and resolved hydrocarbons	207
7.4	Isolation of a monoaromatic hydrocarbon unresolved complex mixture	208
7.4.1	Rotary evaporation of crude oil	208
7.4.2	Fractionation by open column chromatography	208
7.4.3	Normal-phase HPLC fractionation	208
7.5	Investigation into the effect of an isolated monoaromatic unresolved complex mixture upon mussel feeding rate	209
7.5.1	Test substrate	209
7.5.2	Collection and maintenance of mussels (*Mytilus edulis*)	209
7.5.3	Preparation of toxicant solutions	209
7.5.4	Exposure of mussels to toxicant	210
7.5.5	Measurement of feeding rate	210
7.5.6	Tissue analysis	211
7.5.7	External calibration of aromatic unresolved complex mixture hydrocarbons	211
7.6	Synthesis of a model monoaromatic UCM hydrocarbon	212
7.6.1	Synthesis of 3-benzoyl(4'-cyclohexyl)propanoic acid	212
7.6.2	Synthesis of 4-phenyl(4'-cyclohexyl)butanoic acid	213
7.6.3	Synthesis of 7-cyclohexyl-1-tetralone	214
7.6.4	Synthesis of 7-cyclohexyl-1-hydroxy-1-methyltetralin	214
7.6.5	Synthesis of 7-cyclohexyl-1-methenyltetralin and 7-cyclohexyl-1-methyl-3,4-dihydronaphthalene	215
7.6.6	Synthesis of 7-cyclohexyl-1-methyltetralin	216
7.6.7	Isolation of 6-cyclohexyltetralin	216
7.6.8	Isolation of 7-cyclohexyl-1-propyltetralin	217
7.7	Investigations into the effect of 7-cyclohexyl-1-methyltetralin upon mussel feeding rate	217
7.8	Investigations into the aqueous solubility behaviour of model aromatic UCM hydrocarbons	218
7.8.1	Test materials	218
7.8.2	Experimental set-up	218
7.8.3	Generation of solutions	219
7.8.4	Extraction of solutions	219
7.8.5	Effect of salinity on aqueous solubility	220
7.8.6	Effect of temperature on aqueous solubility	220
7.8.7	Modifications for generator column using isolated monoaromatic UCM	220
7.9	Investigations into the photodegradation of model aromatic UCM hydrocarbons	220

7.9.1	Test compounds	220
7.9.2	Preparation of model compound solutions	221
7.9.3	Method of exposure	221
7.9.4	Work-up of irradiated solutions	222
8.0	**Summary and suggestions for future work**	224
8.1	Summary	225
8.2	Suggestions for future work	232
	References	235

List of Figures

1.0	**Introduction**	
Figure 1.1	Gas chromatograms of a) weathered crude oil; b) fresh crude oil (Wraige, 1997)	3
Figure 1.2	Average 'model' aromatic UCM hydrocarbon as proposed by Thomas (1995)	10
Figure 1.3	Examples of gas chromatograms of the aromatic fraction from different environmental matrices exhibiting an unresolved complex mixture	13
Figure 1.4	Scope for Growth (SFG) as part of the individual energy budget, in an ecotoxicological framework (Smaal & Widdows, 1994)	19
Figure 1.5	Relationship between mean Scope for Growth (SFG) and the log concentration of 2- and 3-ring aromatic hydrocarbon in the tissues of *Mytilus edulis* collected from sites in Shetland during the period 1982-1989 (Widdows *et al.*, 1995a).	20
Figure 1.6	Link between reduced scope for growth and 'total toxic hydrocarbons' as calculated from the measurement of aromatic hydrocarbons ('2-3 ringed') by HPLC-UV (Widdows *et al.*, 1995b).	21
Figure 1.7	Gas chromatogram of the aromatic hydrocarbon fraction of mussels (*Mytilus edulis*) from Whitby collected in 1990 (Smith, this study)	23
Figure 1.8	Relationship between the concentration of 'total toxic hydrocarbons' (Widdows *et al.*, 1995) and 'aromatic UCM hydrocarbons' (Wraige, 1997) in *Mytilus edulis* from U.K. east coast sites with reduced Scope for Growth.	25
Figure 1.9	a) Gas chromatogram of the 'aromatic hydrocarbon' fraction separated from contaminated mussels by open column chromatography. b) The relationship between HPLC and GC indicators of the concentrations of aromatic hydrocarbons in mussel tissues.	26
2.0	**Isolation and toxicological assessment of an aromatic UCM**	
Figure 2.1	Isolation of a monoaromatic hydrocarbon unresolved complex mixture	45
Figure 2.2	Normal-phase HPLC chromatogram of aromatic hydrocarbons of Gullfaks produced crude illustrating fractionation	47
Figure 2.3	Gas chromatogram of products of each stage of the monoaromatic UCM isolation procedure	48
Figure 2.4	External calibration graph used to quantify the monoaromatic UCM hydrocarbons accumulated by mussels exposed to toxicant solutions.	51
Figure 2.5	Concentration (aqueous)-response curve for the effect of an isolated monoaromatic hydrocarbon UCM upon mussel feeding rate over 24 h	54
Figure 2.6	Concentration (tissue)-response curve for the effect of an isolated monoaromatic hydrocarbon UCM upon mussel feeding rate over 24 h	54
Figure 2.7	Gas chromatograms of a) monoaromatic hydrocarbon fraction isolated from mussels Whitby U.K. May 1995	57

b) a monoaromatic UCM fraction isolated from Gullfaks North Sea crude oil
c) total organic extract from control mussels
d) total organic extract from mussels exposed to the monoaromatic UCM isolated from Gullfaks oil (b)

3.0 Synthesis of a model monoaromatic UCM hydrocarbon

Figure 3.1	Proposed structure of model monoaromatic UCM hydrocarbons	68
Figure 3.2	Model monoaromatic UCM compounds	70
Figure 3.3	Reaction scheme for the synthesis of model monoaromatic UCM hydrocarbons	71
Figure 3.4	Gas chromatogram of 3-benzoyl(4'-cyclohexyl)propanoic acid (as TMS ester)	73
Figure 3.5	Mass spectrum of 3-benzoyl(4'-cyclohexyl)propanoic acid (as TMS ester)	73
Figure 3.6	Infra-red absorbance spectrum of 3-benzoyl(4'-cyclohexyl) propanoic acid.	74
Figure 3.7	Assignment key for the NMR spectra of 3-benzoyl(4'-cyclohexyl)propanoic acid	76
	a) Proton spectrum	76
	b) ^{13}Carbon spectrum	77
	c) DEPT spectrum	77
Figure 3.8	Gas chromatogram of 4-phenyl(4'-cyclohexyl)butanoic acid (as TMS ester)	79
Figure 3.9	Mass spectrum of 4-phenyl(4'-cyclohexyl)butanoic acid (as TMS ester)	79
Figure 3.10	Infra-red spectrum of 4-phenyl(4'-cyclohexyl)butanoic acid	80
Figure 3.11	Assignment key for the NMR spectra of 4-phenyl(4'-cyclohexyl)butanoic acid	82
	a) Proton spectrum	82
	b) ^{13}Carbon spectrum	83
	c) DEPT spectrum	83
Figure 3.12	Gas chromatogram of 7-cyclohexyl-1-tetralone	85
Figure 3.13	Mass spectrum of 7-cyclohexyl-1-tetralone	85
Figure 3.14	Infra-red spectrum of 7-cyclohexyl-1-tetralone	86
Figure 3.15	Assignment key for the NMR spectra of 7-cyclohexyl-1-tetralone	88
	a) Proton spectrum	88
	b) ^{13}Carbon spectrum	89
	c) DEPT spectrum	89
Figure 3.16	Gas chromatogram of 7-cyclohexyl-1-hydroxy-1-methyltetralin (as TMS ester)	91
Figure 3.17	Mass spectrum of 7-cyclohexyl-1-hydroxy-1-methyltetralin	91
Figure 3.18	Infra-red spectrum of 7-cyclohexyl-1-hydroxy-1-methyltetralin	92
Figure 3.19	Assignment key for the NMR spectra of 7-cyclohexyl-1-hydroxy-1-methyltetralin	94
	a) proton spectrum	94
	b) ^{13}Carbon spectrum	95
	c) DEPT spectrum	95
Figure 3.20	Gas chromatogram of 7-cyclohexyl-1-methenyltetralin and 7-cyclohexyl-1-methyl,3,4-dihydronaphthalene	97
Figure 3.21	Mass spectrum of 7-cyclohexyl-1-methenyltetralin and 7-	97

	cyclohexyl-1-methyl,3,4-dihydronaphthalene	
Figure 3.22	Infra-red spectrum of 7-cyclohexyl-1-methenyltetralin and 7-cyclohexyl-1-methyl,3,4-dihydronaphthalene	98
Figure 3.23	Assignment key for the NMR spectra of 7-cyclohexyl-1-methenyltetralin and 7-cyclohexyl-1-methyl,3,4-dihydronaphthalene	99
	a) Proton spectrum	99
	b) ^{13}Carbon spectrum	100
	c) DEPT spectrum	100
Figure 3.24	Gas chromatogram of 7-cyclohexyl-1-methyltetralin	102
Figure 3.25	Mass spectrum of 7-cyclohexyl-1-methyltetralin	102
Figure 3.26	Infra-red spectrum of 7-cyclohexyl-1-methyltetralin	103
Figure 3.27	Assignment key for the NMR spectra of 7-cyclohexyl-1-methyltetralin	104
	a) Proton spectrum	104
	b) ^{13}Carbon spectrum	105
	c) DEPT spectrum	105
Figure 3.28	Gas chromatogram of 6-cyclohexyltetralin	107
Figure 3.29	Mass spectrum of 6-cyclohexyltetralin	107
Figure 3.30	Gas chromatogram of 7-cyclohexyl-1-propyltetralin	108
Figure 3.31	Mass spectrum of 7-cyclohexyl-1-propyltetralin	108
Figure 3.32	Gas chromatogram showing a typical unresolved complex mixture (UCM) of monoaromatic hydrocarbons isolated from mussels collected from Whitby, U.K. (Wraige, 1997) and the three synthetic alkyltetralins	110
4.0	**Toxicological assessment of a model monoaromatic UCM hydrocarbon**	
Figure 4.1	Concentration (aqueous)-response curve for the effect of 7-cyclohexyl-1-methyltetralin upon mussel feeding rate over 24 h	121
Figure 4.2	Concentration (tissue)-response curve for the effect of 7-cyclohexyl-1-methyltetralin upon mussel feeding rate over 24 h	121
Figure 4.3	Concentration ($\log_{10}$tissue)-response curve for the effect of 7 cyclohexyl-1-methyltetralin upon mussel feeding rate over a 24 h exposure period	123
Figure 4.4	Comparison of concentration (aqueous)-response curves for the model aromatic UCM hydrocarbons, 6-cyclohexyltetralin, 7-cyclohexyl-1-propyltetralin (Wraige, 1997) and 7-cyclohexyl-1-methyltetralin (present study)	125
Figure 4.5	Comparison of concentration ($\log_{10}$aqueous)-response curves for curves for the model aromatic UCM hydrocarbons, 6-cyclehexyltetralin, 7-cyclohexyl-1-propyltetralin (Wraige, 1997) and 7-cyclohexyl-1-methyltetralin (present study)	125
Figure 4.6	Comparison of concentration (tissue)-response curves for the model aromatic UCM hydrocarbons, 6-cyclehexyltetralin, 7-cyclohexyl-1-propyltetralin (Wraige, 1997) and 7-cyclohexyl-1-methyltetralin (present study)	126
Figure 4.7	Comparison of concentration (aqueous)-response curves for the model aromatic UCM hydrocarbons, 6-cyclehexyltetralin, 7-cyclohexyl-1-propyltetralin (Wraige, 1997), 7-cyclohexyl-1-methyltetralin and an isolated monoaromatic UCM (present	127

	study)	
Figure 4.8	Comparison of concentration (log_{10}aqueous)-response curves for the model aromatic UCM hydrocarbons, 6-cyclehexyltetralin, 7-cyclohexyl-1-propyltetralin (Wraige, 1997), 7-cyclohexyl-1-methyltetralin and an isolated monoaromatic UCM (present study)	128
Figure 4.9	Concentration (log_{10}aqueous)-response curve for an isolated monoaromatic UCM upon mussel feeding rate over a 24 hour exposure	129
Figure 4.10	Comparison of concentration (tissue)-response curves for the model aromatic UCM hydrocarbons, 6-cyclehexyltetralin, 7-cyclohexyl-1-propyltetralin (Wraige, 1997), 7-cyclohexyl-1-methyltetralin and an isolated monoaromatic UCM (present study)	130
Figure 4.11	Comparison of concentration (log_{10}tissue)-response curves for the model aromatic UCM hydrocarbons, 6-cyclehexyltetralin, 7-cyclohexyl-1-propyltetralin (Wraige, 1997), 7-cyclohexyl-1-methyltetralin and an isolated monoaromatic UCM (present study)	131
Figure 4.12	Relationship between nominal aqueous concentration and total body burden of 6-cyclohexyltetralin, 7-cyclohexyl-1-propyltetralin (Wraige, 1997), 7-cyclohexyl-1-methyltetralin and an isolated monoaromatic UCM (present study)	132
5.0	**Aqueous solubility studies of model monoaromatic UCM compounds and an isolated monoaromatic UCM**	
Figure 5.1	Illustration of generator column set-up for aqueous solubility determination.	153
Figure 5.2	Comparison of the aqueous solubility of model aromatic UCM hydrocarbons 6-cyclohexyltetralin, 7-cyclohexyl-1-methyltetralin and 7-cyclohexyl-1-propyltetralin as determined by estimation and direct methods under variable environmental conditions	160
Figure 5.3	Gas chromatograms of a) isolated monoaromatic UCM, b) generated solution from isolated monoaromatic UCM	163
Figure 5.4	The relationship between aqueous solubility and biological effect TEC_{50} – a synthesis of the data of Donkin *et al.* (1989, 1991) and Wraige (1997) with the present study	166
6.0	**Photodegradation studies of model monoaromatic UCM compounds**	
Figure 6.1	Spectral distribution of the emission spectra of the most important artificial radiation sources (Roof, 1982)	179
Figure 6.2	Schematic arrangement of photolysis experimental apparatus	180
Figure 6.3	Photodegradation of generated solutions of 6-cyclohexyltetralin irradiated under xenon lamp	184
Figure 6.4	Photodegradation rate constant (k_p) of 6-cyclohexyltetralin irradiated under xenon lamp	184
Figure 6.5	Photodegradation of generated solutions of 7-cyclohexyl-1-methyltetralin irradiated under xenon lamp	187
Figure 6.6	Photodegradation rate constant (k_p) of 7-cyclohexyl-1-methyltetralin irradiated under xenon lamp	187
Figure 6.7	Photodegradation of generated solutions of 7-cyclohexyl-1-	190

	propyltetralin irradiated under xenon lamp	
Figure 6.8	Photodegradation rate constant (k_p) of 7-cyclohexyl-1-propyltetralin irradiated under xenon lamp	190
Figure 6.9	Photodecay of generated solutions of three model aromatic UCM hydrocarbons irradiated under a xenon lamp	192
Figure 6.10	Photodegradation rate constants of three model aromatic UCM hydrocarbons irradiated under a xenon lamp	192
Figure 6.11	UV-Visible spectra (190-500 nm) of model monoaromatic UCM compounds	197
Figure 6.12	Comparison between the UV-Visible spectra (190-500 nm) of model monoaromatic UCM compounds and an isolated monoaromatic UCM	198

List of Tables

1.0	**Introduction**	
Table 1.1	Gravimetric composition of six crude oils from various depositional environments (Revill, 1992)	5
Table 1.2	Summary of aromatic UCM hydrocarbon concentrations reported in environmental samples	13
Table 1.3	Concentrations of 'total toxic hydrocarbons' (Widdows *et al.*, 1995) compared to 'aromatic UCM hydrocarbons' (Wraige, 1997; *Smith, unpublished) in mussels (*Mytilus edulis*) from U.K. east coast sites with reduced Scope for Growth.	24
Table 1.4	Reported concentrations of aliphatic UCMs in environmental water samples	36
2.0	**Isolation and toxicological assessment of a monoaromatic UCM**	
Table 2.1	The effect of an isolated monoaromatic hydrocarbon UCM upon mussel feeding rate over a 24 h exposure period	53
Table 2.2	Summary of the concentration-response data for the effect of an isolated monoaromatic hydrocarbon UCM upon mussel feeding rate, 24 h exposure	55
3.0	**Synthesis of a monoaromatic UCM hydrocarbon**	
4.0	**Toxicological assessment of a monoaromatic UCM hydrocarbon**	
Table 4.1	The effect of 6-cyclohexyltetralin upon mussel feeding rate over a 24 h exposure period	118
Table 4.2	Comparison of effect of 25 $\mu g\ L^{-1}$ 6-cyclohexyltetralin upon mussel feeding rate over a 24 h exposure period from present study with Wraige (1997)	119
Table 4.3	The effect of 7-cyclohexyl-1-methyltetralin upon mussel feeding rate over a 24 h exposure period	120
Table 4.4	Summary of the concentration-response data for the effect of 7-cyclohexyl-1-methyltetralin upon mussel feeding rate	122
Table 4.5	TEC_{50} values for the effect of model aromatic UCM hydrocarbons 6-cyclohexyltetralin, 7-cyclohexyl-1-methyltetralin and 7-cyclohexyl-1-propyltetralin upon mussel ciliary feeding activity	124
Table 4.6	The effect of hydrocarbons on the filter feeding rate of mussels (*Mytilus edulis*) – a synthesis of the data of Donkin *et al* (1989, 1991) and Wraige (1997)* with present study+	138
5.0	**Aqueous solubility studies of model monoaromatic UCM compounds and an isolated monoaromatic UCM**	
Table 5.1	Estimates of log K_{ow} and aqueous solubility for three model monoaromatic UCM hydrocarbons	149
Table 5.2	Reported aqueous solubility of anthracene as determined by generator column method	154
Table 5.3	The effect of temperature and salinity on the reported measurement of aqueous solubility of anthracene determined by	155

	the generator column method	
Table 5.4	Aqueous solubility of anthracene as determined by generator column method	156
Table 5.5	Aqueous solubility of model aromatic UCM hydrocarbons 6-cyclohexyltetralin, 7-cyclohexyl-1-methyltetralin and 7-cyclohexyl-1-propyltetralin as determined by generator column method in distilled water at 25 °C	157
Table 5.6	Aqueous solubility of model aromatic UCM hydrocarbons 6-cyclohexyltetralin, 7-cyclohexyl-1-methyltetralin and 7-cyclohexyl-1-propyltetralin as determined by generator column method in seawater at 25 °C	157
Table 5.7	Aqueous solubility of model aromatic UCM hydrocarbons 6-cyclohexyltetralin, 7-cyclohexyl-1-methyltetralin and 7-cyclohexyl-1-propyltetralin as determined by generator column method in distilled water at 15 °C	158
Table 5.8	Aqueous solubility of model aromatic UCM hydrocarbons, 6-cyclohexyltetralin, 7-cyclohexyl-1-methyltetralin and 7-cyclohexyl-1-propyltetralin as determined by generator column method in seawater at 15 °C	158
Table 5.9	Summary of aqueous solubility data for model UCM hydrocarbons 6-cyclohexyltetralin, 7-cyclohexyl-1-methyltetralin and 7-cyclohexyl-1-propyltetralin.	159
Table 5.10	Factorial reduction in the aqueous solubility of the 'model' aromatic UCM compounds measured in distilled water at 25 °C by salinity and temperature	161
Table 5.11	The effect of hydrocarbons on the filter feeding rate of mussels (*Mytilus edulis*) – a synthesis of the data of Donkin *et al.* (1989, 1991) and Wraige (1997) with present study	167
6.0	**Photodegradation studies of model monoaromatic UCM compounds**	
Table 6.1	Photodegradation of generated aqueous solutions of 6-cyclohexyltetralin in distilled water irradiated under xenon lamp at 25 °C.	183
Table 6.2	Photodegradation of generated solutions of 7-cyclohexyl-1-methyltetralin in distilled water irradiated under xenon lamp at 25°C.	186
Table 6.3	Photodegradation of generated solutions of 7-cyclohexyl-1-propyltetralin in distilled water irradiated under xenon lamp at 25°C.	189
Table 6.4	Photodegradation rate constants (k_p) and half-lives of model aromatic UCM compounds in distilled water under xenon lamp at 25°C	191
Table 6.5	Xenon arc lamp exposure times and equivalent Florida summer sunlight (summer lat. 30°N, 12 h light d^{-1}) days	193
8.0	**Summary and suggestions for future work**	
Table 8.1	Summary of experimental data pertaining to model monoaromatic UCM hydrocarbons and an isolated monoaromatic UCM	231

LIST OF COMMON ABBREVIATIONS

^{13}C NMR	^{13}Carbon nuclear magnetic resonance spectroscopy
^{1}H NMR	Proton nuclear magnetic resonance spectroscopy
CI	Chemical ionisation
DCM	Dichloromethane
DEPT	Distortionless enhancement by polarisation transfer
EI	Electron impact
FID	Flame ionisation detector
GC	Gas chromatography
GC-MS	Gas chromatography -mass spectrometry
HPLC	High performance liquid chromatography
IR	Infra-red
K_{ow}	Octanol-water partition co-efficient
LC_{50}	Concentration of toxicant which results in 50 % mortality
LBB	Lethal body burden
MS	Mass spectrometry
NMR	Nuclear magnetic resonance spectroscopy
PAH	Polycyclic aromatic hydrocarbon
QSAR	Quantitative structure activity relationship
rbf	Round-bottom flask
RSD	Relative standard deviation
SFG	Scope for Growth
SIM	Selected ion monitoring
TEC_{50}	Tissue concentration to reduce toxicant induced response by 50 %
TEL	Total extractable lipid
TLC	Thin layer chromatography
TMS	Trimethylsilyl
TOE	Total Organic Extract
UCM	Unresolved Complex Mixture
UV	Ultra-violet
WAF	Water accommodated fraction
WEC_{50}	Aqueous concentration to reduce toxicant induced response by 50%
WSF	Water soluble fraction

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.
This study was financed by the University of Plymouth in collaboration with Plymouth Marine Laboratory.
Relevant scientific seminars and conferences were attended at which work was often presented and several papers prepared for publication

Publications:

Rowland, S.J., Donkin, P., **Smith, E.L.** & Wraige, E.J. (2001) Aromatic hydrocarbon 'humps' in the marine environment: unrecognised toxicants? *Environmental Science and Technology*, **35**, 2640-2644

Smith, E.L., Wraige, E.J., Donkin, P. & Rowland, S.J. (2001) Hydrocarbon humps in the marine environment: synthesis, toxicity and aqueous solubility of monoaromatic compounds. *Environmental Toxicology and Chemistry*, **20**, 2428-2432

Oral presentations and Conferences attended:

Smith E.L., Rowland, S.J. & Donkin, P. Photooxidation of Unresolved Aromatic Hydrocarbons: Characterisation and Environmental Toxicity (poster presentation); Society of Environmental Chemistry and Toxicology 18th Annual Meeting, Bridging the Global Environment: Technology, Communication and Education, 16-20 November 1997, San Francisco.

Smith, E.L. Photooxidation of Unresolved Aromatic Hydrocarbons: Characterisation and Environmental Toxicity (oral presentation); Departmental seminar, University of Plymouth, 1997

Smith, E.L. The aqueous solubility and octanol-water partition coefficient of highly hydrophobic compounds (oral presentation); Departmental seminar, University of Plymouth, 1998

Smith, E.L. The Perfect Solution? (oral presentation); Departmental seminar, University of Plymouth, 1999

Smith, E.L., Donkin, P. & Rowland, S. Unresolved complex mixtures in the marine environment (oral presentation); Society of Environmental Chemistry and Toxicology 3rd World Congress, Global Environmental Issues in the 21st Century: Problems, Causes and Solutions, Brighton, 20-25 May 2000.
Young Scientist Award for best platform presentation (awarded by Proctor and Gamble)

Signed.....

Dated.....

ACKNOWLEDGEMENTS

In the long journey that has been my PhD there are many people who have helped me in countless ways, I would like to thank a few.

I would like to offer my heart felt thanks to my supervisors Prof. Steve Rowland and Dr Peter Donkin for their continued support and patience which I am sure I have tried on many occasions. I only hope that I may become a good scientist and merit the time and energy that they afforded me.

I would also like to thank Dr Anthony Lewis, Dr Simon Hardy and Colin Woods (Zeneca) for the help they have given me in different aspects of this work.

Prof. John Dearden (Liverpool John Moores University) for supplying aqueous solubility predictions.

Julie Sloane, Roger Srodzinski, Ian Doidge, Andrew Arnold, Andrew Tonkin, and Dr Roger Evans for the technical advice and support they have kindly given me during my time at Plymouth. Similar thanks are due to Sheila Evans and Fred Staff for their kind help during my time at Plymouth Marine Laboratory.

A big shout out to the PEGG gang (past and present) for putting up with my ability to jinx any electrical equipment I come into contact with, and for making my time at Plymouth such an enjoyable one, especially Paul. Also thanks with the help when the chips were down guys – you know who you are.

Finally to my family, for their constant support, advice and encouragement. I hope you can be as proud of me as I am of you. Long live the Smith family motto, 'bollock on'.

Is the cup half empty, or is the cup half full?
Does the sun rise at dawn, or does the earth just spin along?
Or is it all perspective on how we see the world?
How many countless life forms are on a little ball that twirls?

Are we intrinsically separate beings?
Or could we possibly be parts of the same thing?
Water feeds the plants and the sunshine makes them grow,
So how can anyone be an island on their own?

Does life make me happy, or does it bring me down?
Do I have what I need, or do I just want what's around?
Am I seeing the truth, or do I just see wool?
Well is the cup half empty or is the cup half full?

'Twenty Questions' – The Beastie Boys, The Sounds of Science

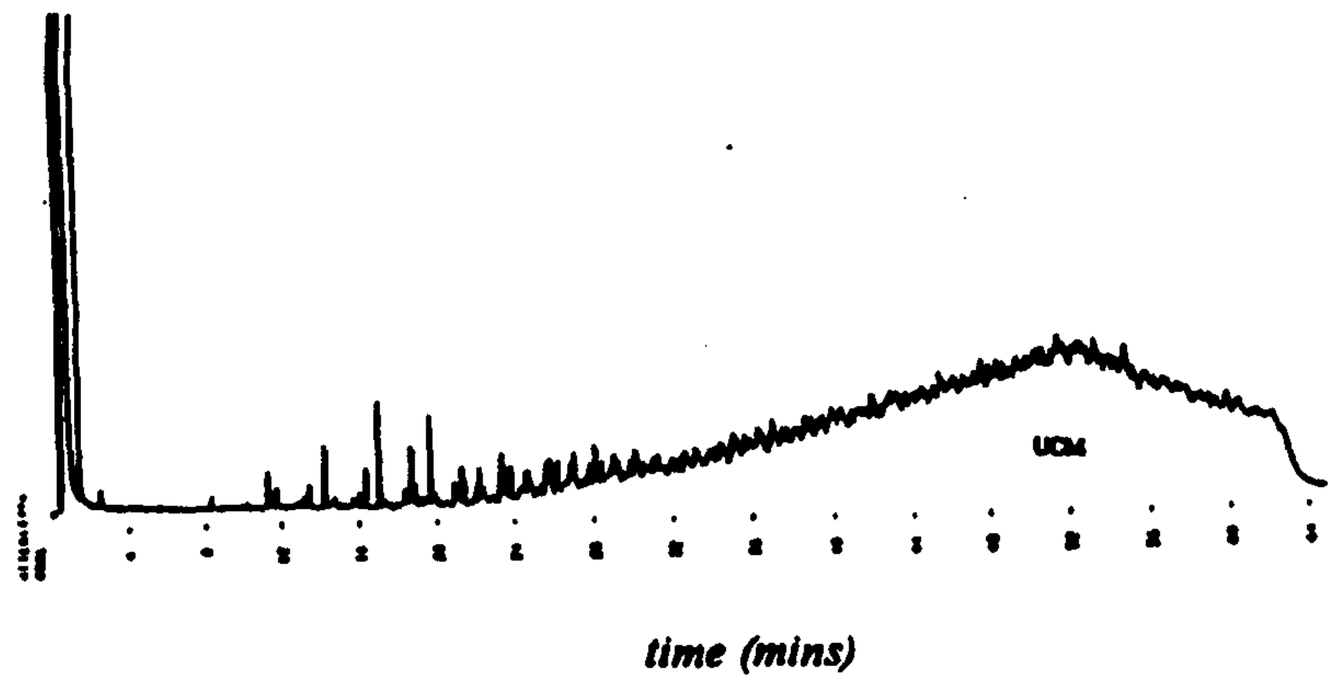
Chapter 1. Introduction

This chapter presents a review of present knowledge of unresolved complex mixtures (UCMs) of aromatic hydrocarbons in the marine environment. The review suggests that UCMs occur in a wide range of environmental samples and often at concentrations well above background. Research carried out to determine the molecular composition of the aromatic UCM is also reviewed. The behaviour and possible detrimental effects of these compounds in the marine environment are discussed, and the rationale behind the work undertaken in this study is presented.

1.1 Unresolved complex mixtures of hydrocarbons

Considerable quantities of crude petroleum and refined petroleum products are introduced into the marine environment each year (estimated 2.5 million tonnes; Clark, 1992) through runoff, industrial and sewage effluents, shipping activities and spillage (Volkman *et al.*, 1992). In fact, residual hydrocarbons arising from the transport and use of petroleum are one of the most common pollutants in the marine environment (Bouloubassi *et al.*, 2001). Crude oils are very complex mixtures of hydrocarbons and substituted derivatives of hydrocarbons. Once released into the marine environment they are rapidly 'weathered', being altered by processes such as evaporation, dissolution, dispersion, photochemical oxidation, microbial biodegradation, water-oil emulsification and adsorption onto suspended particulate materials (Wang & Fingas, 1997). The effect of these processes is to reduce the concentration of volatile constituents, to lower the total hydrocarbon concentrations, and thereby increase the relative proportions of higher molecular weight compounds, including the so-called polar N, S and O-containing residues. Perhaps the most obvious physical result of extensive weathering of crude petroleum is the production of black tarry residues (Atlas, 1995). However, coincident with this overall effect are significant changes in the hydrocarbon profiles of the liquid petroleum components. These are most obvious when the gas chromatographic (GC) profiles of weathered petroleums are examined (Figure 1.1). The total hydrocarbon fraction from unweathered crude petroleum is usually dominated by obvious homologues of resolved *n*-alkanes, with lesser amounts of branched (including isoprenoid) and cyclic alkenes, and alkylaromatics (such as benzenes, naphthalenes and phenanthrenes) (Figure 1.1.b). In contrast, the gas chromatograms of aliphatic and aromatic fractions from weathered oils are dominated by unresolved complex mixtures (UCMs) of liquid hydrocarbons, sometimes known as hydrocarbon 'humps'

a)



b)

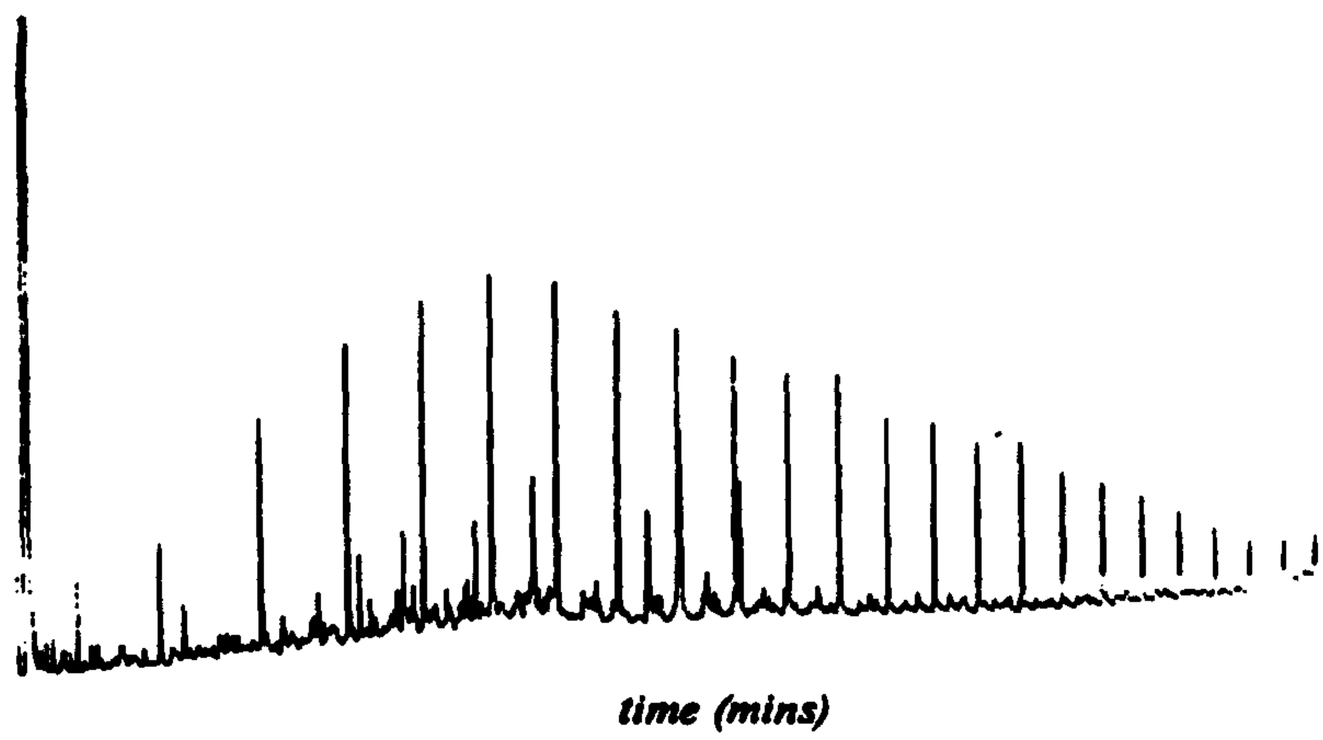


Figure 1.1 Gas chromatograms of a) weathered crude oil; b) fresh crude oil (Wraige, 1997)

(Figure 1.1.a) (Killops & Al-Juboori, 1990). UCMs are common features of gas chromatograms of weathered and biodegraded oils, but due to normalization on the resolved compounds in chromatograms of undegraded oils, UCMs are a less obvious feature of gas chromatograms of fresh crude oils. Nonetheless, UCMs constitute 21-33 % even of fresh crude oils (Table 1.1), as demonstrated by physical isolation of UCMs by clathration of fresh oils, followed by gravimetry of the clathrates (Revill *et al.*, 1992). Furthermore, Revill (1992) was able to produce an UCM of liquid hydrocarbons by heating polythene in the laboratory at 330 °C for 3 days. The products were chemically similar to a proportion of the UCM hydrocarbons of crude and refined lube oils. It therefore seems likely that the UCM derives from the catagenesis of kerogen; ancient geological matter generally considered to form from petrified biological compounds such as proteins and lipids, and other processes associated with oil formation, in the same way as the resolved components, such as the *n*-alkanes, rather than being produced during the weathering process (Killops & Al-Juboori, 1990; Revill, 1992). The implication of these studies is that the potential contribution of UCM hydrocarbons to the environment *via* fresh, refined and weathered petroleum residues is enormous.

Table 1.1. Gravimetric composition of six crude oils from various depositional environments (Revill, 1992)

Oil	% aliphatics	% aromatics	% asphaltenes and polars*	aliphatic urea adduct % of aliphatics	aliphatic urea non adduct+ % of aliphatics	urea non adduct+ % of oil *i.e.* aliphatic UCM
Nigerian Light[1]	52	17	31	37	63	**33**
Kuwait[2]	33	24	43	34	66	**22**
Forties[3]	42	18	40	38	62	**26**
Libyan[2]	50	16	34	44	56	**28**
Iranian Light[2]	40	19	41	37	63	**25**
Tia Juana Pesado[3] (Venezuela) (Biodegraded)	23	35	42	10	90	**21**

* By Difference
\+ Taken to represent UCM

1 Deltaic Source
2 Carbonate Source
3 Marine Source

1.2 Compositional studies of aromatic hydrocarbon UCMs

Among the analytical techniques available for structural determination of crude oil hydrocarbons, gas chromatography (GC) in combination with mass spectrometry (GCMS) is by far the most widely used. Fractionation of crude oil and subsequent GCMS analysis has allowed the characterisation of nearly 300 compounds comprising 'aliphatic', 'aromatic' and so-called biomarker compounds (reviewed by Peters & Moldowan, 1993). Nevertheless, many components of crude oil still remain uncharacterised since the majority cannot be resolved by GC, and these appear as 'humps' or unresolved complex mixtures (UCMs) in gas chromatograms (Figure 1.1.a). UCMs result from the co-elution of complex mixtures of thousands of hydrocarbons with similar physical and chemical properties - though the exact number is unknown (Thompson & Eglinton, 1978).

Although the exact structural composition of any one UCM hydrocarbon has not been determined, a number of studies have attempted to identify structural components of hydrocarbon UCMs, and some have proposed model structures (*e.g.* Killops & Al-Juboori, 1990; Gough, 1989; Revill, 1992; Revill *et al.*, 1992; Thomas, 1995). These studies have been reviewed (Revill, 1992; Thomas, 1995; Wraige, 1997) therefore discussion here is a synopsis of studies relevant to aromatic UCM hydrocarbons.

Killops & Readman (1985) reported fractionation of aromatic components from a total neutral fraction from a recent estuarine sediment exhibiting a high degree of petroleum contamination, using normal phase high performance liquid chromatography (HPLC). The bulk of the unresolved components were present in the third eluting fraction (equivalent to 3 aromatic double bonds) indicating that the aromatic UCM in this sediment was principally monoaromatic in nature. Wraige (1997) also found that the aromatic hydrocarbon UCMs isolated by similar procedures, from mussels from petroleum-impacted sites, were mainly monoaromatic (~80 % as determined by GC and HPLC).

Killops & Al-Juboori (1990) investigated the chemical composition of a UCM from a heavy biodegraded crude oil (West Sak, Alaska). Bulk UCM characteristics were defined using proton (^1H) and carbon (^{13}C)-nuclear magnetic resonance (NMR) spectroscopy, infra-red (IR) spectroscopy, ultra-violet (UV) spectroscopy, elemental analysis, electron impact (EI)-MS, chemical ionisation (CI)-MS, thin layer chromatography (TLC) and various size fractionation techniques. Spectroscopic and spectrometric investigations (IR, NMR and MS) of the bulk total hydrocarbon fraction showed the predominance of aliphatic structures. The proportion of carbon atoms that were aromatic was approximately only 10%. Chromic acid oxidation of the UCM yielded several classes of identifiable compounds amongst which *n*-alkanoic acids in the range C_5-C_{18} predominated. These suggested that *n*-alkyl chains up to ~C_{19} were present in the UCM. The composition of oxidation products suggested that the aromatic compounds comprised chiefly alkyl substituted benzenoid structures with an average molecular weight of 300.

Revill (1992) investigated the aromatic fraction of a different biodegraded crude oil (Tia Juana Pesado, Venezuela) from which most of the resolved components had been removed by in-reservoir bacterial action. Analysis of the aromatic UCM by various methods (elemental analysis, UV, IR and NMR) again produced evidence that for the majority of compounds in the 'aromatic' UCM a large proportion of the carbon was in fact highly 'aliphatic' in nature, and the presence of only one or two aromatic rings. UV spectroscopy showed evidence for both mono- and di-aromatic compounds, and IR spectroscopy indicated highly aliphatic structures, confirmed by ^1H-NMR. Revill *et al.* (1992) suggested that in general the evidence was consistent with alkylated naphthenoaromatic compounds.

Since elemental analysis and spectroscopic studies provided only limited compositional information a number of recent studies (*e.g.* Thomas 1995, Warton, 1999) have used oxidative degradation techniques in conjunction with bulk analytical techniques to characterise aromatic hydrocarbon UCMs. Chemical degradation studies of UCMs

followed by instrumental analysis have allowed partial structural elucidations of some hydrocarbon UCMs. This approach, termed '*retro*-structural analysis' by some workers (Thomas, 1995), involves the use of selective oxidising agents which attack specific structural features in the UCM hydrocarbons giving rise to products, the structures of which can be related back to the parent UCM molecule. For example, it has long been known that aromatic compounds can be selectively degraded using ruthenium tetroxide (RuO_4). RuO_4 is a *regio*-selective oxidant, in theory attacking only *ipso*-carbons on aromatic rings. Alkyl substituents are thus preserved in the oxidation products as carboxylic acids, whereas the aromatic carbons are oxidised further to carbon dioxide. This has recently been successfully applied to aromatic UCMs (Thomas, 1995; Warton *et al.*, 1999). Using this technique to characterise a range of aromatic UCMs from crude and refined oils Thomas (1995) found that oxidation produced organic solvent-soluble acids and other more resolved products, the quantities of which allowed an estimation of the proportion of alkyl substituents on the aromatic rings to be made. Similarly, the proportion of water-soluble acids and diacids gave a measure of the short chain alkyl substituents and alicyclic rings respectively, with the amount of carbon dioxide produced being proportional to the aromatic content. Results indicated that substituted tetralins were quantitatively significant components and allowed the proposition of an average structure for some aromatic UCM compounds (Figure 1.2.)

Upon a similar RuO_4 based chemical oxidation of a monoaromatic UCM from a Cretaceous biodegraded Leatherjacket crude oil from Australia, Warton *et al.* (1999) also found that the dichloromethane (DCM) soluble products were mainly monocarboxylic acids and the water soluble products mainly dicarboxylic acids. They also proposed that the oxidation products represented the alkyl moieties that were attached to aromatic rings in the unoxidised crude oil fractions. The results indicated that species with *n*-alkyl groups attached to the aromatic ring were present in the initial aromatic fraction which confirmed

the findings of Thomas (1995) and earlier studies (*e.g.* Revill, 1992). Observations indicated that compounds present in the original monoaromatic UCM fraction contained straight, branched and isoprenoid side chains, *n*-alkyl chains and *ω*-cyclohexyl groups and bicyclic components attached to monoaromatic compounds. Warton *et al.* (1999) concluded that alkylindane and alkyltetralin structures, as well as components with three or more fused rings, of which one was aromatic, were present in the original UCM. These conclusions were consistent with the work carried out by Revill (1992) and Thomas (1995) and lend support to the occurrence of substituted alkyltetralins in a variety of crude oil UCM aromatic hydrocarbons fractions.

Whilst the individual components of the aromatic hydrocarbon UCM have, as yet, not been characterised it is apparent that there are similarities in the general findings between the studies on their exact nature. For the purposes of this study the general model aromatic UCM structure (Figure 1.2) proposed by Thomas (1995) using the retro-structural analysis approach in conjunction with Fourier transform-ion cyclotron resonance spectroscopy to examine the total oxidation products is considered the best currently available model.

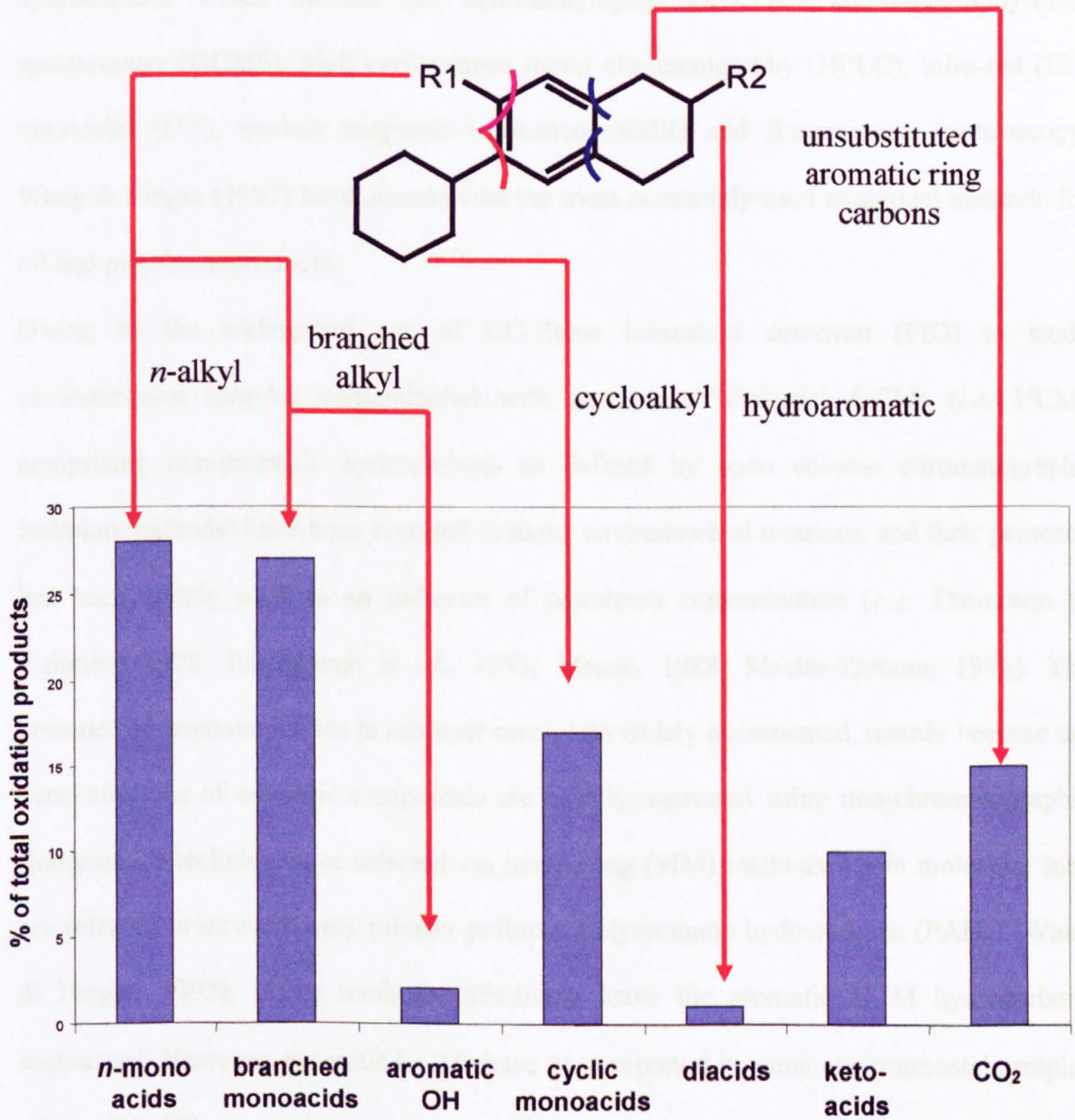


Figure 1.2 Average 'model' aromatic UCM hydrocarbon as proposed by Thomas (1995)

1.3 Environmental occurrences of aromatic UCM hydrocarbons

A wide variety of instrumental techniques are currently used in the analysis of oil hydrocarbons which include gas chromatography (GC), gas chromatography-mass spectrometry (GCMS), high performance liquid chromatography (HPLC), infra-red (IR), ultraviolet (UV), nuclear magnetic resonance (NMR) and fluorescence spectroscopy. Wang & Fingas (1997) have summarised the most commonly used analytical methods for oil and petroleum products.

Owing to the widespread use of GC-flame ionization detection (FID) to study environmental samples contaminated with petroleum 'aliphatic' UCMs (*i.e.* UCMs comprising non-aromatic hydrocarbons as defined by open column chromatography isolation methods) have been reported in many environmental matrices, and their presence has been widely used as an indicator of petroleum contamination (*e.g.* Thompson & Eglinton, 1978; Risebrough *et al.*, 1983; Mason, 1988; Macias-Zamora, 1996). The presence of aromatic UCMs is however much less widely documented, mainly because the concentrations of aromatic compounds are usually measured using non-chromatographic fluorescence techniques, or selected ion monitoring (SIM) methods where molecular ions are selected to measure only priority pollutant polyaromatic hydrocarbons (PAHs) (Wang & Fingas, 1997). These methods effectively leave the aromatic UCM hydrocarbons undetected. However, aromatic UCMs have been reported in some environmental samples where GC-FID or total ion current GCMS methods have been used. Such aromatic hydrocarbons from polluted sediments appear as GC 'humps' which are absent from chromatograms of hydrocarbons of unpolluted sediments (Jones *et al.*, 1983).

Venkatesan *et al.* (1982), Le Dreau *et al.* (1997), and Readman *et al.* (1996) all reported aromatic hydrocarbon UCM concentrations in sediments (Table 1.2). Aromatic UCMs have also been reported in mussels (Soler *et al.*, 1989; Wraige, 1997), urban aerosols

(Aceves & Grimalt, 1993), urban runoff (Bomboi & Hernandez, 1991), fish (Fowler *et al.*, 1993), and dissolved/dispersed in water (Douabul & Al-Shiwafi, 1998). Examples of aromatic UCMs from environmental samples are shown in Figure 1.3. In all of the examples shown UCMs dominate the aromatic fractions, and this is commonly the case in polluted environmental samples such as sediment and biota when appropriate analytical methods are used. The concentrations of aromatic UCMs in some selected environmental samples are summarised in Table 1.2. Such data, whilst limited, suggest that aromatic UCMs are a quantitatively important environmental burden. For example, concentrations of up to 7172 $\mu g\ g^{-1}$ dry weight have been found in mussels from Cape Town Harbour, South Africa (Mason, 1988), accounting for up to 98% of the aromatic compounds present in some samples. However, the toxicological impacts (if any) of the occurrence of these UCMs in organisms and sediments appear to have been virtually ignored.

Table 1.2. Summary of aromatic UCM hydrocarbon concentrations reported in environmental samples

Aromatic UCM concentration ($\mu g\ g^{-1}$ dry weight)	% UCM	Substrate	Region	Reference
1-1400	71-98	sediment	Saudi Arabia	Readman *et al.* (1996)
5.9-80	81-98	sediment	Kuwait	
10-93	30-68	mussels	Oil production platform, Amposta, W. Mediterranean	Soler *et al.* (1989)
1.1-160	82-98	sediment	Arabian Gulf	Fowler *et al.*(1993)
62-210	70-95	bivalves	Arabian Gulf	
2.5-210	34-88	fish	Arabian Gulf	
0.4-66	n.a.	mussels	Ebro Delta, Spain	Risebrough *et al.* (1983)
7172	n.a.	mussels	Entrance to Cape Town harbour, South Africa	Mason (1988)
489		mussels	Sewage outlet, West coast, South Africa	
114-152	83-86	mussels	New Brighton, Mersey, U.K.	Wraige (1997)
102-136	82-88	mussels	Cleethorpes, U.K.	
83-94	81-83	mussels	Teesmouth, U.K.	
365-496	85-90	mussels	Whitby, U.K.	
n.d.		mussels	Whitsand, U.K.	

n.a. not available n.d. not detected

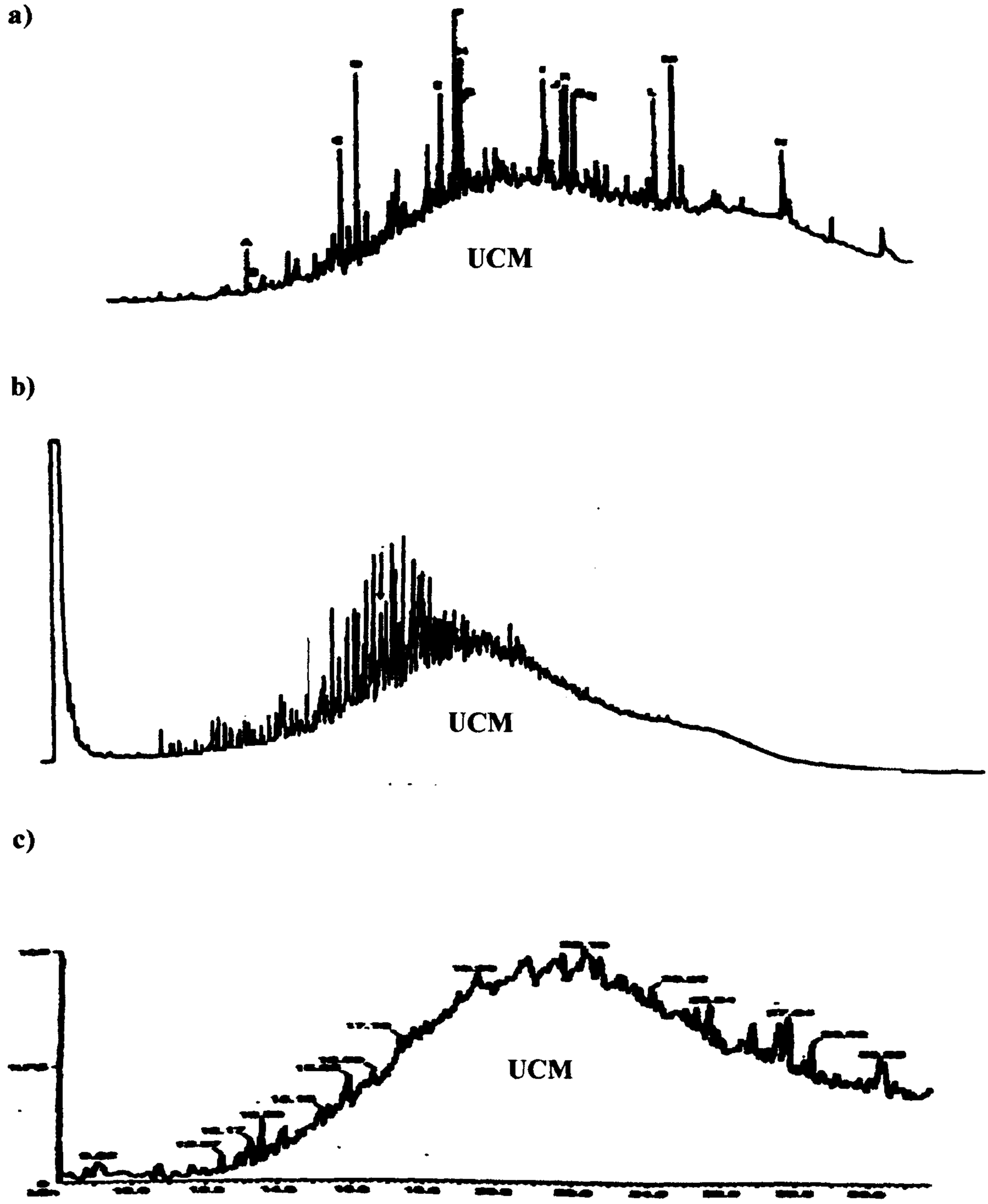


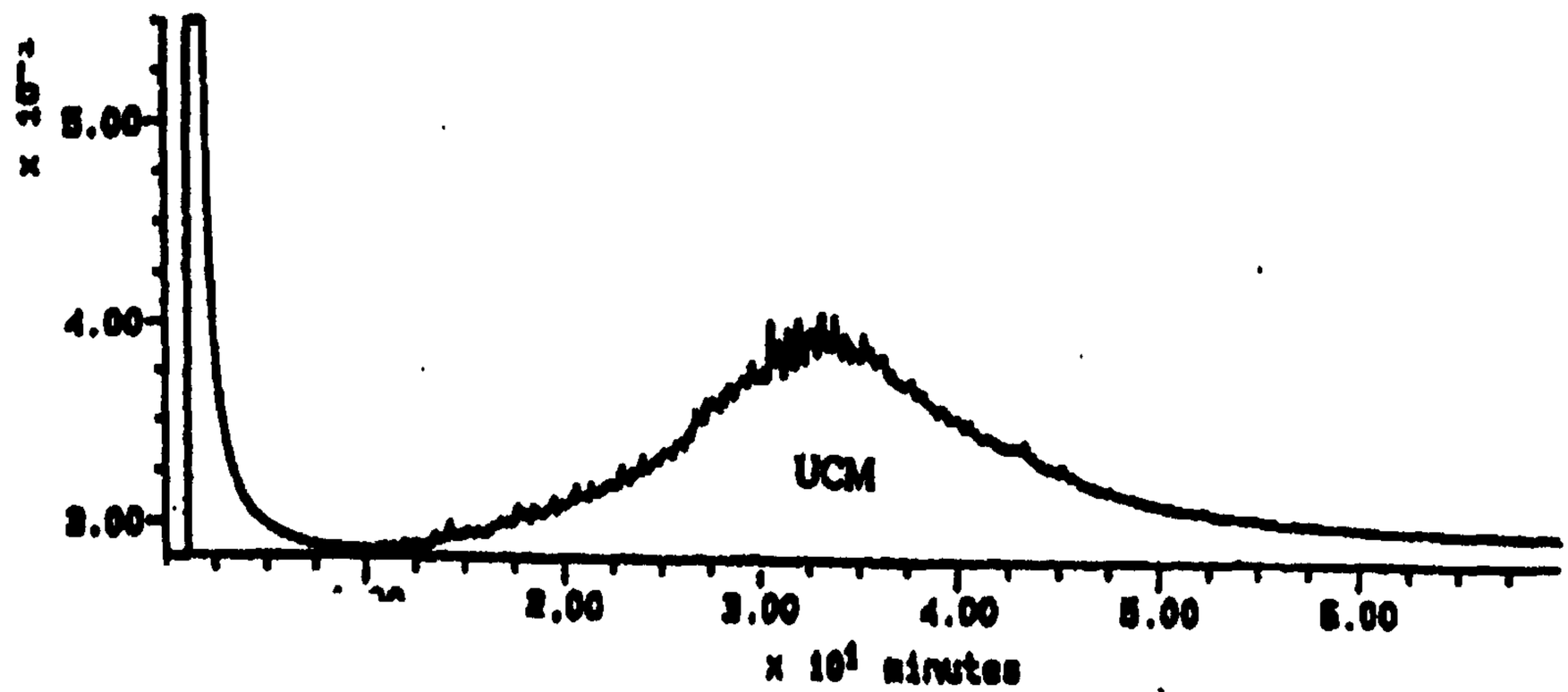
Figure 1.3. Examples of gas chromatograms of the aromatic fraction from different environmental matrices exhibiting an unresolved complex mixture

a) urban aerosol <0.5 μm, Barcelona (Aceves & Grimalt, 1993)

b) urban runoff, Legzpi (Bomboi & Hernandez, 1991)

c) dissolved/dispersed, Arabian Gulf (Douabul *et al.*, 1998)

d)



e)

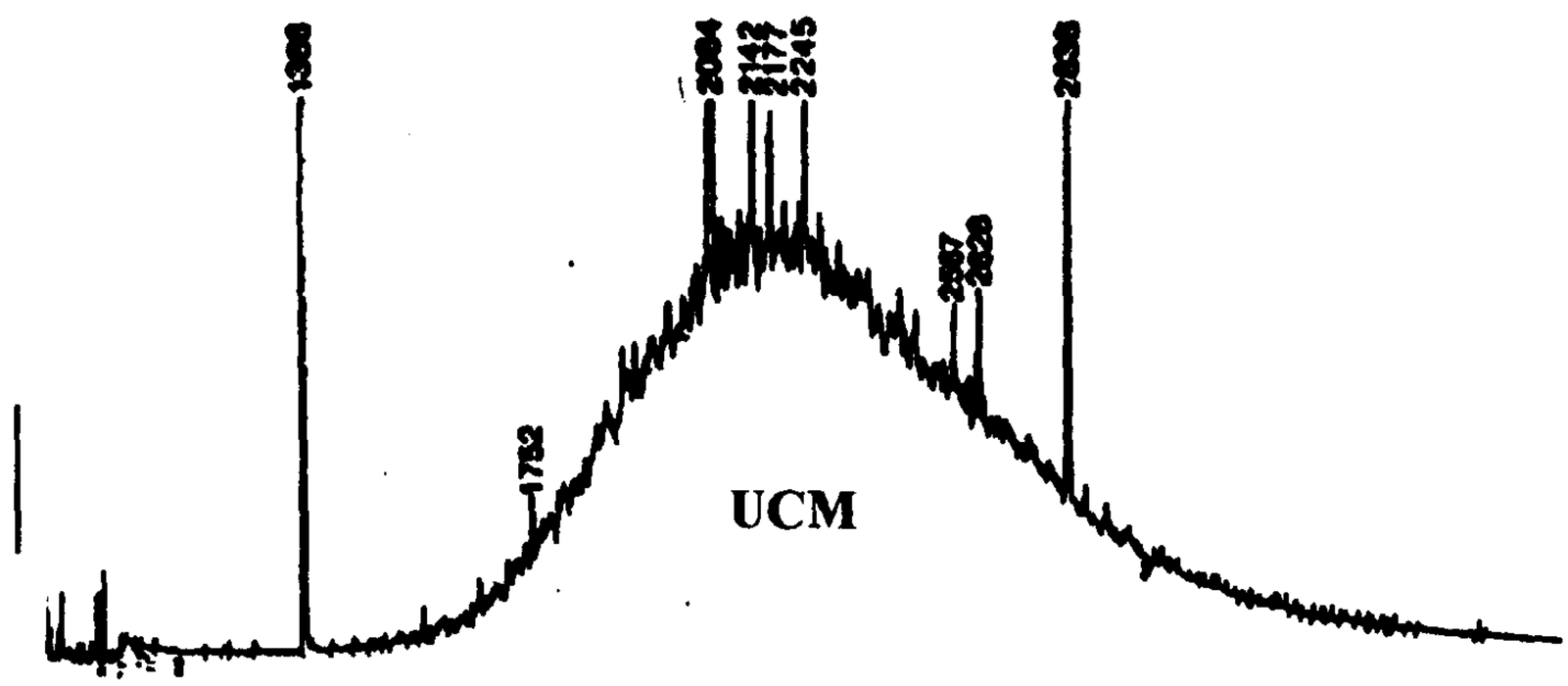


Figure 1.3. (continued.) Examples of gas chromatograms of the aromatic fraction from different environmental matrices exhibiting an unresolved complex mixture

d) sediment, Gulf of Fos, France (Le Dreau *et al.*, 1997)

e) oysters, Mina Abdullah (Anderlini *et al.*, 1981)

1.4 Circumstantial field evidence for the environmental impact of aromatic unresolved hydrocarbons

In the fate and risk assessment of persistent organic contaminants and related compounds in Victoria Harbour, Hong Kong, Connell *et al.* (1998) found that the effects of the concentrations of compounds giving an unresolved complex mixture could not be evaluated due to the lack of environmental guidelines. However, it was suspected that they would add to the adverse effects accounted for by other known contaminants.

Accumulation of contaminants in marine sediments contributes to the degradation of marine systems, which are manifested as toxicity, bioaccumulation, and population and community level disruptions in benthic biota (Burgess & McKinny, 1999). Chemicals can be accumulated in marine organisms *via* direct uptake from the surrounding medium by the gills (bioconcentration), by ingestion of particle-bound chemicals (bioaccumulation) or *via* the food web (biomagnification) (Franke *et al.*, 1994). Uptake of hydrocarbons has been demonstrated from aqueous, dietary and sedimentary pathways. Retention of hydrocarbons in lipophilic cellular compartments may result in disruption in membrane functions or alterations in energetic processes, leading to impairment of the adaptive capability of an organism within its natural habitat (Capuzzo, 1985). However, the presence of a xenobiotic compound in an ecosystem does not in itself indicate an adverse effect. Connection must be established between levels of exposure or tissue concentrations and adverse effects (Farrington, 1991).

Since the introduction of the International 'Mussel Watch' programme (Goldberg, 1978) mussels have been widely used as 'sentinel' organisms to monitor the state of the marine environment (*e.g.* Gray, 1992; Petrovic, 2001), Mussels are sessile, filter feeders and dominant members of coastal and estuarine communities with a wide geographical

distribution. As such they represent a useful tool for monitoring spatial and temporal trends of contamination (extensively reviewed by NAS, 1980).

Marine bivalves are ideally suited to field based monitoring programmes as they remain in a fixed location attached to solid substrates such as rocks. They filter large volumes of water and so can accumulate considerable quantities of pollutants, including petroleum hydrocarbons, within their tissues. Mussels thus represent a substrate in which it is easier to detect and quantify environmental contamination (Burns & Smith, 1991). They are also easy to collect and transport, being relatively unaffected by transplantation and handling stress. Their physiology is well documented and their physiological health can be readily determined in the laboratory or field situations (Widdows & Donkin, 1992). Together these attributes have resulted in the widespread use of mussels as sentinel organisms or 'bioindicators' to monitor the state of the marine environment.

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 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). Direct measurement of growth is not practical in the case of marine bivalves due to the presence of a shell and a large proportion of total production can be lost in the form of gametes when spawning (Widdows, 1985). However, the integration of physiological responses such as feeding, food absorption, respiration, excretion and production, termed Scope for Growth (SFG) can be considered the growth potential of an animal and then represent the bioenergetic status of an organism or population under specific conditions (Widdows, 1985, 1994; Widdows and Donkin, 1992; Smaal & Widdows, 1994). The Scope for Growth technique has been extensively reviewed (Martin *et al.*, 1984; Widdows, 1985; Donkin & Widdows, 1986; Wade *et al.*, 1998) and

has been shown to be consistently reliable as an indicator of general stress in mussels (Gray, 1992).

SFG is a measurement of the energy balance of mussels determined from the rate of food uptake and its assimilation efficiency. This energy balance is strongly indicative of the health of the mussel since a high positive energy balance is achieved only when a wide range of biochemical processes within the organism are functioning correctly. The assessment of SFG under field conditions provides an indication of the physiological condition or health of the animals, which in turn may reflect differences in environmental quality (Donkin & Widdows, 1986). Determination of SFG provides an instantaneous measure of the energy status of animals which can range from maximum positive values under optimal conditions, declining to negative values when the animal is severely stressed and utilising body reserves, *i.e.* when dealing with chronic pollution (Widdows *et al.*, 1995a, 1995b).

The concept of SFG has been used to assess the sub-lethal biological effects of pollutants on a variety of marine organisms and has proved particularly useful when combined with the analysis of chemical contaminants in attempts to identify cause and effect relationships (Figure 1.4). This approach has been successfully applied by Gilfillan *et al.* (1977), Martin *et al.* (1984) and Widdows *et al.* (1990, 1995a 1995b). The coupling of SFG measurement to tissue residue chemistry thus provides a toxicological interpretation of contaminant concentrations in the tissue and enables identification of the cause of effects observed in the field (Widdows & Donkin, 1989).

Widdows *et al.* (1987; 1995 a, b) have demonstrated significant correlations between SFG and the concentration of 2-3 ringed aromatic hydrocarbons in mussels as determined by high performance liquid chromatography with UV detection (HPLC-UV). Widdows *et al.* (1987) exposed *Mytilus edulis* in a mesocosm study to 28 $\mu g\ L^{-1}$ and 125 $\mu g\ L^{-1}$ of oil for 8 months and demonstrated a marked reduction in SFG for mussels exposed to both oil

concentrations, principally caused by reduction in feeding rate and food absorption efficiency. Analysis of tissues revealed a significant correlation between log tissue concentration of aromatic hydrocarbons (2-3 ring aromatics) and SFG.

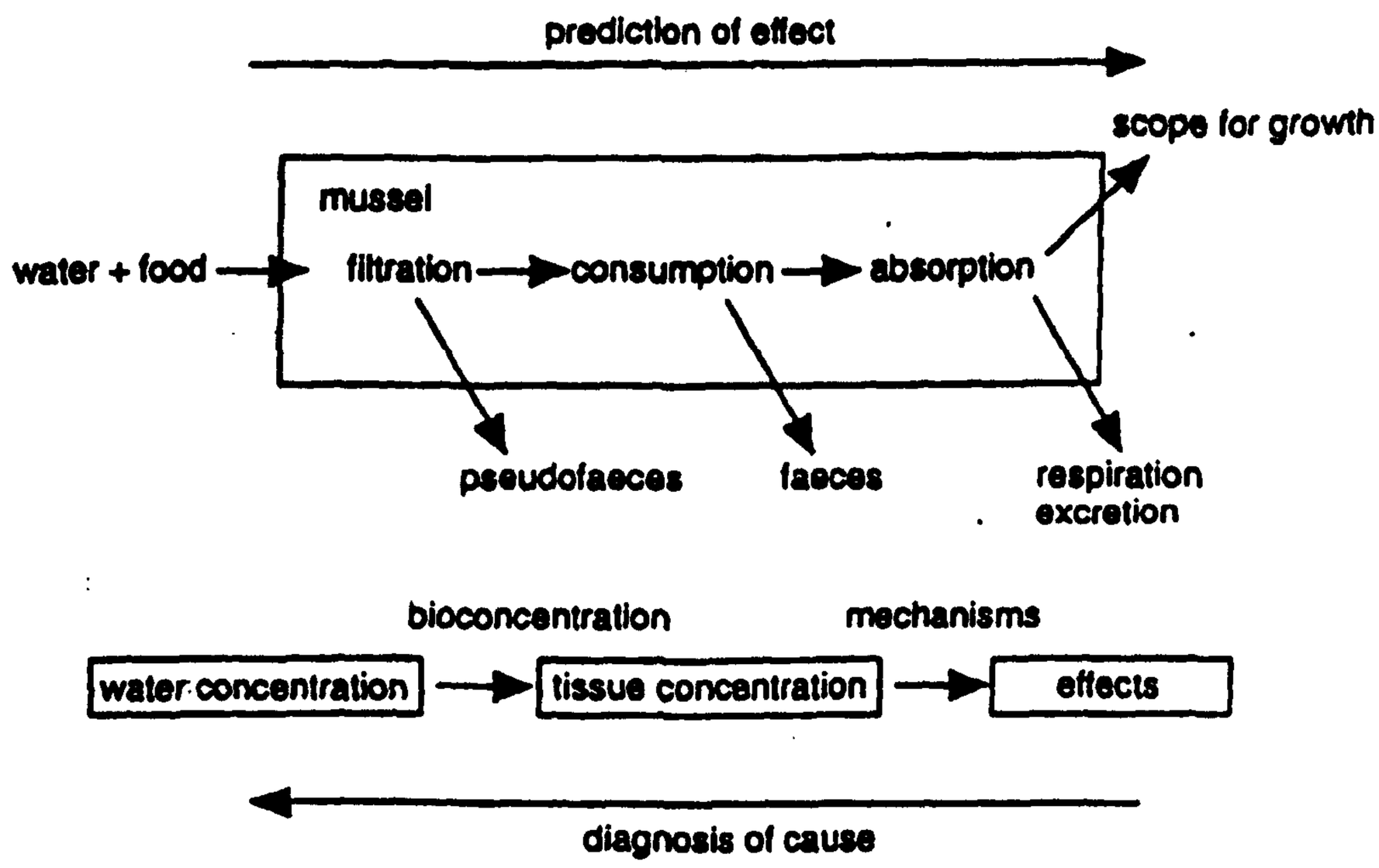


Figure 1.4 Scope for Growth (SFG) as part of the individual energy budget, in an ecotoxicological framework (Smaal & Widdows, 1994)

A similar relationship was demonstrated in an eight-year field study (Widdows *et al.*, 1995a). *Mytilus edulis* was used to monitor the degree of chemical contamination (petroleum hydrocarbons and organics) and the associated sub-lethal biological effects (in terms of Scope for Growth) in the vicinity of the Sullom Voe terminal, Shetland Isles over several years. Widdows *et al.* (1995a) found that the spatial and temporal change in concentration of aromatic hydrocarbons within the mussel reflected the major sources of oil inputs (*i.e.* oil spillages due to tanker loading operations). A significant correlation (r =

+0.89) between the net quantity of oil spilled at Sullom Voe, Shetland, U.K., one month before sampling, and the aromatic hydrocarbons accumulated in mussel tissues a month later, was found. The log of this concentration furthermore displayed a significant negative relationship with SFG (r = -0.83). This relationship was present over three orders of magnitude and without any threshold effect, suggesting a simple mode of toxicity based on the loading of body tissues and the absence of any physiological adaptation. Figure 1.5 shows the inverse relationship between SFG and the log concentration of aromatic hydrocarbons in mussel tissue reported by Widdows *et al.* (1995a). The decline in SFG at most contaminated sites was attributed primarily to low clearance rates or feeding rates, which is the component of the energy budget most responsive to pollution.

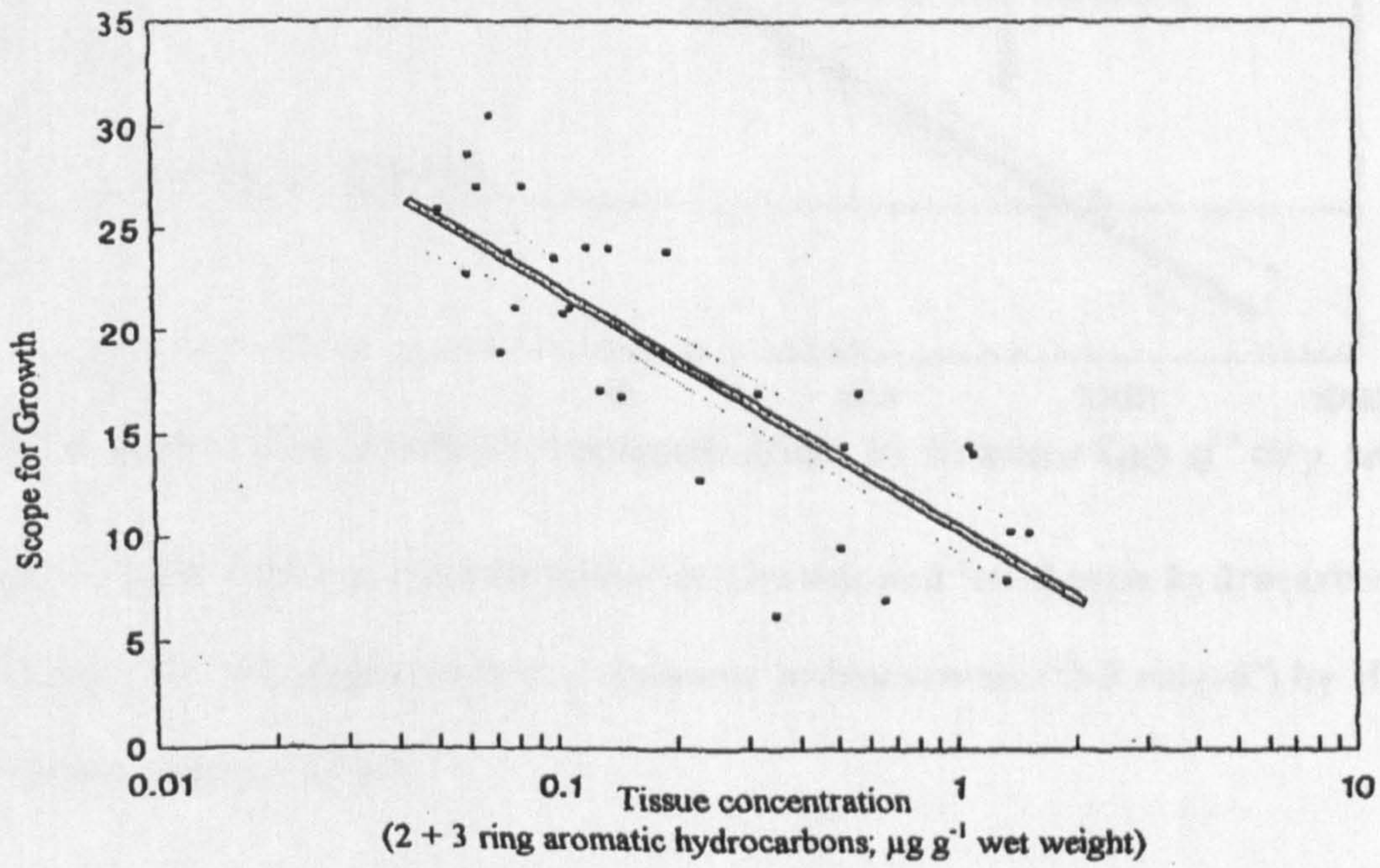


Figure 1.5 Relationship between mean Scope for Growth (SFG) and the log concentration of 2- and 3-ring aromatic hydrocarbon in the tissues of *Mytilus edulis* collected from sites in Shetland during the period 1982-1989 (Widdows *et al.*, 1995a)

Furthermore, Widdows *et al.*, (1995b) found that SFG in mussels in twenty-six east coast U.K. sites, sampled in 1990, was inversely related (r = -0.41) to the log tissue concentration of aromatic hydrocarbons despite diverse sources of pollution (Figure 1.6). Hydrocarbon contamination was measured by HPLC-UV, and expressed as '2-3 ringed aromatics' as this group of compounds was thought to represent the major toxic component of the bioaccumulated petroleum hydrocarbons.

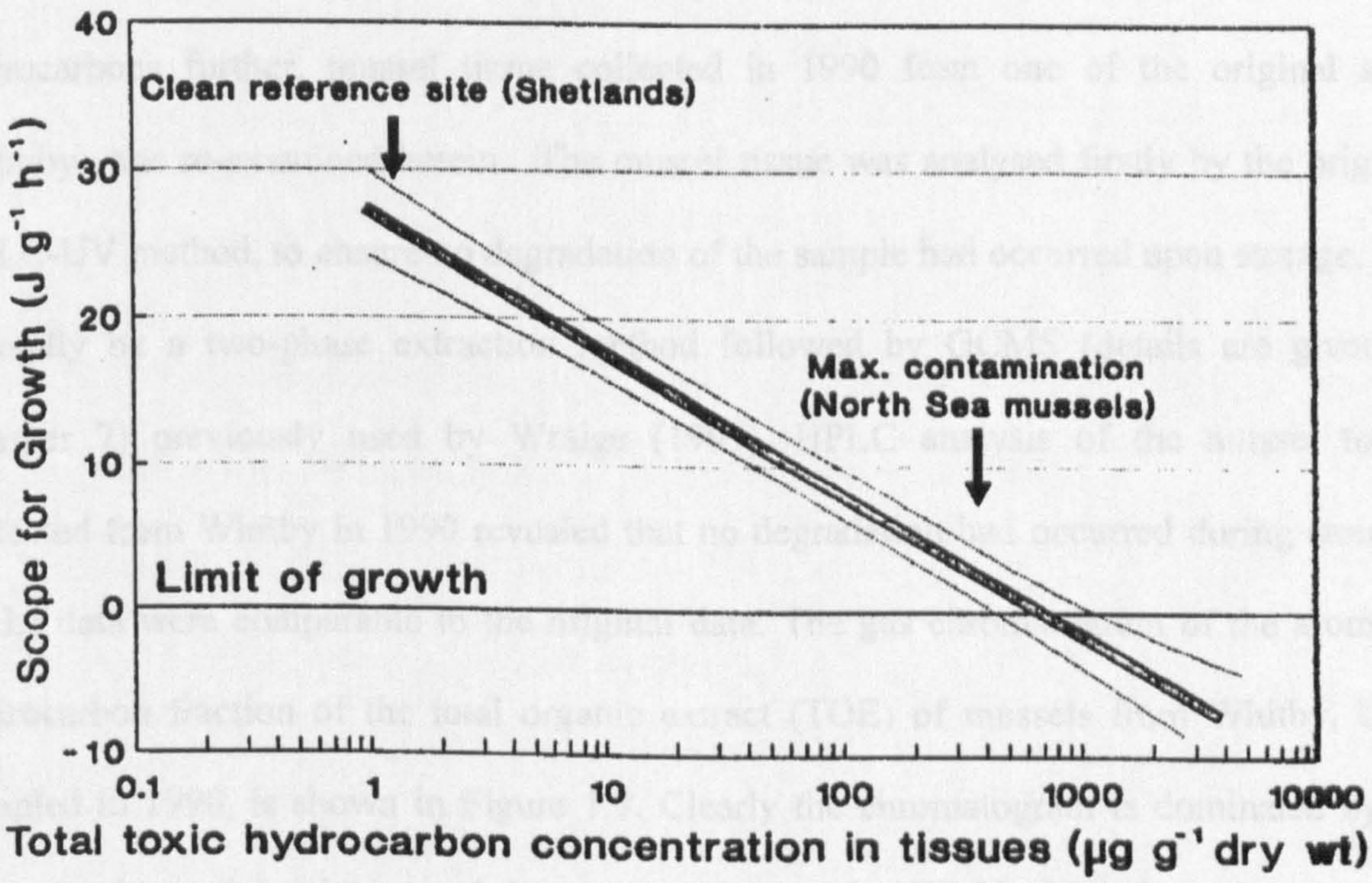


Figure 1.6 Link between reduced Scope for Growth and 'total toxic hydrocarbons' as calculated from the measurement of aromatic hydrocarbons ('2-3 ringed') by HPLC-UV (Widdows *et al.*, 1995b)

However, the HPLC-UV method did not provide a high degree of chromatographic resolution or an absolute measurement of individual compounds but measured groups of compounds with similar physicochemical properties. The gas chromatographic profiles of such HPLC fractions have rarely been reported. However, a gas chromatographic study by

Wraige (1997) indicated that the aromatic fractions of mussels taken from three of the east coast sites (Whitby, Teesmouth and Cleethorpes) in 1995, originally studied by Widdows *et al.* (1995b) by HPLC-UV in 1990, were in fact dominated by UCMs. Thus, it is unclear whether the toxic burden of so-called '2-3 ringed aromatic hydrocarbons' represents compounds which are resolved by GC and identifiable by GCMS (*e.g.* PAH priority pollutants) or whether it might in reality comprise UCMs of aromatic hydrocarbons. To investigate this possible connection between 'total toxic hydrocarbons' (measured as 2- and 3-ringed aromatic hydrocarbons by HPLC) and the concentration of aromatic UCM hydrocarbons further, mussel tissue collected in 1990 from one of the original sites (Whitby) was re-examined herein. The mussel tissue was analysed firstly by the original HPLC-UV method, to ensure no degradation of the sample had occurred upon storage, and secondly by a two-phase extraction method followed by GCMS (details are given in Chapter 7) previously used by Wraige (1997). HPLC analysis of the mussel tissue collected from Whitby in 1990 revealed that no degradation had occurred during storage, as the data were comparable to the original data. The gas chromatogram of the aromatic hydrocarbon fraction of the total organic extract (TOE) of mussels from Whitby, U.K. sampled in 1990, is shown in Figure 1.7. Clearly the chromatogram is dominated by an unresolved complex mixture and the amount of aromatic UCM hydrocarbons found at this site was 396-408 $\mu g\ g^{-1}$ dry weight. Thus, in mussels from Whitby collected in 1990, which showed reduced Scope for Growth, the aromatic compounds were actually dominated by an aromatic UCM, the concentration of which closely matches the 'total toxic hydrocarbon burden' (390 $\mu g\ g^{-1}$) determined by Widdows *et al.* (1995) by HPLC. The concentration also closely matches the concentration of aromatic UCM hydrocarbons (365-496 $\mu g\ g^{-1}$) found in mussels taken from this site in 1995 by Wraige (1997).

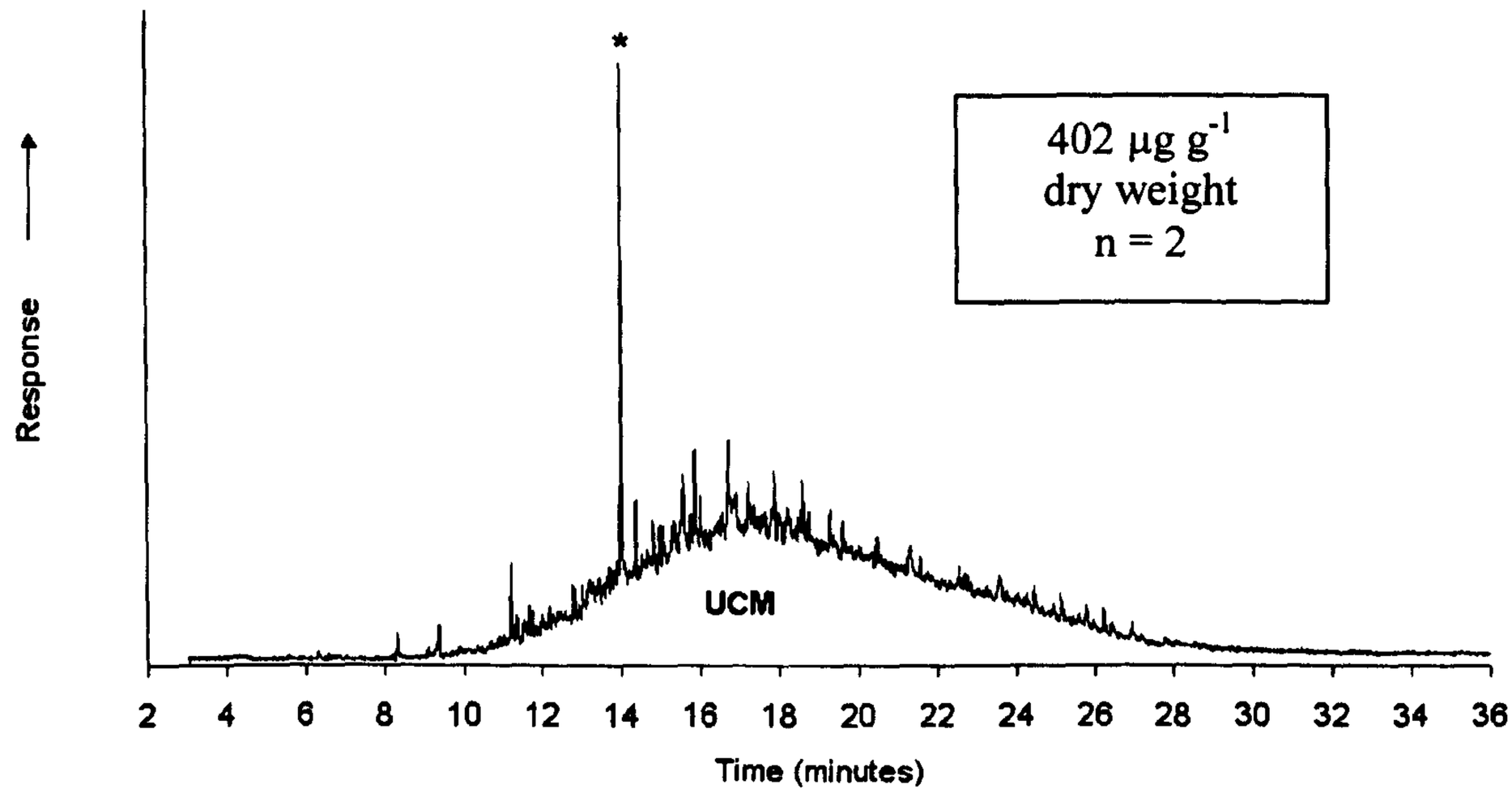


Figure 1.7 Gas chromatogram of the aromatic hydrocarbon fraction of mussels (*Mytilus edulis*) from Whitby, U.K., 1990 (Smith, present study)

*GC details : HP1 (12 m x 0.2 mm id) column, He carrier gas, 40℃-300℃ @ 10℃ min^{-1}, hold 10min; * internal standard d_{10} phenanthrene.*

Although this preliminary analysis was conducted on mussels from only one site, a further correlation was also found (Figure 1.8) between the values of 'total toxic hydrocarbons' determined by Widdows *et al.* (1995b) at sites with reduced SFG and aromatic UCM concentrations by Wraige (1997). These data are shown in Table 1.3. The concentrations of aromatic hydrocarbon UCMs determined by Wraige (1997) ranged from 83-496 µg g^{-1} dry weight, most of which were monoaromatic. No aromatic hydrocarbon UCM was detected in mussels from an unimpacted 'control' site in Cornwall in 1995. The concentrations of resolved aromatic hydrocarbons in these samples were low in comparison (*e.g.* total alkylnaphthalenes 0.053 µg g^{-1} dry weight; Whitby, 1995). The concentrations of aromatic

UCMs correlated well (r^2 adjusted = 0.94, n = 8) with the total toxic hydrocarbon burden as determined by HPLC-UV in mussels from the same sites in 1990 (Figure 1.8.).

Table 1.3 Concentrations of 'total toxic hydrocarbons' (Widdows *et al.*, 1995) compared to 'aromatic UCM hydrocarbons' (Wraige, 1997; *Smith, this study) in mussels (*Mytilus edulis*) from U.K. east coast sites with reduced Scope for Growth

Sample Site	'Total toxic hydrocarbons' ($\mu g\ g^{-1}$) (1990)	Aromatic UCM hydrocarbons ($\mu g\ g^{-1}$) 1990	1995
Teesmouth	77.7	not determined	83, 94
Whitby	390	*396, 408	365, 496
Cleethorpes	124.9	not determined	102, 136

N.B. The term 'total toxic hydrocarbons' is derived from the measurement of 2- and 3-ringed PAHs (by HPLC) which is converted to a total toxic hydrocarbon value (by GC) using a factor of 7.1 (Widdows et al., 1982, 1995).

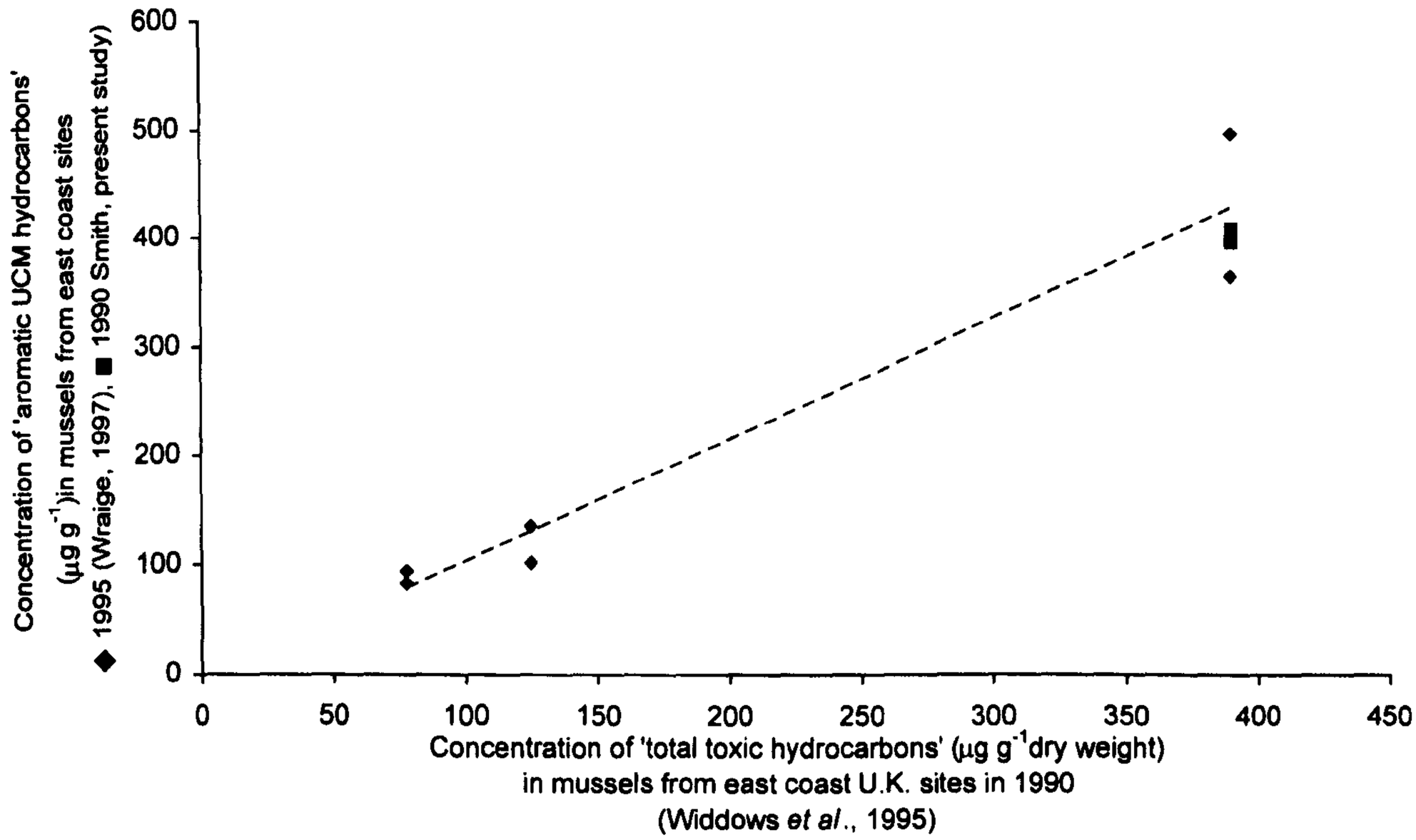
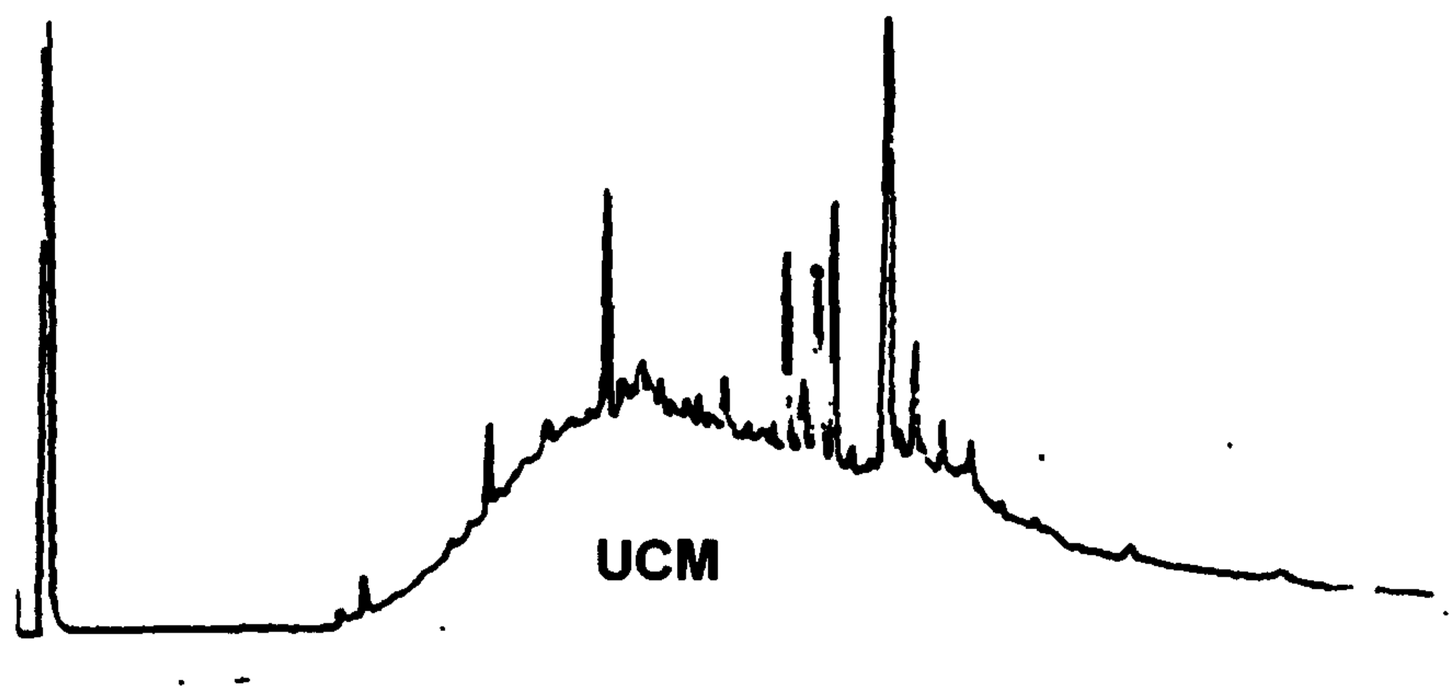


Figure 1.8 Relationship between the concentration of 'total toxic hydrocarbons' (Widdows *et al.*, 1995) and 'aromatic UCM hydrocarbons' (Wraige, 1997; Smith, present study) in *Mytilus edulis* from U.K. east coast sites with reduced 'Scope for Growth'

The re-examination of mussels collected from Whitby in 1990 by the more specific GCMS method herein has thus demonstrated that high concentrations of aromatic UCMs are present (Figure 1.7, this study; Wraige 1997). A review of the literature indicated that at least one other study had noted the relationship between concentrations of aromatic hydrocarbons detected by HPLC and GC. Murray *et al.* (1983) demonstrated a significant relationship between HPLC and GC indicators of the concentrations of aromatic hydrocarbons in mussel tissue ($r = 0.99$, $n = 11$) where gas chromatograms were also dominated by unresolved compounds (Figure 1.9). They suggested that the combined effects of factors such as selective accumulative weathering and biodegradation resulted in an aromatic hydrocarbon fraction in mussels which is a 'complex mixture of polyalkyl and cycloalkyl substituted rings'.

a)



b)

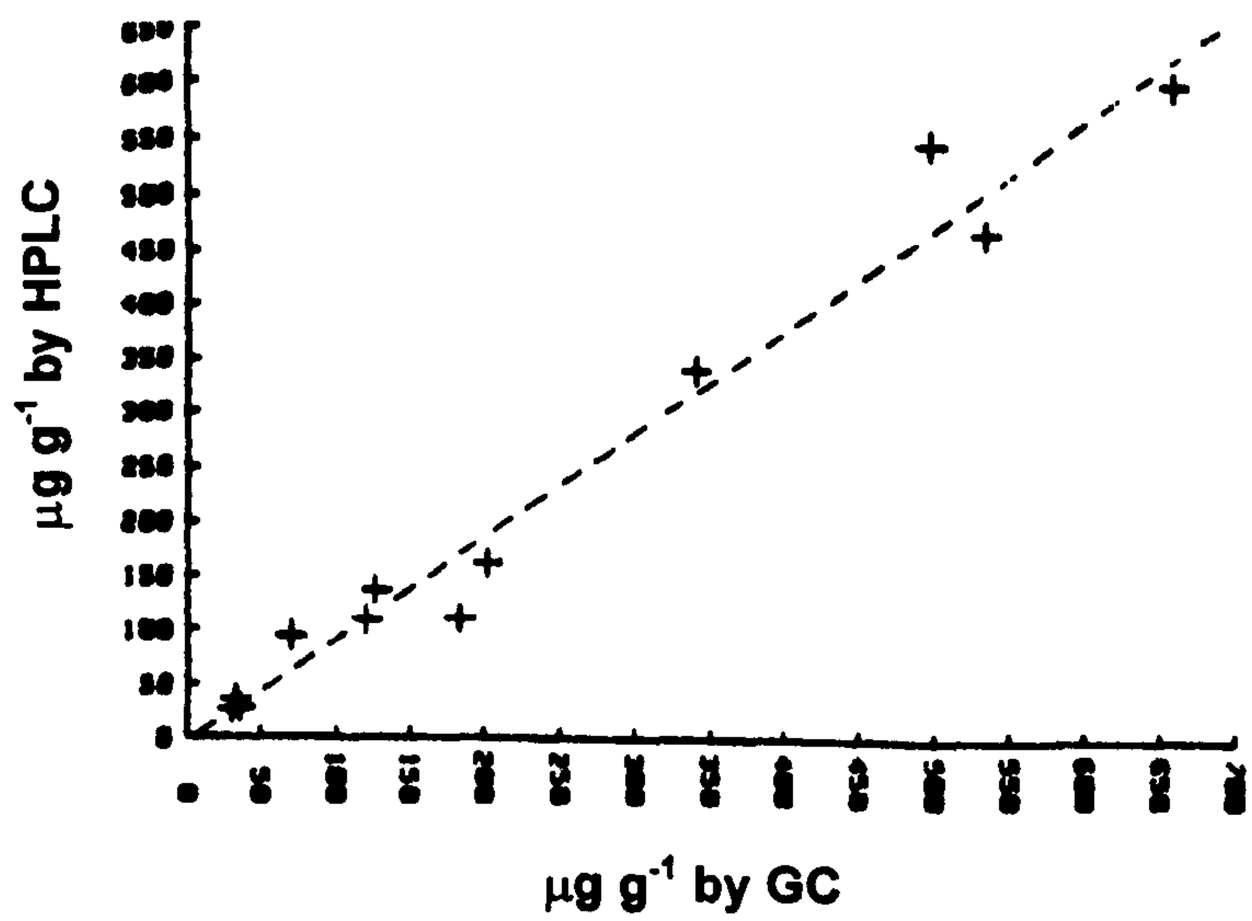


Figure 1.9 a) Gas chromatogram of the 'aromatic hydrocarbon' fraction separated from contaminated mussels by open column chromatography. b) The relationship between HPLC and GC indicators of the concentrations of aromatic hydrocarbons in mussel tissues. Values are presented as µg g^{-1} dry weight. The correlation is significant ($p < 0.001$, $r = 0.99$) and the best line of fit has a slope of 0.93 and intercept of 1.2 µg g^{-1} (Murray *et al.*, 1983)

Another two reports provide further circumstantial evidence that UCMs accumulated in marine organisms may be responsible for the observed impaired health effects (Gilfillan *et al.*, 1977; Gold-Bouchet *et al.*, 1995). Gilfillan *et al.* (1977) measured the long-term effects of an oil spill on populations of the clam *Mya arenaria* from different sites in Casco Bay, Maine. Two years after the spill there was no correlation between toxic response (measured in terms of carbon flux, which is effectively Scope for Growth) and the total body burden of hydrocarbons (dominated by an aliphatic UCM). It was therefore suggested that when dealing with low concentrations of a highly weathered oil the total hydrocarbon content, of either sediments or animal tissues, is not an accurate measure of the stress imparted by the oil. However, the reduction in carbon flux was found to correlate with elevated body burdens of aromatic hydrocarbons. Gilfillan *et al.* (1977) assumed that the activity influencing the carbon flux was contained in the aromatic lower molecular weight portion of the unresolved envelope in the gas chromatogram, and used the ratio of the maximum height of the low molecular weight side of the unresolved envelope to the high molecular weight residual hydrocarbons to indicate the amount of lower molecular weight aromatics present. This peak height ratio correlated directly with the carbon flux data.

Gold-Bouchet *et al.* (1995) correlated UCM hydrocarbon concentrations with adverse effects in wild populations of oysters from Tabasco, Mexico. They suggested that histopathological lesions exhibited in oysters were related to the concentration of hydrocarbon UCMs within the organisms. Concentrations of UCM were linearly correlated with damage in the digestive diverticulum ($r = 0.71$) and damage to the gills ($r = 0.60$). At a concentration of 70 $\mu g\ g^{-1}$ UCM (not stated whether dry or wet weight) 28 % of the population showed damage to the gills, and 45 % of the population exhibited damage in the digestive diverticulum. The authors recognised the difficulty in assigning toxic effects to the UCM due to its complex nature, but no details of the UCM measurement methods were given.

1.5 Responses of marine organisms to petroleum hydrocarbons

The effects of petroleum hydrocarbons on aquatic animals have been reviewed by Connell & Miller (1981), and more specifically in mussels by Bayne *et al.* (1982). Oil entering the marine environment may affect organisms in a number of ways including; direct lethal toxicity, changes in physiological/behavioural activity, effects of direct coating, incorporation of hydrocarbons into organism tissues, and alteration of habitat (Moore & Dwyer, 1974). Marine animals exhibit variations in their sensitivities to oil. For adult stages of a variety of species lethal effects from water-soluble fractions occur in the 1-100 ppm (mg L^{-1}) range, whereas larval and juvenile life stages are usually more sensitive, with lethal concentrations often in the 0.1-1 ppm range (GESAMP, 1993). The lower boiling point, more soluble, aromatic fractions have been consistently implicated as the primary cause of mortality (Moore & Dwyer, 1974).

Biota may also exhibit sub-lethal responses to chronic hydrocarbon contamination such as decreased productivity *via* reduced fecundity, delayed sexual maturity, small mean brood sizes and abnormal embryo development (Neff, 1976). Boylan & Tripp (1971) suggested that changes in the behaviour of marine organisms, such as disruption in normal feeding sequences, sex attraction, host recognition, symbiotic relations, and other intrinsic social interactions, may be caused by the presence of sub-lethal concentrations of soluble oil fractions. Sub-lethal effects of petroleum hydrocarbons are thought to occur in the range of 1-100 ppb (μg L^{-1}) (Moore & Dwyer, 1974). It is believed that low concentrations of hydrocarbons that cause disruption and interfere with physiological and behavioural processes, are also likely to damage reproductive potential and the ability to colonise habitats, increase susceptibility to predation and parasitism, and lead to changes in the populations of individual species, resulting in shifts in species composition and diversity (GESAMP, 1980). Effects, such as suppression of growth, may be expected to impair

ecological efficiency, reduce competitive ability and in some cases increase susceptibility to predation.

Following a spill of crude or refined oil, several physical, chemical and biological processes, collectively called 'weathering', interact to change the physical and chemical properties of the oil, and thereby influence its effect on marine ecosystems. The most important 'weathering' processes include; spreading, evaporation, dissolution, dispersion into the water column, formation of water in oil emulsions, photochemical oxidation, microbial degradation, adsorption to suspended particulate matter and sedimentation to the sea floor (Neff *et al.*, 2000). One effect of these weathering processes is to increase the abundance of the unresolved components within hydrocarbon fractions as the resolved components are preferentially degraded.

The role of weathering in determining petroleum toxicity is not well understood. According to Bobra (1983) there is no simple answer to the question of whether or not weathering reduces the toxicity of oil, as it depends on the definition of toxicity. Weathering results in aqueous solutions which give lower LC_{50} values (the aqueous concentration required to produce a 50% lethality in the test organism), suggesting they are more toxic than solutions of fresh oils. However, weathering of oil also markedly reduces its aqueous solubility, and thus fresh oils can generate aqueous solutions which are more toxic although at higher concentrations.

Soto (1974 and references therein) found, in agreement with observations by other researchers, that extracts from aged or weathered crude oil did not show significant biological effects. However, more recent studies have shown that weathered oil can elicit biological responses (Heintz *et al.*, 1999; Barron *et al.*, 1999). Heintz *et al.* (1999) found that highly weathered oil from the *Exxon Valdez* was more toxic to pink salmon embryos than the unweathered oil. The authors attributed the enhancement in toxicity to elevated concentrations of larger PAHs in the weathered oil compared to the fresh oil. However

when Barron *et al.* (1999) examined the water-accommodated PAH content of three naturally weathered Californian crude oils by SIM GCMS, they unexpectedly found that toxicity to the mysid shrimp (*Mysidopsis bahia)* did not correlate with PAH content. The published total ion current GCMS chromatograms of the oils show that the fractions tested in their study were in fact dominated by UCMs.

In studying the effects of the toxicity of three offshore Australian crude oils and a diesel fuel to marine animals, Neff *et al.* (2000) found that the water-accommodated fractions (WAFs) of a middle weight crude oil and diesel fuels were higher than predicted on the basis of their concentrations of total MAHs (monoaromatic hydrocarbons), PAHs (polyaromatic hydrocarbons), and phenols. This indicated that 'other' components of the WAF contributed to the toxicity, and it was suggested that these components may include the UCM and polar compounds, but no evidence was provided.

Work by Thomas *et al.* (1995) and Payne *et al.* (1995) suggests that the aliphatic UCM is non-toxic. Payne *et al.* (1995) found that the aliphatic component of the complex mixture detected in sediment contaminated with an aliphatic oil-based drilling mud was relatively non-toxic to flounder. The 'aliphatic' UCM in the total ion chromatogram of the muscle extract from the flounder had a Kovats index GC range similar to the cuttings obtained from exploratory wells off Canada and the Arctic using aliphatic hydrocarbon based drilling fluids, suggesting the non-toxic nature of the accumulated UCM. Thomas *et al.* (1995) tested the toxicity of a saturated aliphatic UCM isolated from the hydrocarbon feedstock of Silkolene 150 lubricating oil (1 mg L^{-1}) by measuring mussel feeding rate. The relatively non-toxic nature of the saturated aliphatic UCM compounds was attributed to their low aqueous solubility.

A survey of the literature found no comparable reports on the toxicity of aromatic unresolved complex mixtures. However, Wraige (1997) studied the impact of two synthesised aromatic hydrocarbons considered to represent 'average' structures within an

aromatic UCM. These were found to reduce mussel feeding rate considerably (> 50% compared to controls) during a 24 hour exposure period.

The limited data available thus suggest that aliphatic UCM hydrocarbons may not be toxicologically significant or contribute in only a minor way to the toxic hydrocarbon burden in marine ecosystems. However, the known toxicity of many resolved aromatic hydrocarbons (Donkin *et al.*, 1989; Pelletier *et al.*, 1997), including two model aromatic UCM hydrocarbons suggests that the corresponding aromatic UCM may be more toxic than its aliphatic counterpart and therefore should be studied.

Marine molluscs exhibit various sub-lethal responses to petroleum hydrocarbons including decreased feeding rate and absorption efficiency, and increased respiration (Widdows *et al.*, 1987; Bayne, 1982; Donkin, 1992) which have been used in field monitoring programmes as part of the Scope for Growth measurement (Section 1.4). A reduction in mussel feeding rate is caused by reduced pumping of the lateral cilia of the gill (Axaik & George, 1987). The cilia are under neuronal control (Paparo, 1972), therefore the mechanism of feeding rate reduction is consistent with a non-specific mode of narcotic action (Donkin *et al.*, 1989).

Non-specific narcosis is considered to be a minimum or 'baseline' effect in organisms as many reactive chemicals are more toxic than predicted from non-specific Quantitative Structure Activity Relationships (QSARs) owing to additional modes of toxic action. A review of the classification of modes of toxic action of organic chemicals is given by Verhaar *et al.* (1992).

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 & Opperhuizen, 1995). The exact mechanism(s) of narcosis and the site(s) of toxic action

remain unknown but it appears that the effect occurs when a target site has accumulated a sufficient volume of a chemical to interfere with normal structure and function. Therefore, it is argued that all compounds have the potential for 'baseline' non-specific narcotic activity.

For many groups of compounds, toxicity, expressed in terms of aqueous exposure required to produce an effect, increases through a homologous series until a molecular weight is reached at which the rate of toxicity declines. 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 toxic action to induce a response (Veith *et al.*, 1984; Donkin *et al.*, 1989). For example, the solubility of alkanes decreases until minimum solubility is reached at about C_{10} (decane), at which point a sharp discontinuity in solution occurs. The longer chain alkanes no longer exist in true solution (molecular dispersion) but are present as aggregates in suspension. According to Seeman (1972) these aggregates may be extremely large, since they may be filtered out by 0.45 micron-pored filter, and therefore may not be absorbed into the aqueous compartments of the body. This would lead them to be treated as foreign bodies by the cell membranes which would phagocytose (engulf and ingest the foreign bodies with cells such as white blood cells) or pinocytose (introduce the fluids into a cell by invagination of the cell membrane, followed by formation of vesicles within the cells) the aggregates.

This theory explains the observation that mussels have been shown to accumulate compounds which elicit no toxic response. For example, researchers have detected large quantities of aliphatic hydrocarbons ranging from C_{12}-C_{30} alkanes, and large quantities of aliphatic hydrocarbons in marine mussels. Field studies linking biological effects to contaminant concentration have shown limited correlation between accumulated aliphatic

compounds and the observed impaired health effects. Laboratory studies investigating the effect of aliphatic and aromatic hydrocarbons (Donkin *et al.*, 1989, 1991) demonstrated that the total body concentration required to reduce mussel feeding rate by 50 % was much greater for four ring aromatic hydrocarbons than for mono or diaromatic compounds. This meant that despite efficient accumulation of all the compounds tested a cut-off effect was observed. These authors suggested that this effect was either indicative of a molecular size related cut-off at the site of toxic action or by sequestration of the higher molecular weight compounds within the mussel tissue in a toxicologically unavailable form, such as crystals or lysosomes. Therefore, the theory that only compounds which are present in the bioavailable dissolved phase may interact with the target site is plausible. It was suggested by Shaw (1979) that aquatic organisms take up and store different chemical and size fractions in different ways and that the differences in chemical make-up as a function of droplet size will be reflected in bioavailability. It was proposed that there would be enhancement in the dispersed phase of the more soluble components, meaning that particulate oil that contains alkanes could be taken up as food by filter feeders and small grazers whereas sub-particulates rich in aromatics could enter the circulatory system through the gills. Baumard (1999) also observed different accumulation behaviour of PAHs from sediments by mussels. It was concluded that the more water soluble compounds were preferentially accumulated in the water and entered though the gills in a dissolved form whereas the heavier molecular weight compounds were preferentially absorbed from filtered particles where they passed through the digestive system. According to van Praet (1980) the similarity between the chemical composition of hydrocarbons observed in sediments and related organisms reveals nonselective contamination of invertebrates caused by direct pinocytosis of insoluble hydrocarbons and phagocytosis by polluted feeding. This may also explain why aliphatic UCMs are present in high levels in

mussel tissue but cannot be correlated to toxic effects observed in the field. However how this relates to aromatic UCMs is unknown.

1.6 The bioavailability of UCM hydrocarbons to organisms in the marine environment

Routes of exposure of contaminants to organisms can include whole sediment, overlying water and interstitial water (assuming no feeding), which can further be subdivided into particulate, colloidal and dissolved aquatic phases (Burgess *et al.*, 1999). In the environment it is well established that nonpolar organic contaminants readily associate with particle and colloidal phases, and to a much lesser extent the truly dissolved phase. Despite the majority of these contaminants being associated with the particulate and colloidal phases, the dissolved phase is considered the primary bioavailable phase to aquatic organisms (Burgess *et al.*, 1999)

Hydrocarbons can exist in seawater in a variety of forms: truly dissolved, solubilised in micellar structure (accommodated), hydrocarbon droplets of colloidal dimensions-(accommodated), adsorbed to detrital material, adsorbed onto or incorporated within living particles, and particulate hydrocarbon residues as tar ball flakes. Over the world's oceans the concentrations of hydrocarbons in surface and near surface waters range from 1-100 μg L^{-1}. In open seas, without significant oil contamination, 1 μg L^{-1} is considered typical, whilst in affected areas, such as tanker routes, concentrations up to 500 μg L^{-1} have been reported (Boehm *et al.*, 1980).

Upon analysis of dissolved filterable hydrocarbons (dissolved material, particles of colloids less than 1 μm, and solubilised material present in colloidal micellar structures) found in environmental samples Boehm *et al.* (1980) detected levels in the μg L^{-1} range as

expected. However, further examination of the gas chromatograms of particulate and dissolved hydrocarbons revealed a dissimilar pattern for the aliphatic and aromatic hydrocarbons. An unresolved complex mixture was present in the particulate component of the aliphatic fraction but not the aromatic fraction, which showed a UCM in the dissolved fraction. This suggests that aromatic unresolved components are more soluble than their aliphatic counterparts, which is consistent with observations for resolved aliphatic and aromatic compounds, and indicates that the aromatic unresolved complex mixture is more bioavailable than the aliphatic UCM. This supports work carried out by Soler *et al.* (1989) who examined the concentrations of aliphatic and aromatic UCMs in mussels at offshore oil platforms in the North Sea. They found a consistent increase in the concentrations of unresolved hydrocarbons, both aliphatic and aromatic, in mussels living closer to the leg surface. In the most recently established mussels there was a significant decrease in the concentration of unresolved aliphatic hydrocarbons with water depth as compared to a more even distribution of unresolved aromatic hydrocarbons. The explanation offered by the authors was that on introduction to the sea surface the aromatic compounds, being more soluble, could be rapidly accommodated into the water column.

There are no reports of levels of aromatic UCMs in seawater although there are a few reports of aliphatic UCMs in water samples (Table 1.4). As expected most of the aliphatic UCM is associated with particulate matter rather than being dissolved, although Bayona *et al.* (1991) reported concentrations of 38 $\mu g\ L^{-1}$ in surficial waters and 68 $\mu g\ L^{-1}$ in wastewaters. As aromatic hydrocarbons are more soluble than aliphatic hydrocarbons of comparable molecular weight one would expect the concentrations of dissolved aromatic UCM hydrocarbons to be higher than those reported for aliphatic UCMs.

Although no data were found to substantiate this a few reports have noted the presence of a UCM in the aromatic fraction of aqueous samples. For example, Boylan & Tripp (1971) noted the presence of a high boiling point envelope (UCM) in the aqueous extract of

Kuwait crude oil and suggested that when concentrated and retained by marine mammals this may present a health hazard. Boehm *et al.* (1982) also observed an unresolved complex mixture in water samples taken from the area around the Ixtoc blowout. A gas chromatogram of the water soluble fraction of Ecofisk crude oil studied by Sydnes *et al.* (1985) exhibited a UCM, along with those of the water accommodated fraction of three weathered middle distillate oils exposed to mysids by Barron *et al.* (1999).

Table 1.4 Reported concentrations of aliphatic UCMs in environmental water samples

Dissolved UCM concentration ($\mu g\ L^{-1}$)	Particulate UCM concentration ($\mu g\ L^{-1}$)	Water sample	Region	Reference
63 38 -	2145 1222 13-49	wastewater surficial seawater bottom seawater	Barcelona coast, Mediterranean	Bayona *et al.* (1991)
0.221-0.932 -	- 26-3299*	subsurface surficial spm	Northwestern Black Sea	Maldonado *et al.* (1999)
	30-1100•	0-200m depth	Puget Sound Estuary	Bates *et al.* (1987)
0.01-0.65		surficial seawater	Water marshes of Iraq	Al-Saad *et al.* (1993)

* $\mu g\ g^{-1}$ particulate organic carbon
• $\mu g\ g^{-1}$ dry weight

Theoretically, within a homologous series of hydrocarbons, toxicity is greatest for those compounds with the highest molecular weight (Bobra *et al.*, 1983). However the lower molecular weight components 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). Oudot (1981) suggested that with respect to unresolved complex mixtures their chemical and biological inertness, in conjunction with their low solubility, would limit any potential toxicity, but recognised further investigation was necessary in determining the impact of degraded petroleum.

Aromatic hydrocarbons typically have greater aqueous solubilities than aliphatic hydrocarbons of comparable molecular weight (McAuliffe, 1966; Verscheuren, 1983). Given the solubility and associated toxicity of a number of resolved low molecular weight aromatic hydrocarbons (*e.g.* Donkin *et al*, 1989, 1991) it seems likely that a proportion of the lower molecular weight aromatic UCM would have sufficient aqueous solubility to be considered as non-specific narcotic toxicants. Therefore, it is anticipated that the aromatic UCM may be of greater toxicological significance than the less soluble aliphatic UCM hydrocarbons. The circumstantial evidence provided herein supports this contention but to date this has not been investigated.

Whilst sediment bound UCMs have generally been considered non-bioavailable and fairly inert, uptake by organisms may make a significant contribution to contaminant body burden contributing to narcosis based toxicity attributed to hydrophobic organic compounds. Based on this possibility Le Blanc *et al.* (1999) initiated studies on the chemical and biological bioavailability of UCM compounds. Upon sixty day incubation of New York harbour sediment with XAD resins (used to estimate the desorbable fraction) they found a 5-30% desorption of sediment bound UCM, with the desorbed fraction containing a greater degree of lower molecular weight more soluble hydrocarbons than the sediments. However no details of whether this UCM was aliphatic or aromatic were given.

1.7 Aims of the present study

Aromatic hydrocarbon UCMs are widespread in the marine environment and, when appropriate methods of analysis are used, are shown to dominate the aromatic fractions of marine sediments and biota, constituting approximately 20% of the total hydrocarbon body burden in mussels from a number of impacted sites (Wraige, 1997). However, little attention has been paid to the possible detrimental effects caused by the accumulation of these compounds.

Chapter 1 reviews the existing field evidence for the environmental impact of aromatic unresolved complex mixtures. This evidence suggests a link between the concentrations of aromatic UCMs in mussel tissues and measured reduced Scope for Growth in mussels at petroleum-impacted sites. At present there is no information on the possible effects of accumulation of these compounds within marine organisms, though several workers have raised the possibility that such effects may exist (*e.g.* Neff *et al.*, 2000). The aim of the work described in **Chapter 2** was to isolate a monoaromatic hydrocarbon unresolved complex mixture from a crude oil and to investigate the potential toxicological effects of the UCM using a well accepted mussel feeding rate assay, as feeding rate is a sensitive parameter in the SFG measurement.

The research reviewed in Chapter 1 also shows that a few recent studies have allowed the proposition of tentative structures for some aromatic UCM hydrocarbons. Experimental work with UCMs is complicated by their inherent complex nature, therefore the use of single compounds allows a simpler means of assessing UCM behaviour. The aim of the work described in **Chapter 3** was to synthesise and purify aromatic compounds having structural characteristics similar to those tentatively assigned to the molecular composition of aromatic UCM components, to allow experimental studies to be conducted designed to investigate the behaviour of these compounds in the marine environment.

The toxicity of two 'model' aromatic UCM hydrocarbons, 6-cyclohexyltetralin and 7-cyclohexyl-1-propyltetralin, has been previously studied (Wraige, 1997) using a mussel feeding rate assay. The aim of the work in **Chapter 4** was to assess the toxicity of the compound 7-cyclohexyl-1-methyltetralin synthesised in Chapter 3, by the same method to determine the generality of this effect. A further aim was to gain an insight into the toxicological impact of monoaromatic UCMs (as studied in Chapter 2) by comparing the response of mussels to these 'models'.

Aqueous solubility is an important physicochemical parameter that affects the behaviour of xenobiotics in the marine environment. So-called toxicity 'cut-offs' based on aqueous solubility are also well recognised. Therefore, the aim of the work described in **Chapter 5** was to determine the aqueous solubility of the three model aromatic hydrocarbons, 6-cyclohexyltetralin, 7-cyclohexyl-1-methyltetralin and 7-cyclohexyl-1-propyltetralin. The aqueous solubilities of these compounds were assessed under a variety of conditions (salinity and temperature) to allow an insight into the possible behaviour of these compounds in the marine environment, and to assess their compliance with solubility/toxicity cut-off theory. A further aim was to attempt to generate an aqueous solution of a real monoaromatic UCM isolated from crude oil.

Crude oil is subject to a variety of weathering processes upon entering the marine environment, including biodegradation and photodegradation. UCM hydrocarbons are considered resistant to biodegradation but the influence of photodegradation has not previously been studied. The chemical oxidation of an aliphatic UCM has been shown to increase toxicity (Thomas, 1995). Some aromatic compounds are susceptible to photodegradation in the marine environment and the products may be more water-soluble and more toxic than the parent hydrocarbons. Thus aromatic UCM hydrocarbons may possibly photodegrade in the marine environment and this process may influence their toxicity. Therefore, the aim of the work in **Chapter 6** was to assess the photodegradation

transformation behaviour of the synthesised monoaromatic compounds, 6-cyclohexyltetralin, 7-cyclohexyl-1-methyltetralin and 7-cyclohexyl-1-propyltetralin under simulated environmental conditions.

Chapter 7 presents the experimental details relevant to the previous chapters and **Chapter 8** presents the conclusions of this study as a whole, and suggests areas for future work.

Chapter 2. Isolation and toxicological assessment of a monoaromatic hydrocarbon UCM

Milligram quantities of an unresolved complex mixture of monoaromatic hydrocarbons were isolated from a biodegraded crude oil, Gullfaks (North Sea). The toxic effects of the isolated UCM were determined by a mussel feeding rate assay. The UCM reduced mussel feeding rate by ~40 % over a 24-hour exposure period compared to unexposed controls. The findings indicate that monoaromatic UCM hydrocarbons may have adverse effects in organisms in the marine environment.

2.1 Introduction

One of the most important properties of a toxic pollutant is the extent to which it is able to accumulate in the tissues of organisms. Over a long period of time pollutants present in the environment at low concentrations may accumulate within the body of aquatic species by various mechanisms. The weathering of crude petroleum typically produces oils rich in unresolved complex mixture (UCMs) of both aliphatic and aromatic hydrocarbons. The aliphatic UCM fraction is usually more abundant and is often cited as a measure of petroleum contamination following measurement by GC-FID. Whilst weathered or biodegraded crudes may contain substantial proportions of aromatic UCM hydrocarbons these are rarely measured in environmental samples due to the common practice of monitoring priority pollutants polyaromatic hydrocarbons by SIM GCMS. However aromatic UCMs have been shown to occur in a wide variety of matrices within the marine environment (Venkatesan *et al.*, 1982; Readman, 1986; Soler, 1989; Fowler, 1993, Le Dreau, 1997; Wraige, 1997; Douabul & Alshiwafi, 1998). Although unresolved complex mixtures of aromatic hydrocarbons accumulate in the marine environment, limited attention has been paid to the possible detrimental effects of these compounds.

The physiological responses of mussels, particularly feeding rate, have been used to monitor the impact of hydrocarbons in both field and laboratory studies (Donkin & Widdows, 1986; Widdows *et al.*, 1987; Donkin *et al.*, 1991). Observed adverse health effects in organisms have been found to correlate well with concentrations of aromatic hydrocarbons but poorly with total hydrocarbons data (Gilffilan *et al.*, 1977; Widdows & Donkin, 1989), the latter of which is often dominated by an aliphatic UCM. It is therefore considered that aliphatic unresolved hydrocarbons are accumulated without incurring a toxicological response. This is supported by laboratory assessments. Thomas *et al.* (1995) and Payne *et al.* (1995) found no observable effects when organisms were exposed to aliphatic UCMs. The limited data available thus suggest that aliphatic UCM hydrocarbons

may not be toxicologically significant or contribute in only a minor way to the total toxic hydrocarbon burden. However, the known toxicity of many resolved aromatic hydrocarbons as compared to resolved aliphatic compounds suggests that aromatic UCMs are potentially toxic.

Coincidentally, the use of HPLC-UV methodologies in studies that link the concentrations of accumulated aromatic hydrocarbons to a toxic response (*e.g.* Widdows *et al.*, 1995b) may have led to an underestimation of the importance of aromatic UCMs in impacted marine organisms. The limited evidence made available by the re-examination of a stored sample of mussel tissue by GCMS herein (Chapter 1), from a site shown to have reduced Scope for Growth (Widdows *et al.*, 1995), and analysis of the same population of mussels, collected 5 years after the initial study (Wraige, 1997), certainly supports the contention that some of the aromatic UCM components at least, exhibit toxic effects. These preliminary data indicated that monoaromatic UCMs may dominate the aromatic fractions of tissues of mussels with impaired health and pointed towards a correlation between reduced SFG in mussel populations and the concentration of unresolved aromatic hydrocarbons (Chapter 1). Clearly the toxicity of monoaromatic UCM hydrocarbons requires investigation.

2.2 Aims

The aims of the work carried out in this chapter were to isolate a monoaromatic UCM and to carry out a mussel feeding rate assay using the isolated UCM in order to test the hypothesis that components of the aromatic UCM may be responsible for the reduced Scope for Growth (SFG) found in mussels at petroleum-impacted sites.

2.3 Isolation of a monoaromatic UCM

A major problem in the isolation of a monoaromatic UCM from a fresh crude oil is how to effect efficient removal of the resolved components. Therefore, in the present study an in-reservoir biodegraded produced crude oil was chosen, from which many of the GC resolved components had been removed by natural bacterial action, leaving an oil with a prominent UCM. Since the UCM hydrocarbons are thought to be resistant to biodegradation, at least until most of the resolved components have been removed, the UCM of a biodegraded oil should be similar to that of a fresh crude. Killops & Al-Juboori (1990), Revill *et al.* (1992) and Warton *et al.* (1999) all used biodegraded crude oils in their compositional studies on aromatic UCMs for the same reason. The crude oil used in the current study was a Gullfaks (North Sea) crude. Petroleum from the Gullfaks field has suffered variable degrees of biodegradation in-reservoir mediated by aerobic bacteria (Horstad *et al.*,1990). The particular production oil used herein was very similar, if not identical, to that spilled by the *Braer* in January 1993, which released >84,000 tonnes of the oil into the marine environment around Shetland, U.K. The sample was a produced crude which was provided by the operators of the same terminal (Mongstad, Norway) from which the *Braer* was loaded a few days later. The gas chromatogram of the oil (Figure 2.3a) revealed a small proportion of both C_{6-10} volatile and C_{15-20} semi-volatile resolved compounds overlying an unresolved complex mixture (84 %).

The strategy adopted for the isolation procedure of the monoaromatic UCM was similar to that used by Killops & Readman (1985) and Wraige (1997) for environmental samples. The whole procedure is illustrated in Figure 2.1. Each stage was repeated several times in order to purify a suitable quantity of the monoaromatic UCM for the toxicological assay. Full experimental details are given in Chapter 7. Briefly, Gullfaks (North Sea) produced crude oil was placed in a 50ml round bottomed flask and rotary evaporated (50°C, 10 min).

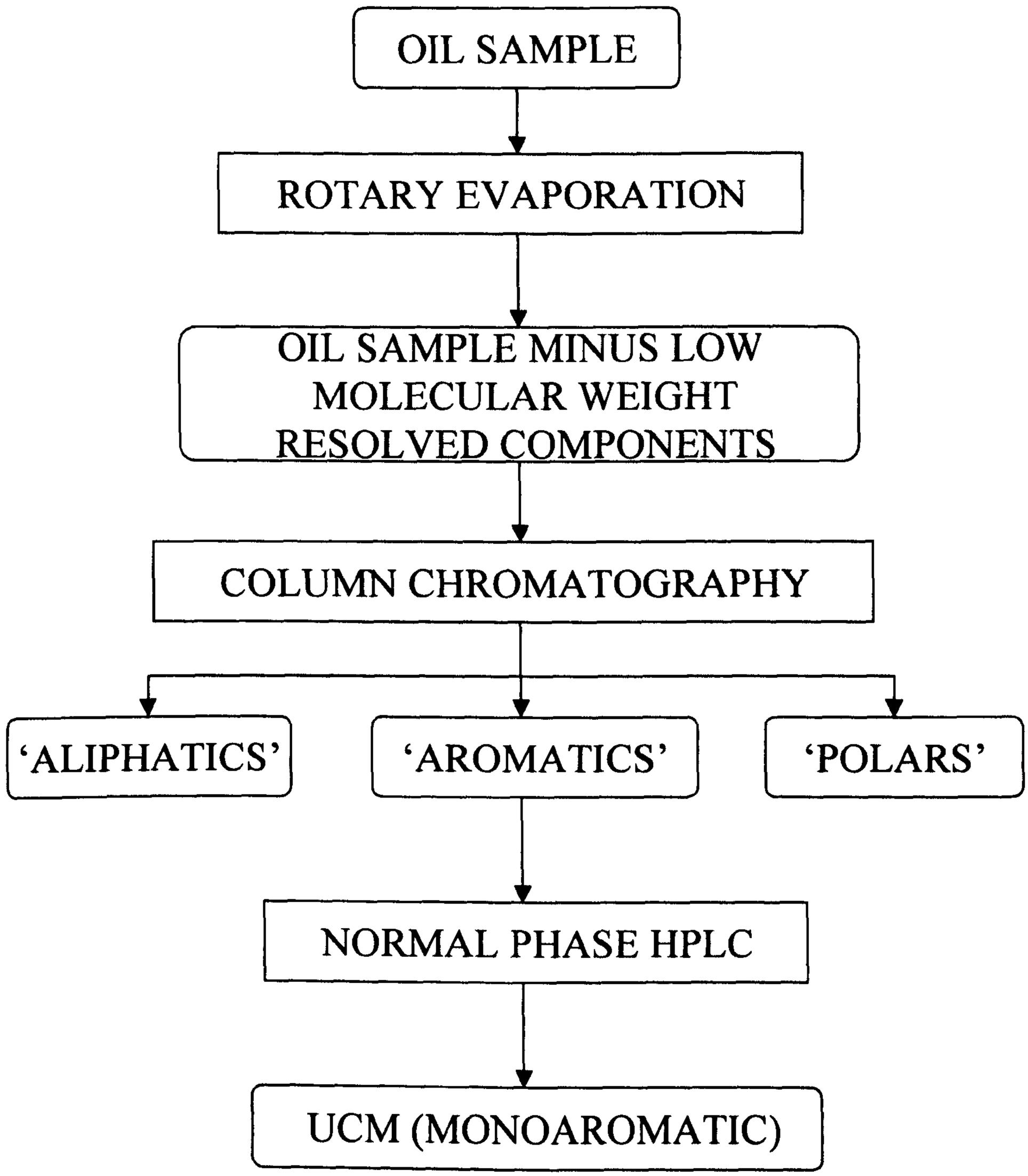


Figure 2.1 Isolation of monoaromatic hydrocarbon unresolved complex mixture

Removal of resolved volatile hydrocarbons was monitored by GCMS. The results of this operation on oil composition are shown in Figure 2.3b. Samples of rotary evaporated oil were fractionated into 'aliphatic' (Figure 2.3c) and 'aromatic' (Figure 2.3d) hydrocarbon fractions using open column chromatography. A high sample:adsorbent ratio (1:200) was employed in conjunction with fully activated silica to achieve good separation of aliphatic and monoaromatic compounds. The method adopted was the same as used by Wraige (1997) in the analysis of UCMs from mussel tissue from petroleum impacted sites as it was designed to effect a good separation between aliphatic and monoaromatic compounds. Typical gas chromatograms of the aliphatic and aromatic hydrocarbon fractions after separation by open column chromatography are illustrated in Figure 2.3.

Preparative normal phase HPLC separation of the 'aromatic' fraction was carried out using three Hypersil 8 μm Hyperprep HSAPS columns fitted with a guard column, and monitored by a UV detector operating at 254nm. The mobile phase/solvent gradient was 100% hexane from 0-40 minutes, changing to 100% DCM from 40-41 minutes, and remaining at 100% DCM from 41-60 minutes. The mobile phase took approximately 20 minutes to pass through the system, hence the chromatogram in Figure 2.2. shows a time from 0-80 minutes. Consecutive fractions were collected beginning at UV absorption minima (as shown in Figure 2.2.), in a manner similar to that of Killops & Readman (1985).

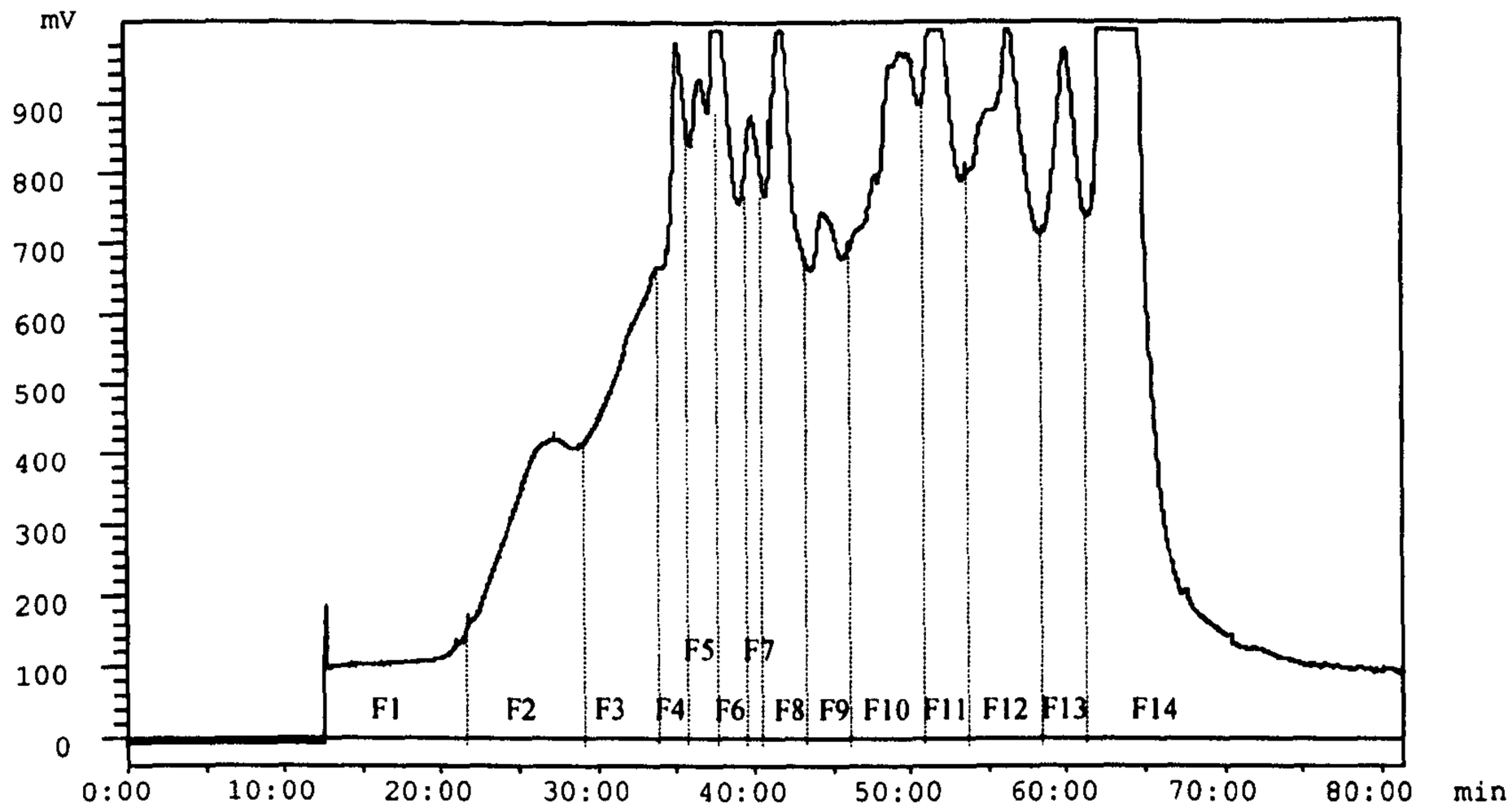
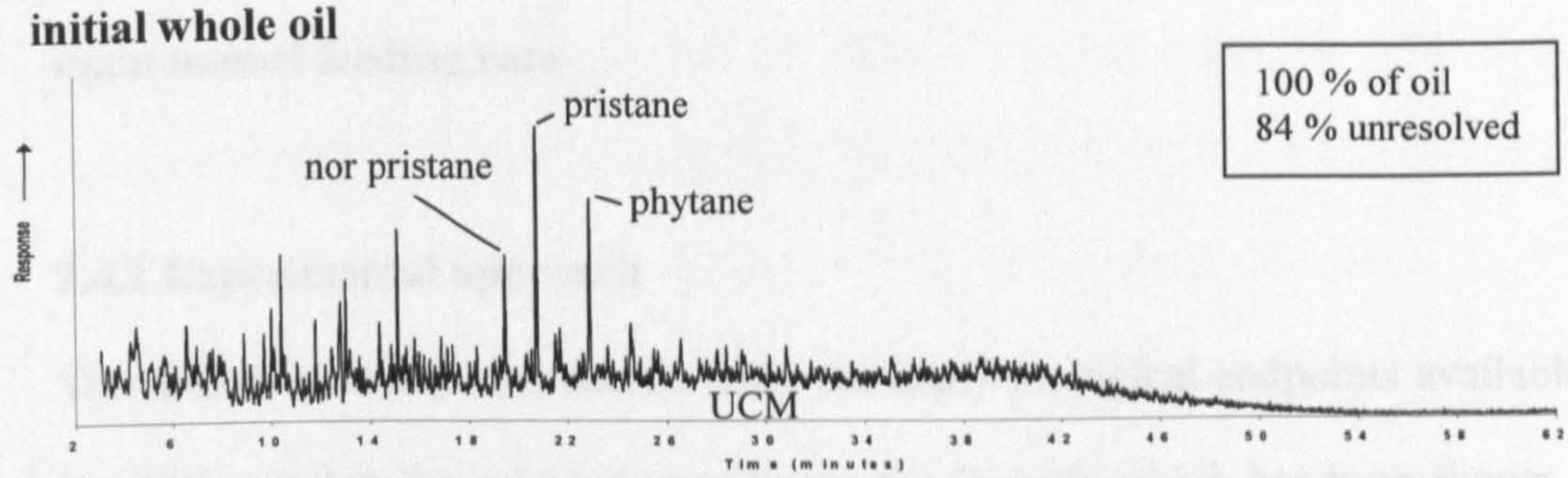


Figure 2.2 Normal-phase HPLC chromatogram of aromatic hydrocarbons of Gullfaks produced crude illustrating fractionation

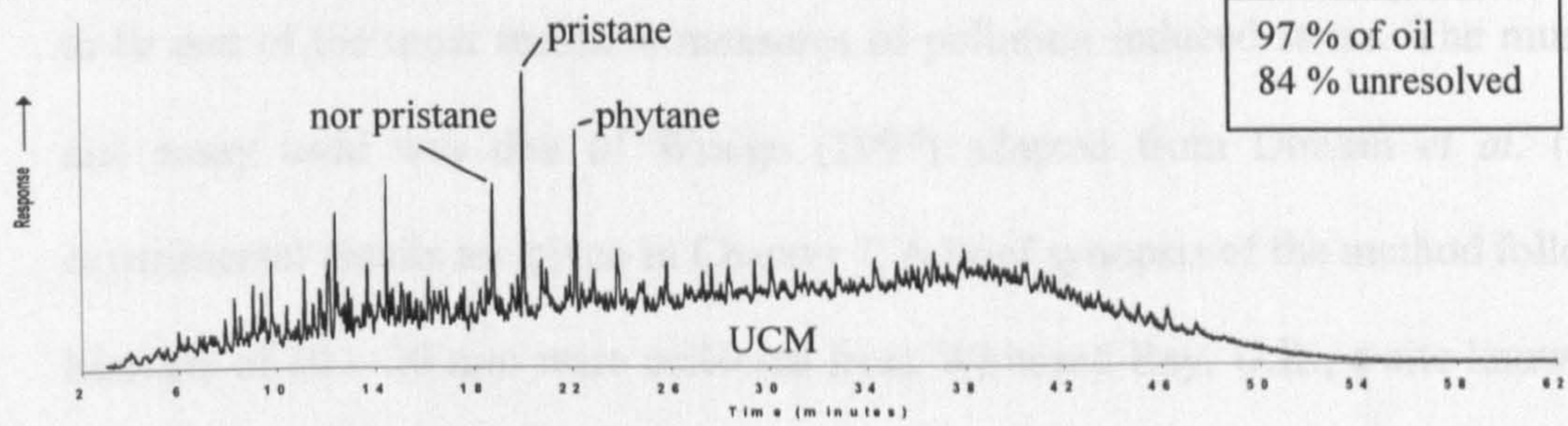
(254 nm; F1 – F14 refer to collected fractions)

The collected fractions were examined by GCMS. Preliminary investigations found that typically 15 mg could be loaded onto the system to yield a monoaromatic fraction (F2, 22-29 min; Figure 2.2) consisting almost entirely of a UCM (> 95 %) and limited resolved compounds (< 5%) (Figure 2.3e). This fraction constituted around 10 % (n = 10) of the whole oil and was found to comprise monoaromatic constituents as evidenced by the presence (GCMS fragmentography) of monoaromatic steroids (*m/z* 253) and alkylbenzenes (*m/z* 91), as observed previously by Killops & Readman (1985). Later eluting fractions contained di- and tri-aromatics such as alkylnaphthalenes (*m/z* 128, 142, 156, 170), alkylphenanthrenes (*m/z* 192) and triaromatic steroids (*m/z* 231) as monitored by GCMS.

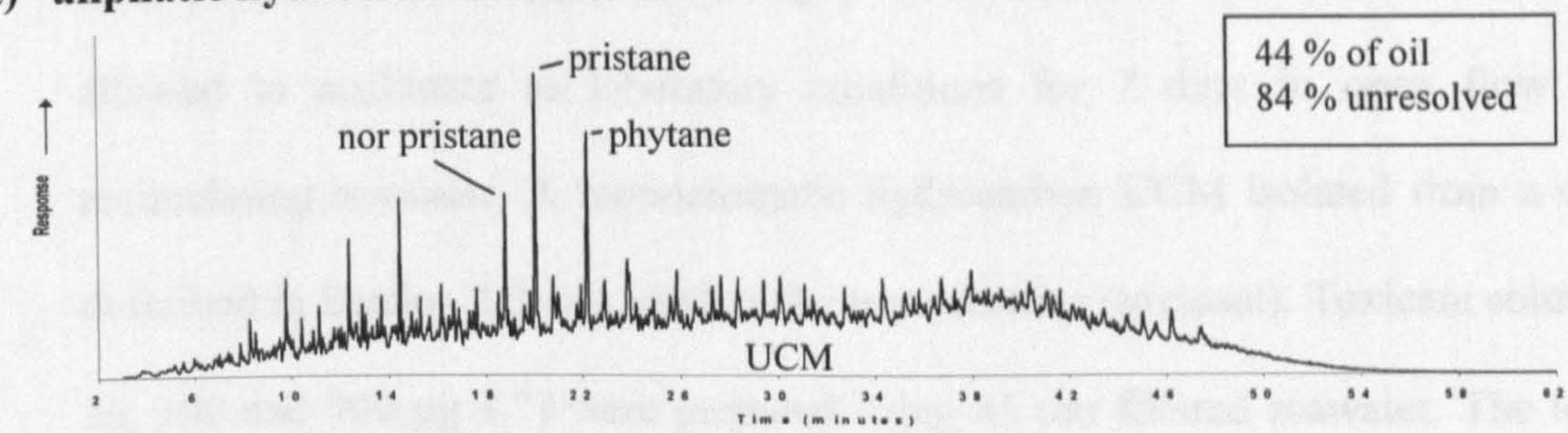
a) initial whole oil



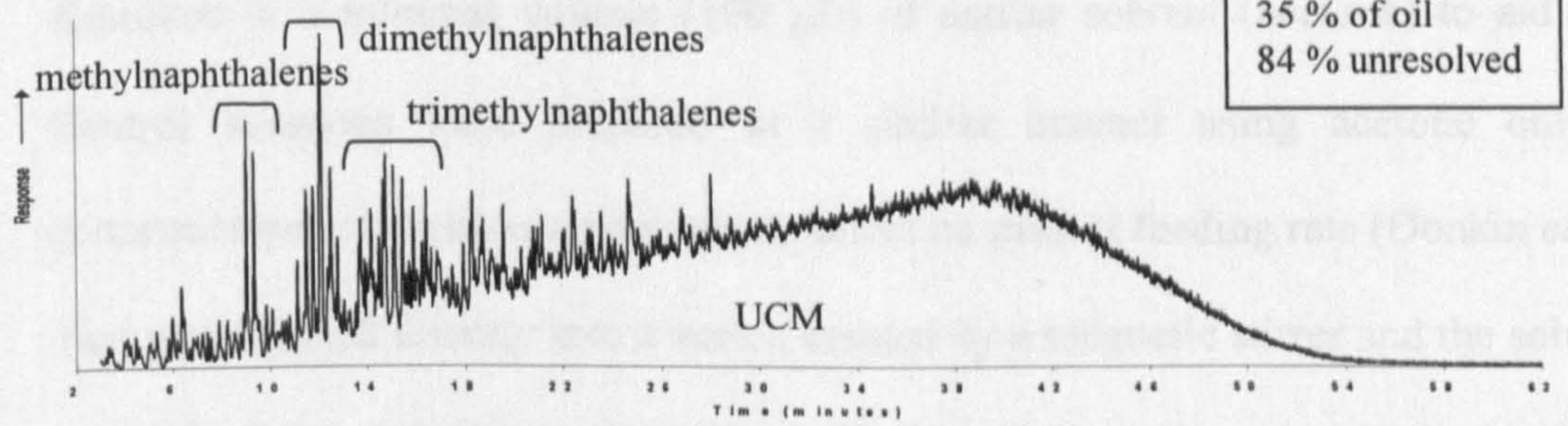
b) rotary evaporated oil



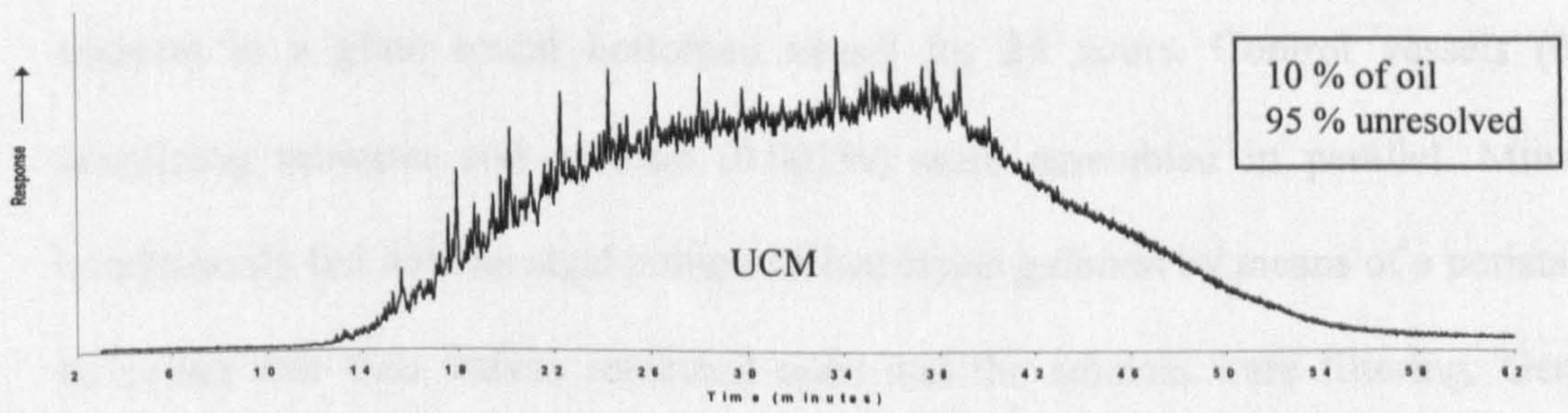
c) aliphatic hydrocarbon fraction



d) aromatic hydrocarbon fraction



e) unresolved complex mixture (monoaromatic hydrocarbons)



GCMS details:HP1 (12m x 0.2mm i.d.) column; He carrier gas; 40℃-300℃ @ 5℃ min^{-1} hold 10 mins

Figure 2.3. Gas chromatograms of products from each stage of the monoaromatic UCM isolation procedure

2.4 Concentration-response studies of the effect of an isolated monoaromatic UCM upon mussel feeding rate

2.4.1 Experimental approach

The mussel bioassay was chosen from the many biological endpoints available because it is widely used in the calculation of Scope for Growth, which has been shown consistently to be one of the most sensitive measures of pollution induced stress. The mussel feeding rate assay used was that of Wraige (1997) adapted from Donkin *et al.* (1989). Full experimental details are given in Chapter 7. A brief synopsis of the method follows.

Mussels of 10 – 20 mm were collected from Whitsand Bay, U.K., a site known to be free from petroleum contamination (Wraige, 1997). Mussels were cleaned of epibionts and allowed to acclimate to laboratory conditions for 7 days in open flow tanks with recirculating seawater. A monoaromatic hydrocarbon UCM isolated from a crude oil as described in Section 2.3 was used as the test substrate (toxicant). Toxicant solutions (0, 25, 50, 100 and 200 $\mu g\ L^{-1}$) were prepared using 45 μm filtered seawater. The toxicant was dissolved in a minimal volume (100 μL) of carrier solvent (acetone) to aid dispersion. Control solutions were prepared in a similar manner using acetone only. At this concentration acetone has no detectable effect on mussel feeding rate (Donkin *et al.*, 1989). This was injected directly into a vortex created by a magnetic stirrer and the solutions were stirred for 2 hours prior to use. Groups of 7 mussels (12 – 14 mm) were exposed to 1.4 L of toxicant in a glass round bottomed vessel for 24 hours. Control vessels (0 $\mu g\ L^{-1}$) containing seawater and acetone (0.001%) were assembled in parallel. Mussels were continuously fed with an algal culture of *Isochrysis galbana* by means of a peristaltic pump to ensure that their valves remained open and the animals were filtering. Gentle water movement was provided by a Teflon magnetic stirrer bar. Duplicate tests were performed at each exposure concentration.

Feeding rates were calculated from the rate at which algal cells were cleared from suspension in the static test system. After exposure, mussels were transferred into individual glass beakers each containing 200 mL of toxicant. A control beaker was also set up containing no mussels. To each vessel a predetermined volume of algal culture was added to give a concentration of 24 – 30000 cells mL^{-1}, and the water gently stirred to ensure an even distribution of algae within the beaker. Mussels were allowed a 30 minute acclimatisation period to open their shells and to resume feeding. After 5 minutes (time 0), a 20 mL aliquot was removed and cell numbers were counted in triplicate, using a model D Coulter counter set to measure particles greater than 3 μm. After a further 15 minutes, another 20 mL aliquot was removed and cell numbers were counted in the same manner. Feeding rates were calculated using the decrease in cell numbers over the 15 minute period using:

$$\textit{Feeding rate}\ (\mathrm{L\ h^{-1}}) = (v \times 60 / t)(\ln M_1 - \ln M_2)$$

Where v = volume of water in beaker (L)

t = time period of measurement (h)

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

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

Mussels were dissected and stored in solvent-rinsed jars at –17 °C pending analysis. Tissue was extracted by alkaline digestion (NaOH), followed by extraction into hexane (x 3). Extracts were concentrated and then analysed by GCMS. The amount of the aromatic UCM accumulated by the animals, and observable in the gas chromatograms, was quantified using an external calibration graph. The monoaromatic UCM isolated from Gullfaks oil was analysed by GCMS over a range of concentrations in triplicate to give a calibration graph. Quantification was performed using Chemstation™ (Hewlett Packard) software. The total area of the chromatogram was calculated using AREASUM parameters. Resolved components were integrated manually as no one set of parameters

adequately quantified the peaks present, and the unresolved component was then calculated from total area in the chromatogram minus the resolved area. The unresolved component present in extracted mussel tissue was calculated from the calibration graph from the calibrated weight of the isolated monoaromatic UCM (Figure 2.4.).

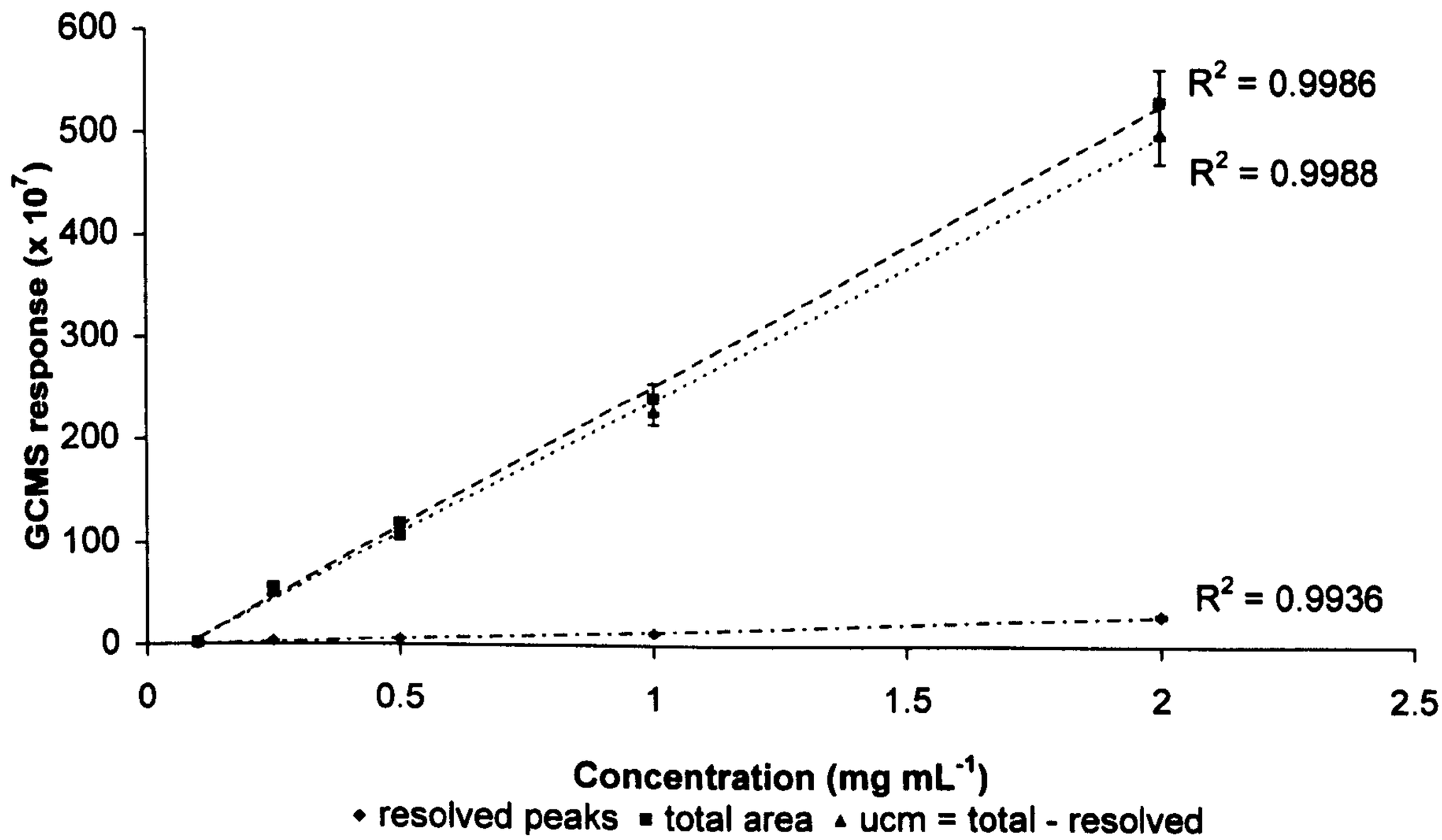


Figure 2.4 External calibration graph used to quantify the monoaromatic UCM hydrocarbons accumulated by mussels exposed to toxicant solutions.

2.5 Results

The isolated fraction (Figure 2.3e) from the crude oil sample was monoaromatic in nature. Extracted ion chromatograms performed on the HPLC fractions showed that Fraction 2 contained no methylbiphenyls or methylnaphthalenes, but monoaromatic compounds such

as a series of monoaromatic steroids (Mackenzie *et al.*, 1982) monitored by ion fragmentography (*m/z* 253). This is consistent with the results of Killops and Readman (1985) who found that the majority of the UCM of a biodegraded Alaskan oil was monoaromatic, and Wraige (1997), who determined that the majority of the aromatic UCM in U.K. mussels was monoaromatic (~80 %) by similar procedures. The aromatic fraction of Gullfaks oil constituted on average 35 % (n = 10) of the total oil sample. The isolated monoaromatic UCM accounted for 30% (n = 10) of the aromatic fraction and thus accounted for about 10 % (n = 10) of the total oil. Since the *Braer* released >84,000 tonnes of a similar crude into the marine environment, the respective monoaromatic UCM entering the environment from that spill could be estimated to be about 8,400 tonnes. The isolated monoaromatic UCM contained < 5 % 'resolved' components. Inspection of the gas chromatogram (Figure 2.3e) reveals that no one component is particularly resolved above the unresolved complex mixture, but the integration procedure, which was found to be consistent between samples (RSD 5.75 %, n = 7), includes any distortions above the basic 'hump' shape as 'resolved'. Mass spectra of the seemingly 'resolved' components were similar to those of the UCM (*i.e.* no distinction of these components could be made) indicating that these were similar structures. This is consistent with similar findings by Warton *et al.*, (1999). Therefore, a monoaromatic unresolved complex mixture had been successfully isolated from a crude oil. Furthermore, the isolated monoaromatic UCM could be used to carry out toxicological studies, to determine if compounds in this mixture, present in the environment in high concentrations, are responsible for observed effects in wild populations of mussels.

Mussels (*M. edulis*) were exposed to aqueous solutions of the isolated monoaromatic UCM for 24 hours to observe its effect on feeding rate. A summary of the effects of aqueous exposure and total body burden of the isolated monoaromatic UCM upon mussel feeding rate are presented in Table 2.1. Feeding rates for both the control and toxicant exposed

animals are expressed as means ± standard deviation (n = 7). Concentration-response curves for the effect of the isolated monoaromatic hydrocarbon UCM on mussel feeding rate over 24 hours, expressed in terms of nominal aqueous concentration and total body burden, are shown in Figures 2.5 and 2.6, respectively. Values are plotted as mean feeding rate ± standard deviation (n = 7) and a reduction in feeding rate is apparent.

Table 2.1 The effect of an isolated monoaromatic hydrocarbon UCM upon mussel feeding rate over a 24 h exposure period

Nominal aqueous concentration ($\mu g\ L^{-1}$)	Total body burden of toxicant ($\mu g\ g^{-1}$ wet weight)	Mussel feeding rate ($L\ hr^{-1}$) mean ± std dev. (n = 7)
0	0	0.41 ± 0.05
	0	0.44 ± 0.07
25	3.1	0.44 ± 0.06
	3.2	0.40 ± 0.05
0	0	0.43 ± 0.05
	0	0.43 ± 0.04
50	10.2	0.39 ± 0.07
	18.3	0.36 ± 0.05
0	0	0.46 ± 0.07
	0	0.45 ± 0.07
100	46.1	0.35 ± 0.05
	70.3	0.35 ± 0.06
0	0	0.44 ± 0.03
	0	0.46 ± 0.04
200	81.0	0.31 ± 0.04
	89.9	0.25 ± 0.05

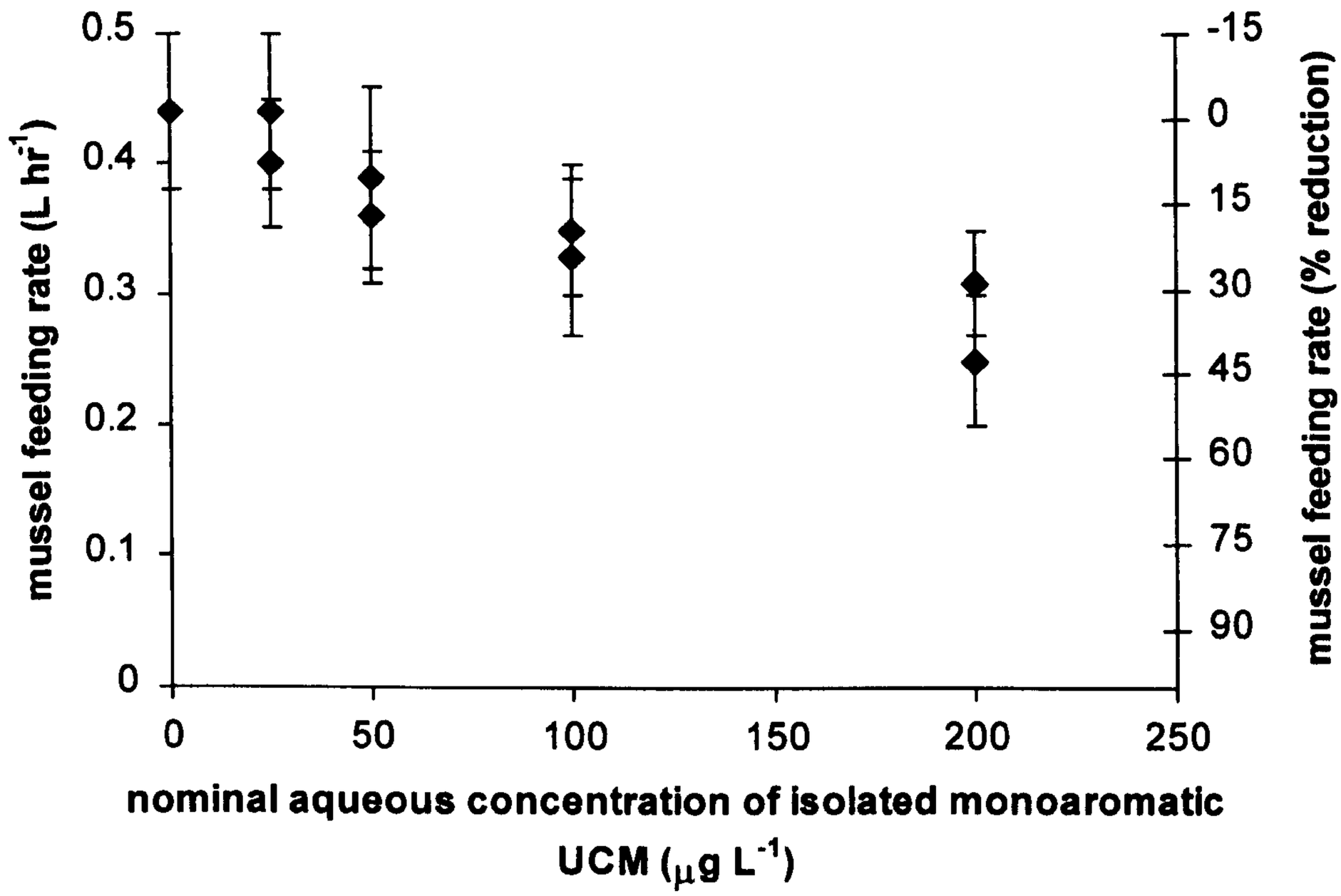


Figure 2.5 Concentration (aqueous)-response curve for the effect of an isolated monoaromatic hydrocarbon UCM upon mussel feeding rate over 24 h

(values plotted as mean ± sd, n=7, except control value exposure 0 µg L^{-1} n=56)

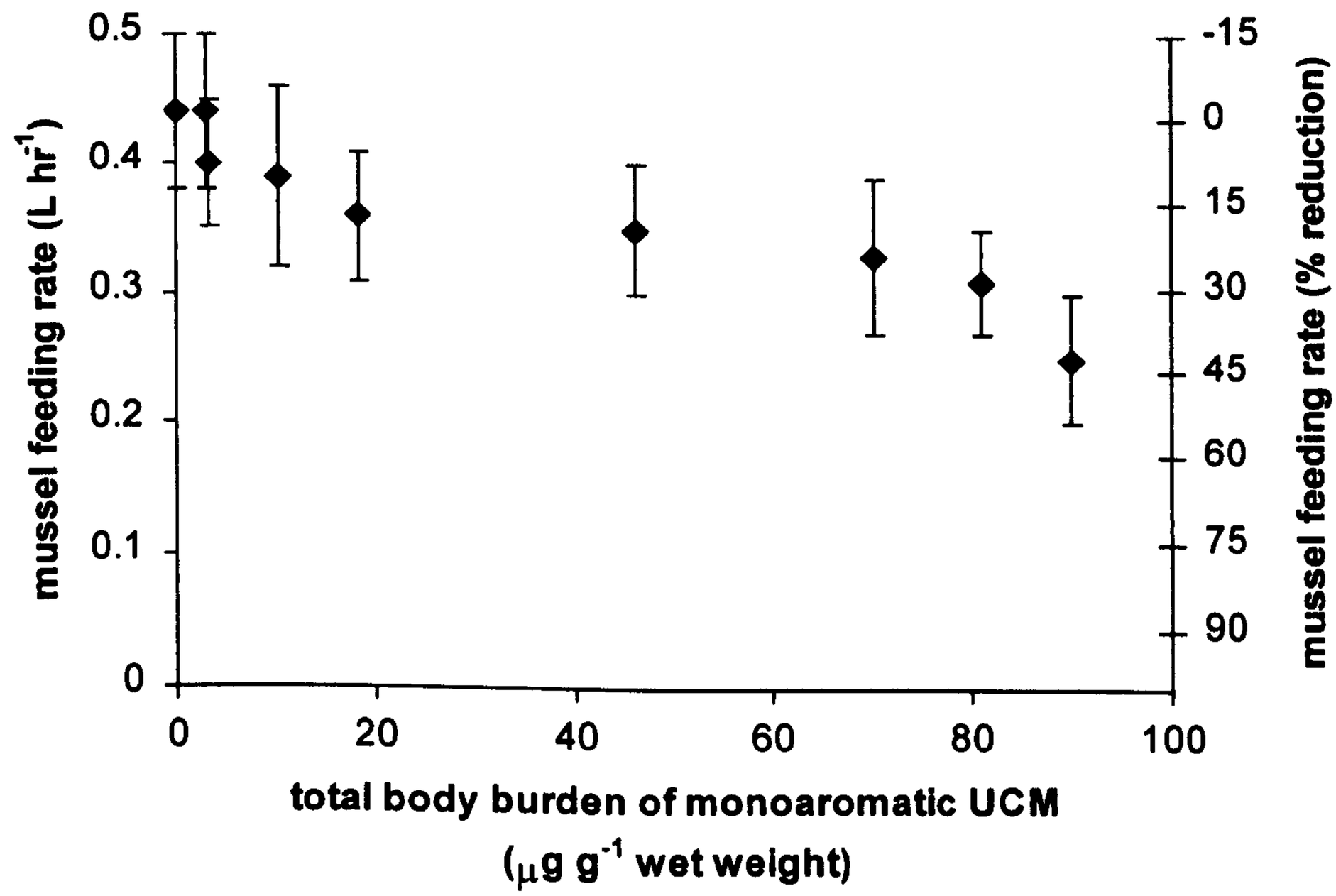


Figure 2.6 Concentration (tissue)-response curve for the effect of an isolated monoaromatic hydrocarbon UCM upon mussel feeding rate over 24 h

(values plotted as mean ± sd, n=7, except control value exposure 0 µg L^{-1} n=56)

Statgraphics 4 was used for all data analysis. Data were tested for normality using standardised skewness and standardised kurtosis. All data were found to be parametric, thus a t-test was used to compare means and ANOVA used to test the distribution of the data. In all statistical analyses differences at the <5% level were considered significant. All the controls that were carried out on different days as part of different exposure tests were found not to be statistically different thus allowing direct comparison of the exposure vessels. Pooled exposure data were compared with pooled control data. All data were normally distributed and duplicate exposures were not significantly different (except at 200 $\mu g\ L^{-1}$) and were therefore pooled. A summary of the pooled data is presented in Table 2.2.

Table 2.2 Summary of the concentration-response data for the effect of an isolated monoaromatic hydrocarbon UCM upon mussel feeding rate, 24 h exposure

Nominal aqueous concentration ($\mu g\ L^{-1}$)	Total body burden of toxicant ($\mu g\ g^{-1}$ wet weight) (mean, n = 2)	Mussel feeding rate ($L\ hr^{-1}$) (mean ± std dev n = 14)	Mussel feeding rate expressed as % of control feeding rate
0	0	0.42 ± 0.06	100
25	3.2	0.42 ± 0.06	100
0	0	0.43 ± 0.05	100
50	14.3	0.37 ± 0.06	86
0	0	0.45 ± 0.07	100
100	58.2	0.34 ± 0.06	76
0	0	0.45 ± 0.04	100
200	81.0	0.31 ± 0.04	68
	89.9	0.25 ± 0.05	55

Feeding rates were also expressed as a percentage of the control value (using a pooled value of the control feeding rates). Expressing results in this manner effectively eliminates the influence of other environmental variables. The results therefore describe a reduction that is attributable to the test substrate. The data show that the monoaromatic hydrocarbon UCM isolated from Gullfaks crude oil reduced mussel feeding rate by ~40 % at the highest nominal concentration of 200 $\mu g\ L^{-1}$. This aqueous concentration resulted in the accumulation of up to 90 $\mu g\ g^{-1}$ wet weight of the UCM in the 24 hour exposure period. This accumulation is equivalent to approximately 400 $\mu g\ g^{-1}$ dry weight which is comparable to the amounts of monoaromatic UCMs found in mussels from impacted sites previously shown to have reduced SFG (*e.g.* Whitby, 392 $\mu g\ g^{-1}$ dry weight).

Figure 2.7d illustrates the accumulation of the isolated monoaromatic UCM within the mussel tissue as compared to control mussels (Figure 2.7c). UCM hydrocarbons are clearly visible in the total organic extract of the mussel tissue from exposed animals (Figure 2.7d), whereas no 'hump' is present in the gas chromatogram of the total organic extract of control animals. This indicates that the accumulation of these components is responsible for the observed effect. The additional peaks present in the total organic extracts of the mussels from the feeding rate experiments are natural lipid compounds and are found in total organic extracts of other mussel tissue. Figure 2.7a shows the monoaromatic fraction isolated from mussels collected from Whitby in 1995. This site is where mussels were previously shown to have reduced Scope for Growth which correlated well with the aromatic content of the tissues (Widdows *et al.*, 1995b). The gas chromatogram of the isolated monoaromatic UCM (Figure 2.7b) exposed to mussels compares well with that isolated from an environmental sample.

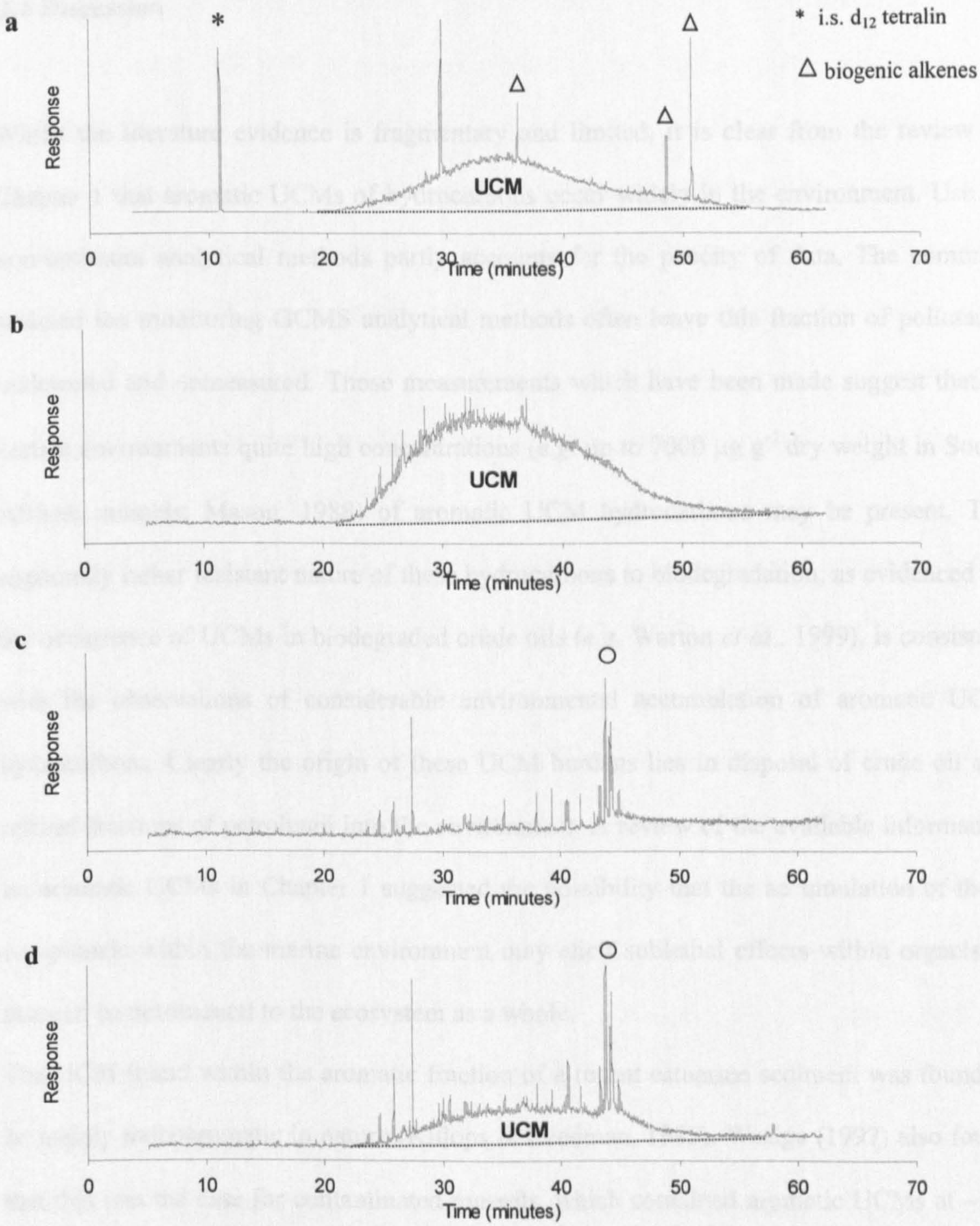


Figure 2.7 Gas chromatograms of
a) monoaromatic hydrocarbon fraction isolated from mussels Whitby U.K. May 1995
b) a monoaromatic UCM fraction isolated from Gullfaks North Sea crude oil
c) total organic extract from control mussels
d) total organic extract from mussels exposed to the monoaromatic UCM isolated from Gullfaks oil (b)
O *The additional peaks in c and d are natural lipid compounds present in the total organic extracts of other mussel tissue. GC conditions: Hewlett Packard MSD 5890, 12m HP-1 column, 40-300 ℃ min^{-1} at 5 ℃ min^{-1}.*

2.6 Discussion

Whilst the literature evidence is fragmentary and limited, it is clear from the review in Chapter 1 that aromatic UCMs of hydrocarbons occur widely in the environment. Use of non-optimum analytical methods partly accounts for the paucity of data. The common selected ion monitoring GCMS analytical methods often leave this fraction of pollutants undetected and unmeasured. Those measurements which have been made suggest that in certain environments quite high concentrations (*e.g.* up to 7000 $\mu g\ g^{-1}$ dry weight in South African mussels; Mason, 1988) of aromatic UCM hydrocarbons may be present. The apparently rather resistant nature of these hydrocarbons to biodegradation, as evidenced by the occurrence of UCMs in biodegraded crude oils (*e.g.* Warton *et al.*, 1999), is consistent with the observations of considerable environmental accumulation of aromatic UCM hydrocarbons. Clearly the origin of these UCM burdens lies in disposal of crude oil and refined fractions of petroleum into the environment. A review of the available information on aromatic UCMs in Chapter 1 suggested the possibility that the accumulation of these compounds within the marine environment may elicit sublethal effects within organisms that can be detrimental to the ecosystem as a whole.

The UCM found within the aromatic fraction of a recent estuarine sediment was found to be mainly monoaromatic in nature (Killops & Readman, 1985). Wraige (1997) also found that this was the case for contaminated mussels, which contained aromatic UCMs at ~10-40 % of the total hydrocarbons, of which ~80 % was monoaromatic. Therefore, a monoaromatic UCM was isolated from produced Gullfaks crude to investigate the possible toxicological properties of these compounds. The monoaromatic UCM was isolated from a sample of Gullfaks (North Sea) produced crude (Figure 2.3) by open column chromatography and HPLC. GC resolved volatile components, such as toluene, present in the produced crude probably due to blending, were removed by rotary evaporation. This oil

is representative of that spilled by the *Braer* in January 1993 into the marine environment around Shetland (>84,000 tonnes) and appears to have been subject to in-reservoir biodegradation. A 'hump' or UCM thus dominates the GC profile of the whole oil. This feature is clearly visible in the chromatogram of the monoaromatic fraction (Figure 2.3) and comprised about 10 % of the whole oil, as determined by gravimetry. The proportion of the isolated monoaromatic UCM of the oil was ~10%. Given that an estimated 84,000 tonnes of a similar oil was spilled from the *Braer* (EGOSS, 1994), an estimated 8,400 tonnes of aromatic UCM was also introduced into the marine environment of the Shetland Islands, U.K.

The isolated monoaromatic fraction from Gullfaks crude oil was exposed to mussels for 24 hours to observe its effect on mussel feeding rate. At the highest nominal aqueous concentration (200 $\mu g\ L^{-1}$) the isolated monoaromatic UCM reduced mussel feeding rate by ~40%. A UCM was clearly visible in the total organic extract of the exposed mussels when compared to untreated controls (Figure 2.7) suggesting that this response was indeed due to the accumulation of these compounds within the mussel tissue at the site of toxic action. The feeding rate was statistically different ($p \leq 0.015$) from control data at all exposure concentrations above 50 $\mu g\ L^{-1}$ and above, corresponding to an accumulation of approximately $\geq$ 15 $\mu g\ g^{-1}$ wet weight (~60 $\mu g\ g^{-1}$ dry weight) of the isolated monoaromatic UCM.

From the gas chromatogram of the total organic extract of mussels exposed to the monoaromatic UCM (Figure 2.7d) it would appear that the accumulated UCM is similar to that of the isolated monoaromatic UCM (Figure 2.7b) to which they were exposed, as the UCM is present over the same retention time range. This suggests that no modification of the UCM occurred during the experiment, i.e. all the monoaromatic UCM components were accumulated in the mussel tissue rather than, for example, those components in the lower molecular weight range. However, at the higher molecular weight range the shape of

the UCM in the mussel tissue extract (Figure 2.7d) is somewhat masked by the presence of naturally occurring lipids, also present in the control mussel tissue extracts (Figure 2.7c). It is possible that the presence of these compounds in the gas chromatograms of tissue extracts may also have slightly interfered with the quantification of the UCM and this may account for the slight difference in the amounts determined to be accumulated by mussels exposed in duplicate to 100 μg L^{-1}; and the difference in feeding rate, despite a similar level of accumulation at duplicate 200 μg L^{-1} exposures. If the experiment were to be repeated it would be interesting to use normal phase HPLC, as in the isolation of the monoaromatic UCM, on the tissue extracts to remove these compounds to determine if this were the case.

It is important to note that the 5% seemingly 'resolved' components in the isolated monoaromatic UCM oil fraction (Figure 2.7b) cannot account for the observed decrease in mussel feeding rates. The tissue effective concentration of typical resolved monoaromatics required to reduced mussel feeding rate by 50 % (TEC_{50}) ranges from 35-94 μg g^{-1} wet weight for 1-phenylpentane to 1-phenyldecane (Donkin *et al.*, 1989), whereas the concentrations of resolved monoaromatic in the mussels exposed to oil was less than 5 μg g^{-1} wet weight. Any resolved peaks found in the tissue extracts of exposed mussels (Figure 2.7d) were also found in the control mussels (Figure 2.7c) indicating that they were either natural compounds present within the mussel tissue, or came from the extraction procedure itself, and so could not be responsible for the reduction in feeding rate. Clearly, the reduction in feeding rate suggests that monoaromatic UCM hydrocarbons are toxic at sublethal concentrations to mussels. It also appears that the isolated monoaromatic UCM as a whole rather than sub-components was responsible for the effect observed.

It could be argued that marine organisms may not encounter levels of 200 μg L^{-1} in the marine environment or that 24 hours was a short exposure time. However, Engelhardt *et al.* (1985) noticed that the level of hydrocarbons accumulated in Arctic bivalves exposed to

dispersed petroleum was dose–time dependent. A 14 day exposure at 50 ppb (parts per billion) achieved a similar body burden to a 6 hour exposure period at 5 ppm (parts per million). The author found that that behavioural abnormalities were related to the total body burden of hydrocarbons, occurring at both a 6-hour exposure to 50-100 ppm and after a 2-week exposure at 50 ppb. This suggests that the exposure of mussels to the isolated monoaromatic UCM at 200 $\mu g\ L^{-1}$ over 24 hours achieving a body burden of ~90 $\mu g\ g^{-1}$ wet weight and a reduction of feeding rate of ~40 % was valid, and that the same response would occur in mussels achieving an equivalent body burden when exposed to a lower aqueous concentration over a longer time period, as might be expected to be the case in environmental situations.

These results have important implications since mussel feeding rate is included in the calculation of the well known Scope for Growth index, widely used as a measure of mussel 'health'. Scope for Growth at 26 east coast U.K. sites has previously been correlated with the concentrations of aromatic hydrocarbons (2-3 ring) as measured by HPLC. However the HPLC method did not differentiate resolved compounds such as PAHs from aromatic UCMs. When some of these sites were later analysed by GCMS they were found to contain high concentrations of aromatic UCMs (Wraige, 1997). The aromatic UCM in east coast mussels ranged from 83-496 $\mu g\ g^{-1}$ of which most was monoaromatic in character (mean 76 %) with none being detected at a clean site (Whitsand). Concentrations of typical resolved priority pollutants were found to be low in comparison, *e.g.* the concentration of alkylnaphthalenes in mussels from Whitby 1995 was only 0.053 $\mu g\ g^{-1}$ dry weight. The levels of aromatic UCMs determined by Wraige (1997) were similar to the levels of 'total toxic hydrocarbons' as calculated by Widdows *et al.* (1995a) for these sites. In fact others have noted that the concentration of aromatic hydrocarbons measured by HPLC correlate well ($r=0.99$, $n=11$) with aromatic UCMs determined by GC in mussel tissue (Murray *et al.*, 1983). Upon reexamination of stored mussels collected from Whitby in 1990 by the

more specific GCMS method (Chapter 1), the high concentrations of aromatic UCMs originally present in 1990 (mean 389 μg g^{-1} dry weight), when the SFG measurement was made, were confirmed. Thus, the effects on SFG originally ascribed to PAHs determined by HPLC (Widdows *et al.*, 1995b) were probably in part, if not entirely, due to aromatic UCMs which to date have been ignored.

This conclusion may reflect a more general analytical problem. In order to increase sample throughput and to minimise costs, several toxicological studies employ the rather unspecific HPLC determination of aromatics. Similarly, owing largely to requirements for measurements of priority pollutant PAHs, workers employing GCMS methods routinely use selected ion monitoring (SIM) procedures which effectively 'filter' out the aromatic UCM burden. It would be useful if, in further studies the aromatic burden at petroleum impacted sites was analysed for, to determine the validity of the potential correlation.

The results obtained herein also suggest that mussels in Cape Town, South Africa contain levels of monoaromatic UCM hydrocarbons (489-7172 μg g^{-1} dry weight aromatic UCM; Mason, 1988) sufficient to induce a biological response. However, extrapolation of this result to reported data of aromatic UCMs from other researchers (although in itself limited due to the lack of reported data), may be limited due to different fractionation processes employed by different workers in the separation of 'aliphatic' and 'aromatic' hydrocarbons. In most studies the aim of the separation of hydrocarbons into 'aliphatic' and 'aromatic' fractions is to study compounds clearly resolved by GC, such as PAHs. Thus, the 'cut' between monoaromatic and aliphatic hydrocarbons is often not clearly defined. In standard column chromatography Wraige (1997) found that monoaromatic compounds were classed with the aliphatic fraction. Killops (1986) also noted that the profiles of unresolved complex mixtures by GC analysis can be severely affected by the fractionation method employed. They observed a larger UCM present in the aliphatic fraction from column chromatography than from HPLC indicating that a proportion of the

aromatic UCM compounds were present in the aliphatic fraction. They suggested that even using HPLC the separation of monoaromatic from aliphatic in terms of unresolved compounds is not complete due to the complex nature of the compounds themselves. This is not surprising when one considers that compositional studies in aromatic UCMs have shown them to contain high amounts of branched and cyclic aliphatic moieties. Thus, inaccuracies in aliphatic/aromatic class separation of the UCM components may significantly affect the gas chromatograms of the resulting fractions and the conclusions drawn from them (Killops, 1986). However, the method used by Wraige (1997) to quantify aromatic UCMs in mussels was designed with this in mind and therefore also used in this study.

Recent reports demonstrate that weathered or highly weathered crude oils characterised by UCMs are indeed toxic to other marine organisms. Barron *et al.* (1999) examined the water accommodated PAH content of three environmentally weathered oils collected from underground plumes of spilled oil at a coastal Californian oilfield by SIM GCMS and reported that toxicity to the mysid shrimp *Mysidopsis bahia* was unexpectedly not correlated with PAH concentration. The published total ion chromatograms of the oils showed that hydrocarbon fractions tested were dominated by aromatic UCMs. Heintz *et al.* (1999) found that highly weathered oil from the *Exxon Valdez* oil spill was more toxic to pink salmon embryos than the unweathered oil. The authors attributed the enhancement of toxicity to elevated concentrations of larger PAHs in the weathered oil. These PAHs were measured by SIM GCMS and a lowering of the recommended legal limits for certain PAHs in seawater was suggested. There is no reason to question their findings but an alternative explanation could be an increased toxic effect owing to the relative enhancement of aromatic UCM compounds brought about by weathering. Neff *et al.* (2000) studied the effects of weathering on the toxicity of three offshore Australian crude oils and a diesel fuel to marine organisms and suggested that the unresolved components may have

contributed to the toxicity they observed from the water accommodated fractions (WAFs) of these oils. They also reported that Hokstad *et al.* (1999) evaluated the chemical composition and Microtox toxicity of (WAFs) of fresh and weathered oils of four crude oils. Identifiable monoaromatic hydrocarbons (MAHs) contributed most to the toxicity of WAFs compared to fresh oils (as in the study by Neff *et al.*, 2000). However, the source(s) of toxicity in the weathered oil was uncertain and may have been due to the presence of UCM compounds. The results of the study herein indicated that monoaromatic unresolved compounds may have been responsible for the observations by these workers.

It was suggested by Glegg & Rowland (1996) that the unresolved monoaromatic hydrocarbons of Gullfaks crude oil would probably add to the burden of toxins found in mussels collected from Sheltand, 3, 6 and 15 months after the *Braer* spill. However, this was not measured in their study. The finding herein that a monoaromatic UCM isolated from Gullfaks crude, similar to that spilt by the *Braer* suggests that an interesting area of further study would be to analyse for monoaromatic UCMs in marine organisms from this area in conjunction with an assessment of their biological impact.

2.7 Summary

Whilst aliphatic UCMs have been demonstrated to be non-toxic, aromatic UCMs have not been studied. Because aromatic UCMs can consist mainly of monoaromatic compounds the overall aim of this work was to conduct a laboratory based assessment into the toxicological impact of monoaromatic UCM hydrocarbons.

An initial aim was therefore to isolate a monoaromatic unresolved complex mixture with which to conduct a toxicological assay. This was successfully achieved using open column chromatography and normal phase HPLC of Gullfaks crude oil. The monoaromatic UCM contained < 5 % resolved compounds and accounted for ~10 % of the initial oil sample.

Characterisation using mass spectrometry showed that the isolated unresolved complex mixture was monoaromatic in nature. No ions for diaromatic compounds were found and these compounds were present in other HPLC fractions. Ions present in the mass spectra of the synthetic model monoaromatic UCM hydrocarbons (see Chapter 3) were also found in the mass spectral analysis of the isolated complex mixture, lending further support to the proposed structures. Suitable quantities (50 mg) were isolated with which to carry out toxicological assessment. As the Gullfaks oil was almost identical to that spilt by the *Braer* the fractionation procedure used herein isolated a monoaromatic UCM that is representative of those found entering the marine environment.

The next aim was to determine what effect an isolated monoaromatic UCM had on mussel feeding rate, a sensitive parameter in the Scope for Growth measurement. At the highest nominal aqueous concentration (200 $\mu g\ L^{-1}$) the mussels accumulated 90 $\mu g\ g^{-1}$ wet weight tissue and feeding rate was decreased by ~40 % compared to controls within 24 h. It is assumed that mussel exposure to a lower concentration over a greater exposure time would also achieve a similar body burden and thus might produce similar effects, but this requires further investigation. This accumulation was equivalent to approximately 350 $\mu g\ g^{-1}$ dry weight, which is comparable to levels found within the marine environment. This level is also comparable to the levels of 'total toxic hydrocarbons' (390 $\mu g\ g^{-1}$ dry weight) found at Whitby where reduced Scope for Growth had been reported (Widdows *et al.*, 1995b).

Results presented in this chapter support evidence (Chapter 1) that the aromatic UCM is a significant environmental burden and a forgotten pollutant within the marine environment, and suggests that the unexplained toxicity found by workers studying weathered oil may have been due, in part, to these monoaromatic UCM compounds. Experiments carried out with other UCMs and other biological endpoints should identify whether the toxicity of monoaromatic UCMs is common to many oils and other marine organisms.

Chapter 3. Synthesis of a model monoaromatic UCM hydrocarbon

Recent studies of the compositional nature of aromatic unresolved complex mixtures have suggested that substituted alkyltetralins may be present. Therefore, 7-cyclohexyl-1-methyltetralin has been synthesised herein, in good purity and yield, by a seven stage synthetic pathway. Synthetic intermediates were characterised by GC, GCMS, IR and NMR. Two further compounds, 7-cyclohexyltetralin and 7-cyclohexyl-1-propyltetralin, were also isolated pure from the crude reaction products of a previous study. These synthetic compounds represent valuable test substrates for the investigation of environmental aromatic UCM behaviour.

3.1 Introduction

Although crude oil is the major energy resource of the Western world, a substantial proportion of the hydrocarbons present in petroleum has yet to be identified. The number of compounds in such a mixture is very large. For example, the number of structures possible for alicyclic alkanes increases exponentially with carbon number, so for C_{30} alkanes there are 4.11×10^9 possible structures (Altgelt & Boduszynski, 1994). Whilst most research has focused on the GC resolvable hydrocarbon components, which are thus identifiable by GCMS, the unresolved components, known as the unresolved complex mixture (UCM), are in fact very abundant as a group. These unresolved components are seen as a 'hump' in GC chromatograms and constitute a significant environmental burden in petroleum impacted sites (see Chapter 1).

Since the pioneering attempts of American Petroleum Institute scientists to unravel the composition of Ponca City crude oil in the 1950s, comparatively few studies of the major unresolved hydrocarbons of crude oil have been published. Instead, detailed knowledge of the chromatographically resolved hydrocarbons of petroleum has been accrued (*e.g.* Peters & Moldowan, 1993; Altgelt & Boduszynski, 1994). Conventional instrumental techniques alone have been unable to elucidate the composition of these UCMs. However a few recent investigations into the composition of the UCM have been carried out.

These recent studies have shown that it is the monoaromatic fraction of the aromatic UCM that is quantitatively important (Killops & Al-Juboori, 1990; Revill, 1992; Thomas, 1995; Warton, 1999). This also appears to be the case for pollutant aromatic UCM hydrocarbons accumulated in sediments (Killops & Al-Juboori, 1990) and mussel tissue (Wraige, 1997). Most recent studies of the unresolved hydrocarbon composition have been based on the principle of oxidative or pyrolytic degradation of UCM hydrocarbons followed by deuteration or derivatisation of the resulting UCM 'fragments', and analysis of the resulting products by GCMS (*e.g.* Warton, 1999) or ICR (inductively coupled resonance)-

MS (Thomas, 1995). These products have indicated structural information about the original components and a so-called '*retro*-structural' approach has suggested that amongst these monoaromatic compounds alkyl substituted tetralins may be important. "*Retro*-structural analysis" involves the reconstruction of the products of oxidation to reveal the original molecule or 'average' molecule. Whilst Gough (1989) proposed alkylbenzene structures with branched side chains, Revill (1992), Thomas (1995) and Warton (1999) all considered that the oxidation products were more consistent with the general structure of an alkylcyclohexyltetralin where R (see Figure 3.1) represents a straight or branched alkyl chain. Oxidation of such compounds generates normal and branched aliphatic acids and diacids, as observed for aromatic UCMs (Sturt, 2000)

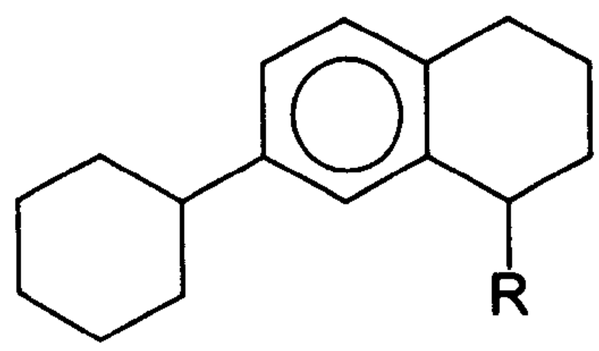


Figure 3.1 Proposed structure of model aromatic UCM hydrocarbons (based on Thomas, 1995 and Warton, 1999)

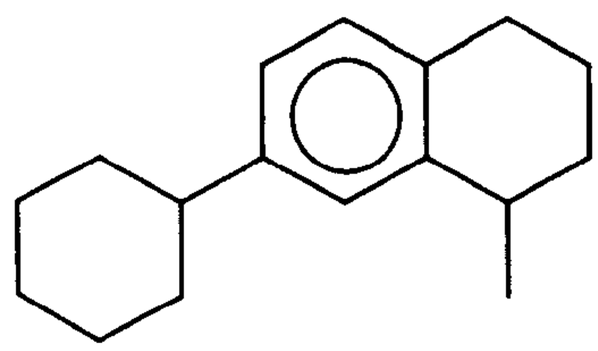
3.2 Aims

Previous characterisation of aromatic UCMs, principally by oxidative methods, followed by GCMS and ICR-MS, has allowed several structures of model aromatic UCM hydrocarbons to be proposed (Gough, 1989; Revill, 1992; Thomas, 1995 and Warton, 1999). Such compounds are not available from commercial sources, and little if anything, is known about the environmental behaviour of hydrocarbons of this type. The aim of this work was therefore to: synthesise suitable quantities of such hydrocarbons to assess the potential toxic effects to the mussel, *Mytilus edulis* (Chapter 4); to allow investigation of the aqueous solubility of UCM components (Chapter 5); and to allow investigations into the effects of photolytic attack on model UCM hydrocarbons (Chapter 6), in order to gain an insight into the behaviour of monoaromatic UCMs in the environment.

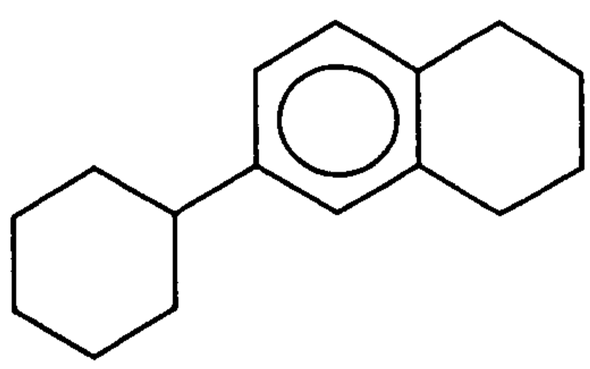
3.3. Synthetic Strategy

The 'model' monoaromatic UCM hydrocarbon, 7-cyclohexyl-1-methyltetralin (**A**, Figure 3.2) was synthesised using the synthetic pathway shown in Figure 3.3. This approach has previously been applied by Wraige (1997) to the synthesis of 6-cyclohexyltetralin and 7-cyclohexyl-1-propyltetralin and by Sturt (2000) to the synthesis of 6-cyclohexyltetralin, 1-(3'-methylbutyl)-7-cyclohexyltetralin and 1-*n*-nonyl-7-cyclohexyltetralin. The method used was a modification of the Haworth synthesis which is commonly used to synthesise aromatic hydrocarbons (Vogel, 1989; March, 1985). This involved the combination of an aromatic hydrocarbon with an aliphatic dibasic acid by a Friedel-Crafts acylation reaction to form a keto acid. The keto acid may then be reduced to an acid by the Clemmensen reduction, the product of which is cyclised to a tetralone using polyphosphoric acid. Addition of the alkyl substituent to the cyclised carbonyl may be performed by means of a

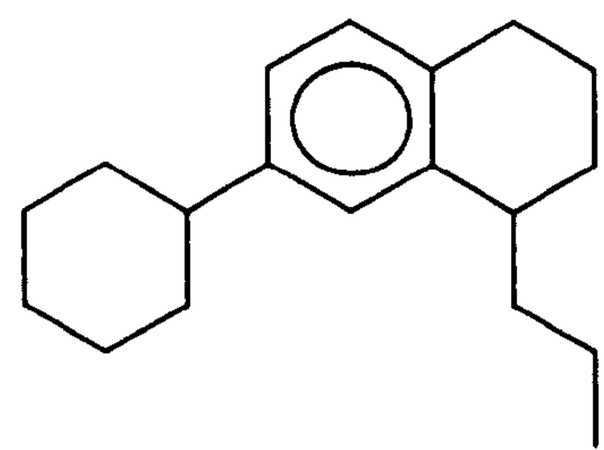
Grignard reaction producing a tertiary alcohol, which may be dehydrated *via* an acid-catalysed reaction. The alkene mixture so formed is easily hydrogenated to yield the target compound, in this case 7-cyclohexyl-1-methyltetralin. The products of each stage of the synthetic pathway were characterised by GC, GCMS, infrared spectroscopy (IR) and nuclear magnetic spectroscopy (NMR). In interpreting MS, IR and NMR spectra, correlation charts and chemical shift data were consulted (*e.g.* Smith & Busch, 1999; Stuart, 1996; Breitmaier, 1993). It was important that the identity and purity of the final product were assigned as small amounts of impurities may have influenced subsequent experimentation. Two further compounds, 6-cyclohexyltetralin (**B**, Figure 3.2) and 7-cyclohexyl-1-propyltetralin (**C**, Figure 3.2) were also purified in milligram quantities for subsequent aqueous solubility and degradation studies from previously synthesised crude reaction mixtures (Wraige, 1997). Full experimental details are given in Chapter 7.



(A) 7-cyclohexyl-1-methyltetralin



(B) 6-cyclohexyltetralin



(C) 7-cyclohexyl-1-propyltetralin

Figure 3.2 Model monoaromatic UCM compounds

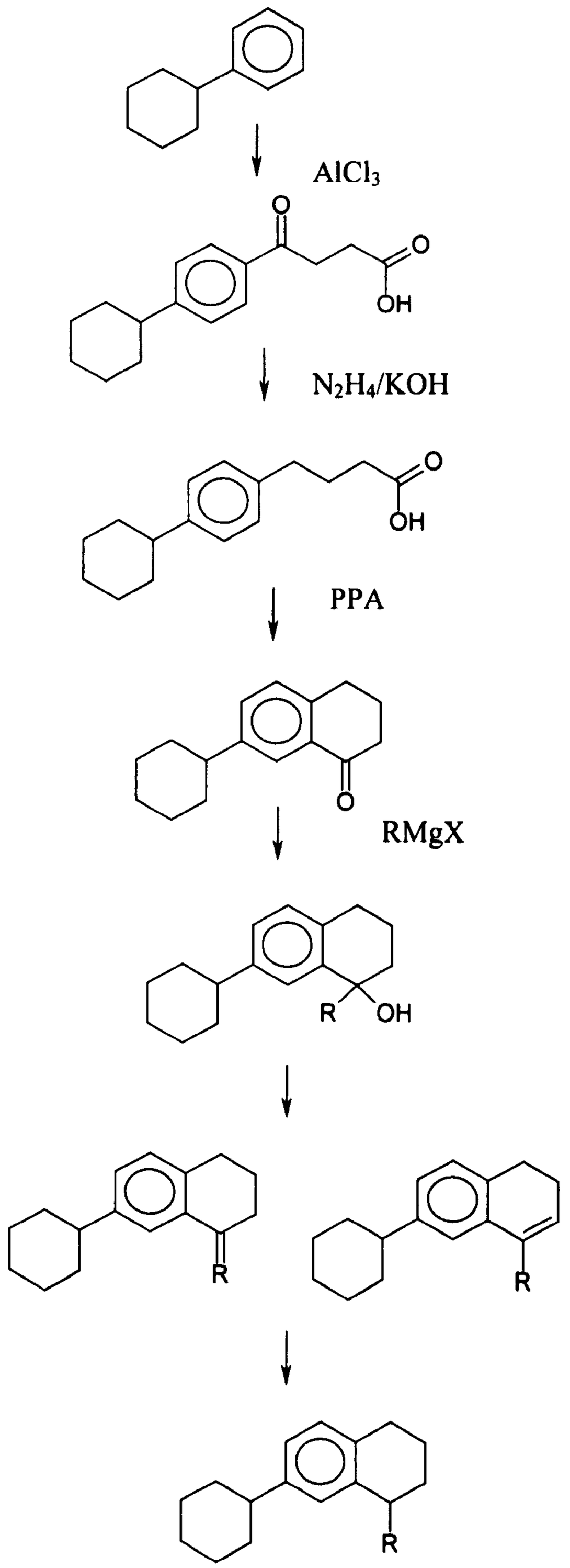
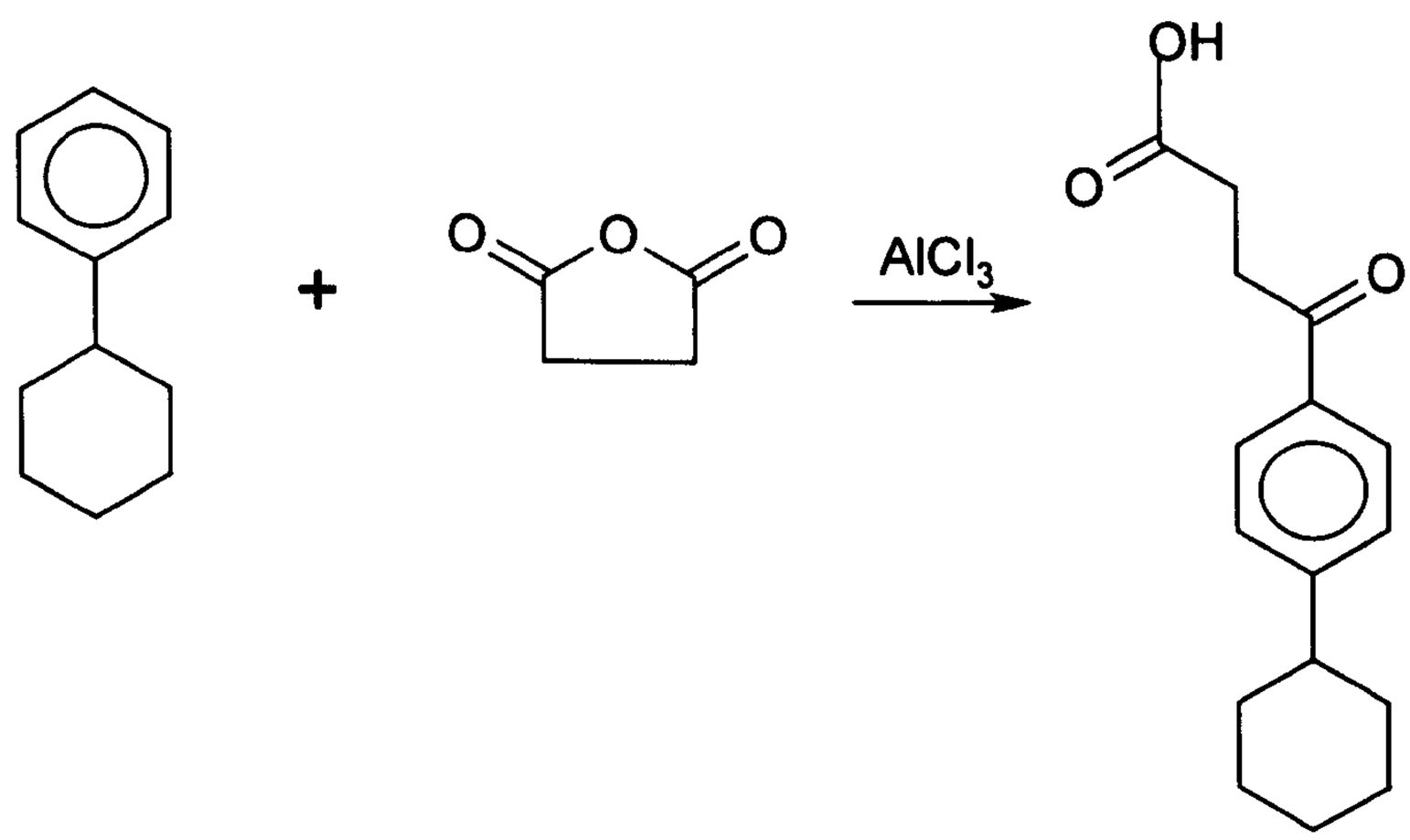


Figure 3.3 Reaction scheme for the synthesis of model monoaromatic UCM hydrocarbons

3.4.Synthesis of 3-benzoyl(4'-cyclohexyl)propanoic acid

3.4.1 Preparation

3-benzoyl(4'-cyclohexyl)propanoic acid (**I**) was formed in good yield (65%) *via* a Friedel-Crafts acylation of phenylcyclohexane using succinic anhydride, with aluminium chloride as a catalyst, and tetrachloroethane as the solvent. A total ion chromatogram of 3-benzoyl(4'cyclohexyl)propanoic acid as the trimethylsilyl (TMS) ester is shown in Figure 3.4. Integration of the peak areas showed the crude keto acid (**I**) to be >98% pure by GC with no impurity > 1%. Therefore further purification was deemed unnecessary.



I

3.4.2 Characterisation

Confirmation of the structure was provided by mass spectrometry where the molecular ion ($M^{+\cdot}$, *m/z* 332) was observed in the electron impact mass spectrum of the TMS ester (Figure 3.5). The ease of loss of the methyl group of the TMS group accounts for the relatively low intensity of the molecular ion, and fragmentation produces the ion at *m/z* 317 ($M^{+\cdot}$-CH_3), characteristic of TMS esters. The base peak at *m/z* 187 results from benzylic cleavage to leave the cyclohexylbenzoyl fragment.

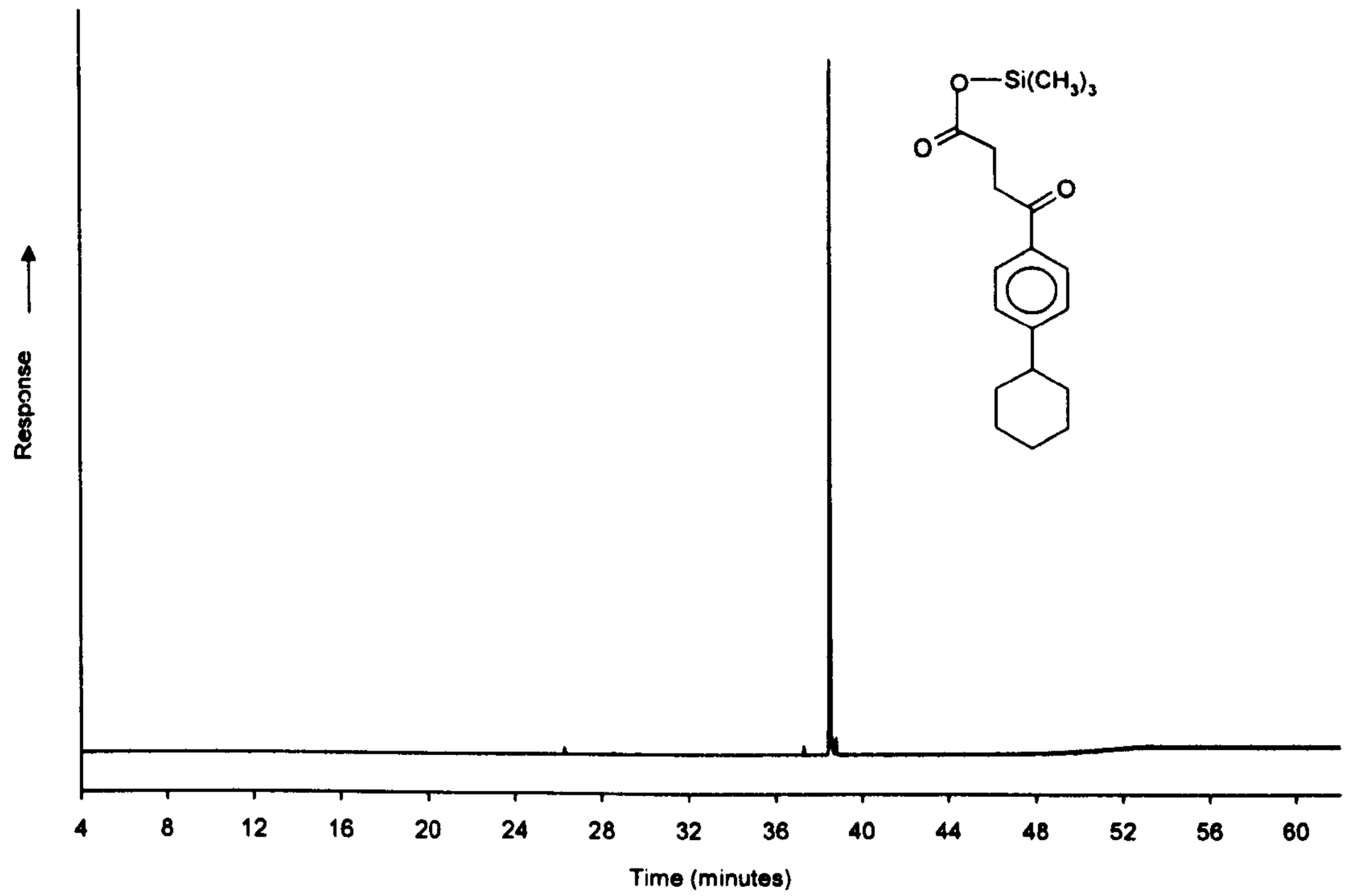


Figure 3.4 Gas chromatogram of 3-benzoyl(4'-cyclohexyl)propanoic acid (as TMS ester)

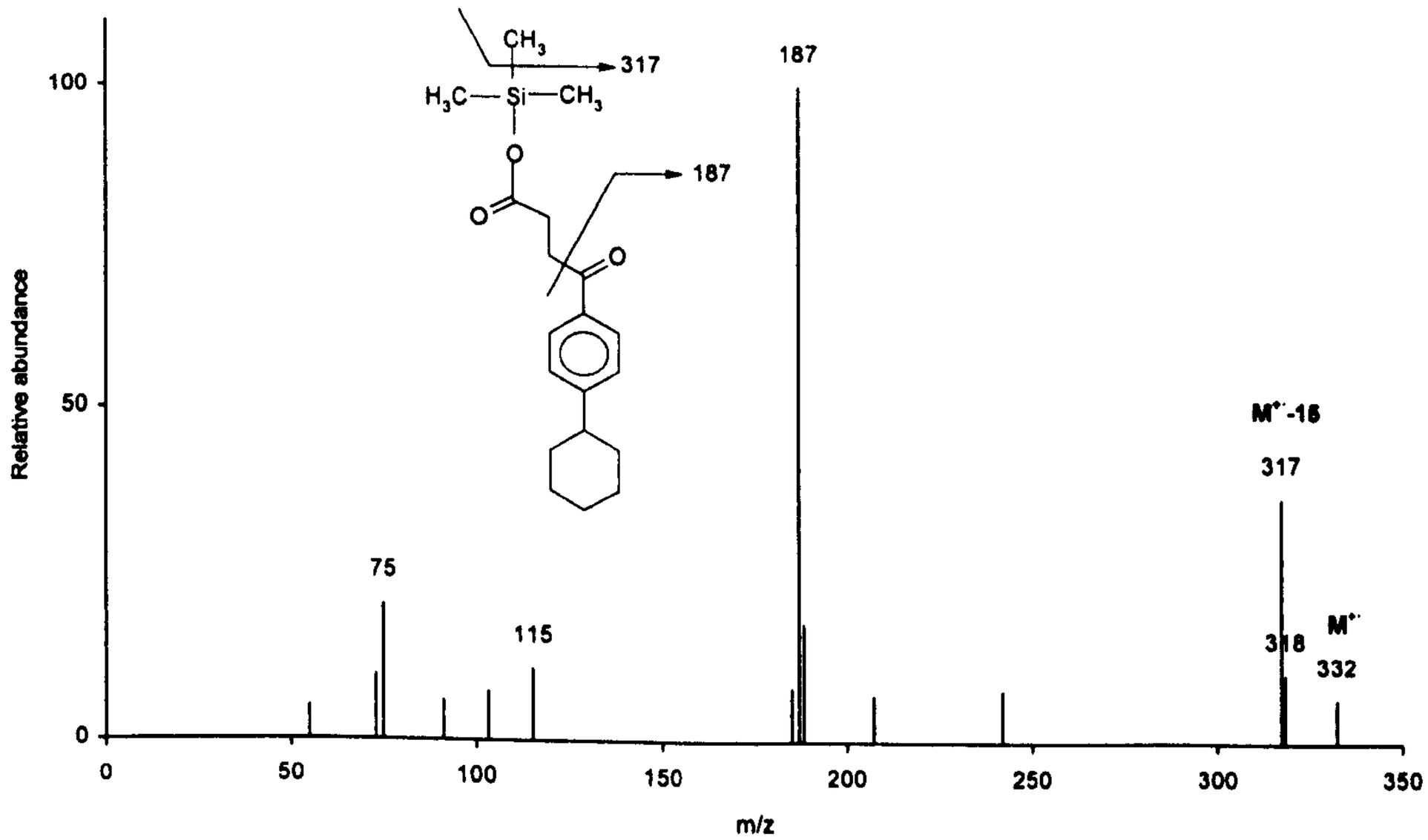


Figure 3.5 Mass spectrum of 3-benzoyl(4'-cyclohexyl)propanoic acid (as TMS ester)

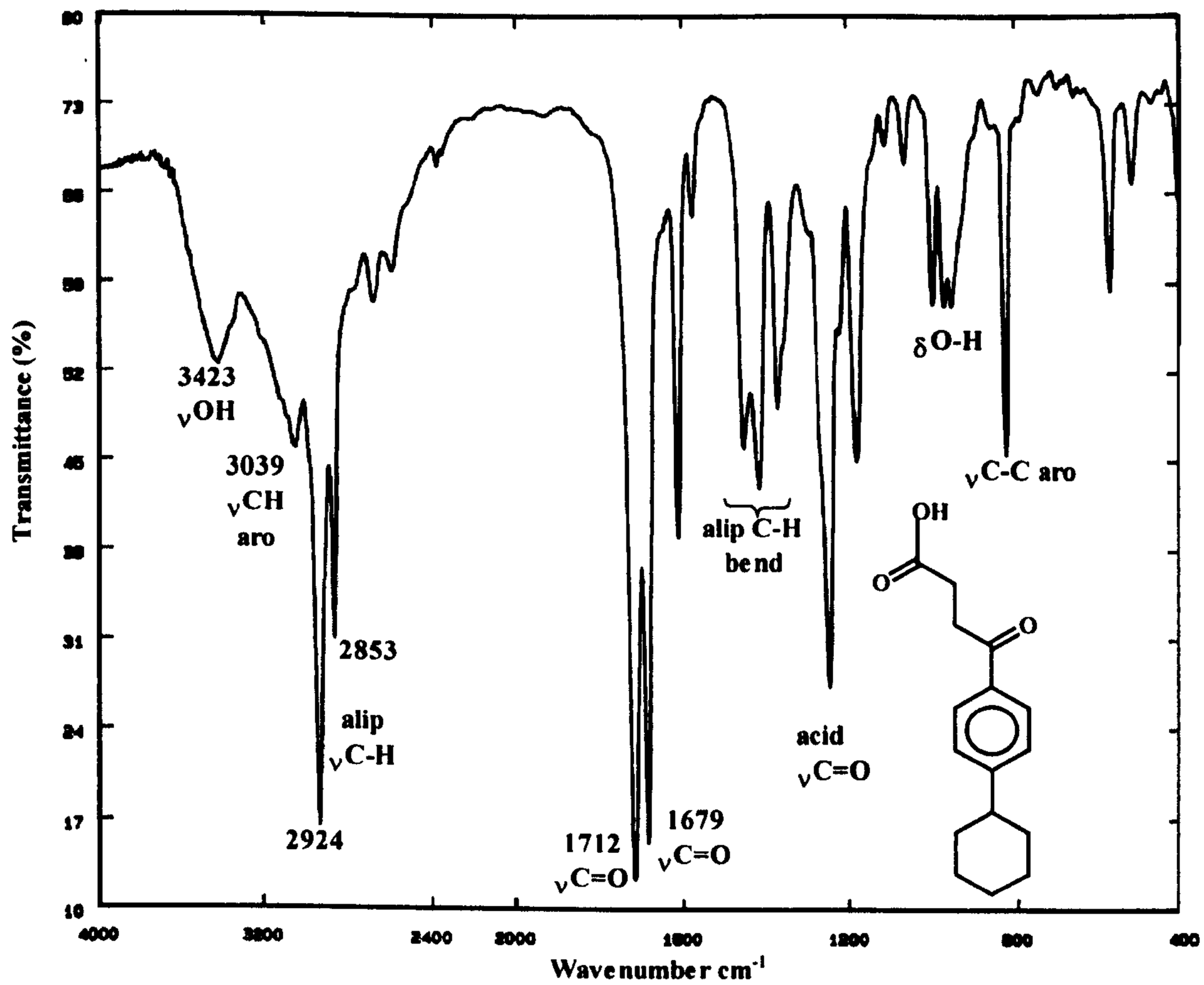


Figure 3.6 Infra-red absorbance spectrum of 3-benzoyl(4'-cyclohexyl)propanoic acid (KBr disc).

Figure 3.6 illustrates the infra-red spectrum of (**I**) and shows a characteristic broad absorption band at 3423 cm^{-1} owing to the OH stretch of the carboxylic acid group. The keto group (C=O) absorption at 1679 cm^{-1} is shifted to a lower absorption than the C=O stretch of the carboxylic acid group (1712 cm^{-1}) due to conjugation with the adjacent aromatic ring. The other features of this spectrum are strong bands at 2924 cm^{-1} and 2853 cm^{-1} due to aliphatic C-H stretching vibrations, and a weaker band at 3039 cm^{-1} due to the aromatic C-H stretch. Peaks in the spectrum below 1600 cm^{-1} may be tentatively assigned as due to C-C stretch in the aromatic ring (*para*-substituted at 819 cm^{-1}), the aliphatic C-H bend (~1350 – 1500 cm^{-1}), the C-O stretch of the carboxylic acid group (~1200-1300cm^{-1}), and the OH band of the acid group (938 cm^{-1}).

The ^{1}H NMR spectrum (Figure 3.7a) exhibited two doublets between 7 and 8 ppm corresponding to the aromatic protons **f** and **g**, confirming the presence of a 1,4-di-substituted benzene. The methylene protons of the acid side chain (**b**, **c**) are present as two triplets (3.3 ppm, 2.8 ppm). The resonance from **i** was observed downfield from the other cyclohexyl protons as a broad multiplet at 2.6 ppm due to the proximity of the benzene ring. The other methylenic cyclohexyl protons (**j**, **k**, **l**) were observed as two multiplets at 1.8 and 1.4 ppm probably due to the axial and equatorial positioning on the cyclohexyl ring. The ^{13}C-NMR spectrum of 3-benzoyl(4'-cyclohexyl)propanoic acid (Figure 3.7b) revealed 12 resonances corresponding to 16 carbon atoms. The two carbonyl carbons (**a** and **d**) are present at the characteristic chemical shifts of 179 ppm (**a**) and 197 ppm (**d**). There are four aromatic resonances relating to the six carbons in the aromatic ring (**e**, **f**, **h**, **g**). The *ipso* carbons on the aromatic ring were assigned as **e** and **h**, with the deshielding effect of the carbonyl carbon **d** causing the downfield shift of **e** (154 ppm) in comparison to **h** (134 ppm). The other four aromatic carbons were accounted for by **f** and **g** at 127 and 128 ppm, with each resonance representing two magnetically equivalent carbons, indicated by the increased intensity of their signal. Six carbon resonances were present in the aliphatic region. The resonance at 45 ppm was attributed to the tertiary carbon (**i**) in the cyclohexyl ring, shifted furthest downfield owing to its proximity to the aromatic ring. The methylene carbons **j** and **k** were assigned on the basis of their intensity, and the methylene carbons on the butanoic side chain (**b**, **c**) were apparent at 33 ppm and 28 ppm. The remaining methylene carbon **l** of the cyclohexyl ring was present at 26 ppm. These assignments were supported by the DEPT sequence (Figure 3.7c) which exhibits resonances caused by five methylene carbons (**b**, **c**, **j**, **k**, **l**), three methine (**f**, **g**, **h**,) and four quaternary carbons (**a**, **d**, **e**, **h**).

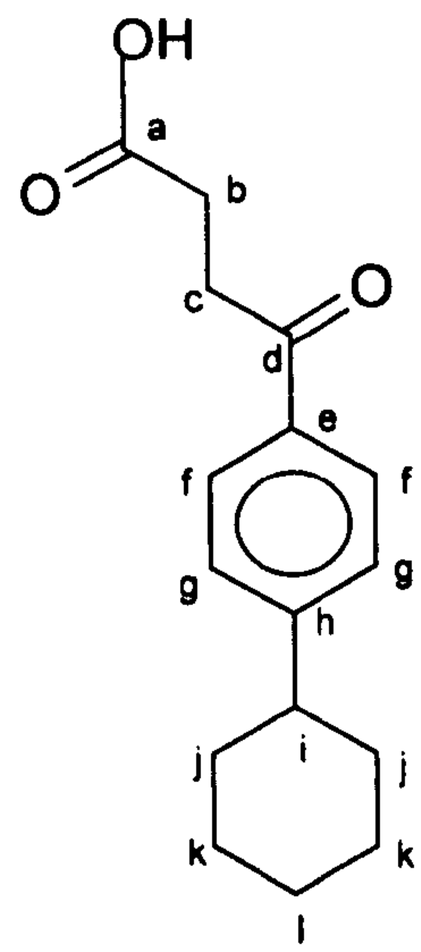


Figure 3.7 Assignment key for the NMR spectra of 3-benzoyl(4'-cyclohexyl)propanoic acid

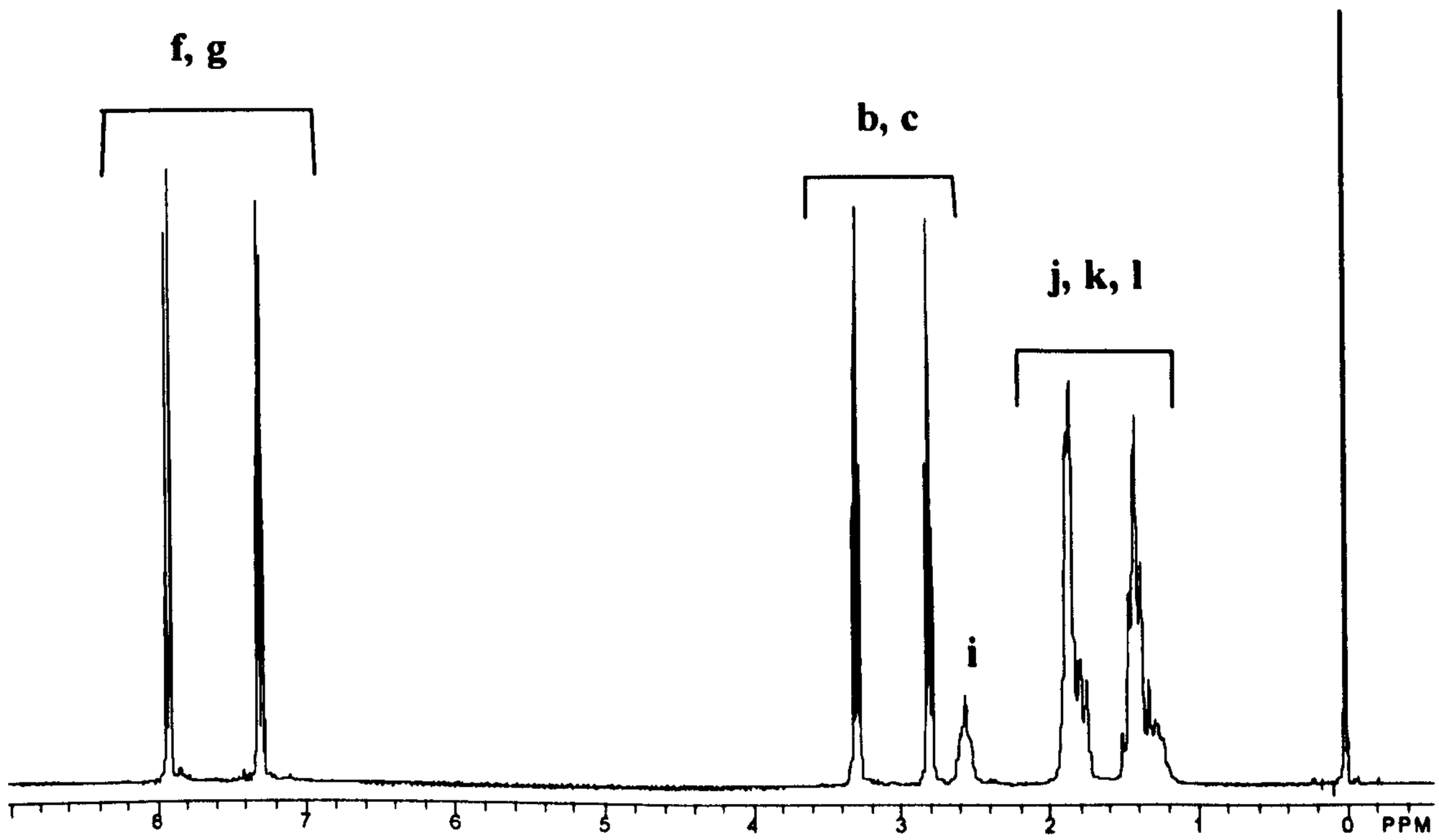


Figure 3.7a Proton NMR spectrum of 3-benzoyl(4'-cyclohexyl)propanoic acid

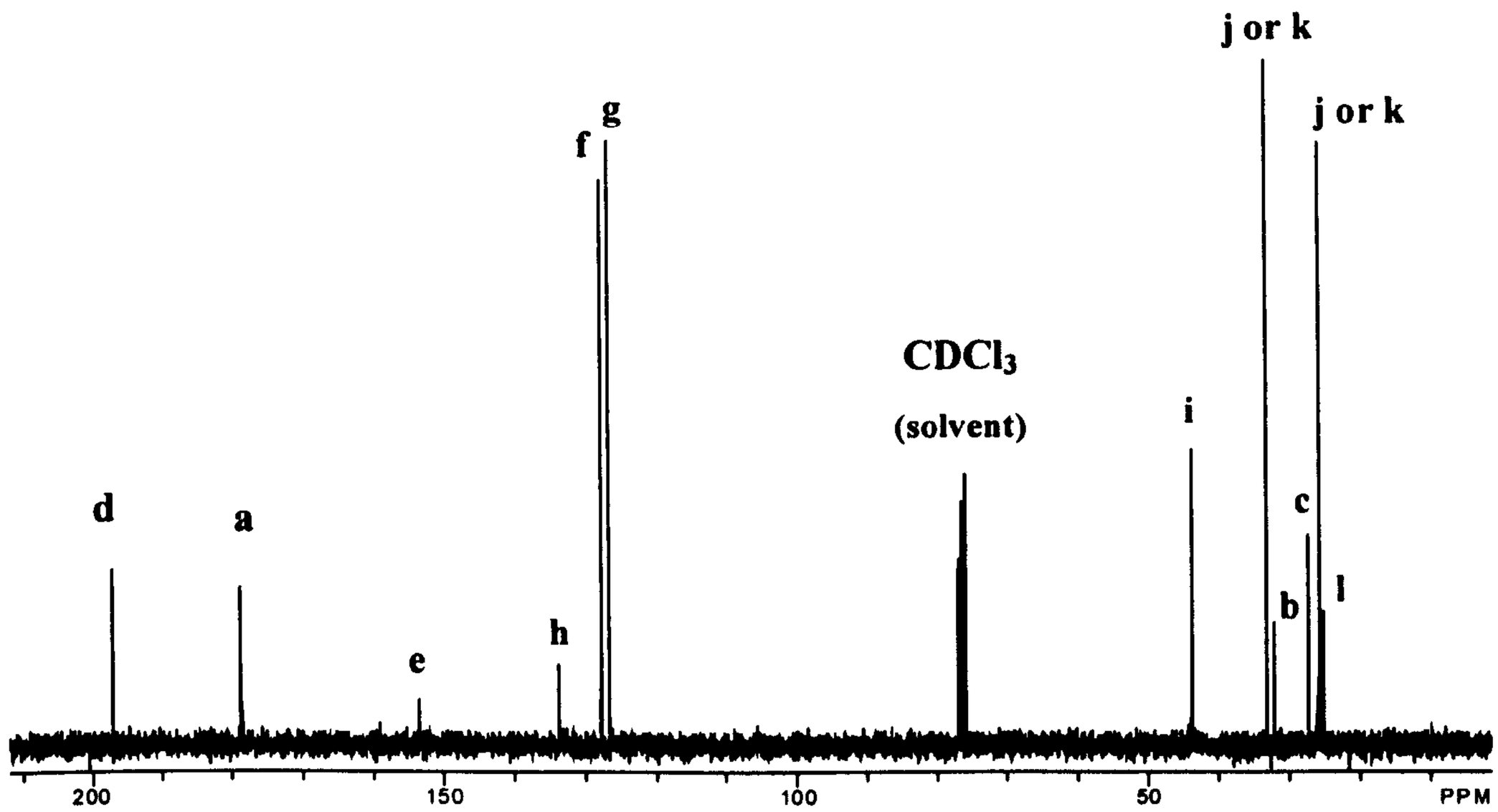


Figure 3.7b ^{13}Carbon NMR spectrum of 3-benzoyl(4'-cyclohexyl)propanoic acid

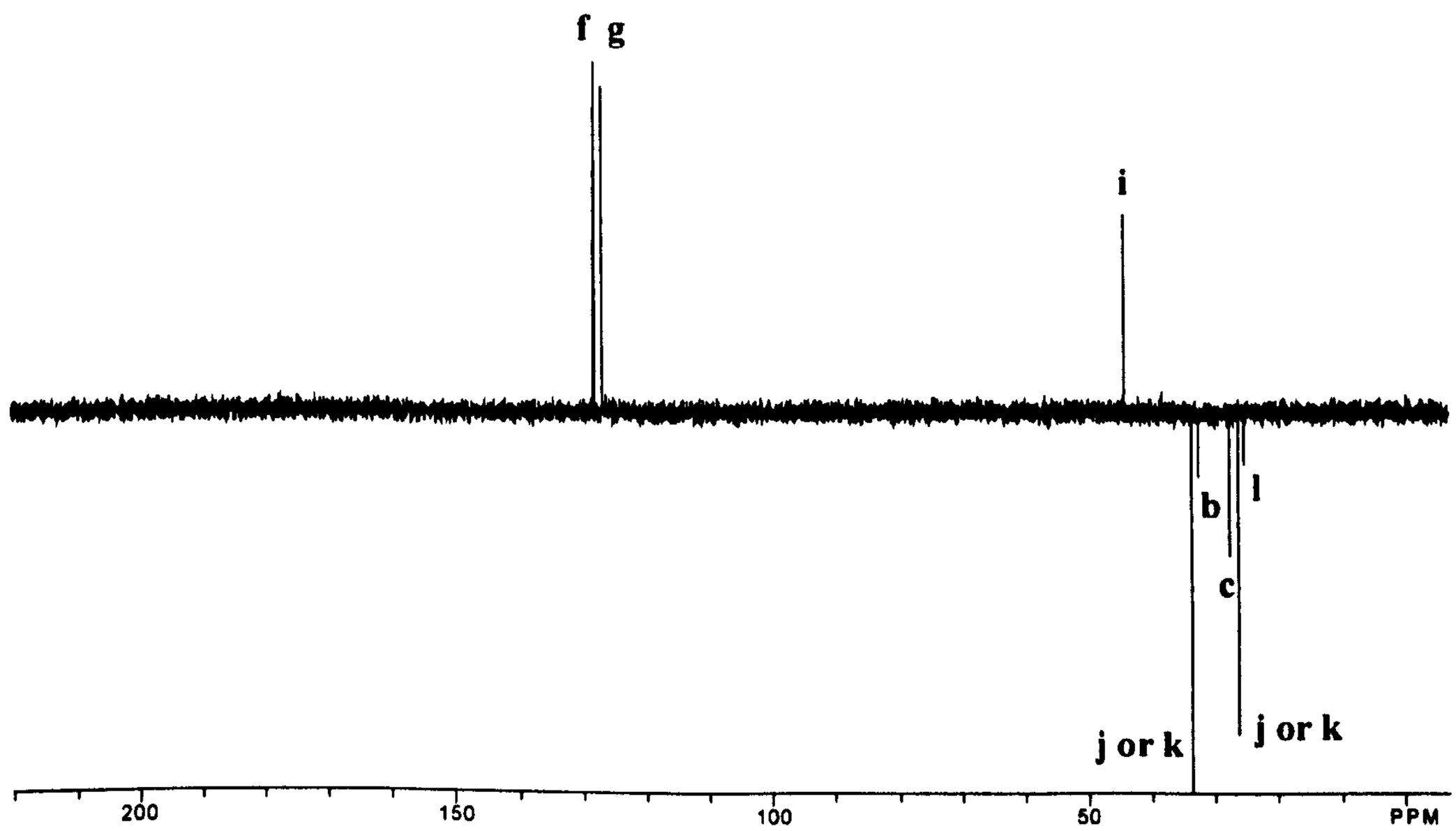
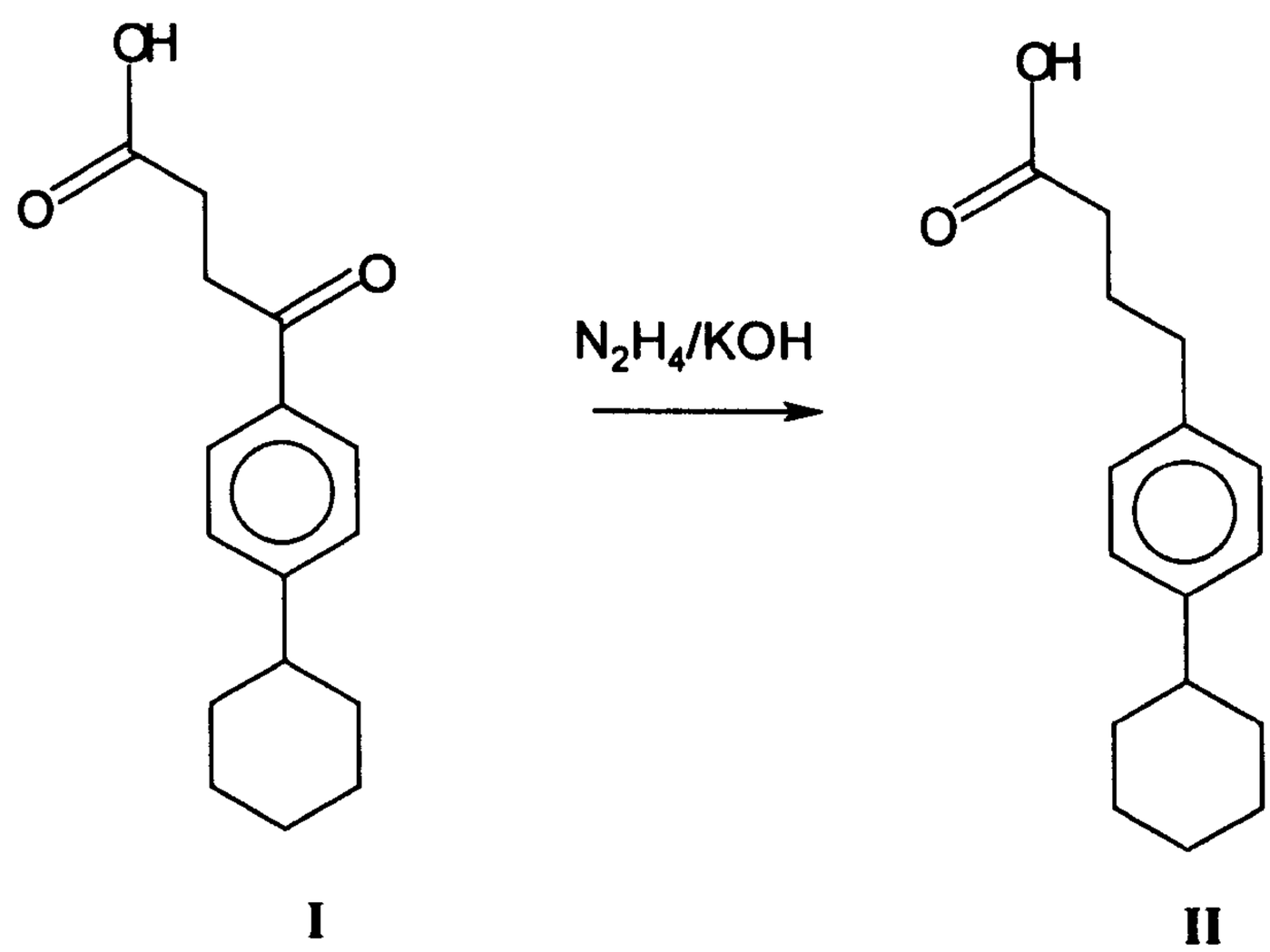


Figure 3.7c DEPT NMR spectrum of 3-benzoyl(4'-cyclohexyl)propanoic acid

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

3.5.1 Preparation

3-benzoyl(4'-cyclohexyl)propanoic acid (**I**) was converted into 4-phenyl(4'-cyclohexyl)butanoic acid (**II**) *via* a Huang-Minlon modification of a Wolff-Kischner reduction (Huang-Minlon, 1946) using hydrazine hydrate with potassium hydroxide and ethylene glycol as the solvent. 4-phenyl(4'-cyclohexyl)butanoic acid was obtained in good yield (83 %) and purity (>98 % by GC) as evidenced by the gas chromatogram of the product as the TMS ester (Figure 3.8.)



3.5.2 Characterisation

The mass spectrum (Figure 3.9.) indicated a relatively weak molecular ion $M^{+\cdot}$ at *m/z* 318, and an ion at *m/z* 303, which corresponds to $M^{+\cdot}$-15, *i.e.* the loss of a methyl group from the TMS group. These were accompanied by the base peak *m/z* 186 resulting from γ-H rearrangement (cleavage of the butanoic acid substituent), and the ion at *m/z* 117 ($COOTMS^{+}$) from β cleavage (cleavage at the esteric carbon). This fragmentation pattern is commonly known as the McLafferty rearrangement and occurs in compounds containing an unsaturated functionality, in this case the carbonyl group, along with a γ-H.

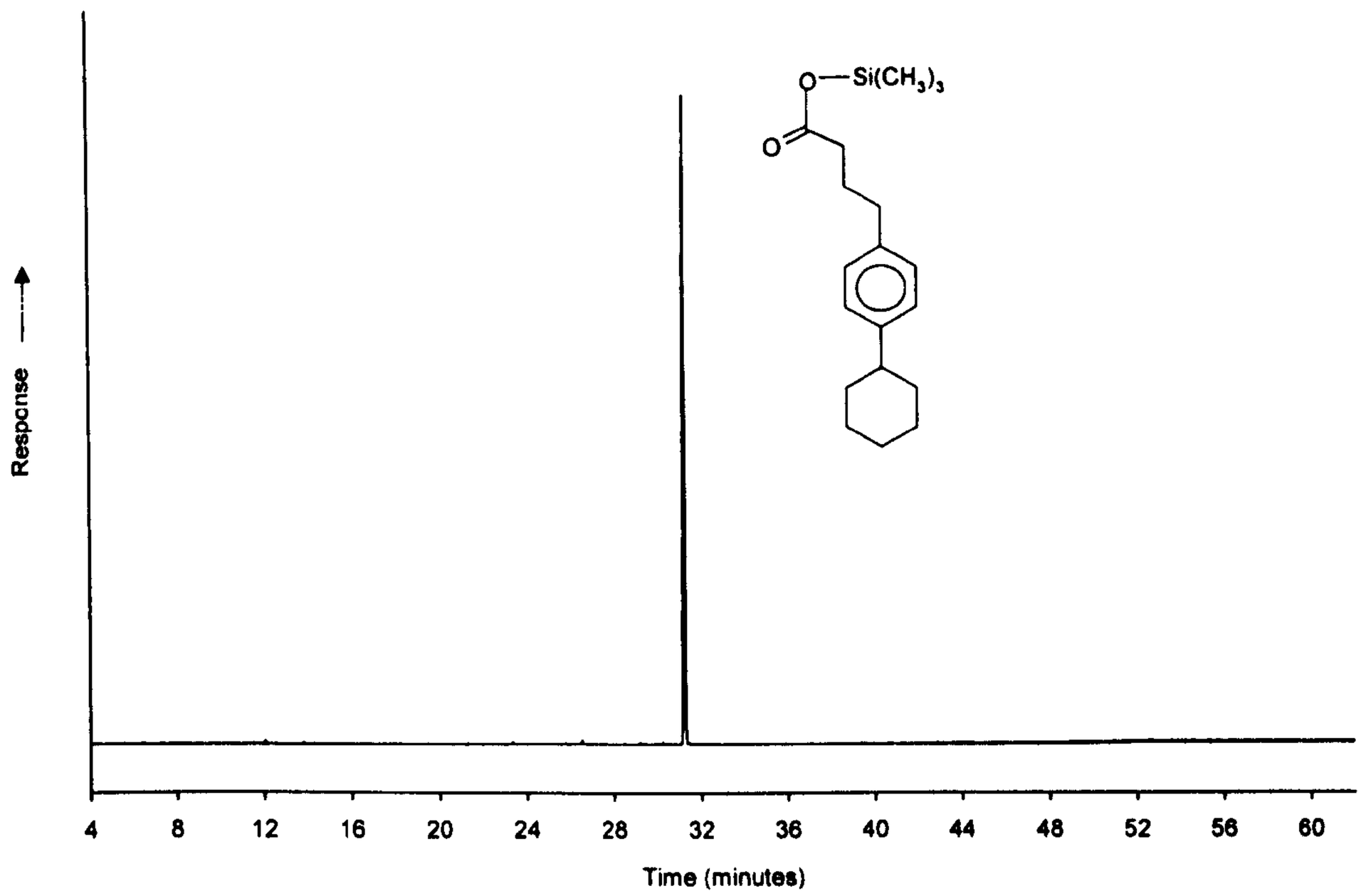


Figure 3.8 Gas chromatogram of 4-phenyl(4'-cyclohexyl)butanoic acid (as TMS ester)

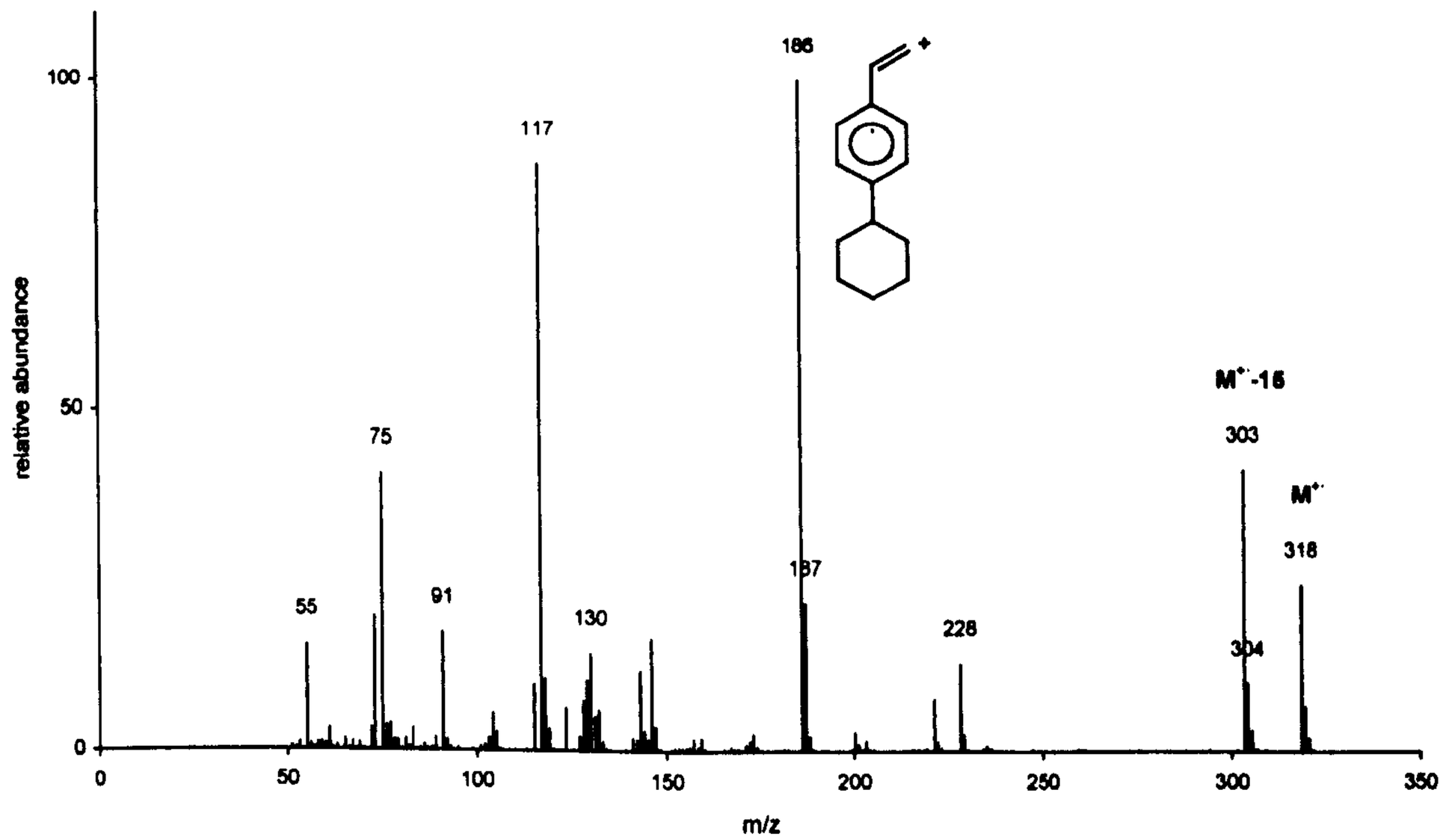


Figure 3.9 Mass spectrum of 4-phenyl(4'-cyclohexyl)butanoic acid (as TMS ester)

The IR spectrum presented in Figure 3.10 was similar to that of 3-benzoyl(4'-cyclohexyl)propanoic acid (Figure 3.6) but revealed that reduction of the keto group had indeed occurred, indicated by the loss of absorption previously assigned to the keto group at 1679 cm^{-1}.

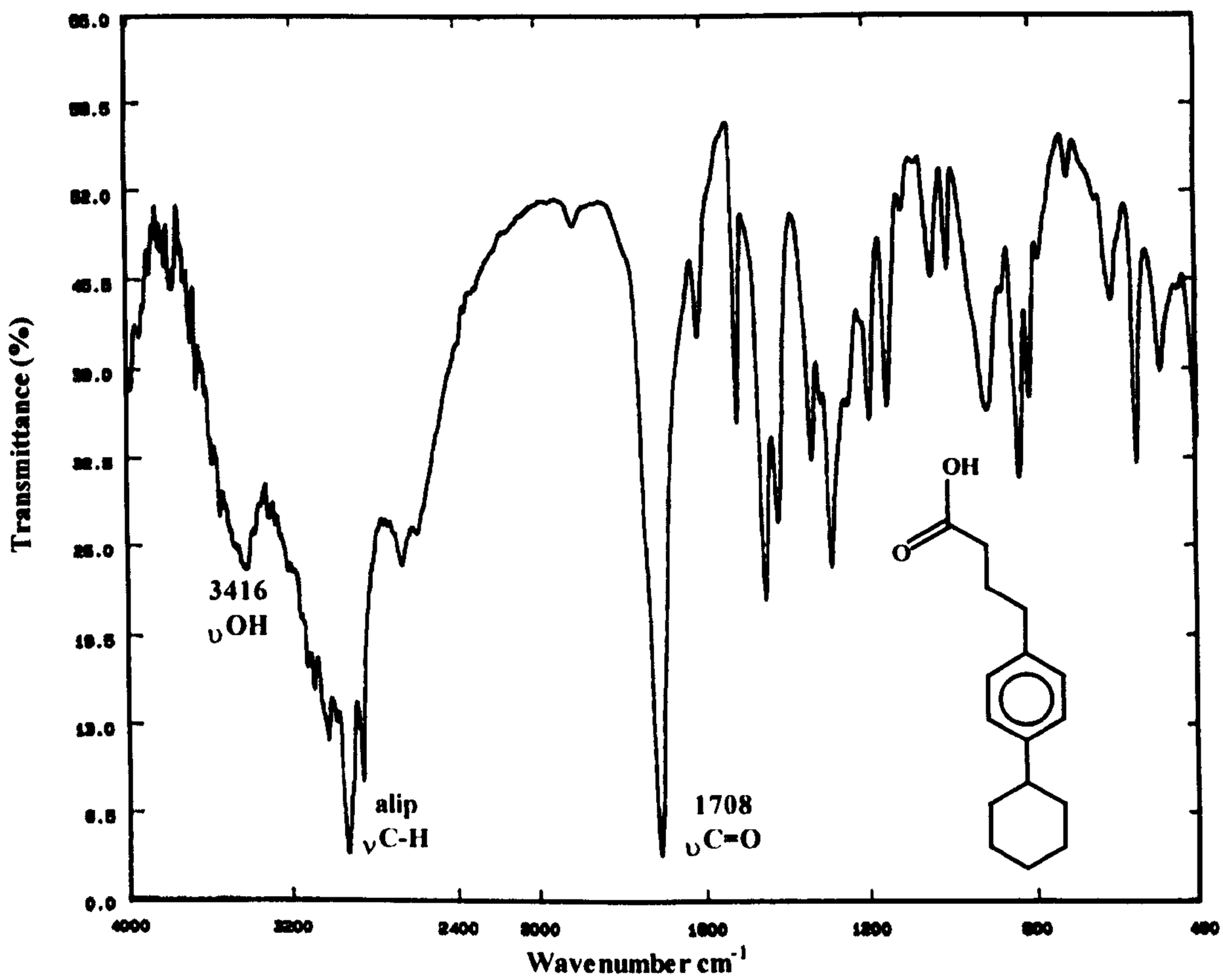


Figure 3.10 Infra-red spectrum of 4-phenyl(4'-cyclohexyl)butanoic acid (KBr disc).

Further evidence for the reduction of 3-benzoyl(4'-cyclohexyl)propanoic acid is provided by the NMR spectra (Figure 3.11). The ^1H NMR spectrum together with assignments is shown in Figure 3.11a. In the aromatic region a multiplet (7.3 ppm) represents the protons **f** and **g**. In the aliphatic region the two triplets at 2.6 and 2.3 ppm are due to the protons **b** and **d** of the butanoic side chain. These have been assigned by their relative proximity to the carboxylic acid group and the benzene ring. The proton **c** is present as a quintet at 1.9 ppm and the hydrogen **i** of the cyclohexyl ring is seen as a broad multiplet at 2.4 ppm

whilst the remaining protons of the cyclohexyl ring (**j**, **k**, **l**) are seen as two multiplets at 1.8 and 1.4 ppm.

The absence of the resonance at 197 ppm in the ^{13}C NMR spectrum (Figure 3.11b) is consistent with the absence of the carbonyl carbon. The adjacent aromatic carbon **e** has been shifted upfield to 146 ppm as there is no longer a carbonyl carbon to exert any effect, whereas the resonance due to **h** is present at 138 ppm, and **f** and **g** resonate at 126 and 127 ppm. In the aliphatic region **c** has also been shifted upfield as it no longer experiences the effect of a carbonyl group. The methine carbon (**i**) of the cyclohexyl group, easily distinguished by the DEPT sequence (Figure 3.11c), was again found at 44 ppm. The other aliphatic carbons are found between 20 and 40 ppm and were assigned based on their distance from the aromatic ring or carboxylic acid group. These assignments are supported by the DEPT sequence which exhibits resonances caused by six methylene carbons (**b**, **c**, **d**, **j**, **k**, **l**), three methine carbon (**f**, **g**, **i**) and four quaternary carbons (**a**, **e**, **h**).

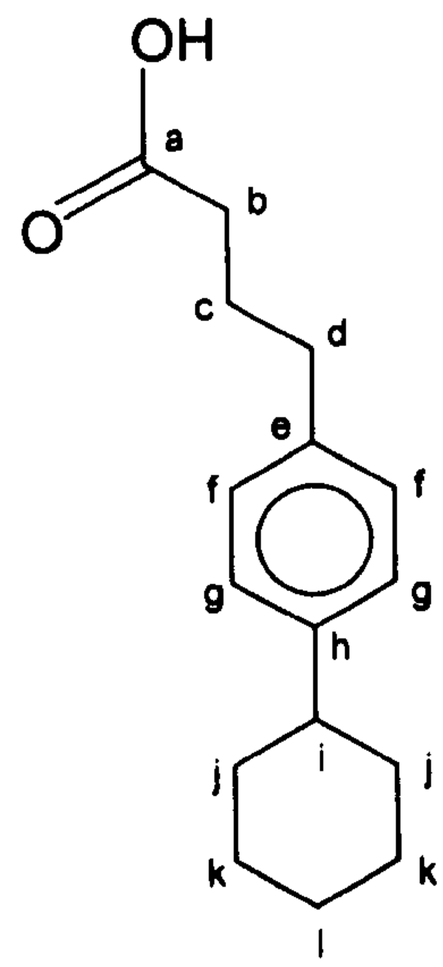


Figure 3.11 Assignment key for the NMR spectra of 4-phenyl(4'-cyclohexyl)butanoic acid

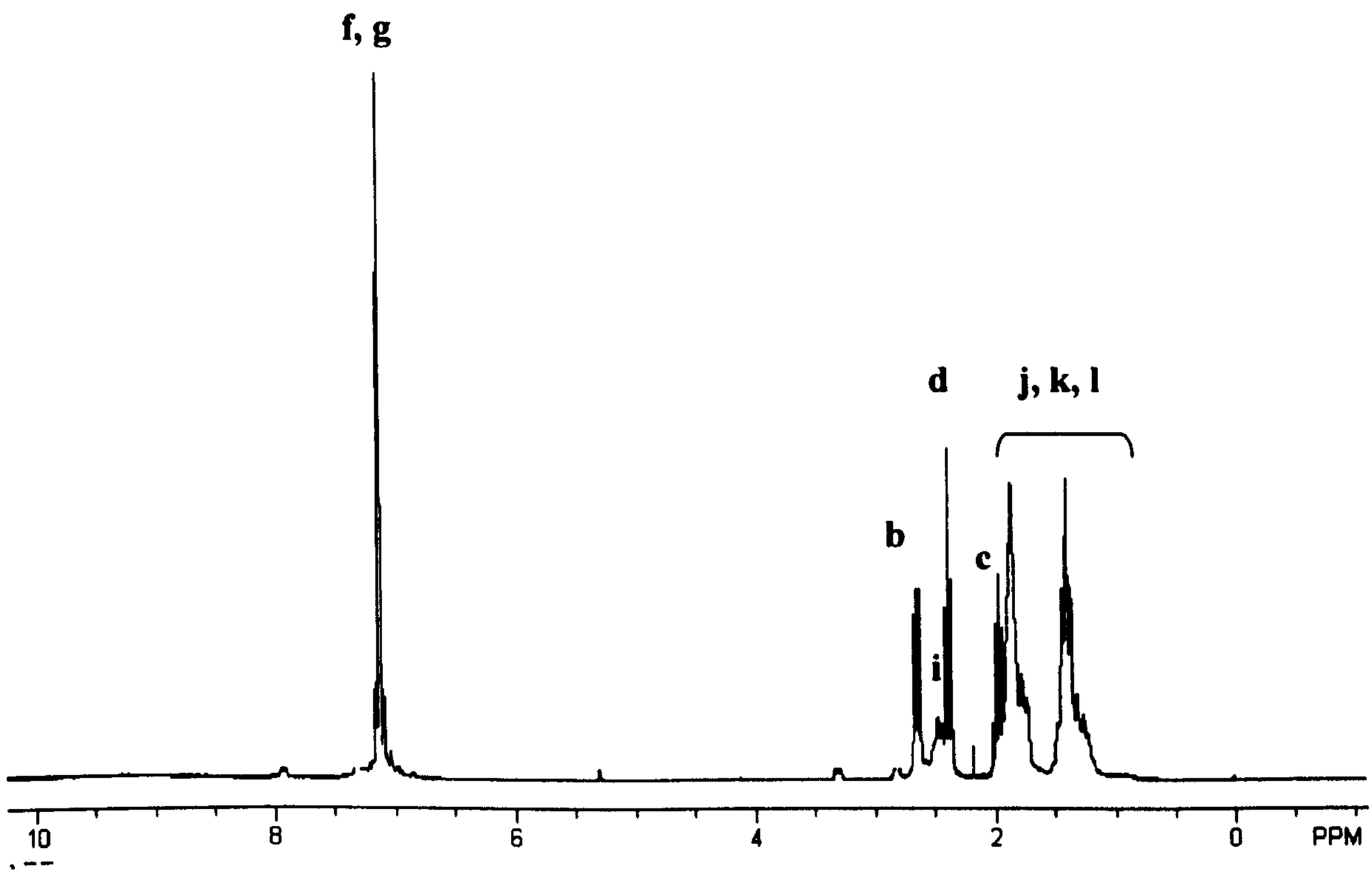


Figure 3.11a Proton NMR spectrum of 4-phenyl(4'-cyclohexyl)butanoic acid

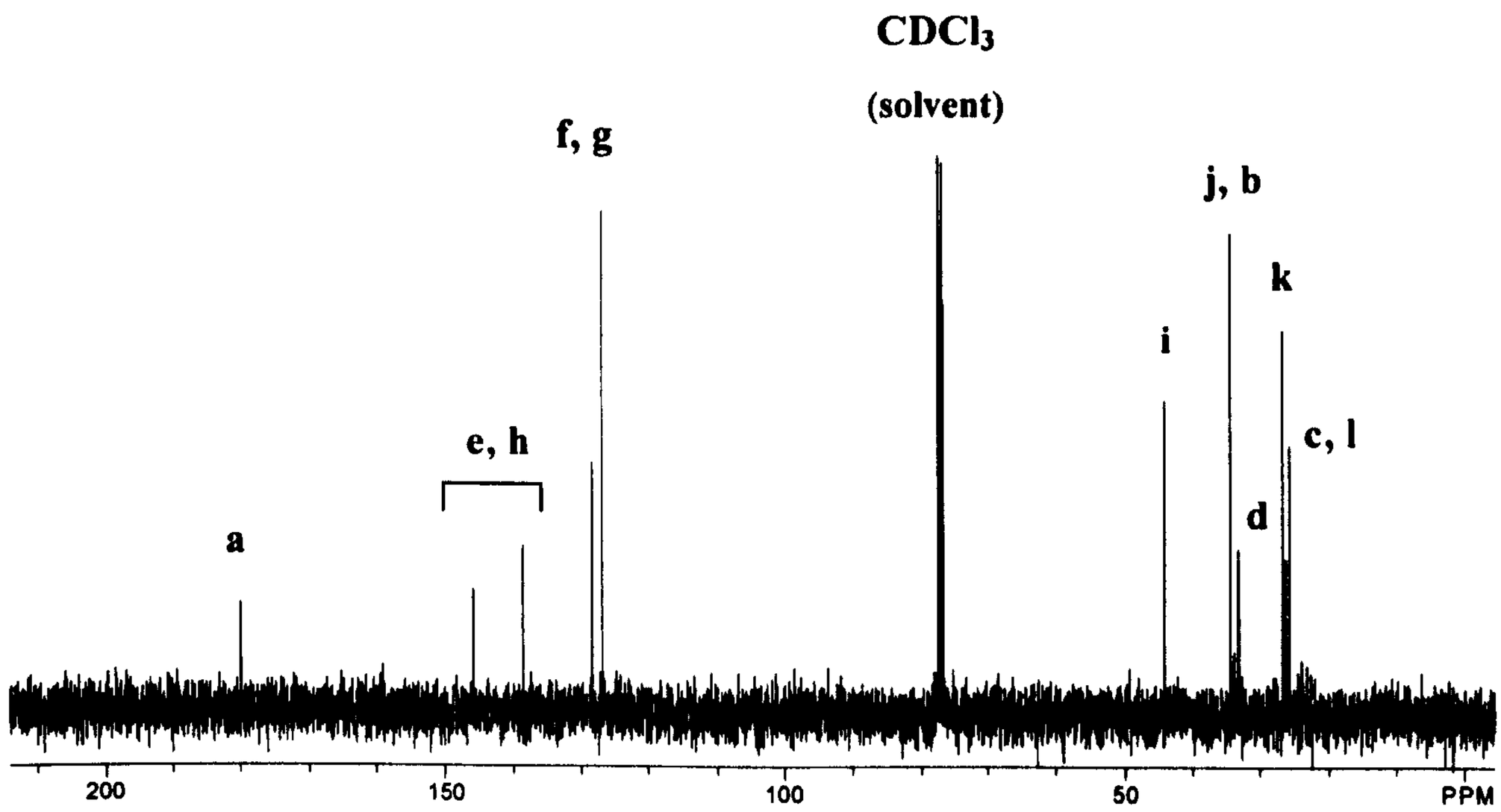


Figure 3.11b ^{13}Carbon NMR spectrum of 4-phenyl(4'-cyclohexyl)butanoic acid

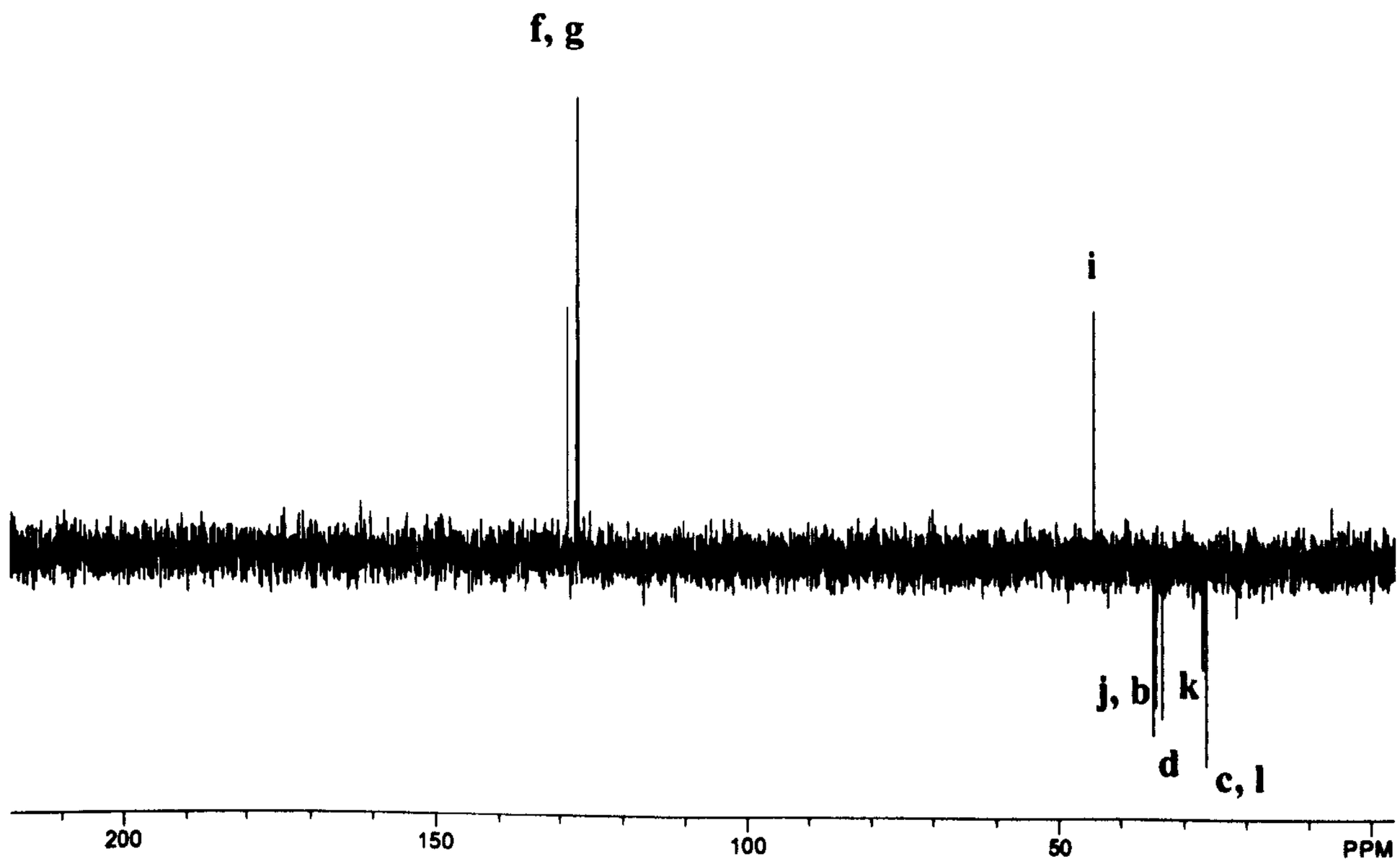
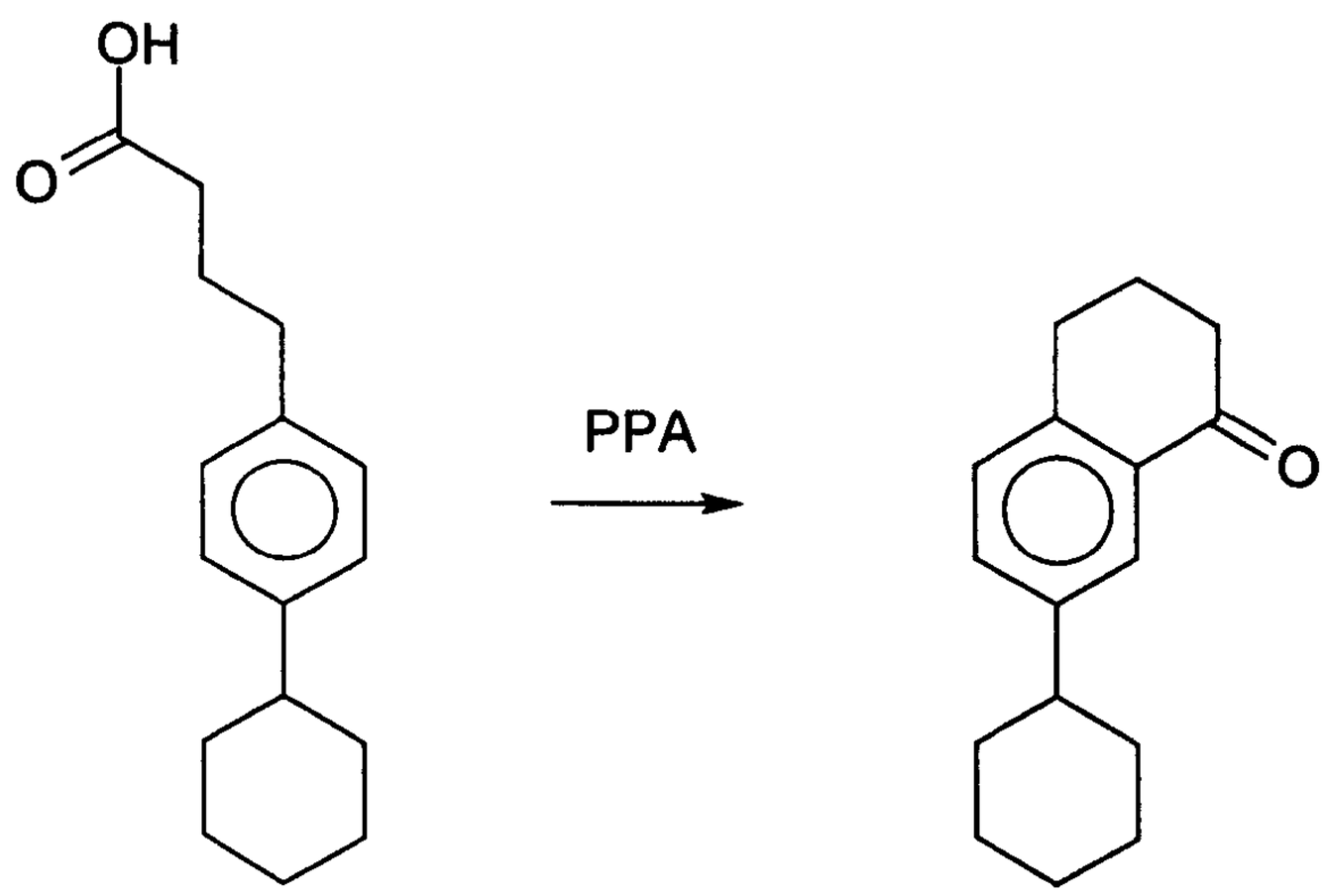


Figure 3.11c DEPT NMR spectrum of 4-phenyl(4'-cyclohexyl)butanoic acid

3.6 Synthesis of 7-cylcohexyl-1-tetralone

3.6.1 Preparation

Synthesis of 7-cyclohexyl-1-tetralone (**III**) was achieved by cyclisation of 4-phenyl(4'-cyclohexyl)butanoic acid (**II**) with hot polyphosphoric acid (March, 1985; Vogel, 1989). The gas chromatogram (Figure 3.12) shows the product was formed in reasonable purity (>94 % by GC), and the reaction proceeded with high yield (80%).



II **III**

3.6.2 Characterisation

The mass spectrum of 7-cylohexyl-1-tetralone (**III**) (Figure 3.13) showed the molecular ion $M^{+\cdot}$ at *m/z* 228 (base peak), along with diagnostic ions *m/z* 200 ($M^{+\cdot}$-CO) and *m/z* 185 ($M^{+\cdot}$-CH_3O).

Analysis by IR spectroscopy revealed the loss of absorptions at 3423 cm^{-1} and 1708 cm^{-1}, indicative of the carboxylic acid group, and the introduction of an absorption at 1685 cm^{-1} consistent with the presence of a ketone group (νC=O). The infra red spectrum of the tetralone (Figure 3.14) also showed spectral bands expected from the aliphatic and aromatic features of the compound. From the aliphatic part of the molecule the strong C-H

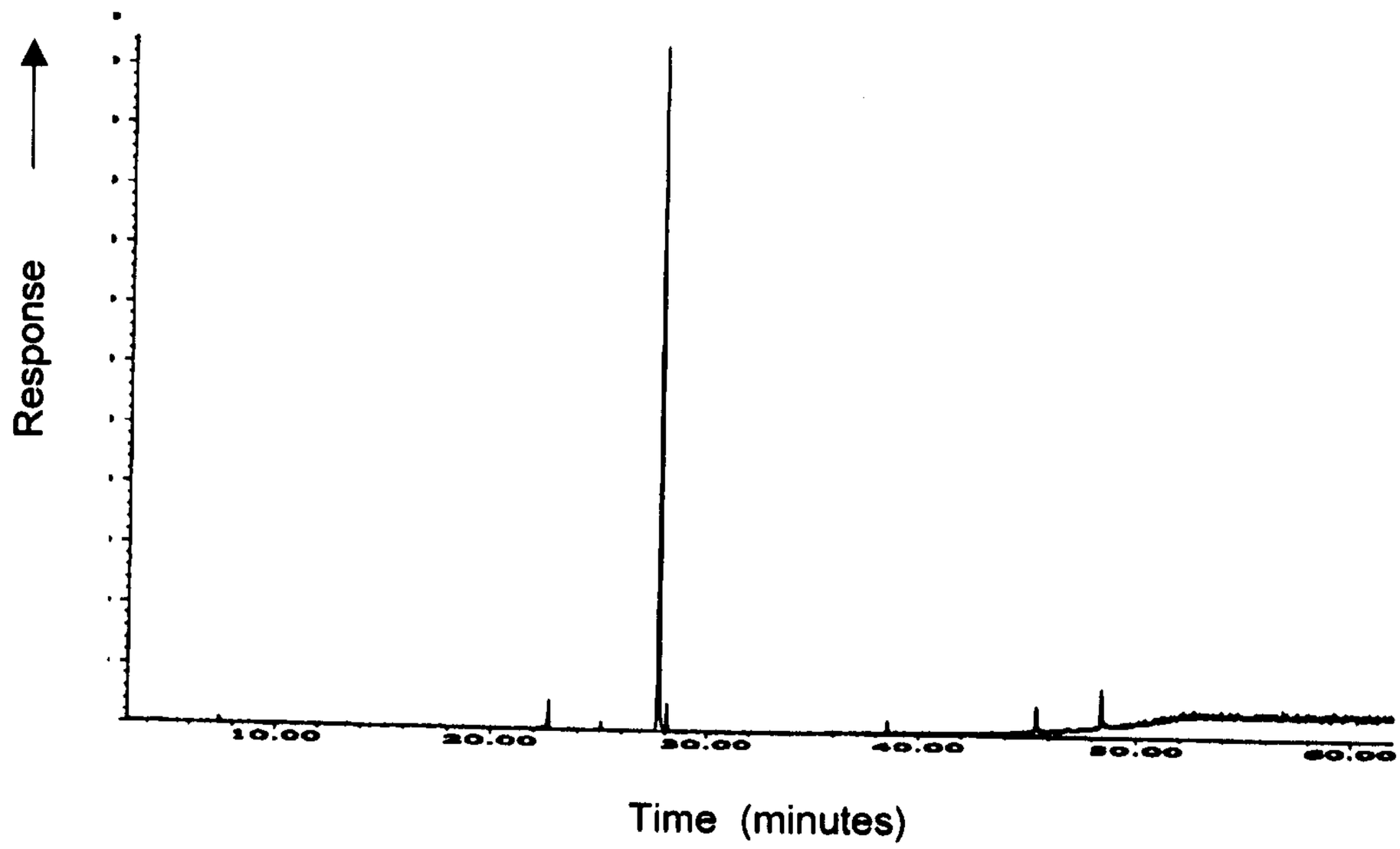


Figure 3.12 Gas chromatogram of 7-cyclohexyl-1-tetralone

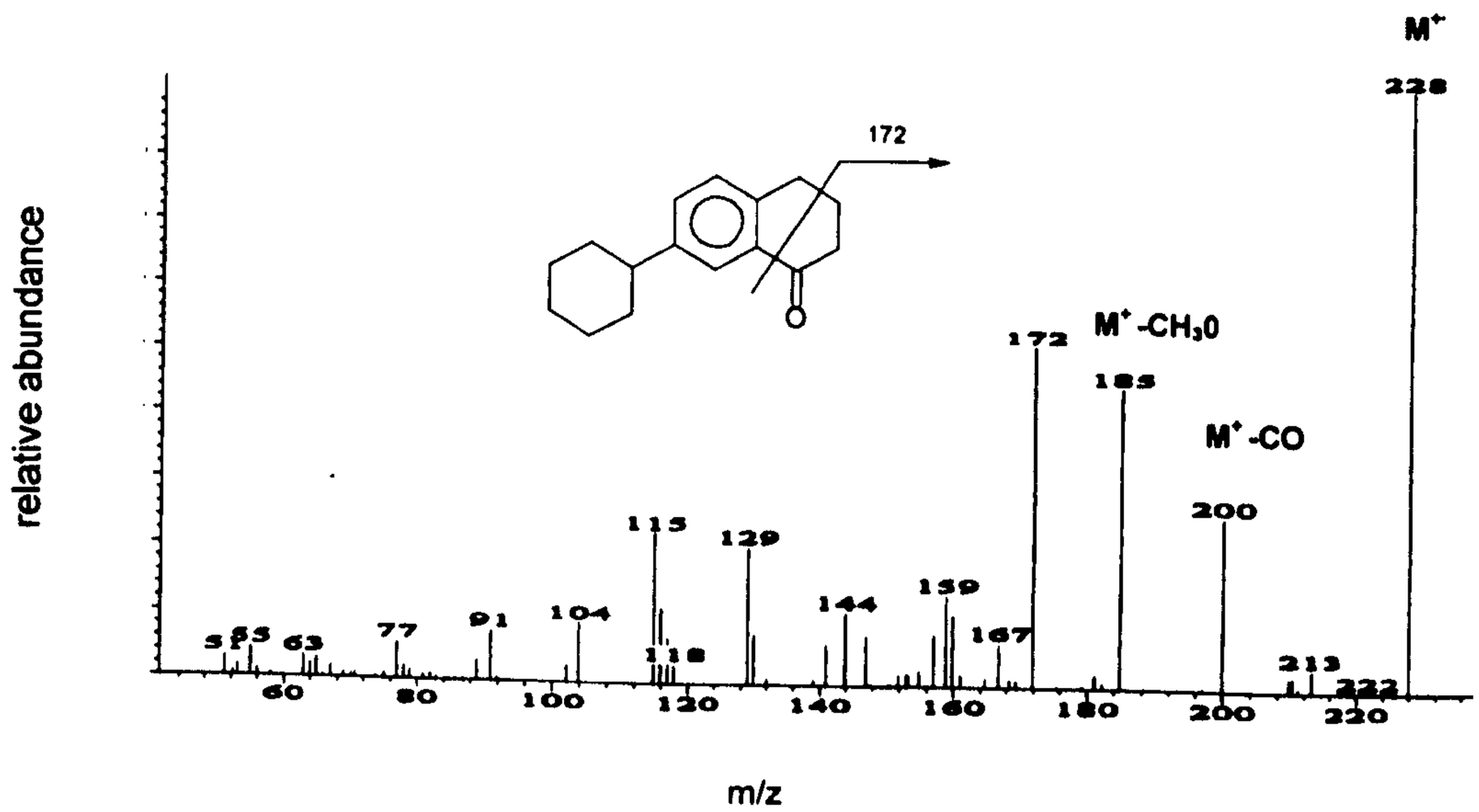


Figure 3.13 Mass spectrum of 7-cylohexyl-1-tetralone

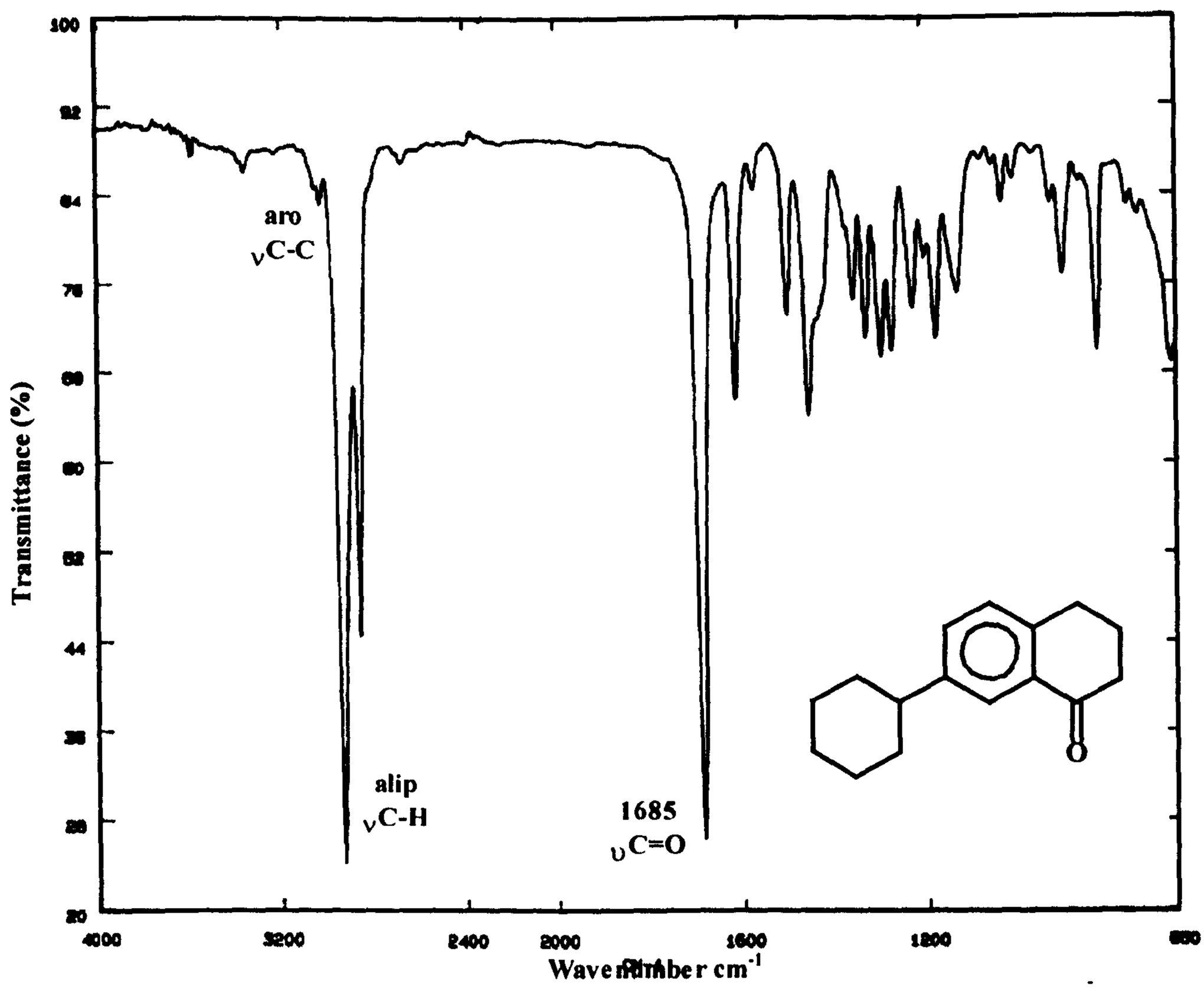


Figure 3.14 Infra-red spectrum of 7-cylohexyl-1-tetralone (liquid film using NaCl disc)

stretches at 2923 and 2850 cm^{-1} are visible, and the weaker band at 3032 cm^{-1} due to the aromatic C-H stretch is present.

The ^{1}H-NMR spectrum is presented in Figure 3.15a together with the assignments. The aromatic protons **i**, **j** and **g** are found between 7 and 8 ppm. The singlet corresponding to **g** is present at 7.9 ppm, shifted downfield from the doublets corresponding to **i** and **j** at 7.3ppm and 7.1 ppm respectively, due to the shielding effect of a carbonyl in a β position. In the aliphatic region the two triplets assigned to **b** and **d** at 2.9 and 2.7 ppm are shifted downfield compared to the quintet at 2.1 ppm corresponding to **c** due to the proximity of

both the carbonyl and the benzene ring. The two multiplets at 1.8 and 1.4 ppm represent the protons of the cyclohexyl moiety (**l**, **m**, **n**), except for **k** which is present further downfield, because of the deshielding effect of the adjacent aromatic ring, as a broad multiplet at 2.5 ppm.

The ^{13}C-NMR spectrum (Figure 3.15b) contained thirteen resonances and was interpreted in conjunction with the DEPT sequence (Figure 3.15c). The carbonyl carbon **a** was present at the characteristic chemical shift of 199 ppm in the ^{13}C spectrum but absent from the DEPT sequence, consistent with a quaternary carbon. In the aromatic region there were 5 resonances attributed to the 6 carbons in the benzene ring. Those at 147 ppm and 142 ppm were not present in the DEPT sequence, *i.e.* were quaternary carbons, and were therefore assigned to **e**, **f** and **h**. It is likely that the resonance at 147 ppm was due to **f** owing to the influence of the adjacent carbonyl group, and that **e** and **h** resonate at 142 ppm due to their similar chemical environments. Thus, the resonances at 132 ppm, 129 ppm and 125 ppm correspond to the remaining aromatic carbons **g**, **i**, and **j**. Likewise, the methine carbon present at 44 ppm can be allocated to the resonance of carbon **k** of the cyclohexyl ring. Remaining carbons present in the cyclohexyl ring were present in the region between 20 and 40 ppm and were tentatively assigned on the basis of intensity and proximity to the ketone group.

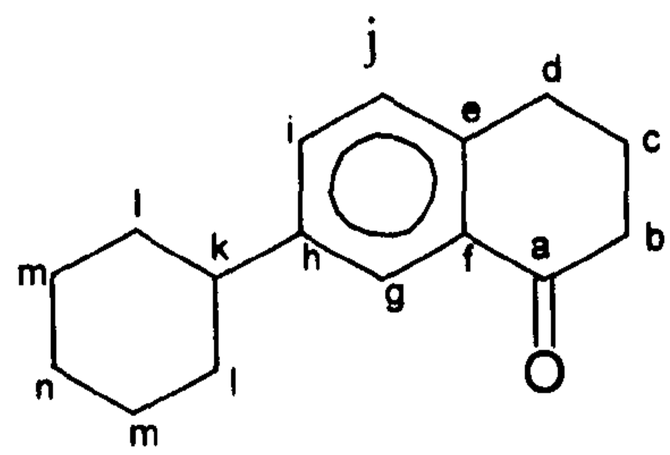


Figure 3.15 Assignment key for the NMR spectra of 7-cylohexyl-1-tetralone

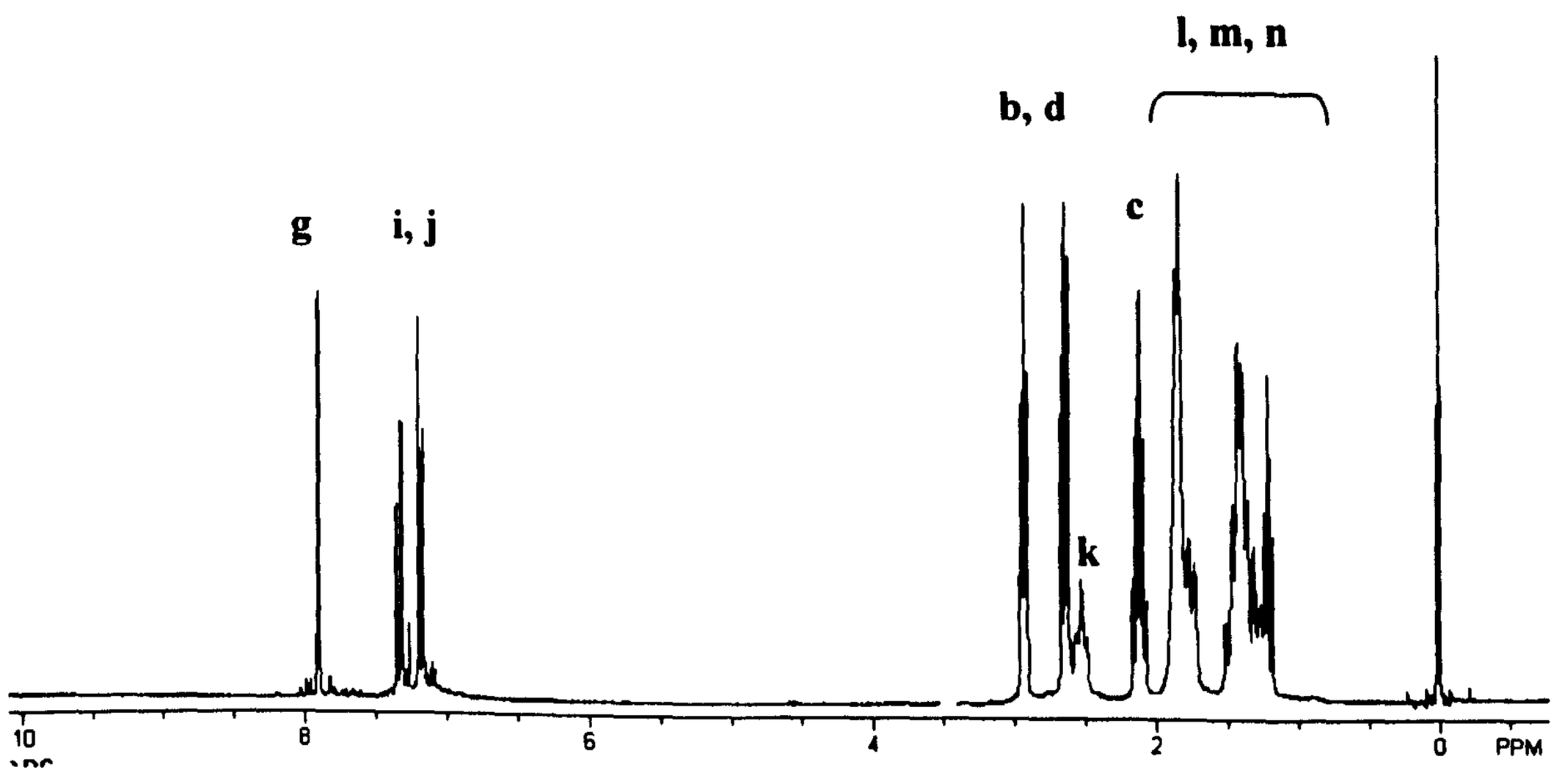


Figure 3.15a Proton NMR spectrum of 7-cylohexyl-1-tetralone

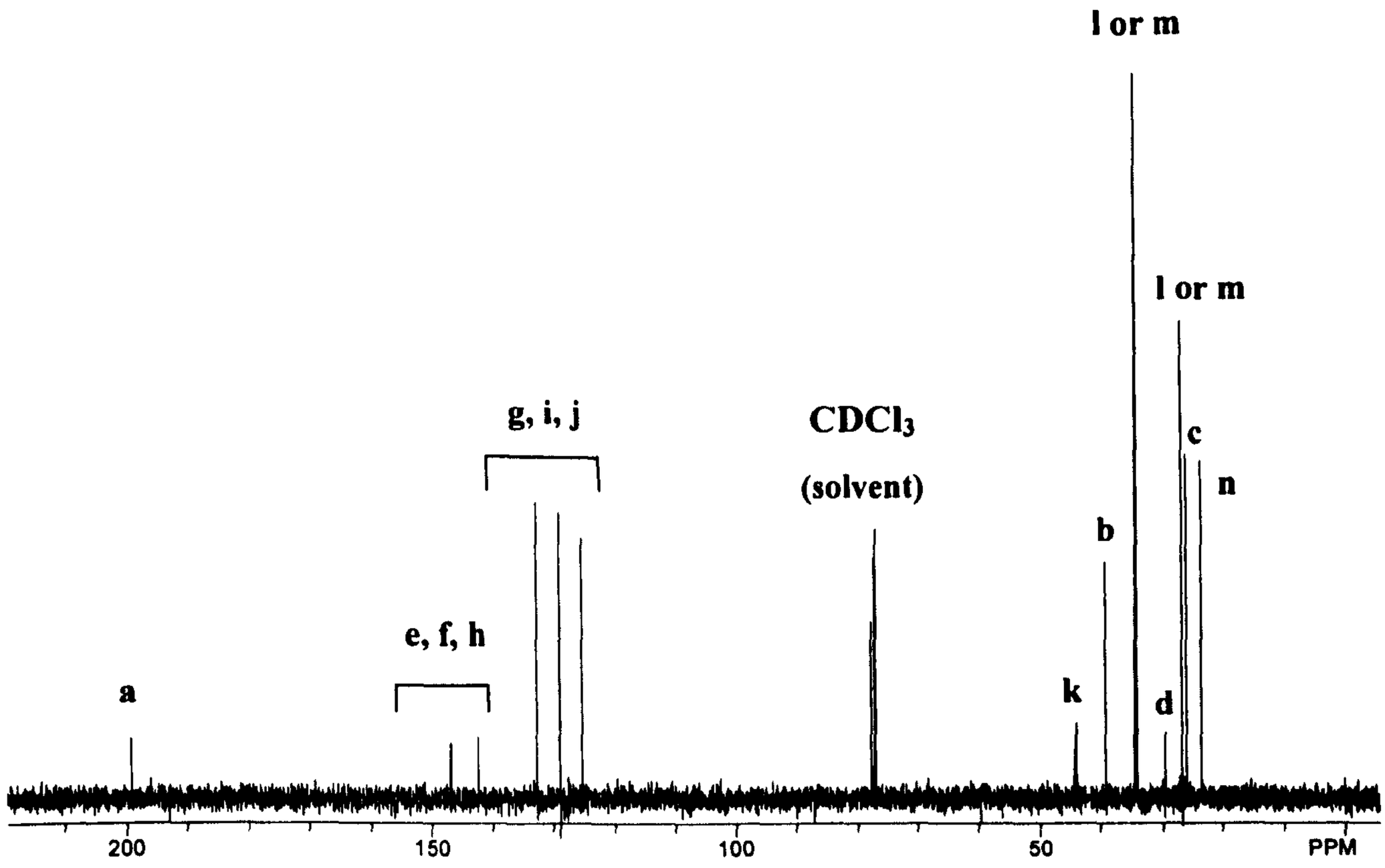


Figure 3.15b ^{13}Carbon NMR spectrum of 7-cylohexyl-1-tetralone

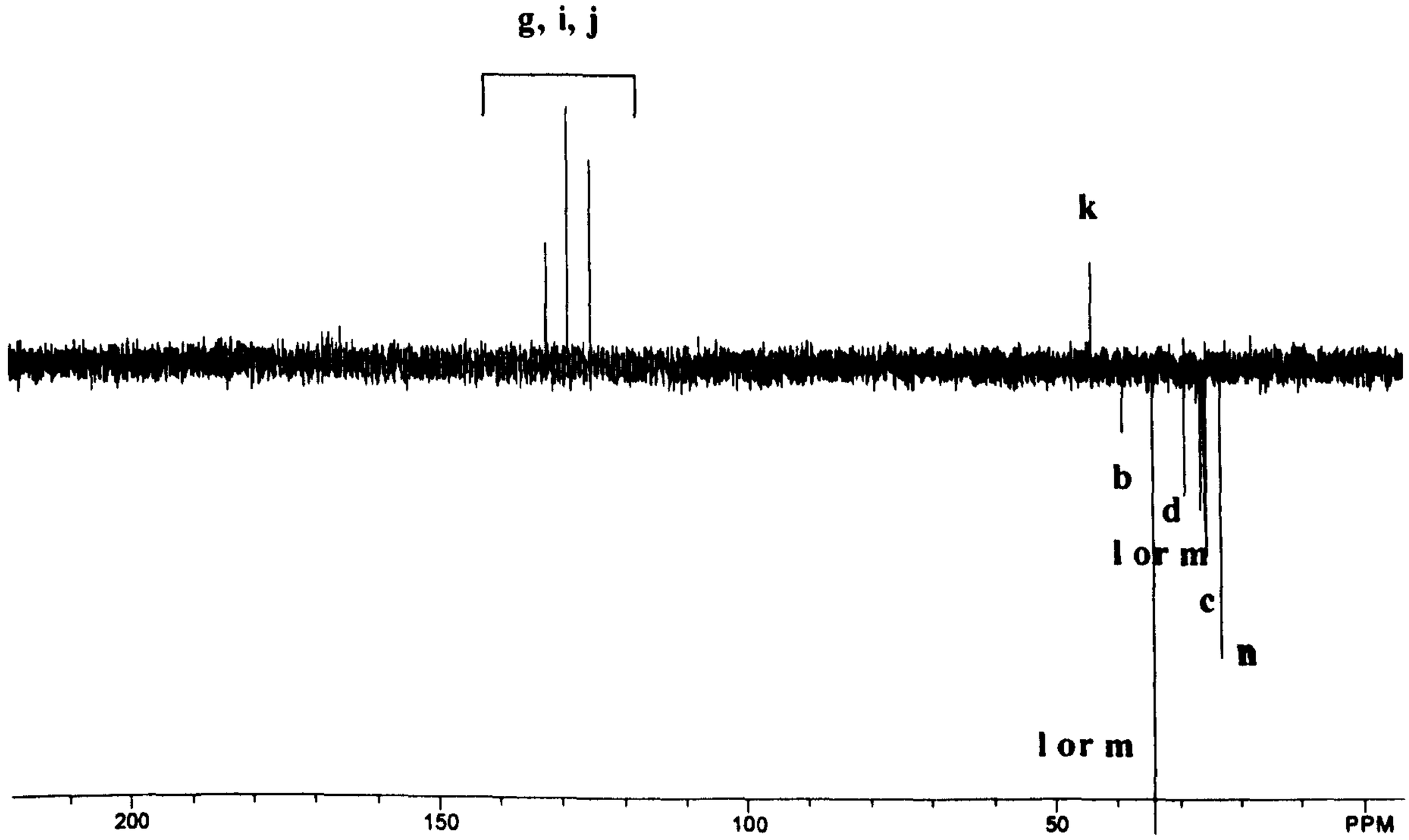
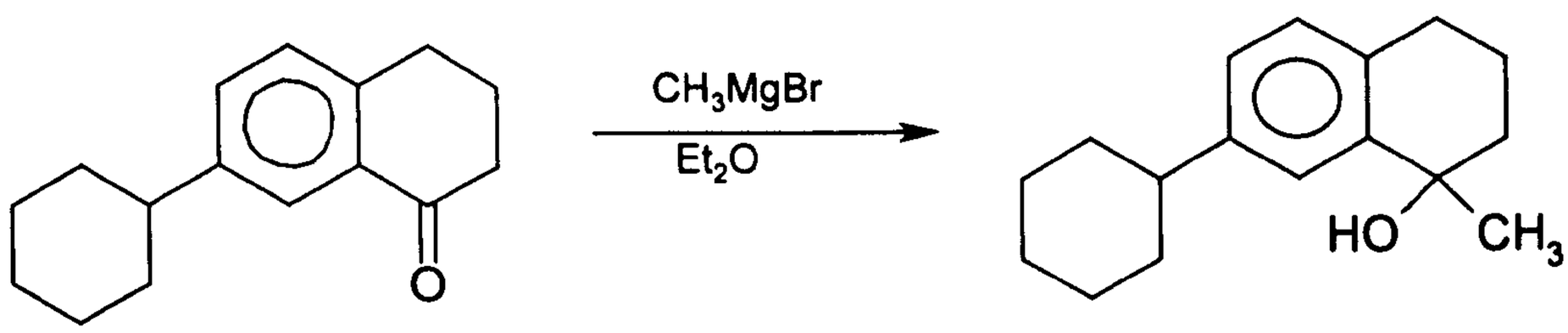


Figure 3.15c DEPT NMR spectrum of 7-cylohexyl-1-tetralone

3.7 Synthesis of 7-cyclohexyl-1-hydroxy-1-methyltetralin

3.7.1 Preparation

An alkyl substituent was added to the tetralone *via* a Grignard reaction, a method which has been widely used previously (Vogel, 1989). This involved nucleophilic attack of the carbonyl by methyl magnesium bromide which reacted to form the tertiary alcohol. Grignard reactions are extremely moisture sensitive, therefore moisture was excluded by carrying out the reaction under a blanket of nitrogen, using glassware that had been oven dried at 120°C overnight, and employing calcium chloride guard tubes.



III **IV**

3.7.2 Characterisation

7-cyclohexyl-1-hydroxy-1-methyltetralin (**IV**) was synthesised in good yield (78 %) and a purity of 94 %. The gas chromatogram of the TMS ester of the reaction product is presented in Figure 3.16. The reaction products were identified using mass spectrometry (Figure 3.17). The target alcohol was identified by the diagnostic ions *m/z* 301 ($M^{+\cdot}$-CH_3) either from loss of a methyl group from the trimethylsilyl derivative or loss of the newly added methyl group, and the peak at *m/z* 226 ($M^{+\cdot}$- $HO^{+}TMS$). IR analysis of the reaction products (Figure 3.18) showed a band at 3366 cm^{-1}, characteristic of an OH stretch, indicating that the Grignard reaction was successful.

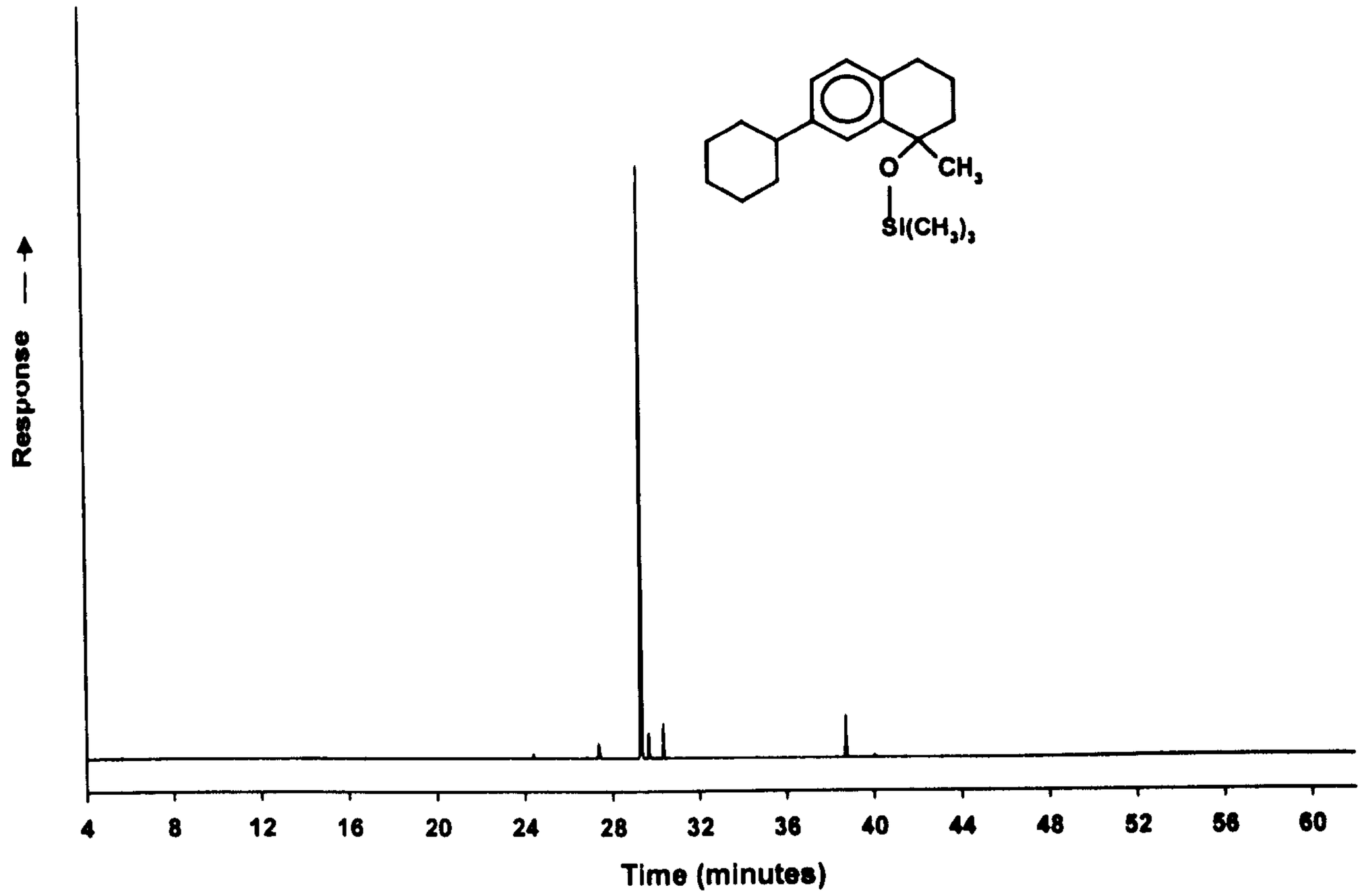


Figure 3.16 Gas chromatogram of 7-cyclohexyl-1-hydroxy-1-methyltetralin (as TMS ester)

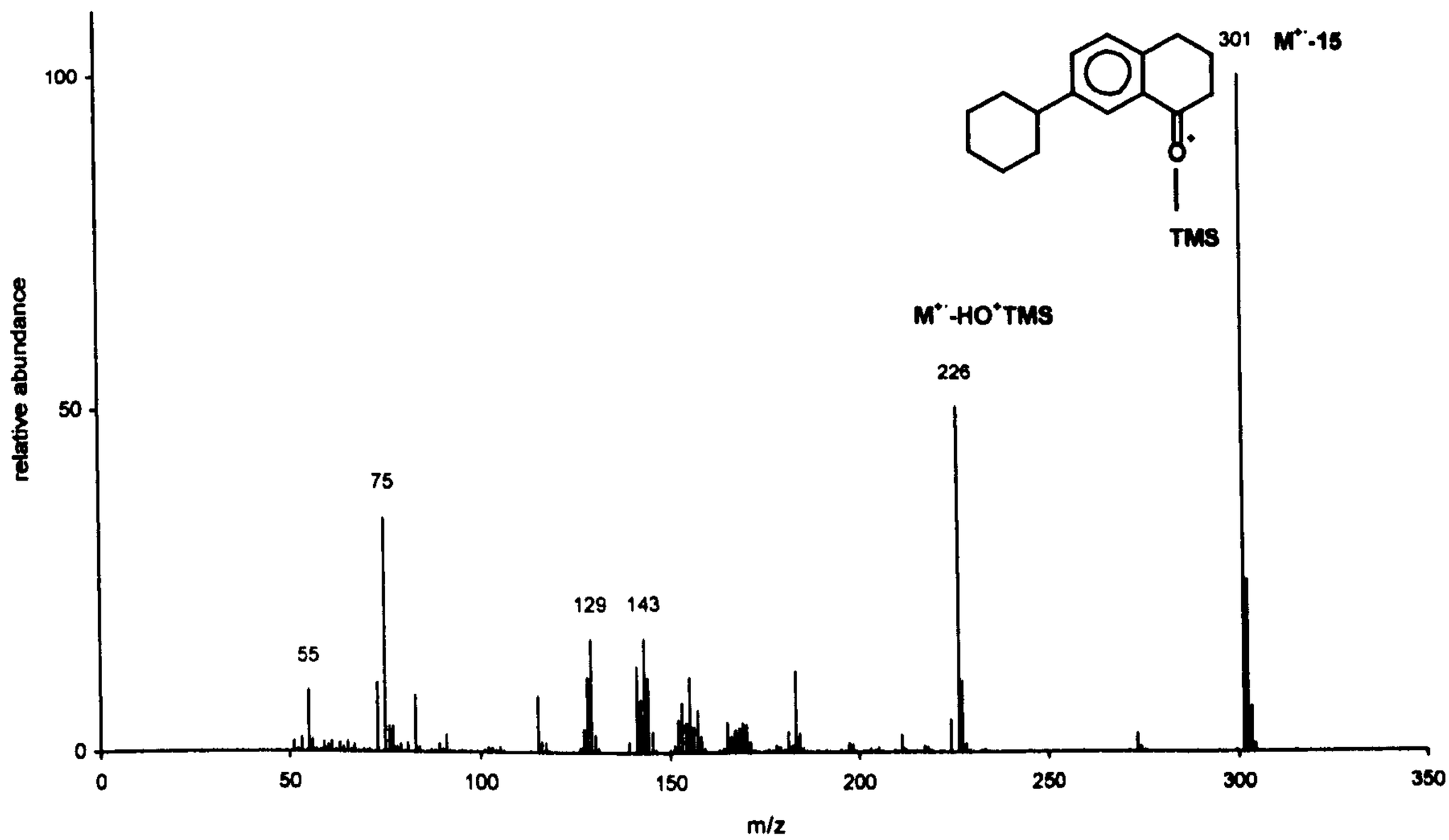


Figure 3.17 Mass spectrum of 7-cyclohexyl-1-hydroxy-1-methyltetralin

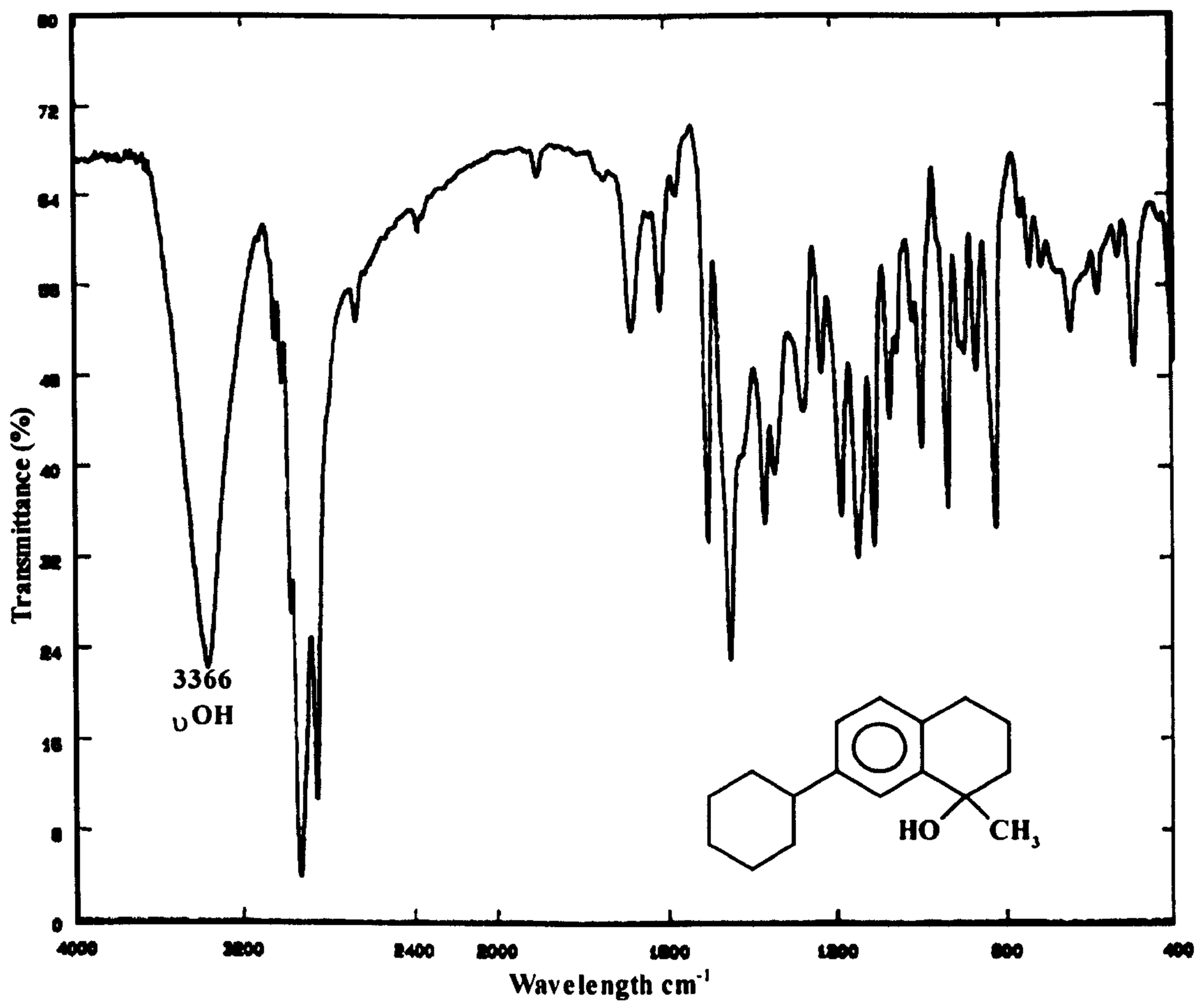


Figure 3.18 Infra-red spectrum of 7-cyclohexyl-1-hydroxy-1-methyltetralin (liquid film using NaCl disc)

The ^{1}H, ^{13}C and DEPT sequence spectra all show that the Grignard reaction was successful and the methyl chain had been added to the cyclohexyltetralin 'base' structure in the β position. The ^{1}H spectrum (Figure 3.19a) indicates the introduction of the methyl group by a singlet present at 1.5 ppm (**a**). The aromatic protons **i**, **j** and **h** are present with the singlet representing **g** shifted downfield due to the hydroxyl group in the β position. The protons at positions **e** and **l** present at 2.4 and 2.7 ppm are shifted downfield from the rest of the aliphatic protons present as a broad multiplet between 1-2 ppm due to the proximity of the aromatic ring. ^{13}C-NMR and DEPT studied in combination allowed full characterisation of

the reaction product (Figure 3.19b, c). The key feature of the ^{13}C and DEPT spectra is the introduction of a methyl carbon in the aliphatic region of the spectrum which can be assigned to the carbon of the methyl substituent (**a**). The downfield shift of **b** compared to the rest of the carbons is due to the deshielding effect of the neighbouring hydroxyl group. The 6 carbons of the aromatic ring are found between 120-150 ppm. The quaternary carbons f, g and k are not present in the DEPT sequence leaving the three resonances for **i**, **j** and **h**. The other CH carbon (l) is distinguished by the DEPT sequence at 44 ppm. The rest of the CH_2 carbons (**c**, **d**, **e**, **m**, **n**, **o**) are present as 6 resonances in the aliphatic region with **e** being shifted downfield due to the proximity of the aromatic ring.

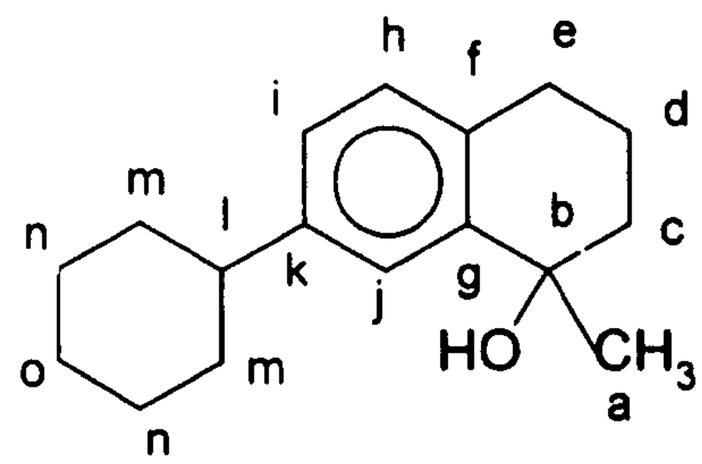


Figure 3.19 Assignment key for the NMR spectra of 7-cyclohexyl-1-hydroxy-1-methyltetralin

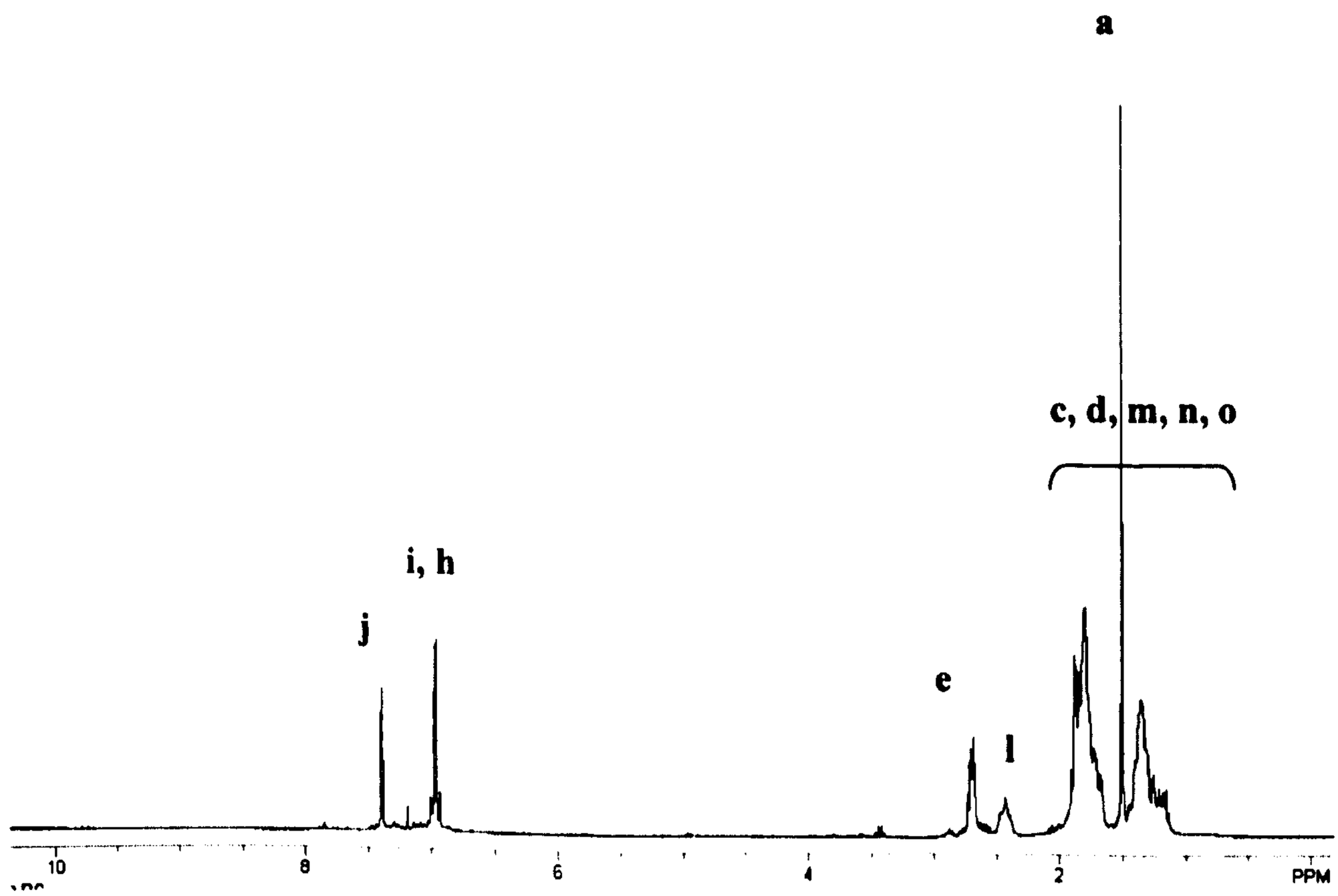


Figure 3.19a Proton NMR spectrum of 7-cyclohexyl-1-hydroxy-1-methyltetralin

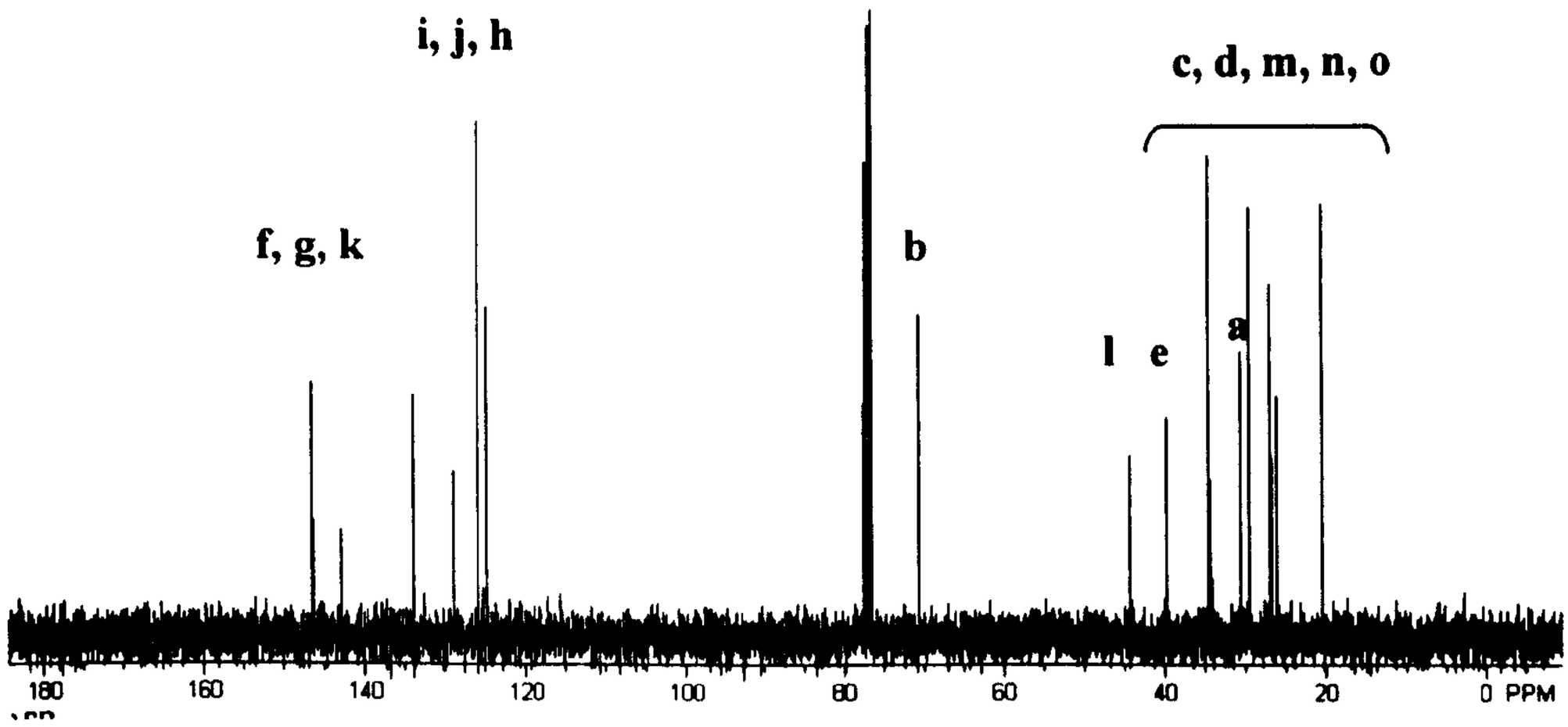


Figure 3.19b ^{13}Carbon NMR spectrum of 7-cyclohexyl-1-hydroxy-1-methyltetralin

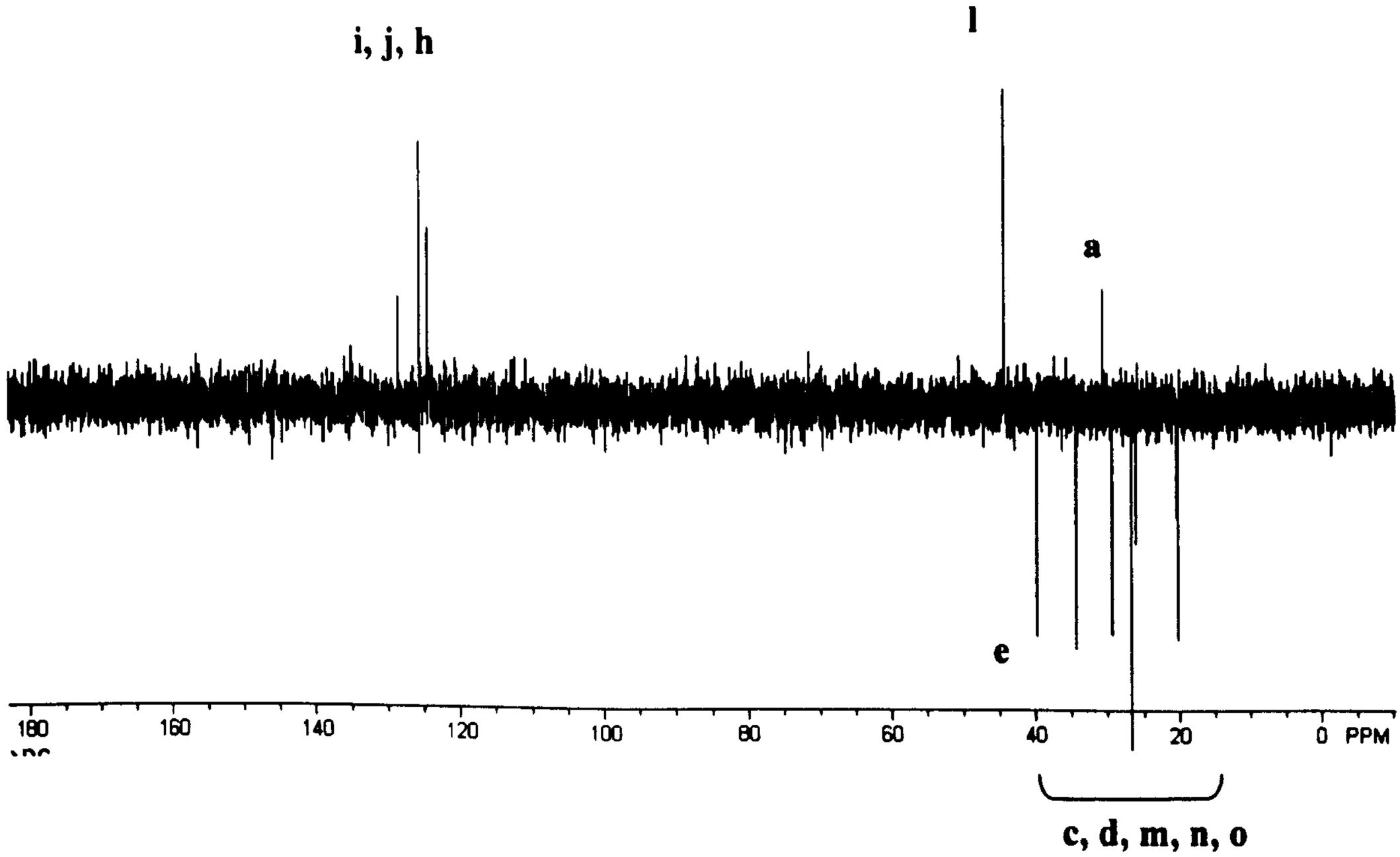
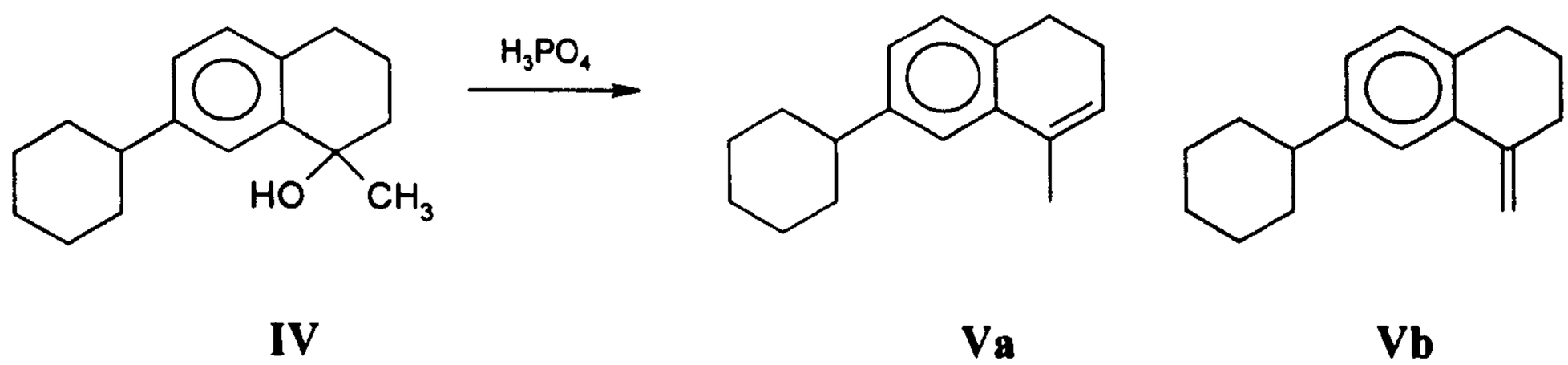


Figure 3.19c DEPT NMR spectrum of 7-cyclohexyl-1-hydroxy-1-methyltetralin

3.8 Synthesis of an isomeric mixture of 7-cyclohexyl-1-methyl-3,4-dihydronaphthalene (Va) and 7-cyclohexyl-1-methenyltetralin (Vb)

3.8.1 Preparation.

The alcohol, 7-cyclohexyl-1-hydroxyl-1-methyltetralin (**IV**) was dehydrated to an isomeric mixture of alkenes, 7-cyclohexyl-1-methyl-3,4-dihydronaphthalene (**Va**) and 7-cyclohexyl-1-methyenyltetralin (**Vb**) using orthophosphoric acid. The crude reaction products (thick brown oil) were further purified using open column chromatography on fully activated silica and elution with hexane resulting in a clear oil.



3.8.2 Characterisation

The gas chromatogram of the reaction products (Figure 3.20) contained one major peak. The mass spectrum (Figure 3.21) demonstrated a molecular ion at *m/z* 226 along with a peak at *m/z* 224 ($M^{+\cdot}$-2). It is likely that 7-cyclohexyl-1-methyl-3,4-dihydronaphthalene is the dominant product as this places the double bond in the alicyclic moiety which is the more stable of the two positions. This was confirmed by NMR spectroscopy. Confirmation that the alcohol (IV) had been dehydrated was provided by IR spectroscopy. The IR spectrum of the purified alkene mixture (Figure 3.22) no longer exhibited an absorption band at 3366 cm^{-1} due to the OH group but instead a small band is present at 3021 cm^{-1} relating to νC=C.

The ^1H NMR spectra exhibits a singlet at 2.1 ppm relating to the carbon **a** of the methyl group substituent and a multiplet at 5.8 ppm relating to the alkene proton **c**, thus indicating that 7-cyclohexyl-1-methyl-3,4-dihydronaphthalene (**Va**) was the dominant product. The

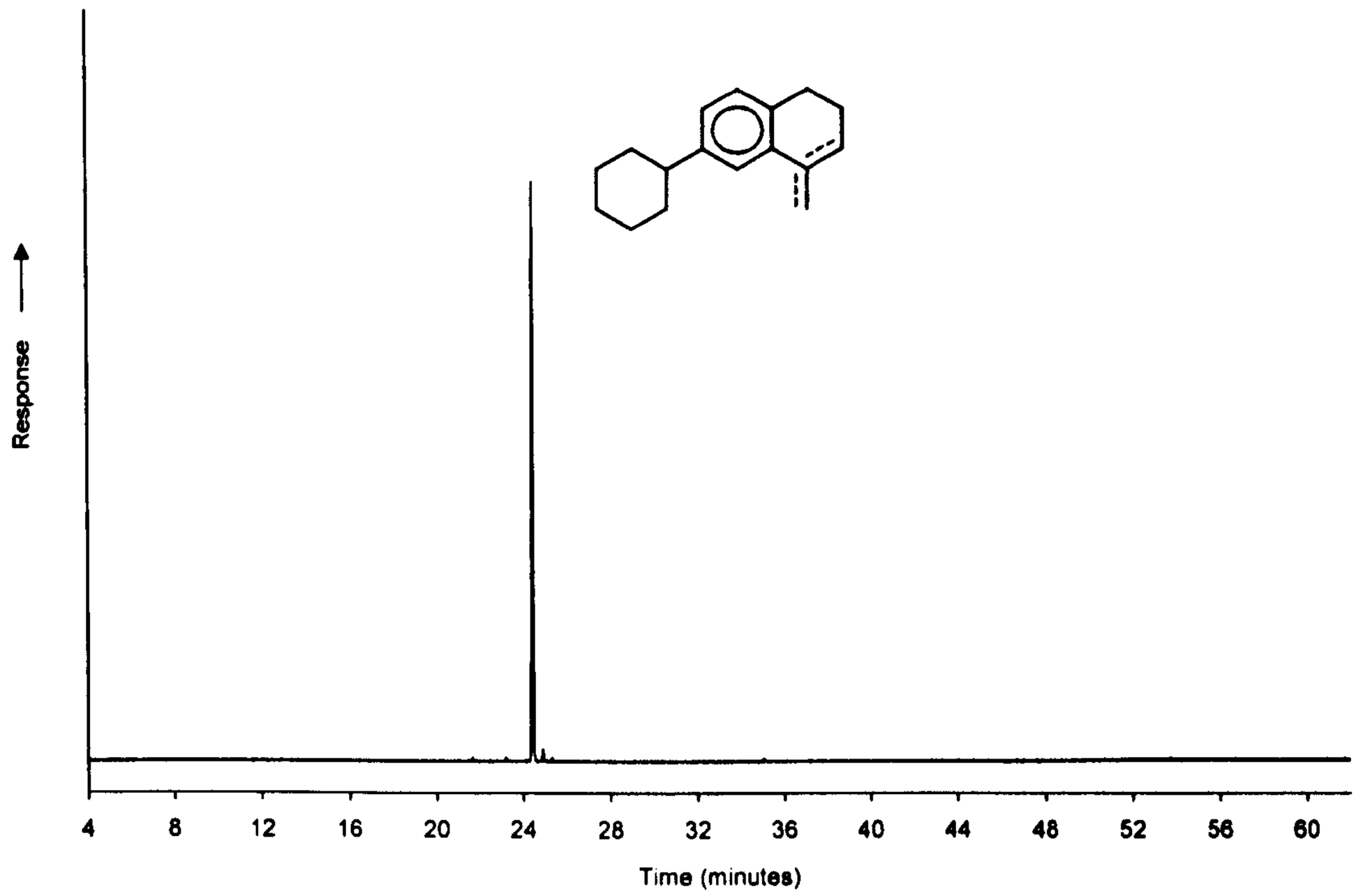


Figure 3.20 Gas chromatogram of 7-cyclohexyl-1-methenyltetralin and 7-cyclohexyl-1-methyl-3,4-dihydronaphthalene.

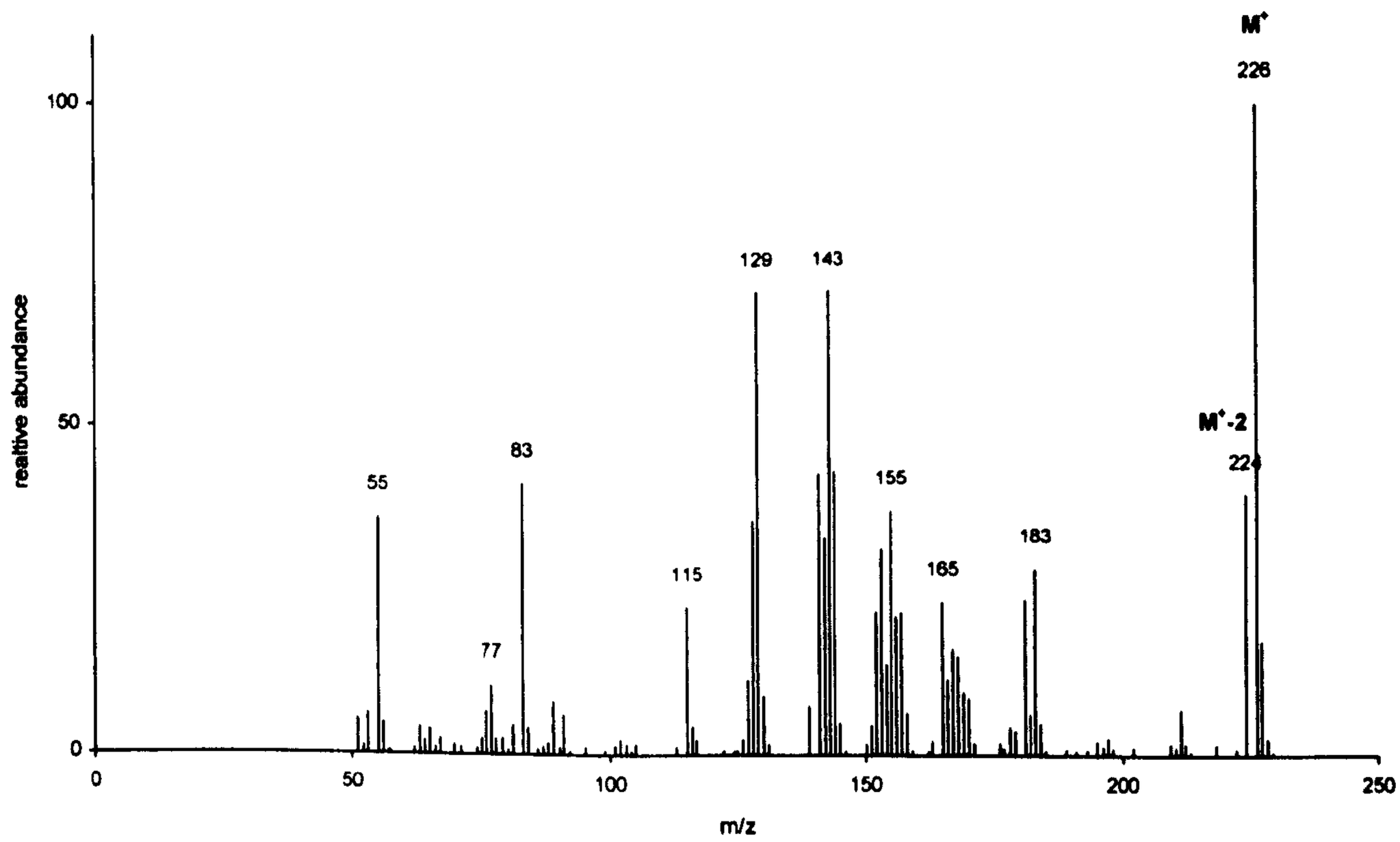


Figure 3.21 Mass spectrum of 7-cyclohexyl-1-methenyltetralin and 7-cyclohexyl-1-methyl-3,4-dihydronaphthalene.

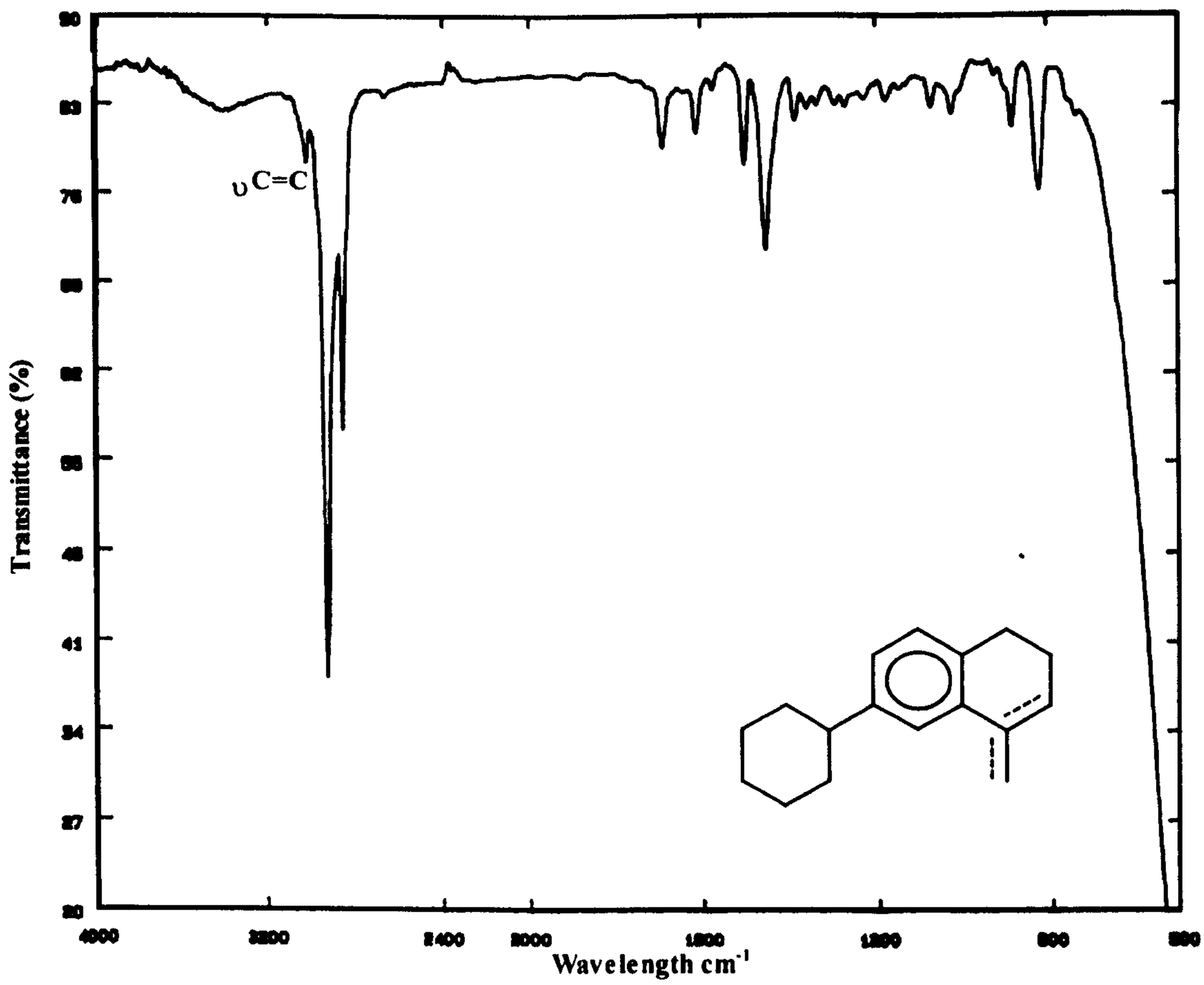


Figure 3.22 Infra-red spectrum of 7-cyclohexyl-1-methenyltetralin and 7-cyclohexyl-1-methyl-3,4-dihydronaphthalene (liquid film using NaCl disc)

singlet due to **a** has also been shifted downfield due to its proximity to the double bond. The aromatic protons (**i**, **h** and **j**) are present as a multiplet at 7 ppm and the aliphatic protons (**m**, **n** and **o**) are found as two broad multiplets between 1.1 and 2 ppm. The triplet at 2.7 ppm accounts for the proton at position **e** and the protons for **d** and **l** are seen as two multiplets between 2.2 and 2.6 ppm. Although the ^{13}C spectrum (Figure 3.23b) shows 6 resonances between 130 and 160 ppm, which would account for the six carbons of the aromatic ring, the DEPT sequence exhibits three CH carbons, relating to **h**, **i** and **j**, and a further CH carbon relating **c**. In the aliphatic region the CH carbon (**l**) and CH_3 carbon (**a**) were identified at 44 ppm and 28 ppm using the DEPT sequence. As a quarternary carbon,

b is missing from the DEPT sequence. The rest of the CH_2 carbons are only seen as 4 resonances suggesting that overlapping has occurred.

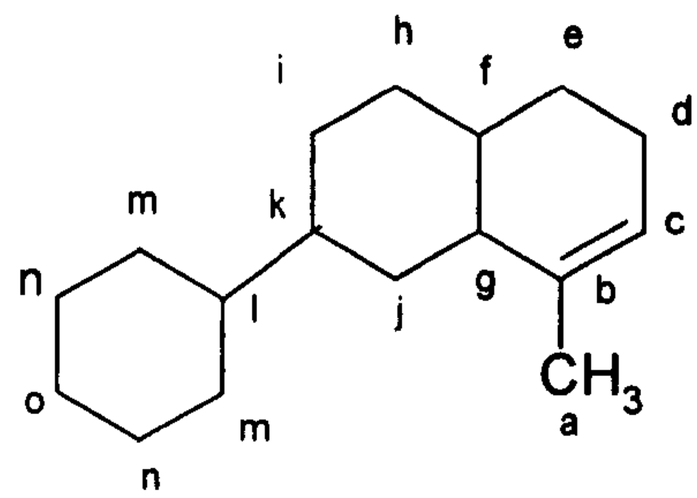


Figure 3.23 Assignment key for the NMR spectra of 7-cyclohexyl-1-methenyltetralin and 7-cyclohexyl-1-methyl-3,4-dihydronaphthalene.

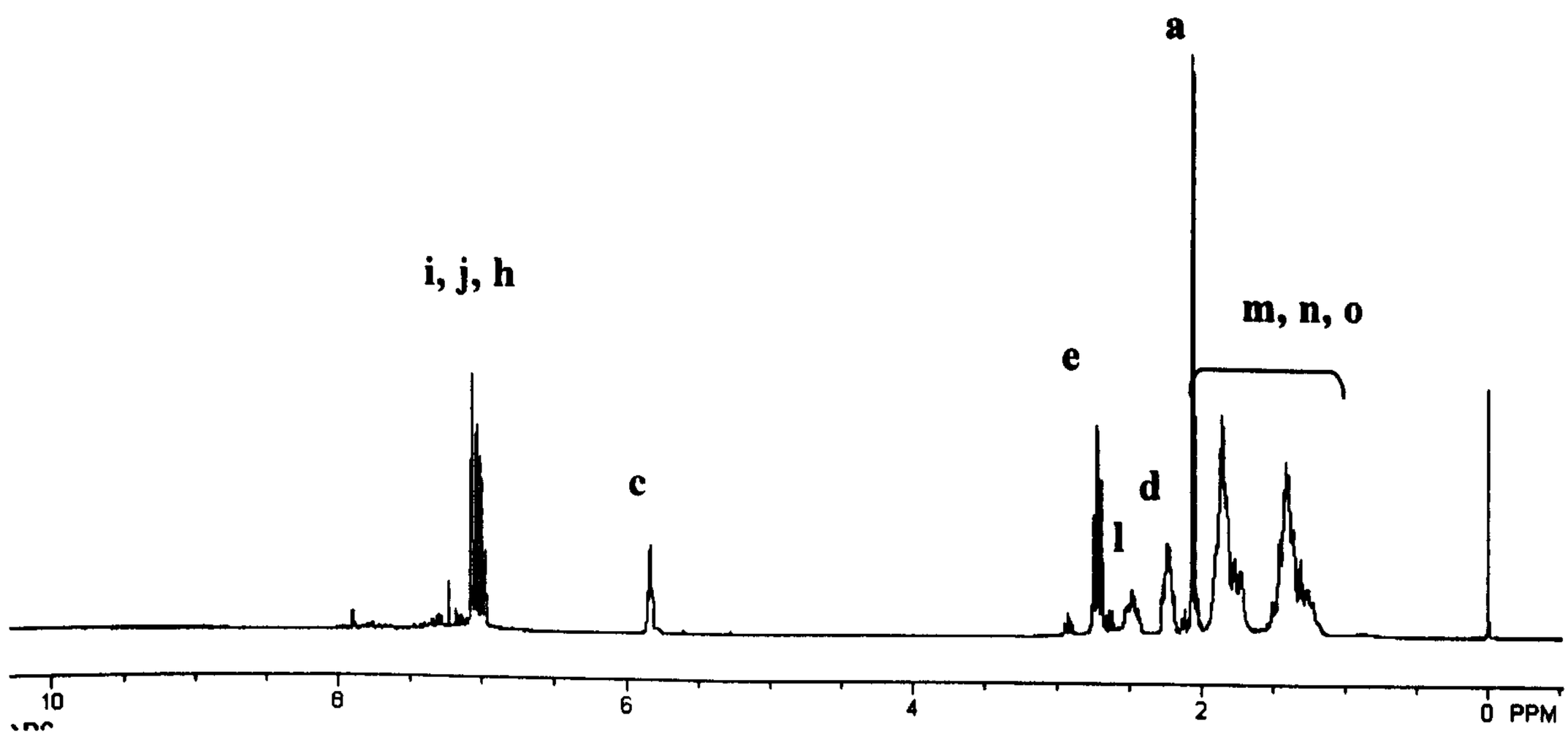


Figure 3.23a Proton NMR spectrum of 7-cyclohexyl-1-methenyltetralin and 7-cyclohexyl-1-methyl-3,4-dihydronaphthalene.

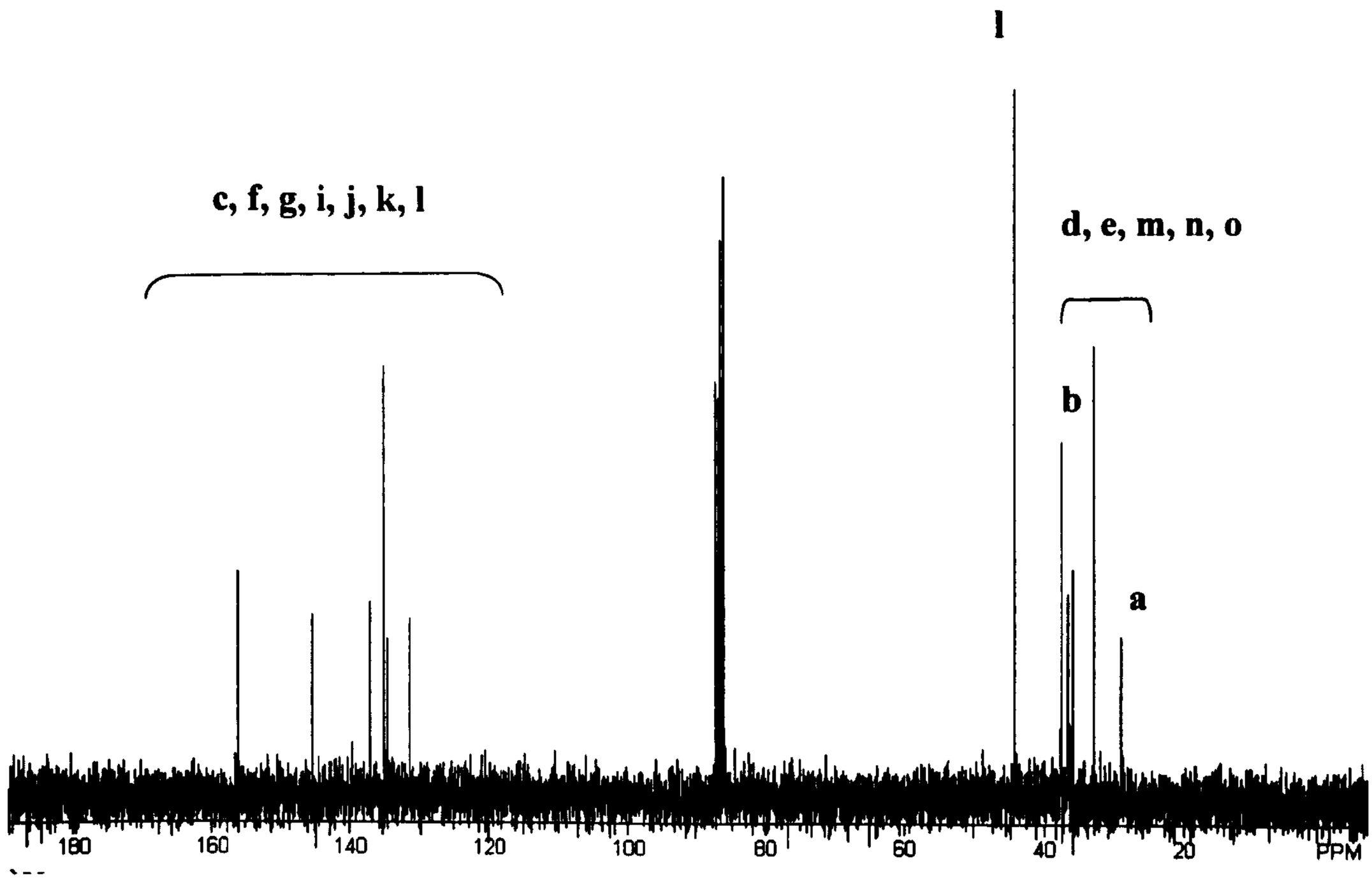


Figure 3.23b ^{13}Carbon NMR spectrum of 7-cyclohexyl-1-methenyltetralin and 7-cyclohexyl-1-methyl-3,4-dihydronaphthalene.

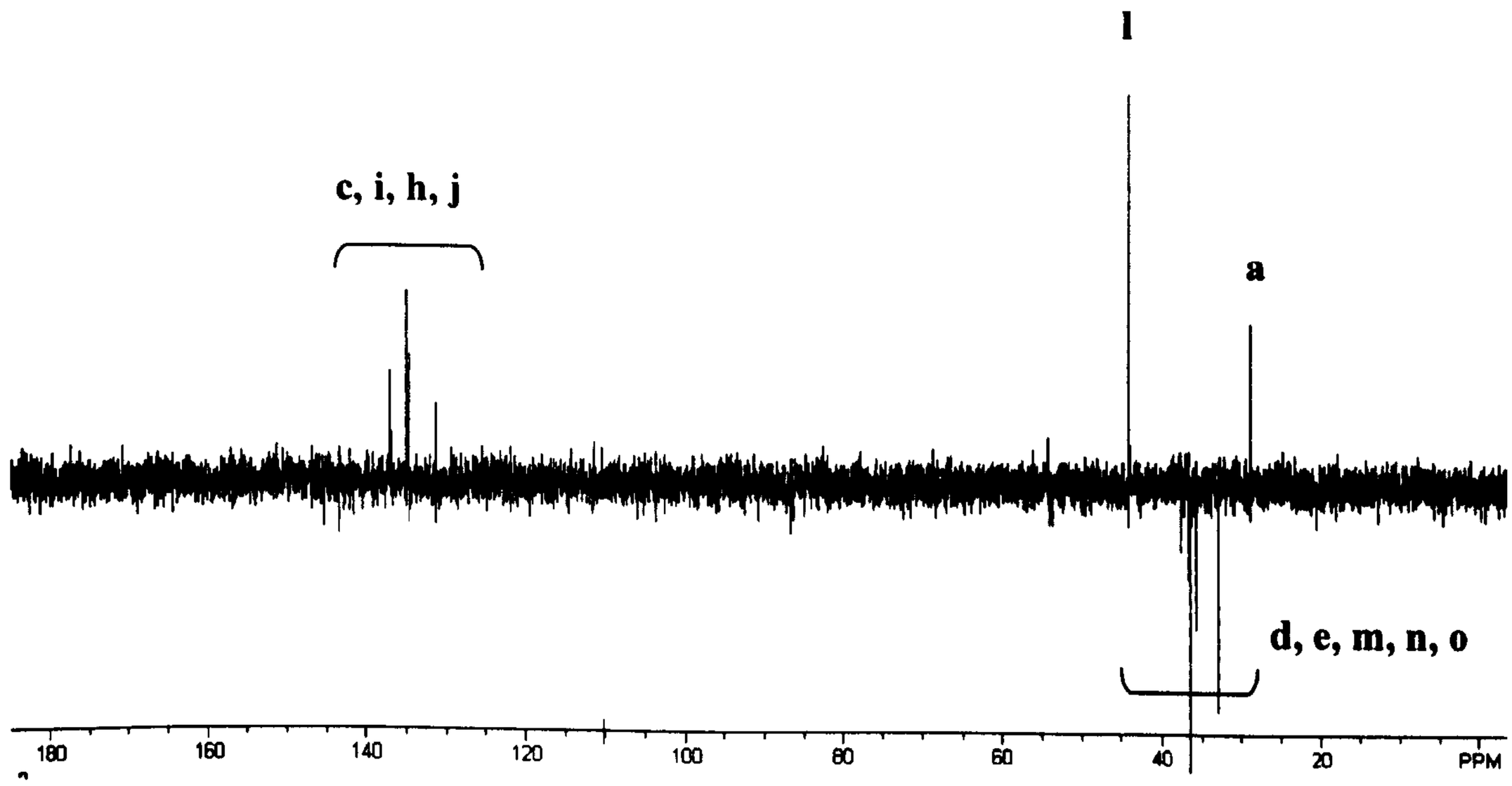
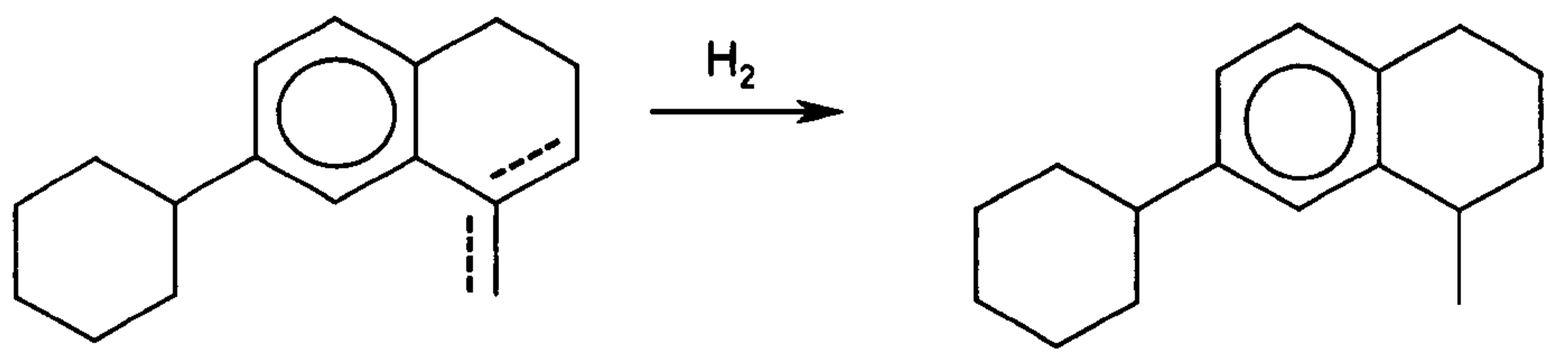


Figure 3.23.c DEPT NMR spectrum of 7-cyclohexyl-1-methenyltetralin and 7-cyclohexyl-1-methyl-3,4-dihydronaphthalene

3.9 Synthesis of 7-cyclohexyl-1-methyltetralin

3.9.1 Preparation

The purified alkenes (**V**) were hydrogenated to the target compound, 7-cyclohexyl-1-methyltetralin (**VI**), by bubbling hydrogen through a solution of the alkenes in the presence of Adam's catalyst. Isolation of the target compound from any diaromatic compounds formed was achieved by Ag^+ open column chromatography. 7-cyclohexyl-1-methyltetralin was synthesised in good yield (75 %) and purity (>99% by GC) as shown in Figure 3.24.



V **VI**

3.9.2 Characterisation

The mass spectrum of 7-cyclohexyl-1-methytetralin presented in Figure 3.25 shows the molecular ion $M^{+\cdot}$ at *m/z* 228, and an ion at *m/z* 213 ($M^{+\cdot}$-15) corresponding to cleavage at the tertiary centre leading to loss of a methyl group. Figure 3.26 illustrates the relatively simple infrared spectrum of 7-cyclohexyl-1-methyltetralin. Aliphatic C-H stretches were observed at 2924 and 2850 cm^{-1} and the aromatic C-H stretch was visible at 3001 cm^{-1}, although they are poorly resolved. The group of bands around 1450 cm^{-1} are likely to be due to δC-H deformations of CH_2 and CH_3 groups in the molecule, with the absorption at 816 cm^{-1} probably being due to the aromatic substitution. Confirmation of the synthesis of 7-cyclohexyl-1-methyltetralin was provided by ^{13}C, DEPT and ^1H NMR spectroscopy. The ^1H NMR spectrum is presented in Figure 3.27.a. The aromatic protons (**h, i, j,**) are present as a multiplet at 7 ppm. The single small peak at 7.24 ppm is due to residual chloroform.

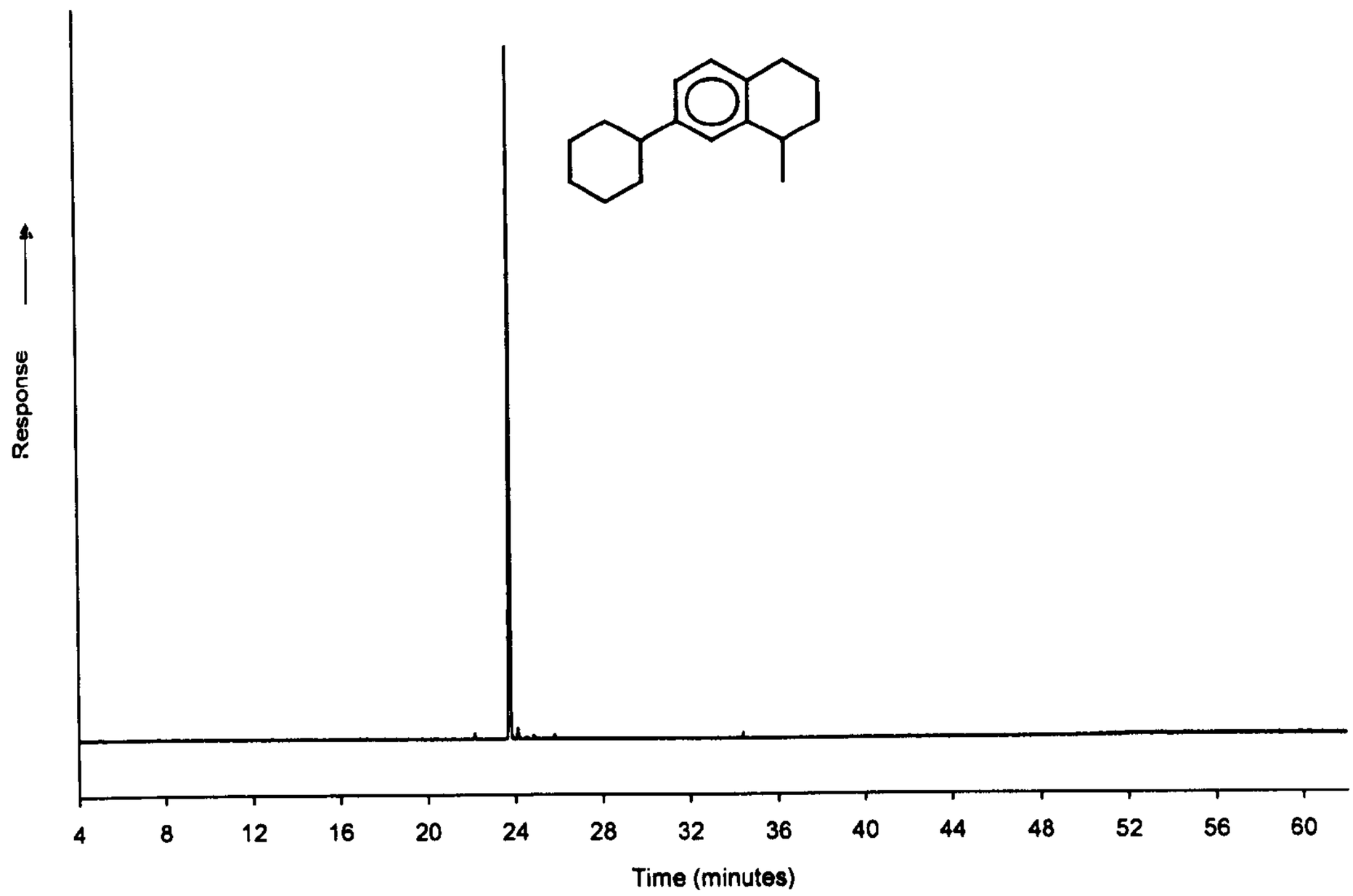


Figure 3.24 Gas chromatogram of 7-cyclohexyl-1-methyltetralin

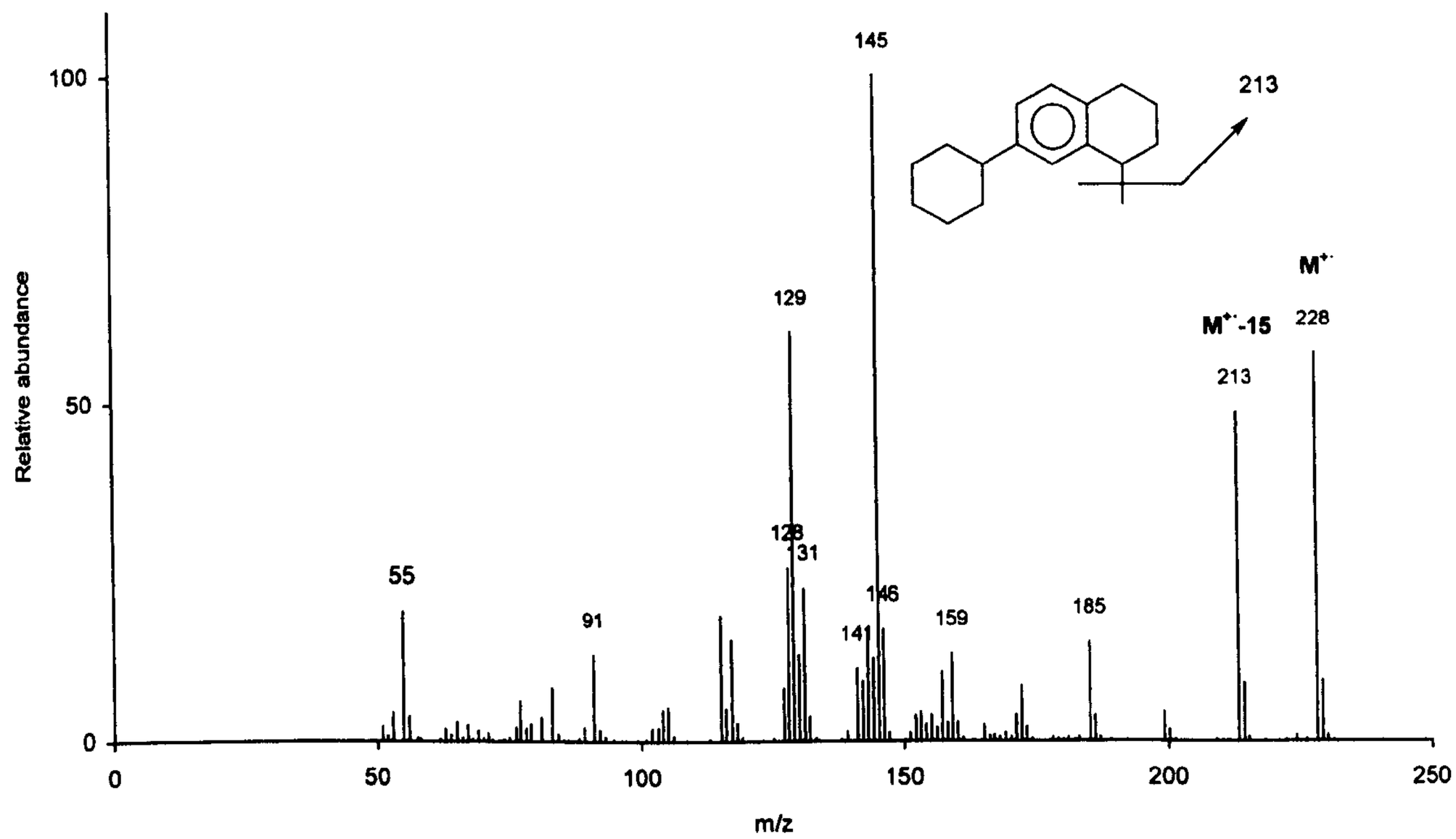


Figure 3.25 Mass spectrum of 7-cyclohexyl-1-methyltetralin

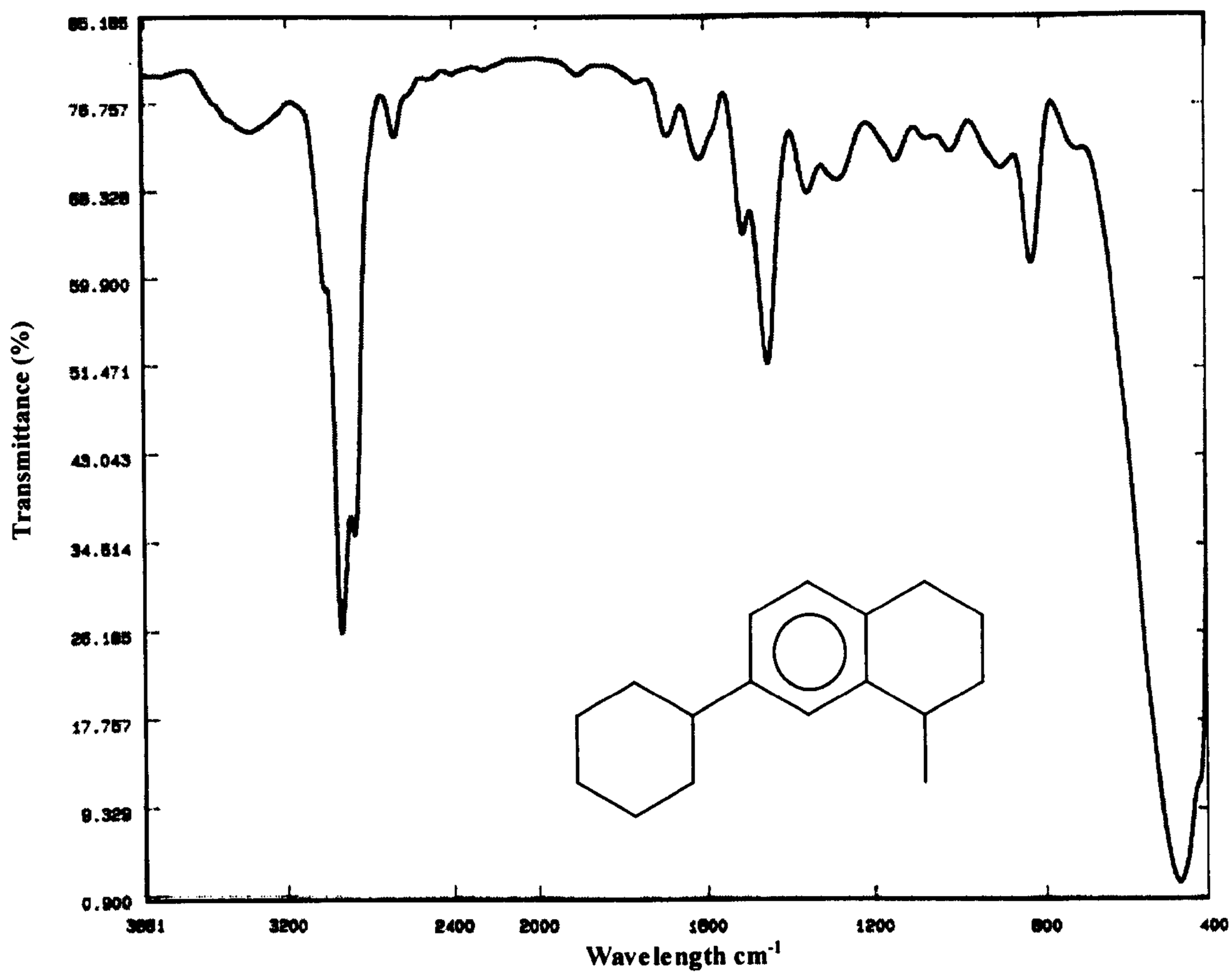


Figure 3.26 Infra-red spectrum of 7-cyclohexyl-1-methyltetralin (liquid film using NaCl disc)

The doublet at 1.3 ppm is due to the protons of the methyl group (**a**). The multiplets at 2.5, 2.7 and 2.9 ppm are likely to be due to the protons **l**, **b** and **e**, shifted downfield from the rest of the protons in the aliphatic region due to the adjacent aromatic ring. As the multiplet at 2.7 ppm is approximately twice the size of the other two it is more than likely to be **e**, the larger signal accounting for the two protons present. The rest of the protons cannot be distinguished but are accounted for by two broad multiplets from 1.4-1.9 ppm.

The ^{13}C spectrum in Figure 3.27.b. revealed the six resonances in the aromatic region of the aromatic ring. The quaternary carbons **f**, **g**, and **h** at 145, 142 and 134 ppm can be distinguished *via* the DEPT sequence (Figure 3.27.c.). Thus **h**, **i** and **j** are present at 129, 127 and 125 ppm. In the aliphatic region of the spectrum the DEPT sequence was also

used to determine the methine carbons **b** and **l** (33 and 44 ppm) and the methyl carbon **a** (22 ppm). The carbons **b** and **l** are shifted downfield due to the effect of the adjacent aromatic ring. Remaining methylene carbons between 15 and 40ppm **c**, **d**, **e**, **m**, **n**, **o** could not be unequivocally assigned and have therefore been grouped together.

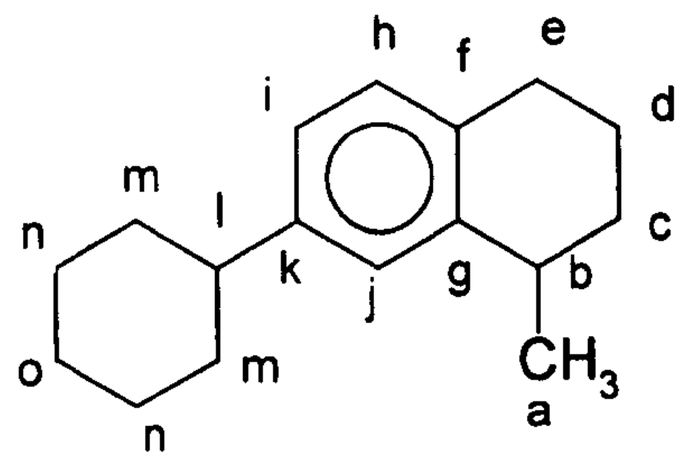


Figure 3.27 Assignment key for the NMR spectra of 7-cyclohexyl-1-methyltetralin

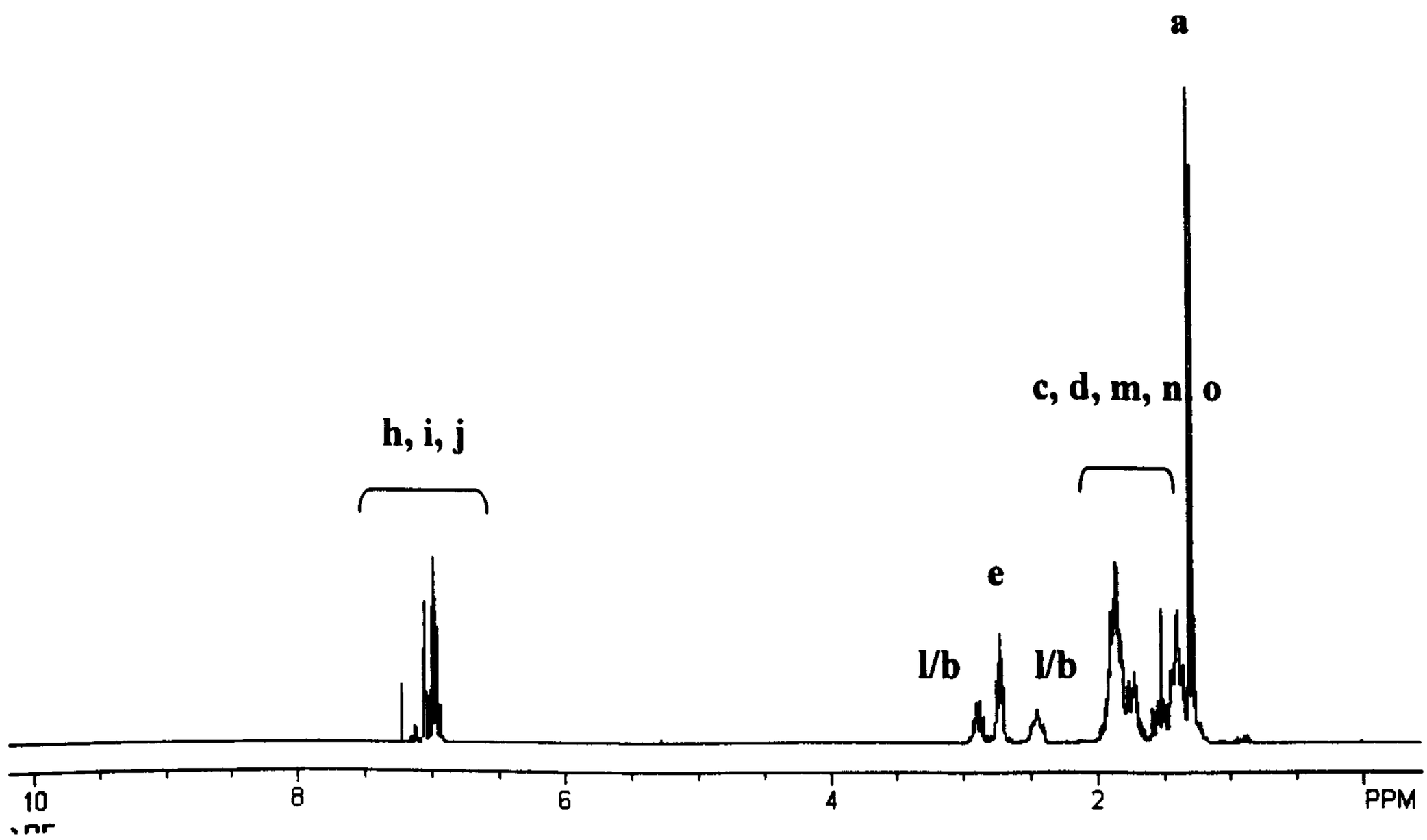


Figure 3.27a Proton NMR spectrum of 7-cyclohexyl-1-methyltetralin

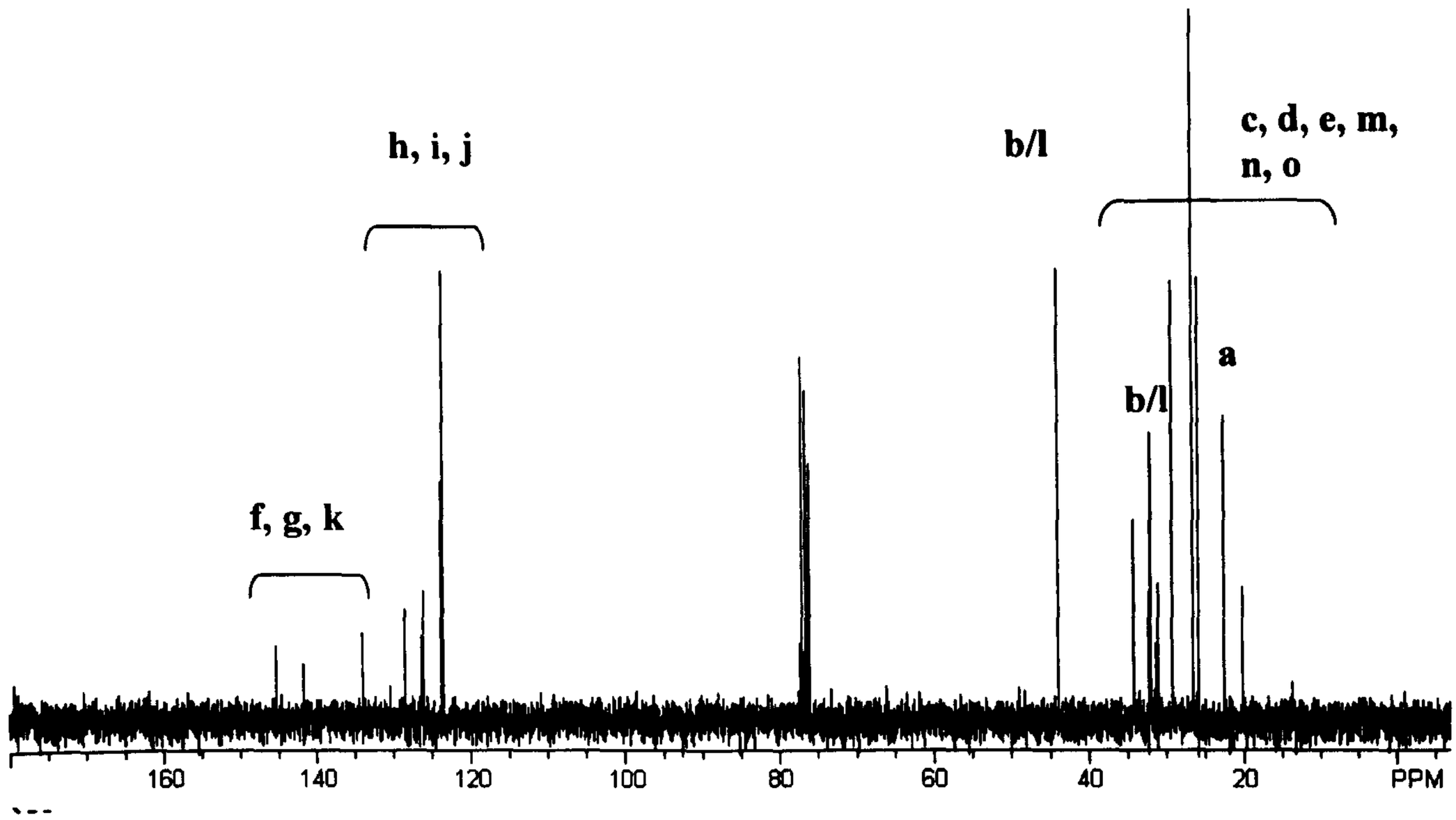


Figure 3.27b ^{13}Carbon NMR spectrum of 7-cyclohexyl-1-methyltetralin

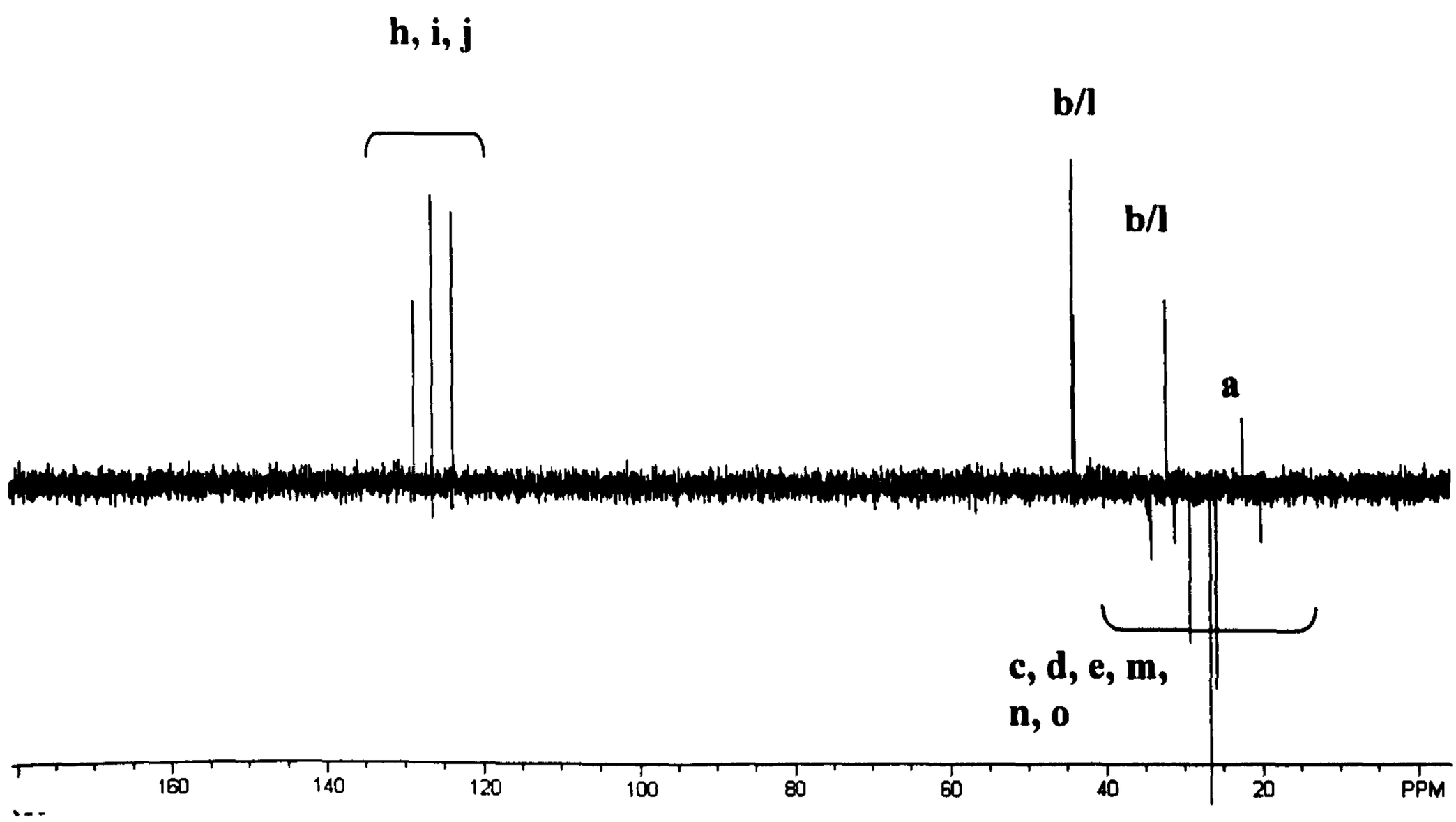


Figure 3.27c DEPT NMR spectrum of 7-cyclohexyl-1-methyltetralin

3.10 Isolation of 6-cyclohexyltetralin and 7-cyclohexyl-1-propyltetralin

Crude reaction products were provided by Wraige (1997) for the isolation of two further model aromatic UCM hydrocarbons. 6-cyclohexyltetralin was purified from crude reaction products by Ag^+ silica chromatography. The gas chromatogram in Figure 3.28 shows the purity of 6-cyclohexyltetralin (>98 %). The mass spectrum (Figure 3.29) of 6-cyclohexyltetralin showed an intense molecular ion at *m/z* 214. Also present was an abundant ion at *m/z* 171 corresponding to a loss of 43 ($M^{+\cdot}$-43), typical of the simple cleavage of aliphatic chains and resulting in the cyclohexyl substituted tropylium ion at *m/z* 171. These are consistent with previous assignments of Wraige (1997). The crude alkene mixture of 7-cyclohexyl-1-propenyltetralin and 7-cyclohexyl-1-propyl-3,4-dihydronaphthalene provided by Wraige (1997) was hydrogenated and purified in the same manner as 7-cyclohexyl-1-methyletralin (Section 3.9.1). The gas chromatogram in Figure 3.30 demonstrates that this compound which was synthesised with a yield of 80% was >98 % pure. The mass spectrum in Figure 3.31 exhibited the molecular ion $M^{+\cdot}$ at *m/z* 256 and the base peak *m/z* 213 ($M^{+\cdot}$-43) corresponding to cleavage at the tertiary centre and loss of the propyl chain. IR and NMR was also performed on these samples. The spectra were the same as those reported by Wraige (1997) where full characterisation had already been documented.

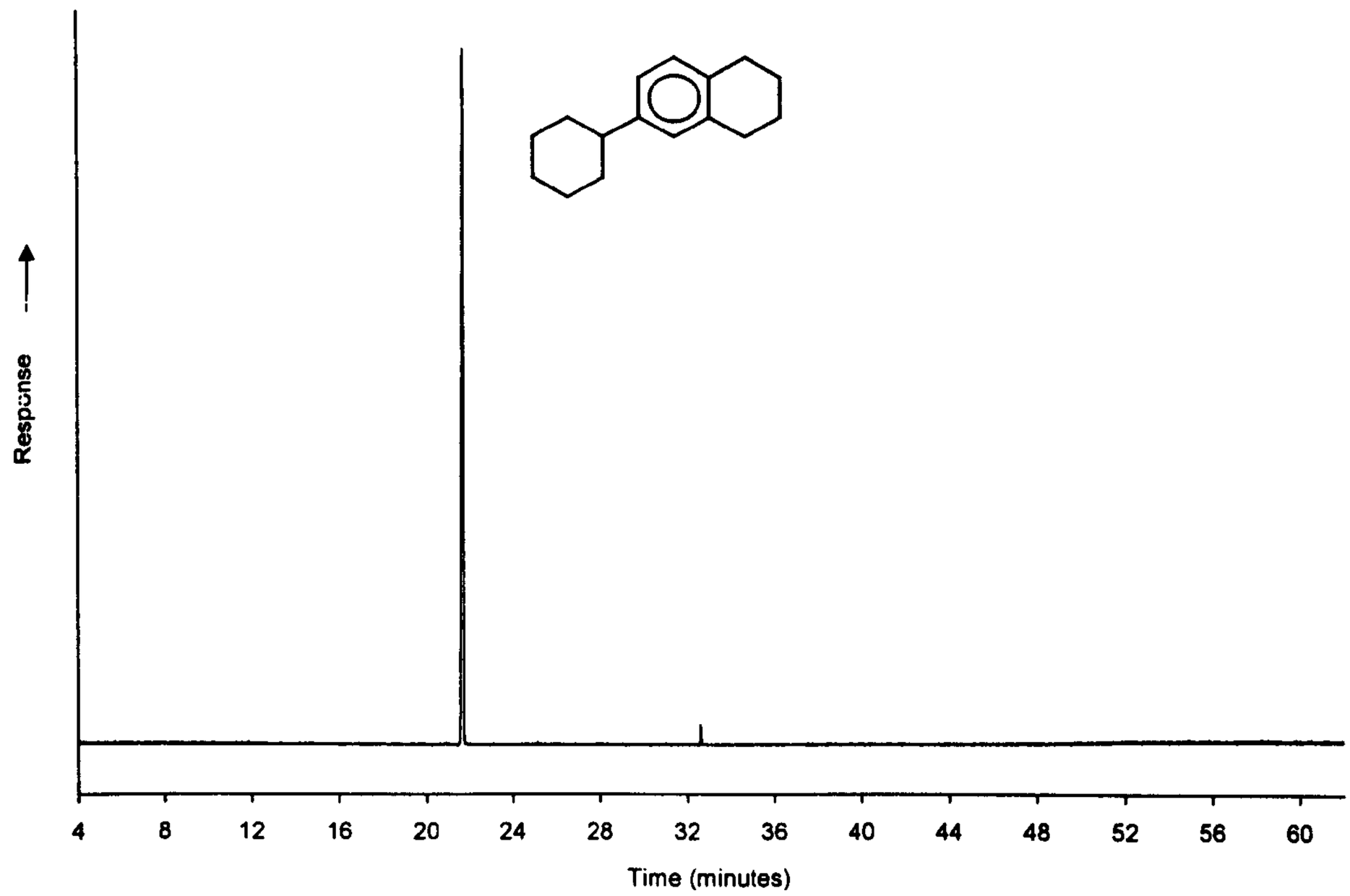


Figure 3.28 Gas chromatogram of 6-cyclohexyltetralin

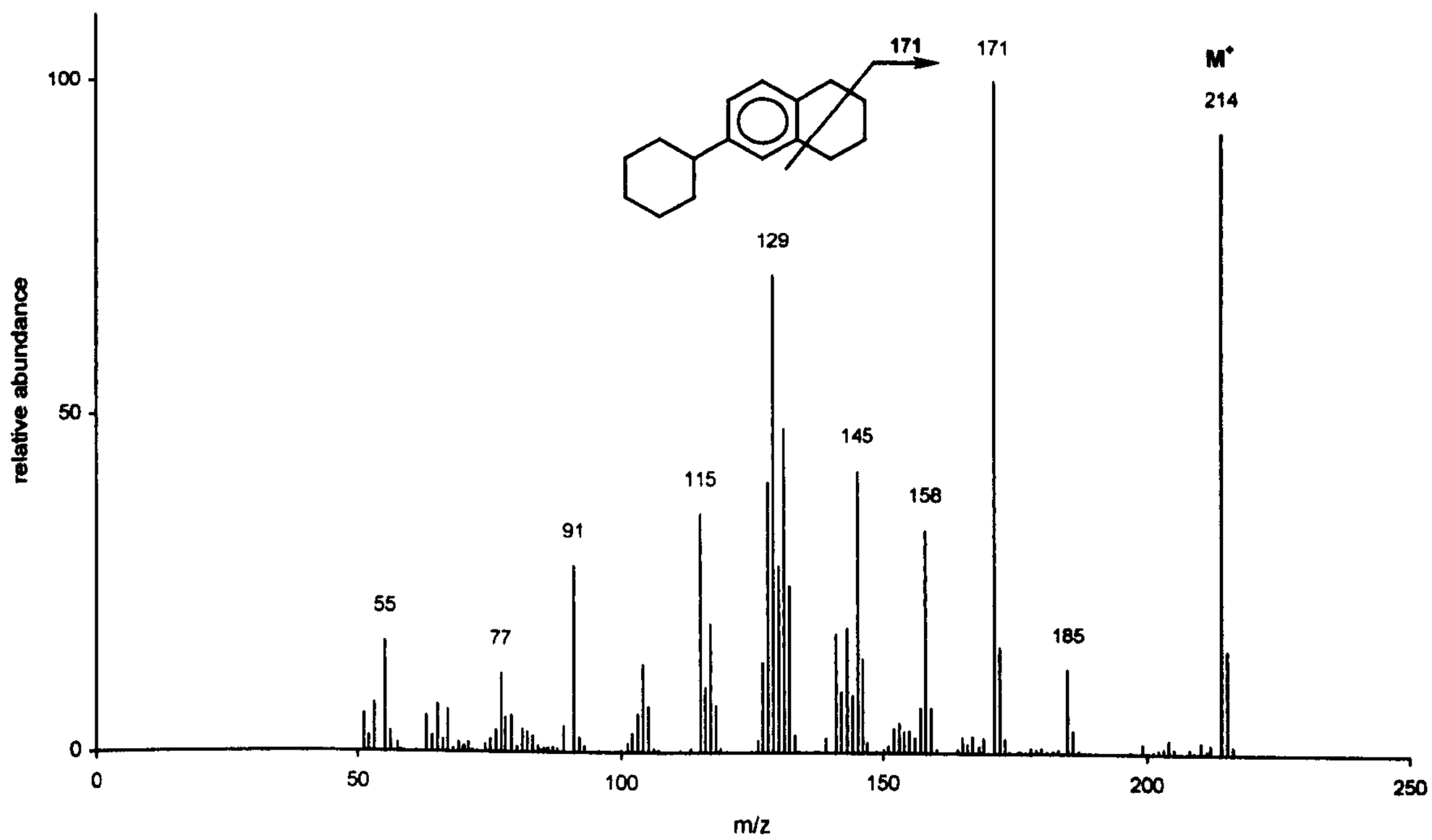


Figure 3.29 Mass spectrum of 6-cyclohexyltetralin

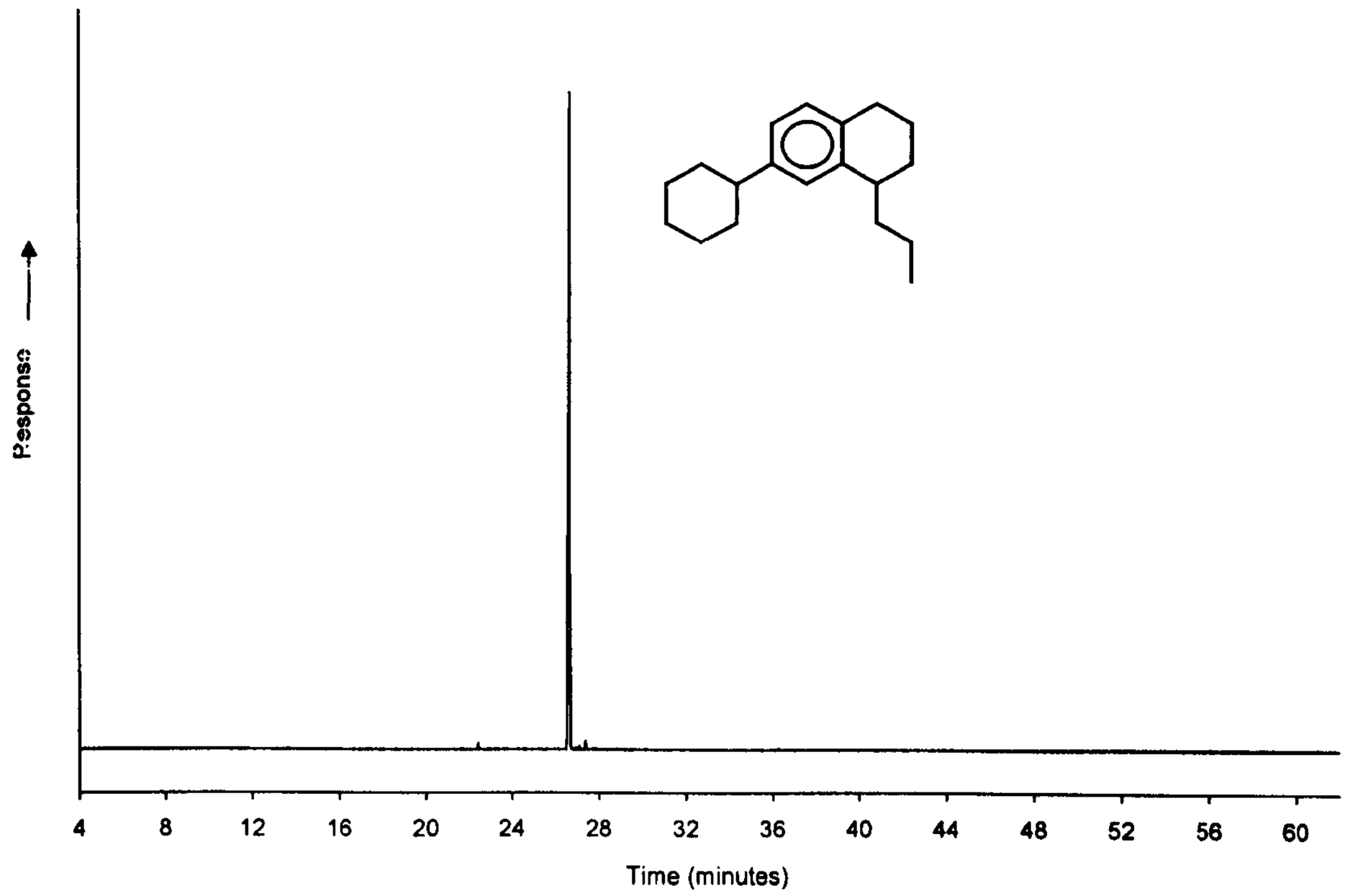


Figure 3.30 Gas chromatogram of 7-cyclohexyl-1-propyltetralin

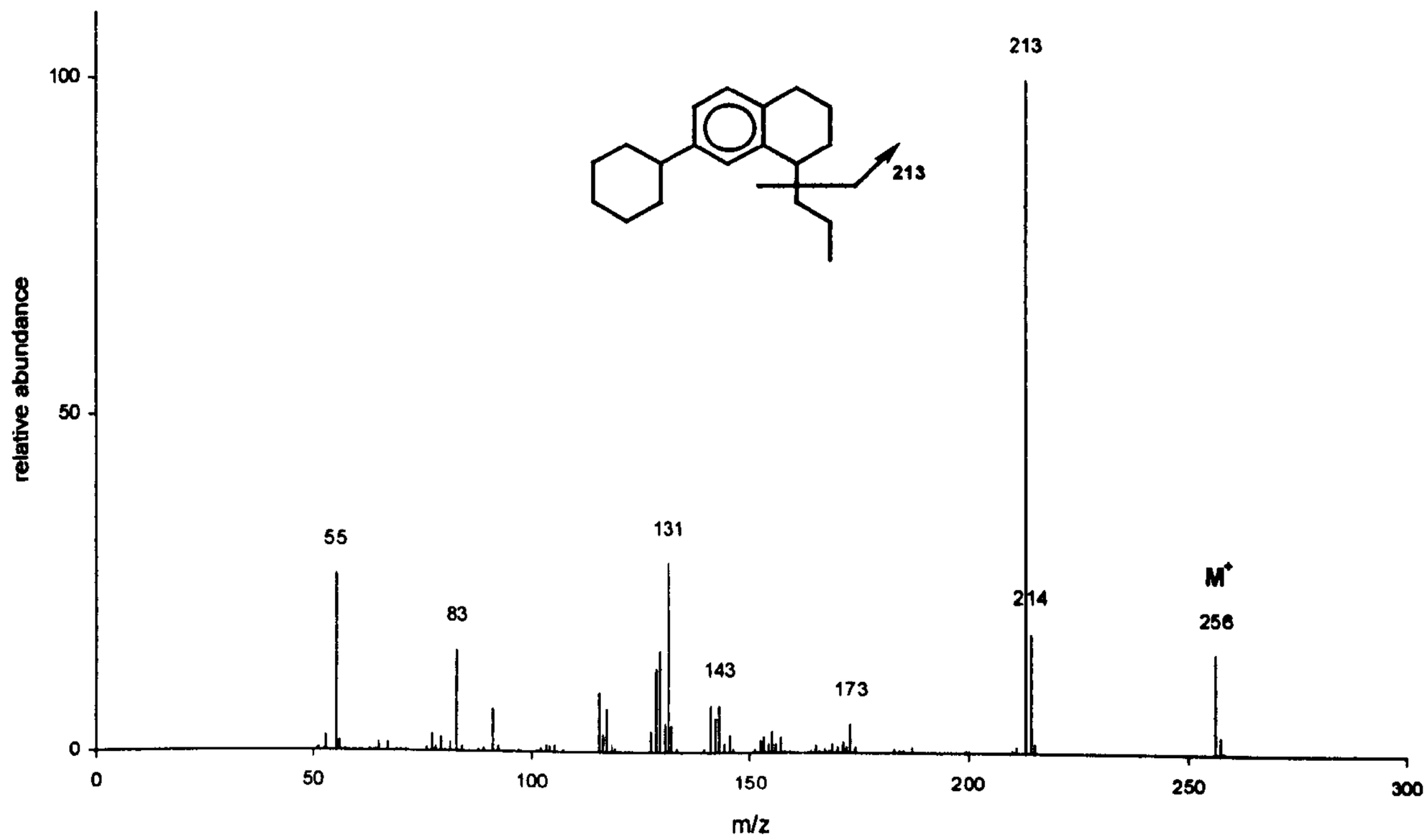


Figure 3.31 Mass spectrum of 7-cyclohexyl-1-propyltetralin

3.11 Summary

7-cyclohexyl-1-methyltetralin has been synthesised in good yield (~500 mg) and purity (>99% by GC). Intermediates at each stage of the synthetic pathway were characterised by GC, GCMS, IR and NMR. Pure 6-cyclohexyltetralin and 7-cyclohexyl-1-propyltetralin were also isolated from crude reaction products supplied by Wraige (1997). These synthesised compounds are thought to be good models for the components present within the aromatic UCM as suggested by previous characterisation studies. To illustrate how the alkyltetralin compounds synthesised as 'model' monoaromatic UCM hydrocarbons compare to an environmental aromatic UCM, Figure 3.32 shows the gas chromatograms of these compounds compared to the monoaromatic fraction isolated from mussels collected at a petroleum impacted site. The retention times of the compounds are within the range of the extracted monoaromatic UCM indicating they may have similar characteristics. Indeed, ions present in the mass spectra of these synthetic model compounds were found in the mass spectral analysis of the isolated monoaromatic UCM, further lending support to the proposed structures.

These model monoaromatic UCM hydrocarbons are unavailable from commercial sources and were synthesised in quantities large enough to allow thorough structural characterisation, and pure enough for subsequent toxicological (Chapter 4), aqueous solubility (Chapter 5), and photodegradation studies (Chapter 6) to be made. These should increase our understanding of the fate, effects and reactions of aromatic UCM hydrocarbons in the marine environment.

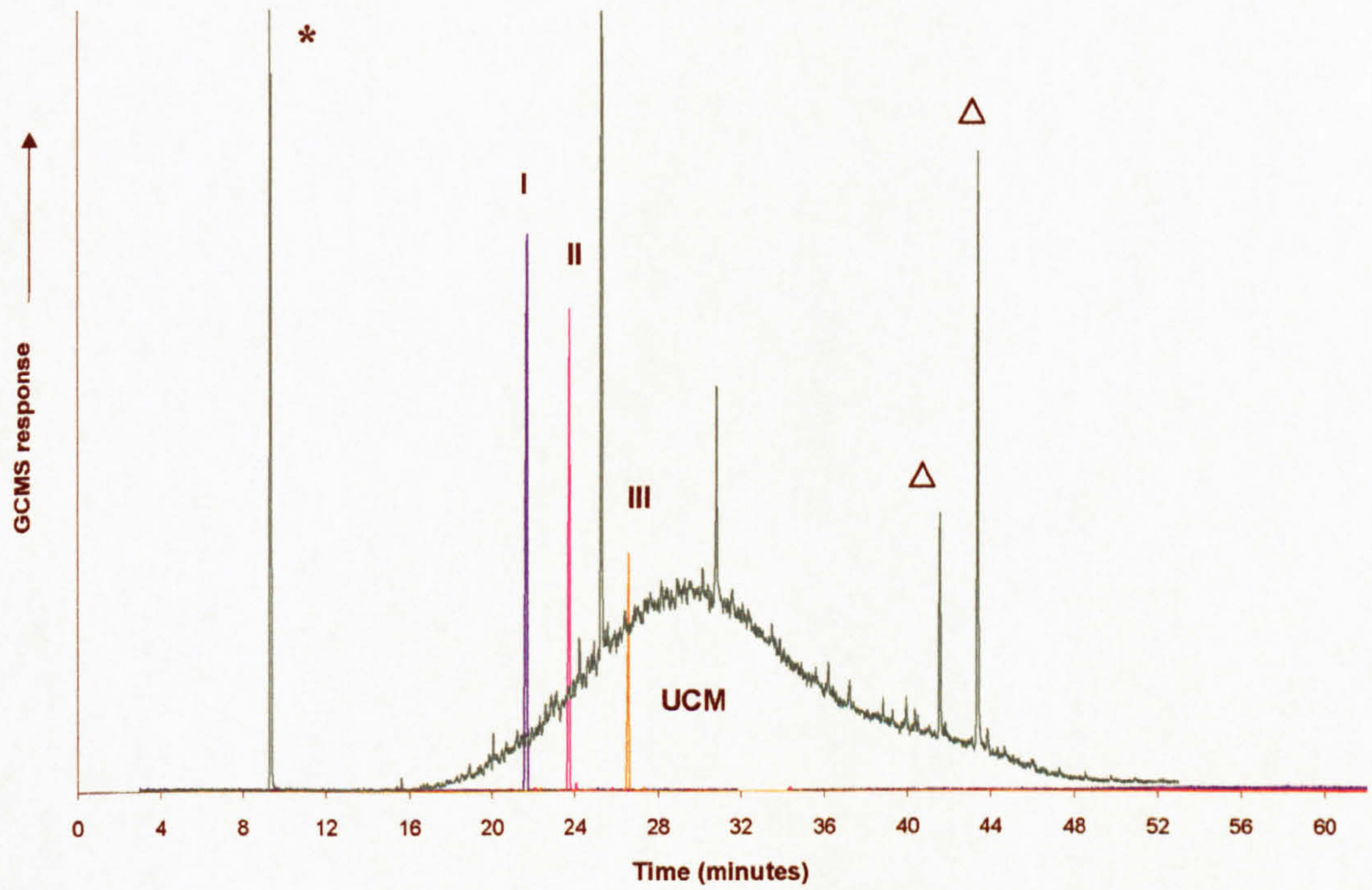


Figure 3.32 Gas chromatograms showing a typical unresolved complex mixture (UCM) of monoaromatic hydrocarbons isolated from mussels collected from Whitby, U.K. (Wraige, 1997) and the three synthetic alkyltetralins (I = 6-cyclohexyltetralin, II = 7-cyclohexyl-1-methyltetralin, III = 7-cyclohexyl-1-propyltetralin)

*** = internal standard, d12 tetralin Δ = biogenic tetraenes.**

GCMS details:HP1 (12m x 0.2mm i.d.) column; He carrier gas; 40℃-300℃ @ 5℃ min^{-1} hold 10 min

Chapter 4. Toxicological assessment of a model monoaromatic UCM hydrocarbon

7-cyclohexyl-1-methyltetralin, synthesised as part of this study, was assessed for its potential toxic effects using a mussel feeding rate assay, and was found to reduce mussel feeding rate significantly over a 24 hour exposure period. These results were consistent with those obtained previously for 6-cyclohexyltetralin and 7-cyclohexyl-1-propyltetralin by Wraige (1997) and were compared to the isolated monoaromatic UCM (Chapter 2).

4.1 Introduction

The effects of petroleum hydrocarbons on aquatic animals have been reviewed by Connel & Miller (1981), and more specifically in mussels by Bayne *et al.* (1982). Lethal effects occur in the 1-100 mg L^{-1} range whereas sub-lethal effects of petroleum are thought to occur in the 1-100 μg L^{-1} range. The ecotoxicological testing of crude oils and petroleum products has been reviewed by Betton (1994). Lethal toxicity is expressed as the LC_{50} value, which is defined as the exposure concentration of a chemical at which a 50% mortality is observed for the population being studied. The LC_{50} values of contaminants with the same mode of toxic action, *e.g.* narcosis, have been inversely correlated to their hydrophobicity (Könemann, 1981; Veith *et al.*, 1983). The lethal body burden (LBB) or critical body residue has been proposed as an alternative for, or as an addition to, the LC_{50} value to quantify toxicity. The LBB is defined as the molar concentration in the organism at lethality. It is hypothesised that the LBB does not depend on hydrophobicity and reflects the intrinsic toxicity of the chemical.

The chemicals usually associated with this hypothesis of constant effective tissue concentrations are non-polar narcotics, such as hydrocarbons. These do not affect any specific organ, organ system or biochemical pathway but cause a reversible disjunction called narcosis. The foremost theories propose that narcotic molecules affect sites in the cell membranes, so that the membranes become inoperative, inducing paralysis and subsequent death, but the exact mechanism of narcosis is unknown. If it is assumed that all the membranes have a similar number of sites of toxic action, which are equally sensitive, and all compounds causing non-specific narcosis are equipotent, the anaesthetic effect in organisms should occur, as a result, at the same tissue concentration of the compound (Pawlisz & Peters, 1993).

Therefore, for acute toxicity to aquatic organisms, individual compounds are considered equally toxic on the basis of their internal molar concentration (Peterson, 1994). The difference in measured toxicity amongst hydrocarbons results from their equilibrium partitioning behaviour between water and organisms. Thus, the toxicity of a compound is related to the internal dose received by an organism. The advantage in the measurement of the LBB is that it is assumed to be less dependent on experimental conditions, *e.g.* the time of exposure, exposure concentration and attainment of equilibrium conditions. Pawlisz & Peters (1993) suggested that an average value of 3.1 mmol kg^{-1} was a useful approximation of the lethal internal burden for narcosis when estimating toxicity for the internal concentration of unstudied narcotic compounds.

The usefulness of lethal toxicity data is limited for the prediction of the ecological impact of petroleum hydrocarbons in chronically polluted situations. In contrast, 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 (Axiack, 1991). Whilst lethal body burden data have been reported in the literature and been established to occur at a relatively constant body burden of 2-8 mmol kg^{-1} (McCarty *et al.*, 1986), only a limited number of studies have reported body residue data for sublethal toxicological endpoints (Call *et al.*, 1985; McCarty *et al.*, 1986; Mortimer & Connel, 1994). However, in these studies the body burdens of toxicants were not analysed, but calculated from the knowledge of aqueous solubility coupled with bioconcentration factors When Donkin *et al.* (1989, 1991) investigated the effect of aliphatic and aromatic hydrocarbons on mussel feeding rate they measured the accumulated body burden of the toxicant in the mussels at the end of each feeding rate determination, and demonstrated that the TEC_{50} (the tissue concentration when a 50 % effect is observed) occurred at a relatively constant tissue concentration of toxicant of 0.1-0.5 mmol kg^{-1}. This was true of the compounds in the log K_{ow} range 2.5-5.0, irrespective of chemical structure or

physicochemical properties. Thus, the difference found in the water concentration based toxicity of these compounds was attributed to differences in bioconcentration factors.

Donkin *et al.* (1989, 1991) observed that compounds with a log K_{ow} larger than 5 were not toxic to mussels. The observation of a cut-off in toxicity has been previously reported (Konemann, 1981; Hermens *et al.*, 1984), but these observations may have been due to experimental conditions as they were based on aqueous solubility data. Nonetheless, Donkin *et al.* (1989, 1991) showed that a cut-off in toxicity occurred despite efficient accumulation of compounds.

Upon exposure to a monoaromatic UCM isolated from Gullfaks crude oil, mussel feeding rate was found to decrease by ~40% of the control mussels at the highest aqueous exposure of 200 $\mu g\ L^{-1}$ (Chapter 2), which suggested that monoaromatic UCM hydrocarbons may be responsible for toxicological effects observed in the field where unresolved monoaromatic hydrocarbons dominate the aromatic fractions of impacted sites. The gas chromatogram of the mussel tissue extracts from exposed mussels indicated that the UCM accumulated within this tissue was the same as the isolated monoaromatic UCM it was exposed to, in that the 'humps' were present over a comparable retention time. However the cut-off in toxicity for compounds with a $\log K_{ow} > 5$ despite accumulation into mussel tissue observed by Donkin *et al.* (1989, 1991), suggests that some of the monoaromatic UCM compounds may have been accumulated without causing an effect. As this monoaromatic UCM is inherently complex in nature, interpretation of the data in comparison to other toxicological studies is hindered by this and the lack of knowledge on its physicochemical and molecular characteristics.

Current available evidence based on spectroscopic and degradative studies, reviewed in Section 1.2, suggests than alkyltetralins may be quantitatively important. A study by Wraige (1997) using 6-cyclohexyltetralin and 7-cyclohexyl-1-propyltetralin as model aromatic UCM hydrocarbons, found these compounds to be toxic to mussels, resulting in

reduced feeding rates. 6-cyclohexyltetralin reduced mussel feeding rate by 80 % of the control feeding rate at the highest exposure concentration of 100 μg L^{-1} where it was accumulated to ~150 μg g^{-1} wet weight mussel tissue during the 24 hour exposure period. 7-cyclohexyl-1-propyltetralin was accumulated to ~213 μg g^{-1} wet weight mussel tissue at the highest nominal aqueous concentration of 100 μg L^{-1} and reduced feeding rate by ~65 % of the control feeding rate after a 24 hour exposure. TEC_{50} values were determined at 0.206 and 0.539 mmol kg^{-1} for 6-cyclohexyltetralin and 7-cyclohexyl-1-propyltetralin. Wraige (1997) concluded that these results were consistent with those reported by Donkin *et al.* (1989, 1991) who demonstrated that the TEC_{50} of a range of aliphatic and aromatic hydrocarbons occurred at a relatively constant tissue concentration of toxicant at 0.1-0.5 mmol kg^{-1}, and suggested that this indicated that some of the aromatic UCM might be capable of eliciting a similar effect. Although these model compounds have yet to be unequivocally identified as hydrocarbon components of UCMs they appear at least to contain structural features consistent with the present limited knowledge of monoaromatic UCM composition (*e.g.* Thomas, 1995).

4.2 Aims

Previous research has shown that proposed model monoaromatic UCM hydrocarbons 6-cyclohexyltetralin and 7-cyclohexyl-1-propyltetralin were toxic to the mussel *Mytilus edulis* as measured by ciliary feeding activity (Wraige, 1997). Furthermore, toxicological testing of an isolated monoaromatic UCM using the same assay as that in the present study (Chapter 2) showed that this too reduced mussel feeding rate. The work described in the present chapter aimed to investigate the generality of the toxicity of these alkyltetralins and provide a study of the effects of decreasing size of the alkyl substituents on toxicity. By defining more clearly the molecular size – toxicity relationships for this class of

compounds an indication would be provided as to which sub-components of the aromatic UCMs are toxic.

4.3 Experimental Approach

The experimental approach was effectively the same as Donkin *et al.* (1989) and Wraige (1997) so that results could be compared to other aromatic hydrocarbons and two previously studied 'model' monoaromatic UCM hydrocarbons. This method was also employed in Chapter 2 to assess the effects of a monoaromatic hydrocarbon unresolved complex mixture isolated from a crude oil.

In successive experiments mussels were exposed to 6-cyclohexyltetralin (25 $\mu g\ L^{-1}$) and 7-cyclohexyl-1-methyltetralin (12.5, 25, 50 and 100 $\mu g\ L^{-1}$). Toxicant solutions were prepared by adding the toxicant in acetone to filtered seawater (0.001% v/v) to give the required exposure concentration. Solutions were stirred for 2 hours prior to use. Controls (0 $\mu g\ L^{-1}$) comprised filtered seawater and acetone only (0.001% v/v) which have previously been shown to elicit no effect (Donkin *et al.*, 1989; Wraige, 1997; Smith, present study). These controls were prepared and carried out in parallel to each exposure experiment. Groups of seven mussels, shell length 12mm, were exposed to 1.4 L toxicant or control solutions in glass beakers for 24 hr. Two vessels were prepared for each toxicant concentration. Gentle water movement was maintained by using a magnetically driven Teflon stirrer bar (10mm) and care was taken to position the animals as far away from the stirrer bar as possible. The animals were fed with an algal culture (*Isochrysis galbana*) for the duration of the exposure period in order to ensure that their valves remained open and the animals were filtering. For the purposes of determining feeding rate, animals were transferred from the exposure vessel into individual 250 mL glass beakers each containing 200 mL toxicant solution at the same exposure concentration. 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 (a volume pre-determined to give a cell concentration of 24000-30000 cells mL^{-1}) was then added to each beaker and the water gently stirred manually using a glass rod to ensure an even distribution of algae within the beaker. An aliquot (20 mL) was then immediately taken from each beaker and the cell count determined in triplicate per aliquot using a Coulter Counter set to measure particles greater then 3 μm in diameter. A further aliquot was taken after 15 minutes and the decline in cell concentration over 15 minutes calculated. Mussels subjected to the same hydrocarbon exposure levels were grouped together, dissected and extracted to determine the body burdens of accumulated compounds. Immediately prior to extraction an internal standard was added to the tissue for quantitation. Tissue was extracted by alkaline digestion (NaOH), followed by extraction into hexane (x 3). Extracts were then concentrated and analysed by GCMS. Compounds were quantified using integration software and internal standard calibration.

4.4 Results

Data were tested for normality using standardised skewness and standardised kurtosis. All data were found to be parametric, thus a t-test was used to compare means and ANOVA used to test the distribution of the data. In all statistical analyses differences at the <5% level were considered significant. Statgraphics 4 was used for all data analysis.

The aim of the first experiment was to repeat the measurement of toxicity of 6-cyclohexyltetralin reported by Wraige (1997), to establish the reproducibility of the assay between different operators. Mussels were exposed to 25 μg L^{-1} 6-cyclohexyltetralin. This was the aqueous exposure concentration which had previously caused accumulation of this

compound within the mussel tissue to the extent that feeding rate was reduced by 50 % (Wraige, 1997).

Feeding rates for both control and toxicant animals are expressed as means ± standard deviations (n = 7) and as a percentage of the control value (Table 4.1) No significant difference was found between each of the duplicate exposures or between controls, therefore the data from each exposure concentration were pooled (Table 4.2). The data in Table 4.2 show that 6-cyclohexyltetralin reduced feeding rate by 52 % compared to controls. These results are consistent with those of Wraige (1997) who found a 47 % decrease in 24 hours at this aqueous exposure concentration. The feeding rate of the control animals in the present study was slightly lower than that of Wraige (1997) but no significant difference was noted between the feeding rate of exposed animals from the present study and that of Wraige (1997) when compared as a reduction of the control value. The lower control values may be attributable to seasonal variation in the feeding rate of mussels.

Table 4.1 The effect of 6-cyclohexyltetralin upon mussel feeding rate over a 24 h exposure period

Nominal aqueous concentration ($\mu g\ L^{-1}$)	Total body burden ($\mu g\ g^{-1}$ wet weight)	Mussel feeding rate ($L\ hr^{-1}$) Mean ± sd (n = 7)	Mussel feeding rate expressed as a % of control feeding rate
0	0	0.43 ± 0.05	100
	0	0.43 ± 0.05	100
25	39.5	0.22 ± 0.05	51
	36.7	0.23 ± 0.06	53

Table 4.2 Comparison of effect of 25 μg L^{-1} 6-cyclohexyltetralin upon mussel feeding rate over a 24 h exposure period from present study with Wraige (1997)

Nominal aqueous concentration (μg L^{-1})	Total body burden (μg g^{-1} wet weight)	Mussel feeding rate (L hr^{-1}) Mean ± sd (n = 14)	Mussel feeding rate expressed as a % of control feeding rate	Reference
0	0	0.43 ± 0.05	100	
25	38.1	0.23 ± 0.05	52	Present study
0	0	0.49 ± 0.04	100	
25	41.5	0.23 ± 0.06	47	Wraige (1997)

A summary of the results indicating the effects of aqueous exposure and total body burden of mussels exposed to 7-cyclohexyl-1-methyltetralin (synthesised in the present study; Chapter 3) upon mussel feeding rate is presented in Table 4.3. Concentration-response curves for the effect of 7-cyclohexyl-1-methyltetralin on mussel feeding rate over 24 hours, expressed in terms of nominal aqueous exposure and total body burden, are shown in Figures 4.1 and 4..2 respectively. Values are plotted as mean feeding rate ± standard deviation (n = 7) except for pooled control values (n = 56). A significant (70 %) reduction in feeding rate was observed over the 24 hour exposure period.

Table 4.3 The effect of 7-cyclohexyl-1-methyltetralin upon mussel feeding rate over a 24 h exposure period

Nominal aqueous concentration ($\mu g\ L^{-1}$)	Total body burden of toxicant ($\mu g\ g^{-1}$ wet weight)	Mussel feeding rate ($L\ hr^{-1}$) mean ± std.dev. (n = 7)
0	0	0.40 ± 0.06
	0	0.46 ± 0.07
12.5	10.8	0.35 ± 0.06
	12.8	0.30 ± 0.05
0	0	0.41 ± 0.07
	0	0.45 ± 0.05
25	30.1	0.31 ± 0.05
	35.5	0.28 ± 0.07
0	0	0.45 ± 0.05
	0	0.41 ± 0.07
50	55.5	0.22 ± 0.06
	59.3	0.16 ± 0.07
0	0	0.41 ± 0.04
	0	0.45 ± 0.05
100	140.2	0.14 ± 0.05
	151.1	0.10 ± 0.06

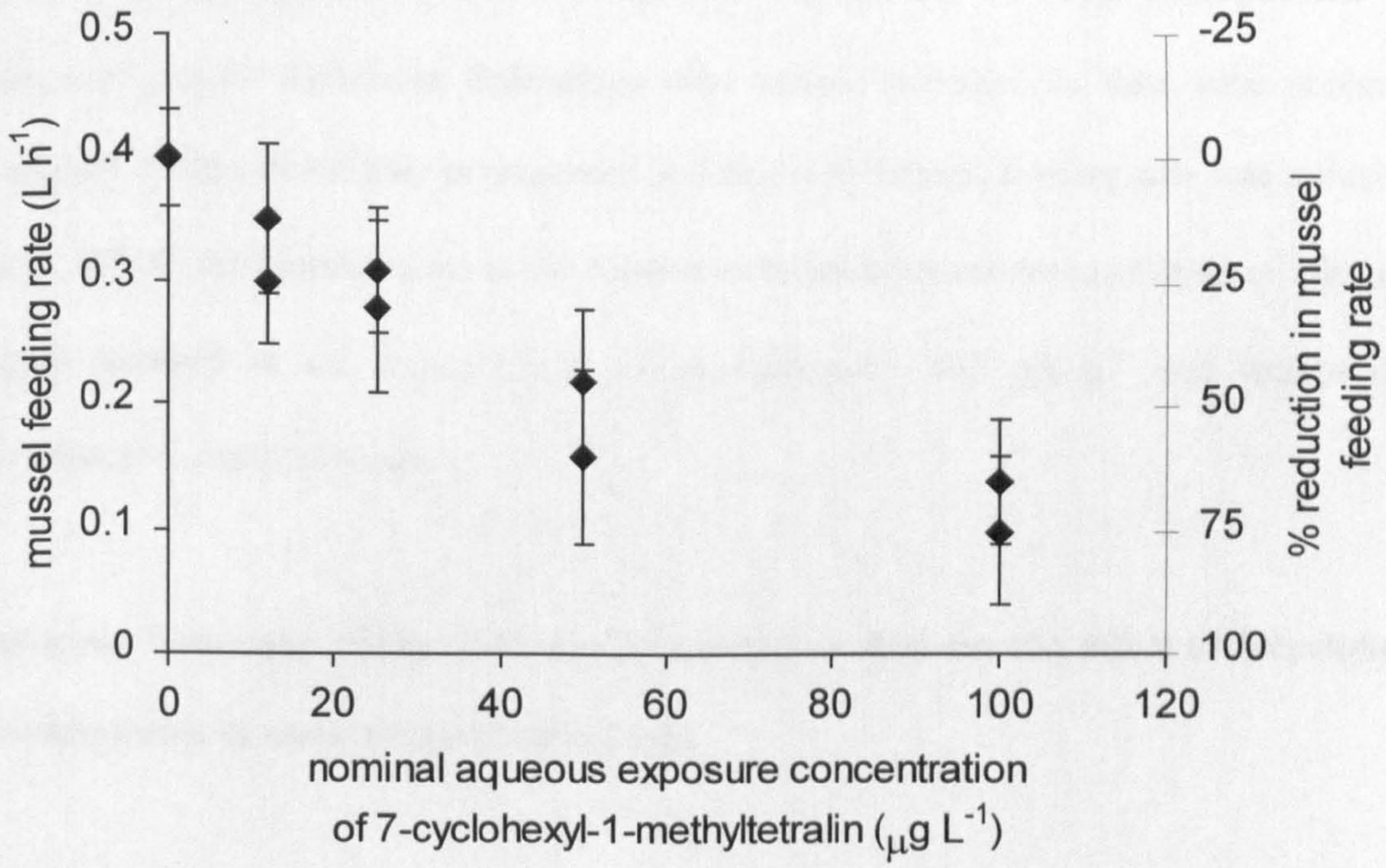


Figure 4.1 Concentration (aqueous)-response curve for the effect of 7-cyclohexyl-1-methyltetralin upon mussel feeding rate over 24 h

(values plotted as mean ± sd, n=7, except control value exposure 0 μg L^{-1} n=56)

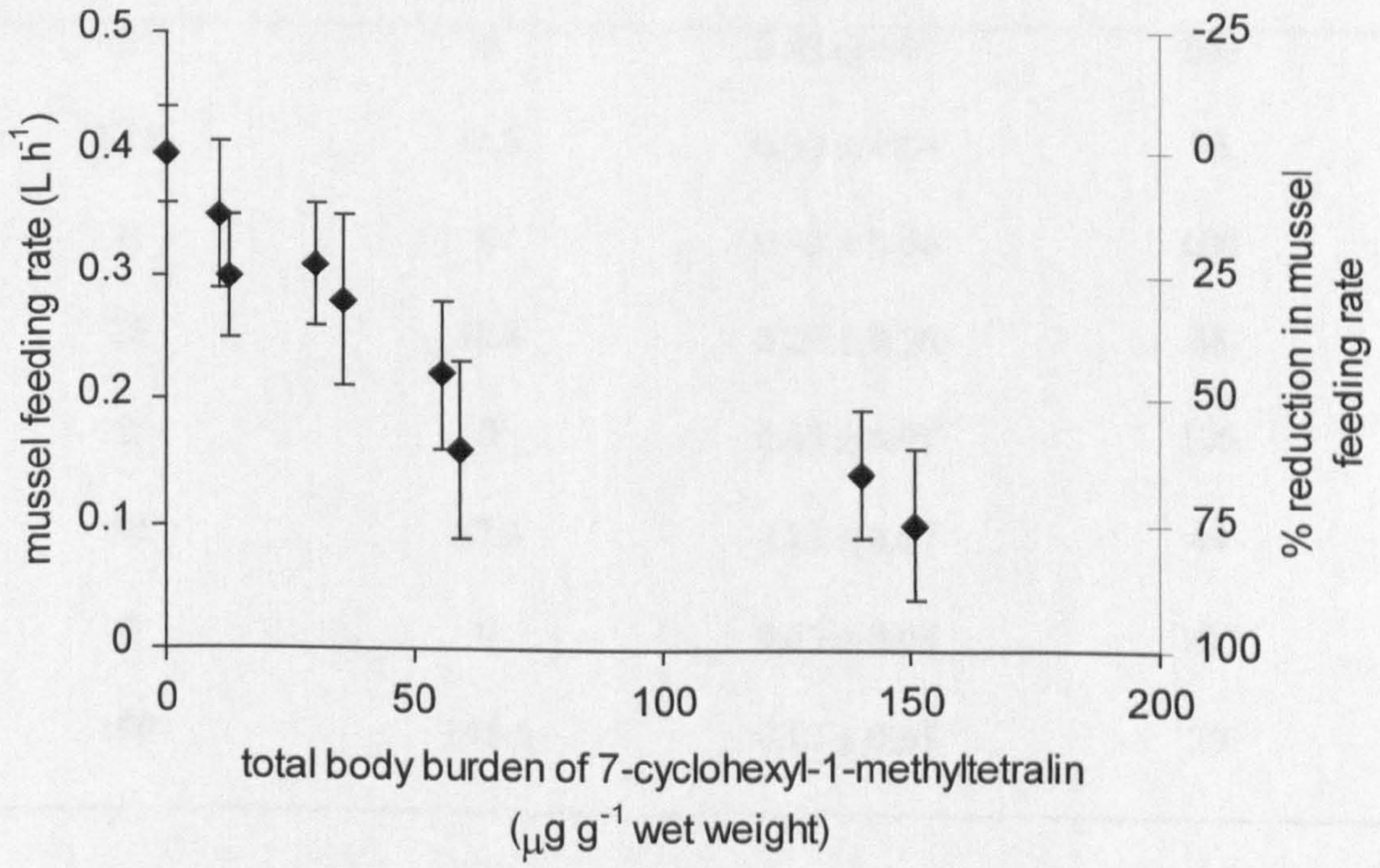


Figure 4.2 Concentration (tissue)-response curve for the effect of 7-cyclohexyl-1-methyltetralin upon mussel feeding rate over 24 h

(values plotted as mean ± sd, n=7, except control value exposure 0 μg L^{-1} n=56)

Mussel feeding rates measured for replicate experiments at each concentration were compared and no significant differences were found, therefore the data were pooled. A summary of the pooled data is presented in Table 4.4. Mussel feeding rate was reduced by up to 70% of the control value at the highest nominal aqueous concentration of 100 μg L^{-1} which resulted in an accumulation of approximately 145 μg g^{-1} wet weight of 7-cyclohexyl-1-methyltetralin.

Table 4.4 Summary of the concentration-response data for the effect of 7-cyclohexyl-1-methyltetralin upon mussel feeding rate

Nominal aqueous concentration (μg L^{-1})	Total body burden of toxicant (μg g^{-1} wet weight) mean (n = 2)	Mussel feeding rate (L hr^{-1}) mean ± std.dev. (n = 14)	Mussel feeding rate expressed as % of control feeding rate
0	0	0.43 ± 0.07	100
12.5	11.8	0.33 ± 0.06	76
0	0	0.43 ± 0.06	100
25	32.8	0.29 ± 0.06	68
0	0	0.43 ± 0.07	100
50	57.4	0.19 ± 0.07	44
0	0	0.43 ± 0.05	100
100	145.6	0.12 ± 0.05	29

Donkin *et al.* (1989, 1991) obtained TEC_{50} estimates for aliphatic and aromatic hydrocarbons by applying the relationship $y = \alpha + \beta \log_{10} x$ fitted by least squares regression. The data obtained in the present study was treated the same way and the pooled body burden data transformed by $\log_{10}$ was plotted against feeding rate (Figure 4.3.) A TEC_{50} value for the effects of 7-cyclohexyl-1-methyletralin upon mussel feeding rate was obtained by applying the relationship $y = \alpha + \beta \log_{10} x$ fitted by least squares regression of data in Table 4.4. A TEC_{50} estimate of 58 $\mu g\ g^{-1}$ ($mg\ kg^{-1}$) wet weight was obtained which translates to a TEC_{50} value of 0.254 $mmol\ kg^{-1}$.

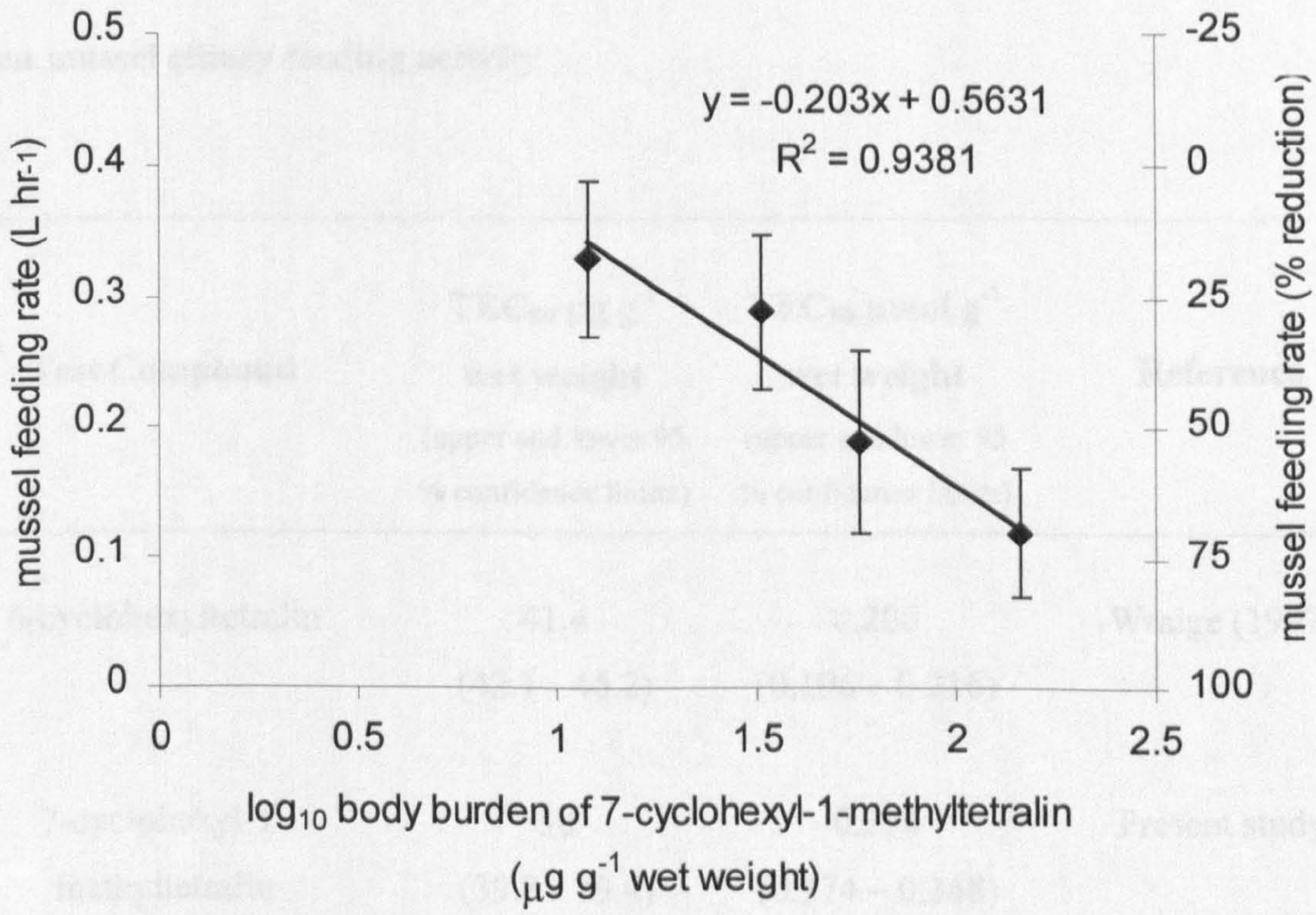


Figure 4.3 Concentration ($\log_{10}$tissue)-response curve for the effect of 7 cyclohexyl-1-methyltetralin (7-CHT) upon mussel feeding rate over a 24 h exposure period

The TEC_{50} value of 0.254 mmol kg^{-1} is compared with results from Wraige (1997) in Table 4.5. To further compare the effect of 7-cyclohexyl-1-methyltetralin upon mussel feeding rate with those observed by Wraige (1997) for 6-cyclohexyltetralin and 7-cyclohexyl-1-propyltetralin, concentration-response curves for both nominal aqueous concentration and total body burden were plotted (Figures 4.4 - 4.6). Mussel feeding rate is expressed as a percentage of the control to eliminate any differences due to the feeding rate of controls. This enabled the direct comparison of the three compounds, which represent a homologous series of compounds with increasing alkyl chain length.

Table 4.5 TEC_{50} values for the effect of model aromatic UCM hydrocarbons 6-cyclohexyltetralin, 7-cyclohexyl-1-methyltetralin and 7-cyclohexyl-1-propyltetralin upon mussel ciliary feeding activity

Test Compound	TEC_{50} µg g^{-1} wet weight (upper and lower 95 % confidence limits)	TEC_{50} µmol g^{-1} wet weight (upper and lower 95 % confidence limits)	Reference
6-cyclohexyltetralin	41.4 (42.1 - 46.2)	0.206 (0.196 – 0.216)	Wraige (1997)
7-cyclohexyl-1-methyltetralin	58 (39.8 - 79.4)	0.254 (0.174 – 0.348)	Present study
7-cyclohexyl-1-propyltetralin	138 (91.0 - 204.2)	0.539 (0.355 – 0.797)	Wraige (1997)

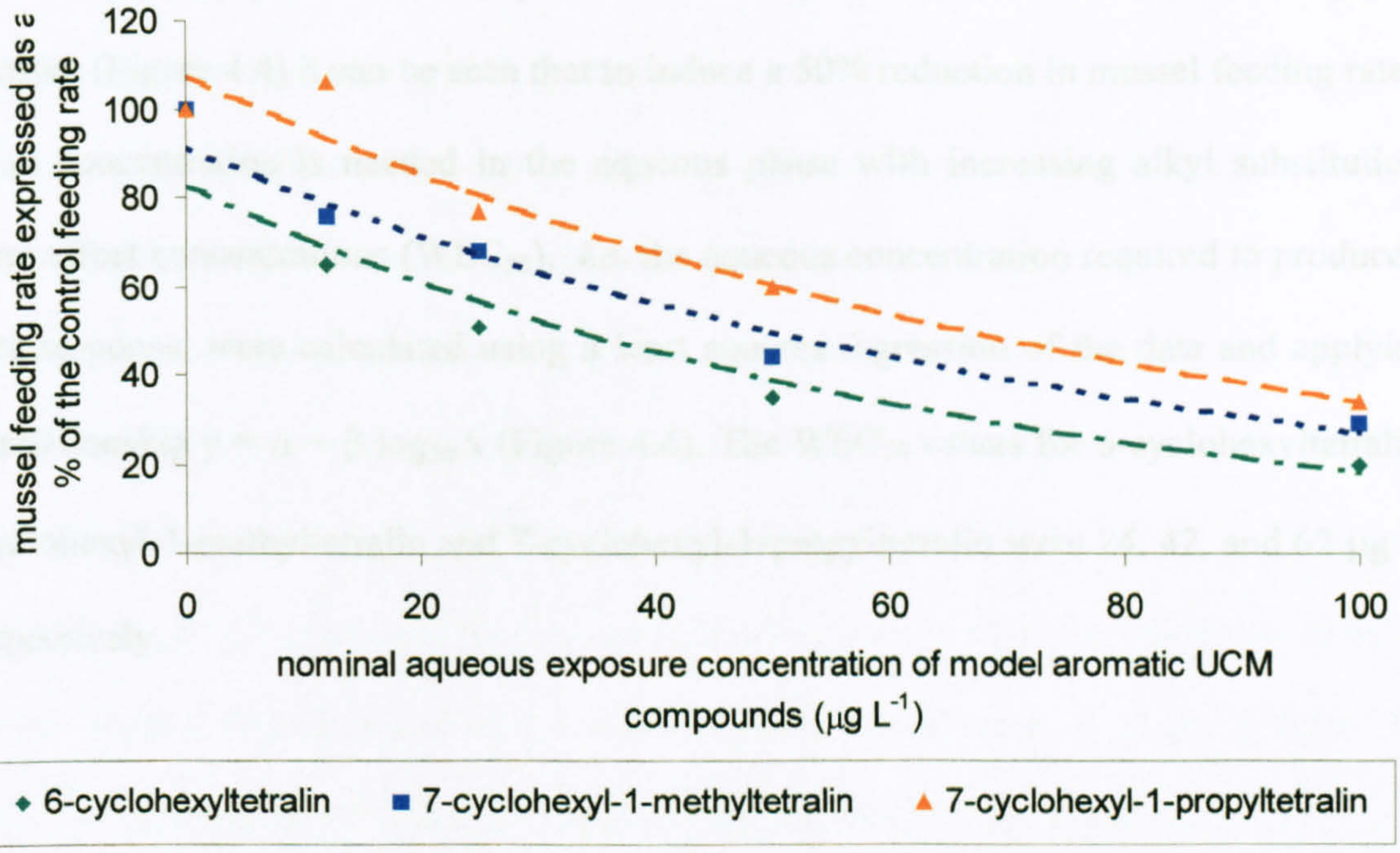


Figure 4.4 Comparison of concentration (aqueous)-response curves for the model aromatic UCM hydrocarbons, 6-cyclohexyltetralin, 7-cyclohexyl-1-propyltetralin (Wraige, 1997) and 7-cyclohexyl-1-methyltetralin (present study)

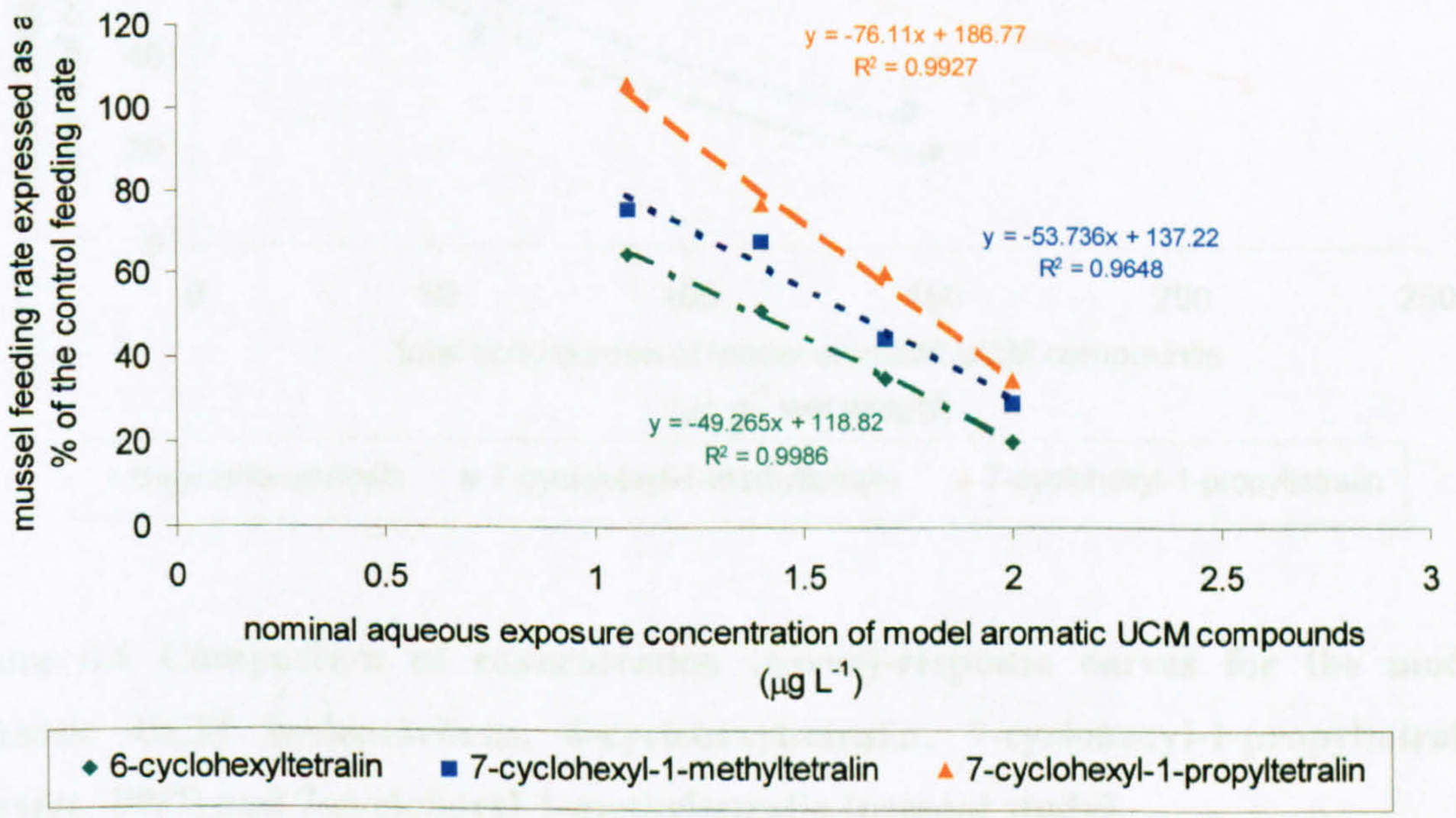


Figure 4.5 Comparison of concentration (log_{10}aqueous)-response curves for curves for the model aromatic UCM hydrocarbons, 6-cyclohexyltetralin, 7-cyclohexyl-1-propyltetralin (Wraige, 1997) and 7-cyclohexyl-1-methyltetralin (present study)

When the three synthesised compounds are compared in terms of nominal aqueous exposure (Figure 4.4) it can be seen that to induce a 50% reduction in mussel feeding rate a higher concentration is needed in the aqueous phase with increasing alkyl substitution. Water effect concentrations (WEC_{50}), *i.e.* the aqueous concentration required to produce a 50 % response, were calculated using a least squares regression of the data and applying the relationship $y = \alpha + \beta \log_{10} x$ (Figure 4.6). The WEC_{50} values for 6-cyclohexyltetralin, 7-cyclohexyl-1-methyltetralin and 7-cyclohexyl-1-propyltetralin were 24, 42, and 62 $\mu g\ L^{-1}$ respectively.

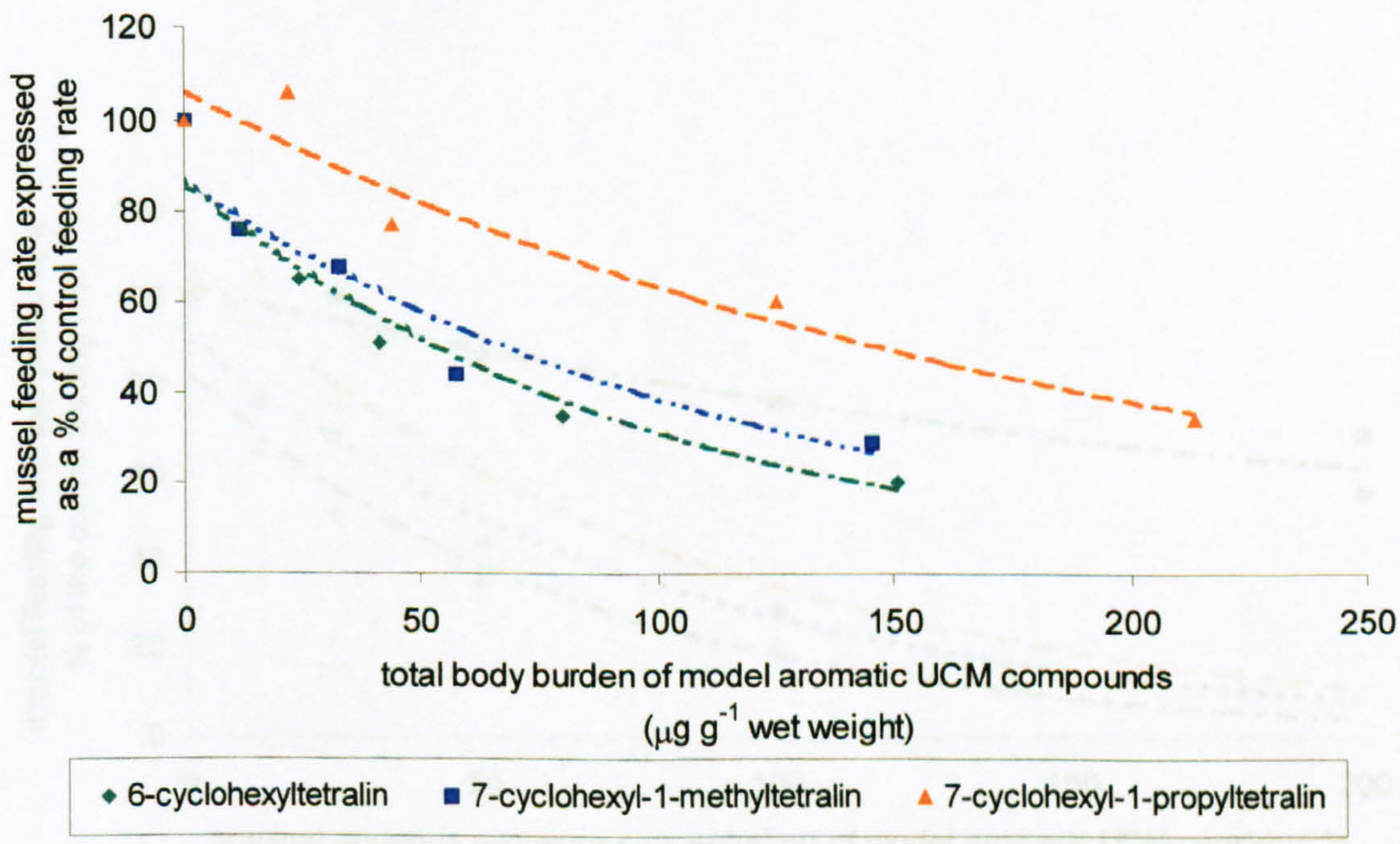


Figure 4.6 Comparison of concentration (tissue)-response curves for the model aromatic UCM hydrocarbons, 6-cyclehexyltetralin, 7-cyclohexyl-1-propyltetralin (Wraige, 1997) and 7-cyclohexyl-1-methyltetralin (present study)

When the effects of the three synthesised compounds upon mussel feeding rate are compared in terms of total body burden (Figure 4.5) a higher body burden is required to achieve a 50 % reduction in feeding rate with increasing chain length on the 'base'

cyclohexyltetralin structure. To achieve a 50 % reduction in mussel feeding rate 6-cyclohexyltetralin, 7-cyclohexyl-1-methyltetralin and 7-cyclohexyl-1-propyltetralin were accumulated to 44, 58 and 138 μg g^{-1} respectively, *i.e.* 7-cyclohexyl-1-propyltetralin was accumulated to more than double the amount of the other two compounds.

A further aim of this study was to compare the behaviour of the 'model' monoaromatic UCM hydrocarbons, 6-cyclohexyltetralin, 7-cyclohexyl-1-methyltetralin and 7-cyclohexyl-1-propyltetralin with that of the isolated monoaromatic UCM (Chapter 2). Therefore, graphs were plotted comparing the concentration response to mussel feeding rate in terms of both aqueous exposure and body burden to gain an understanding of the effect of the isolated monoaromatic UCM as compared to resolved compounds (Figures 4.7-4.12).

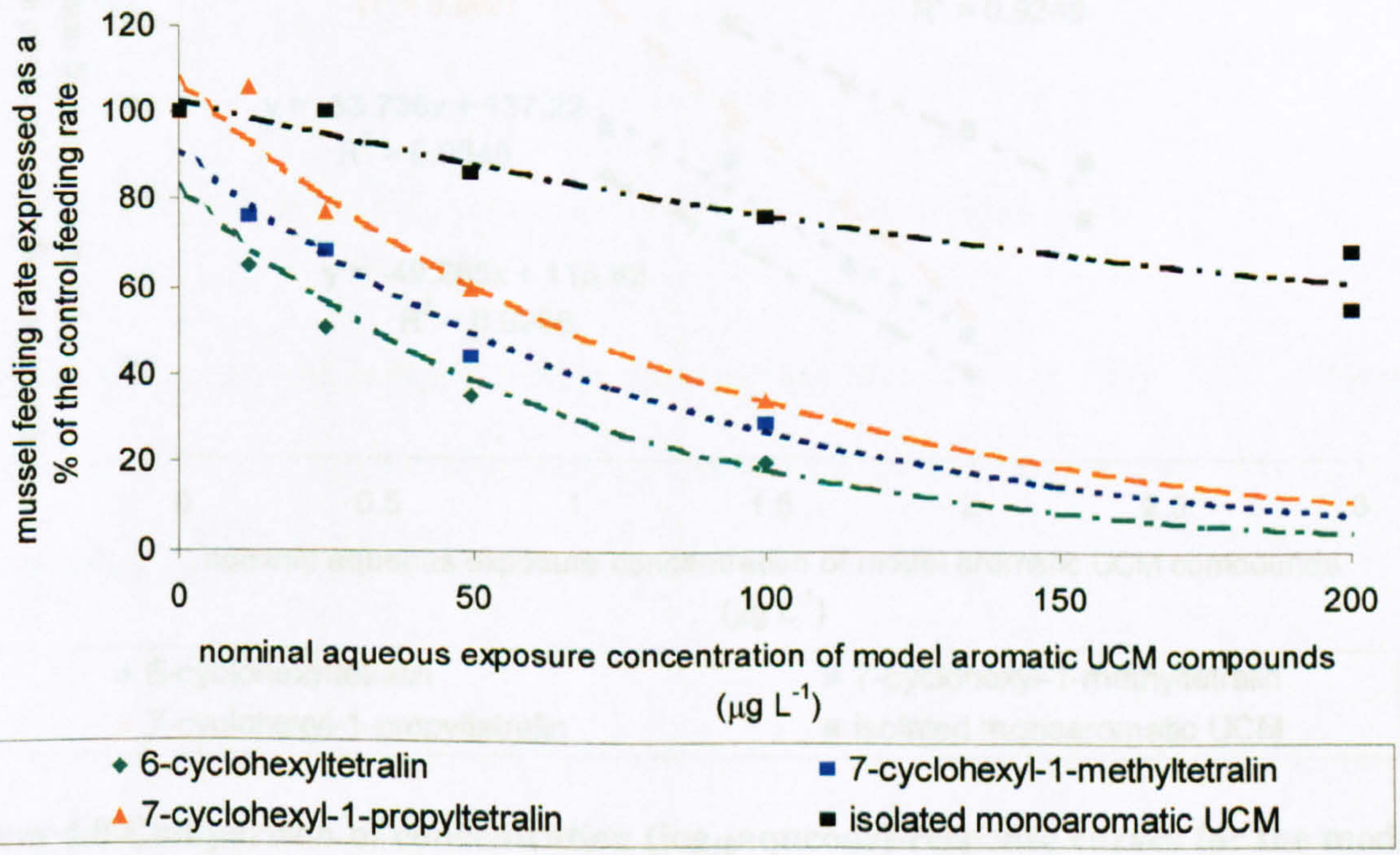


Figure 4.7 Comparison of concentration (aqueous)-response curves for the model aromatic UCM hydrocarbons, 6-cyclehexyltetralin, 7-cyclohexyl-1-propyltetralin (Wraige, 1997), 7-cyclohexyl-1-methyltetralin and an isolated monoaromatic UCM (present study)

In terms of aqueous exposure, a greater aqueous concentration of the isolated monoaromatic unresolved complex mixture was required to induce a response in the 24 hour exposure period in comparison to the model monoaromatic UCM hydrocarbons (Figure 4.7), *i.e.* the isolated monoaromatic UCM was not as toxic in terms of WEC_{50} compared to the model UCM hydrocarbons. The WEC_{50} of the isolated monoaromatic UCM was calculated by plotting the $\log_{10}$ nominal aqueous concentration against feeding rate and applying the same relationship as previously applied to the model UCM hydrocarbons (Figure 4.8). The isolated monoaromatic UCM appears to follow a similar trend to that of 6-cyclohexyltetralin and 7-cyclohexyl-1-methyltetralin, whereas 7-cyclohexyl-1-propyltetralin demonstrated a much sharper gradient.

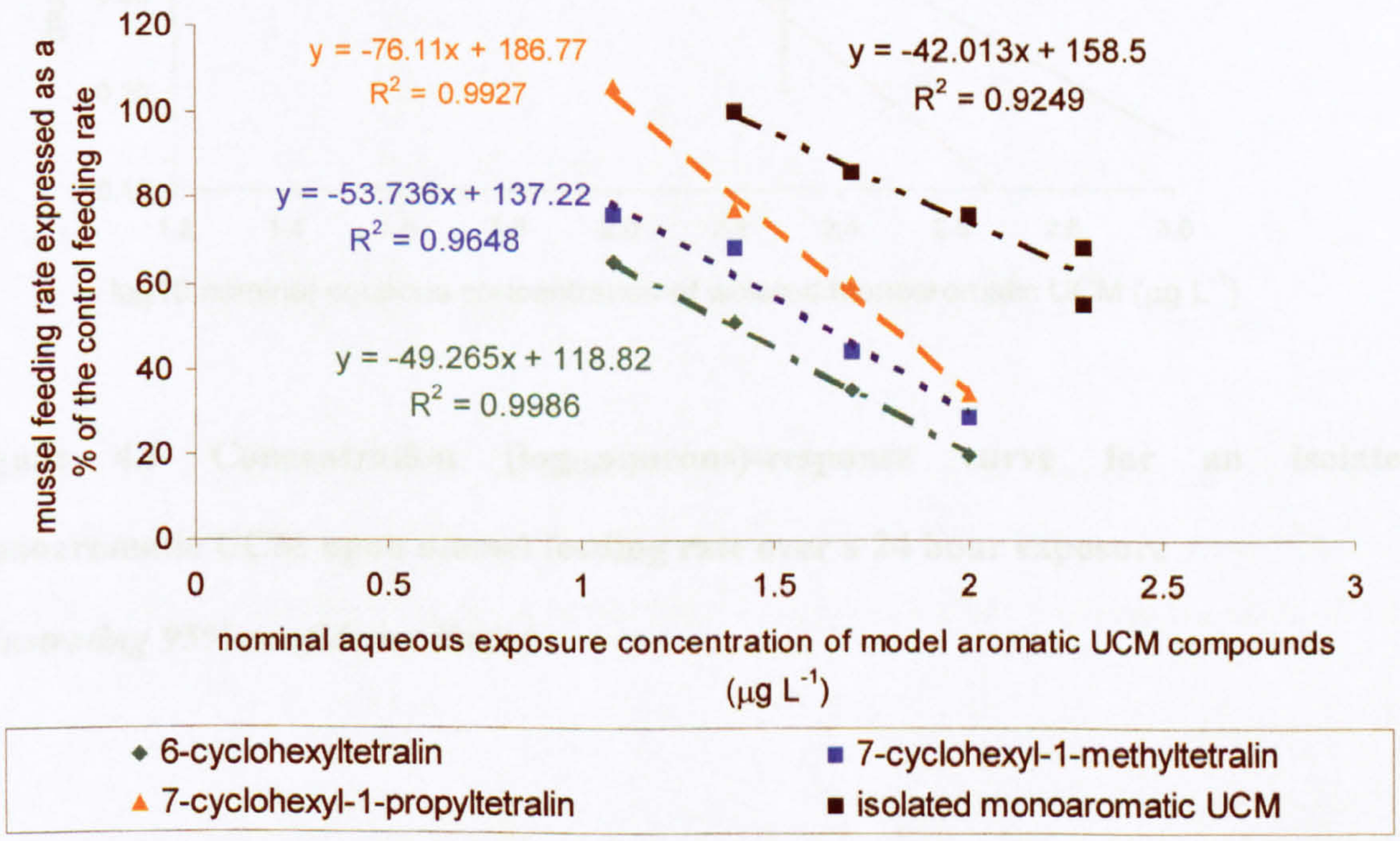


Figure 4.8 Comparison of concentration ($\log_{10}$aqueous)-response curves for the model aromatic UCM hydrocarbons, 6-cyclehexyltetralin, 7-cyclohexyl-1-propyltetralin (Wraige, 1997), 7-cyclohexyl-1-methyltetralin and an isolated monoaromatic UCM (present study)

The WEC_{50} for the isolated monoaromatic UCM was calculated at a mussel feeding rate of 0.22 L h^{-1} to be 518 μg L^{-1}. However, as seen in Figure 4.9 the 95 % confidence limits are large in this range due to the extrapolation of the data, and therefore the accuracy of this approach may be somewhat limited.

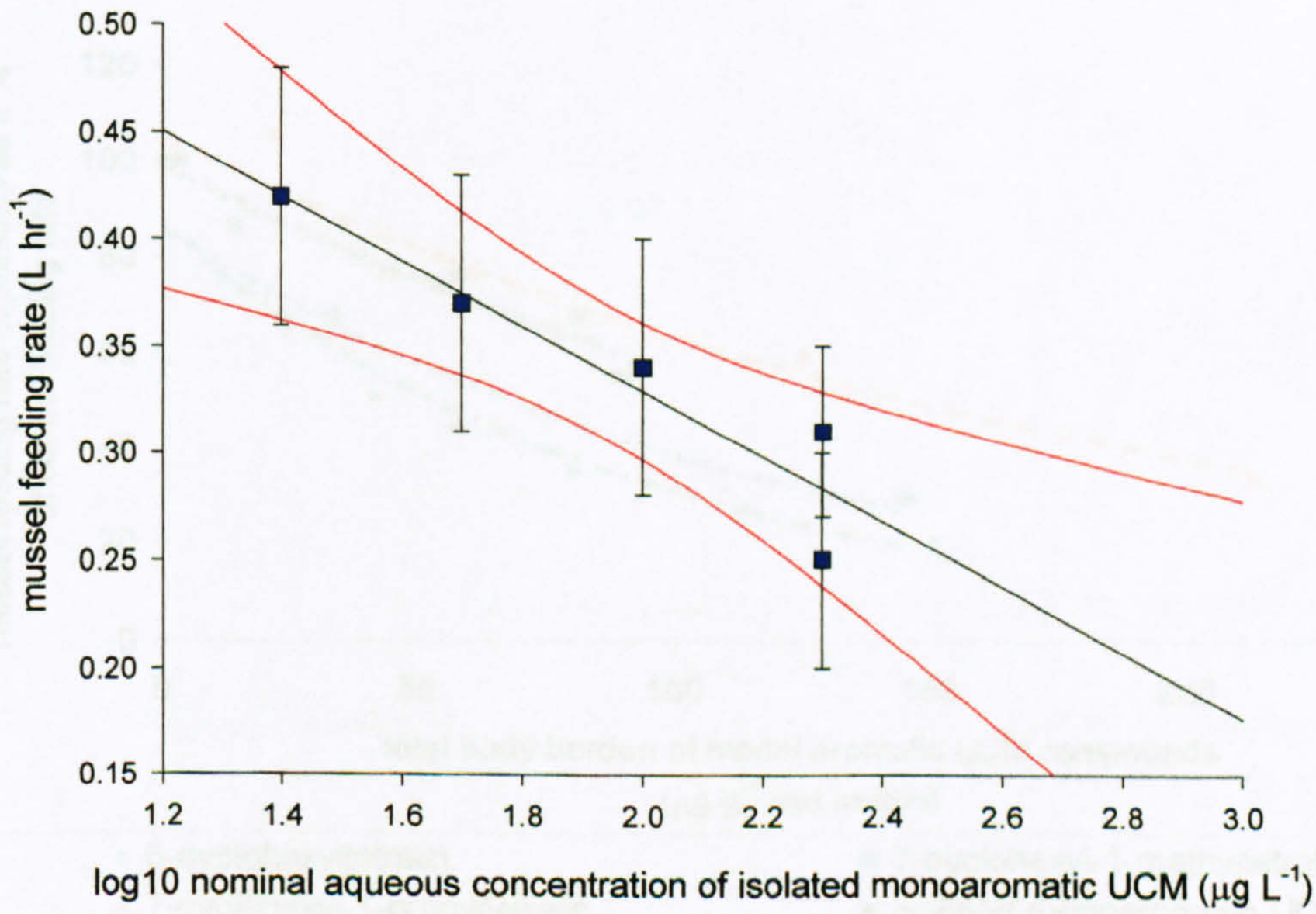


Figure 4.9 Concentration (log_{10}aqueous)-response curve for an isolated monoaromatic UCM upon mussel feeding rate over a 24 hour exposure ***(illustrating 95% confidence limits)***

When the response in feeding rate is plotted against total body burden (Figure 4.10) it can be seen that the isolated monoaromatic is comparable to the model compounds. The tissue concentrations required to induce a response are only slightly greater for the isolated UCM than for 6-cyclohexyltetralin and 7-cyclohexyl-1-methyltetralin but less than that for 7-cyclohexyl-1-propyltetralin. At an accumulation of 75 μg g^{-1} wet weight, mussel feeding

rate was reduced by 60, 53, 28 and 35 % for 6-cyclohexyltetralin, 7-cyclohexyl-1-methyltetralin, 7-cyclohexyl-1-propyltetralin and the isolated monoaromatic UCM, respectively. This indicates that if the isolated monoaromatic UCM was accumulated to the same extent as the model compounds a comparable reduction in feeding rate would be observed.

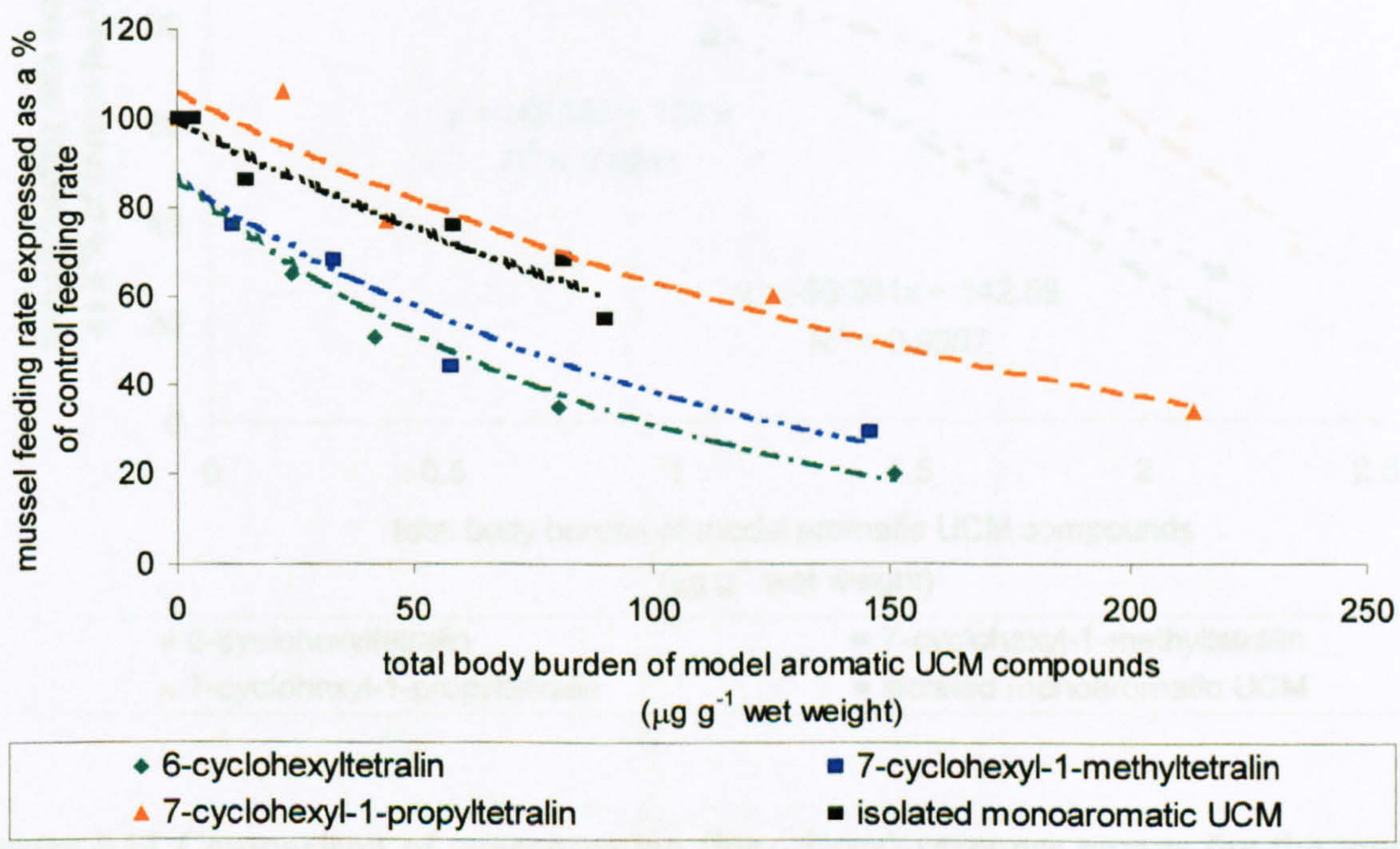


Figure 4.10 Comparison of concentration (tissue)-response curves for the model aromatic UCM hydrocarbons, 6-cyclehexyltetralin, 7-cyclohexyl-1-propyltetralin (Wraige, 1997), 7-cyclohexyl-1-methyltetralin and an isolated monoaromatic UCM (present study)

From the log plot of mussel feeding rate versus body burden (Figure 4.11) an estimate of the TEC_{50} of 500 μg g^{-1} wet weight was calculated. This is much higher than those determined for the model UCM compounds (7-cyclohexyl-1-propyltetralin, 138 μg g^{-1} wet weight). Calculation of the TEC_{50} in this manner was also subject to large variability, similar to the determination of the WEC_{50} value (see Figure 4.9), in that a 50 % reduction

in mussel feeding rate was not reached during the 24 hour exposure period and thus confidence limits due to the extrapolation of the data are large.

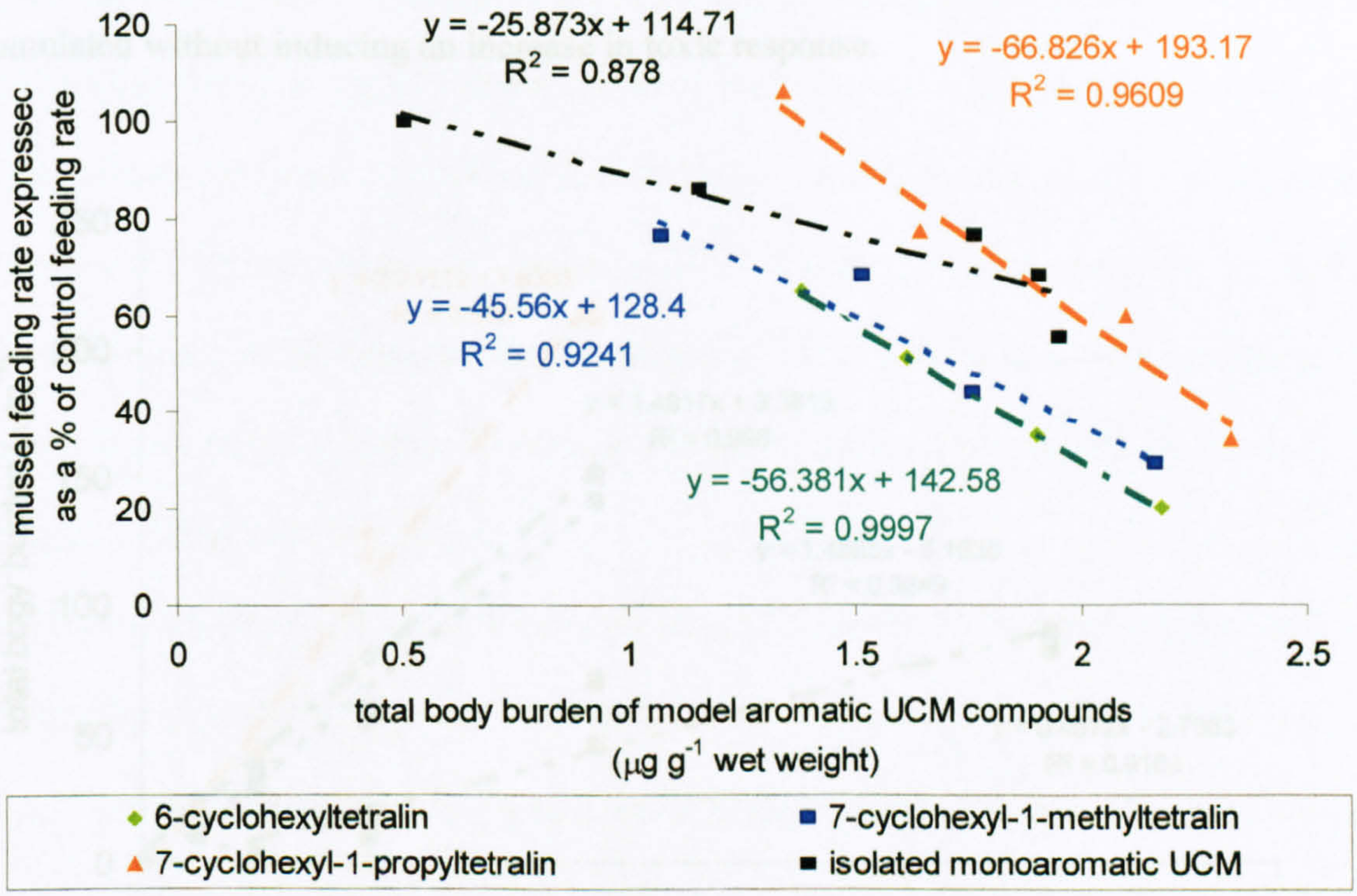


Figure 4.11 Comparison of concentration (log_{10}tissue)-response curves for the model aromatic UCM hydrocarbons, 6-cyclehexyltetralin, 7-cyclohexyl-1-propyltetralin (Wraige, 1997), 7-cyclohexyl-1-methyltetralin and an isolated monoaromatic UCM (present study)

To examine the uptake of 6-cyclohexyltetralin, 7-cyclohexyl-1-methyltetralin, 7-cyclohexyl-1-propyltetralin and the isolated monoaromatic UCM by mussels during the 24 hour exposure period a graph of nominal aqueous exposure against total body burden was plotted (Figure 4.12). 7-cyclohexyl-1-propyltetralin accumulated to the greatest extent within mussels exposed to toxicant solutions, whereas the isolated monoaromatic UCM accumulated the least. At the highest nominal aqueous concentration of 200 μg L^{-1} mussel

accumulated approximately 90 μg g^{-1} whilst the model compounds achieved this in 24 hours at a nominal aqueous concentration of 50 μg L^{-1}. Figure 4.12 also shows that the accumulation of 6-cyclohexyltetralin and 7-cyclohexyl-1-methyltetralin from the aqueous solution are very similar, but larger amounts of 7-cyclohexyl-1-propyltetralin were accumulated without inducing an increase in toxic response.

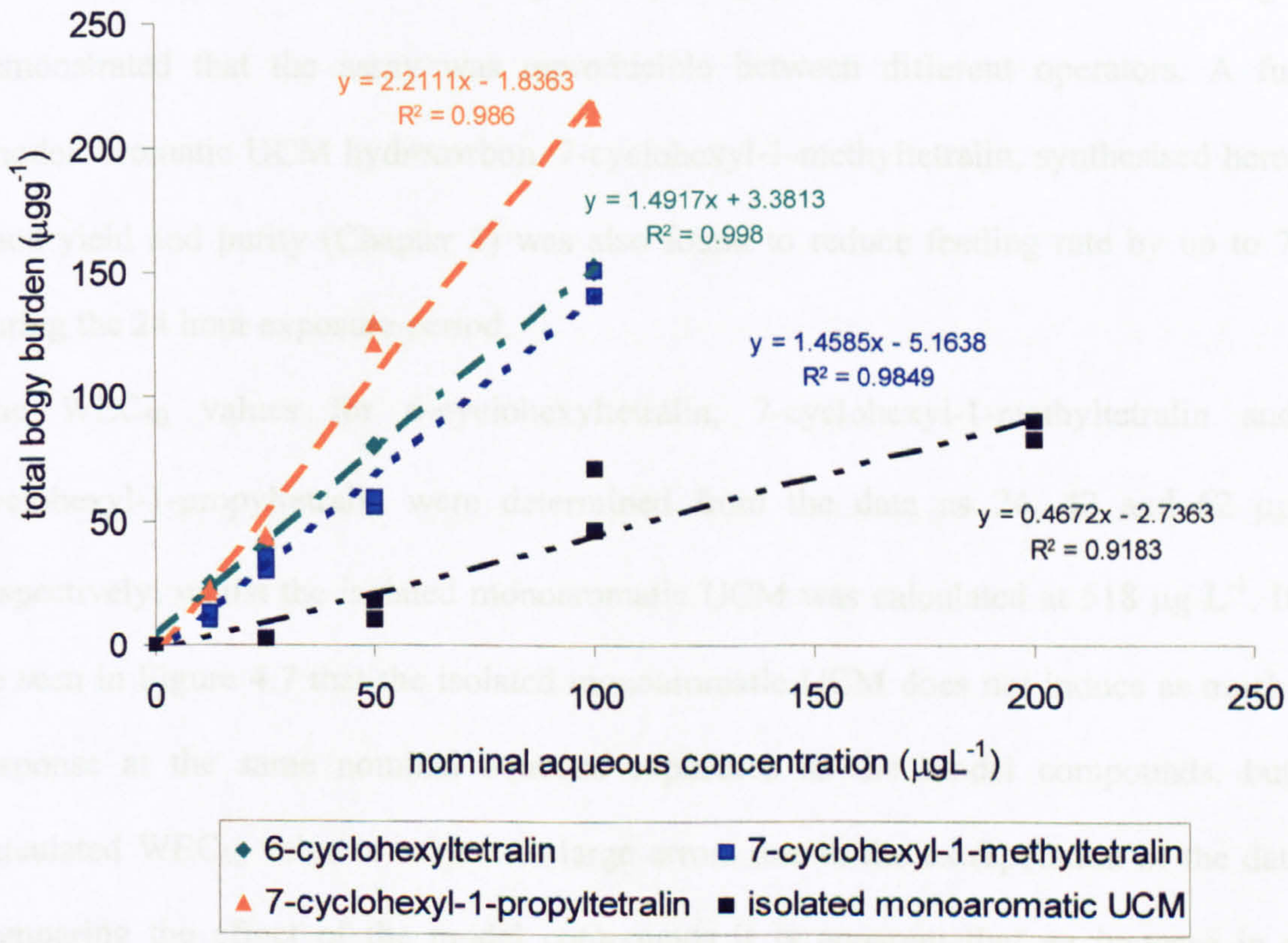


Figure 4.12 Relationship between nominal aqueous concentration and total body burden of 6-cyclohexyltetralin, 7-cyclohexyl-1-propyltetralin (Wraige, 1997), 7-cyclohexyl-1-methyltetralin and the isolated monoaromatic UCM (present study)

4.5 Discussion

Previous work carried out with 6-cyclohexyltetralin and 7–cyclohexyl-1-propyltetralin, proposed as 'model' aromatic UCM hydrocarbons, found them to be toxic as measured by mussel feeding activity (Wraige 1997). A repeat experiment carried out with 7-cyclohexyltetralin at 50 $\mu g\ L^{-1}$, which was previously shown to induce a 50% reduction in mussel feeding rate over 24 hour exposure (Wraige, 1997), confirmed these findings and demonstrated that the assay was reproducible between different operators. A further 'model' aromatic UCM hydrocarbon, 7-cyclohexyl-1-methyltetralin, synthesised herein in good yield and purity (Chapter 3) was also found to reduce feeding rate by up to 70 % during the 24 hour exposure period.

The WEC_{50} values for 6-cyclohexyltetralin, 7-cyclohexyl-1-methyltetralin and 7-cyclohexyl-1-propyltetralin were determined from the data as 24, 42 and 62 $\mu g\ L^{-1}$ respectively; whilst the isolated monoaromatic UCM was calculated at 518 $\mu g\ L^{-1}$. It can be seen in Figure 4.7 that the isolated monoaromatic UCM does not induce as much of a response at the same nominal aqueous exposures as the model compounds, but the calculated WEC_{50} value is subject to large errors due to the extrapolation of the data. In comparing the effect of the model compounds it is apparent that an increase in alkyl substitution increases the nominal aqueous concentration required to achieve a 50% response, thus 7-cyclohexyl-1-propyltetralin has the highest WEC_{50}. As the UCM has a greater WEC_{50} this may be indicative of the isolated monoaromatic UCM containing compounds similar to the models but with greater chain lengths as hypothesised by compositional studies (*e.g.* Thomas, 1995).

There has been much criticism about the practicality and usefulness of water concentration as a measure of potential toxicity in environmental quality objectives (van den Heuvel, 1991) It has been demonstrated that the internal potency of many organic chemicals, in

particular those acting by non-specific narcosis, occurs at a relatively constant level, the difference in observed toxicity being due to difference in uptake kinetics.

A reduction in mussel feeding rate is caused by reduced pumping of the lateral cilia of the gill (Axaik & George, 1987). The cilia are under neuronal control (Paparo, 1972), thus the mechanism of feeding rate reduction is consistent with a non-specific mode of narcotic action (Donkin, 1989). If narcosis occurs when the internal narcotic concentration reaches some constant then the time required to reach this state should be unimportant (Pawlisz & Peters, 1993). Therefore, exposure was related to feeding rate in terms of body burden as well as aqueous exposure. The TEC_{50} value calculated for 7-cyclohexyl-1-methyltetralin was 58 $\mu g\ g^{-1}$ (0.254 mmol kg^{-1}). The effect observed for this synthesised compound was comparable to data from Wraige (1997) who observed TEC_{50} values of 41.4 $\mu g\ g^{-1}$ (0.206 mmol kg^{-1}) and 138 $\mu g\ g^{-1}$ (0.539 mmol kg^{-1}) for two other model UCM compounds, 6-cyclohexyltetralin and 7-cyclohexyl-1-propyltetralin. The data are also comparable with data from Donkin *et al.* (1989, 1991). These authors found that a range of aliphatic and aromatic hydrocarbons reduced the feeding rate of mussels and had TEC_{50} values in the 16-82 $\mu g\ g^{-1}$ (0.1-0.43 mmol kg^{-1}) range. This demonstrated that for compounds with a log K_{ow} of 2.65-5.0 the TEC_{50} value for feeding rate reduction occurred at a relatively constant tissue concentration, indicating that differences in aqueous concentration based toxicity values of these compounds actually reflect differences in bioaccumulation.

Although a much higher aqueous concentration of the isolated monoaromatic UCM was required to reduce the feeding rate of mussels, in terms of tissue concentration the isolated UCM tested appeared to follow a similar trend to the model compounds (Figure 4.10). A comparable reduction in feeding rate is observed at similar accumulated tissue concentrations, for example, at an accumulation of 75 $\mu g\ g^{-1}$ wet weight, mussel feeding rate was reduced by 60, 53, 28 and 35 % for 6-cyclohexyltetralin, 7-cyclohexyl-1-methyltetralin, 7-cyclohexyl-1-propyltetralin and the isolated monoaromatic UCM,

respectively. This observation is consistent with current theories that for any chemical causing toxicity primarily by narcosis the toxicant concentrations should be relatively constant at the site of toxic action. However the TEC_{50} value calculated for the isolated monoaromatic UCM was 500 $\mu g\ g^{-1}$, which is much greater than for the model compounds although this may have been due to the extrapolation of the data outside of the range of the observed response.

Differences in toxicity based aqueous data may have been influenced by differences in bioaccumulation. A difference in uptake between the model aromatic hydrocarbons and the isolated monoaromatic UCM was observed. 6-cyclohexyltetralin and 7-cyclohexyl-1-methyltetralin showed a similar relationship between total body burden and nominal aqueous concentration, 7-cyclohexyl-1-propyltetralin showed a greater uptake, compared to the monoaromatic UCM which showed a slower uptake despite a higher exposure concentration. This suggests that the UCM was slow to reach equilibrium within the tissue. This is not necessarily surprising with a 24 hour exposure period. However one would perhaps expect a similar relationship to occur through the series of model compounds, but in fact the opposite was observed. Another possibility is that not all of isolated UCM may have been bioavailable to the mussels, and only that which was present in a truly soluble form entered into the tissues in the 24 hour exposure period. Upon examination of the gas chromatograms of the tissue extract of the exposed mussels, the 'hump' accumulated within the tissues does not appear to be different from the original test substrate. One might expect to see a shift in the profile of the UCM if this was the case, although this cannot be ruled out as the higher molecular weight range was slightly masked by the presence of the natural compounds present in the mussel extract. This emphasises the difficulty in working with such complex mixtures of undefined compounds, *i.e.* how does one detect subtle changes within the UCM profile. It would certainly be useful to carry out the toxicological assessment of the isolated monoaromatic UCM over a longer exposure time period and

possibly higher exposure concentrations to more accurately assess the importance of these effects on the uptake and toxicity of the monoaromatic UCM compounds, and more accurately determine values for WEC_{50} and TEC_{50}.

If a cut-off effect, as demonstrated by Donkin, (1989, 1991) was observed for the UCM one would expect to see greater accumulation of these compounds within organisms without inducing a response. However, in terms of tissue concentration the isolated UCM tested appeared to follow a similar trend to the model compounds (Figure 4.10). A comparable reduction in feeding rate is observed at similar accumulated tissue concentrations, for example, at an accumulation of 75 $\mu g\ g^{-1}$ wet weight, mussel feeding rate was reduced by 60, 53, 28 and 35 % for 6-cyclohexyltetralin, 7-cyclohexyl-1-methyltetralin, 7-cyclohexyl-1-propyltetralin and the isolated monoaromatic UCM, respectively. This indicates that if the isolated monoaromatic UCM was accumulated to the same extent as the model compounds a comparable reduction in feeding rate would be observed. However a log plot of the body burden against mussel feeding rate shows that the reduction in feeding rate per increment of accumulated pollutant is not as marked for the isolated monoaromatic UCM as for the model monoaromatic UCM compounds. This possibly suggests that not all of the monoaromatic UCM contributed to the toxic response and thus a larger quantity is needed to accumulate in total for a similar amount to accumulate at the site of toxic action as the model compounds.

Hansch *et al.* (1989) suggested that the use of weight of toxicant per unit weight of animal as a means of expressing concentration for toxicological purposes should have been abandoned years ago, and advocated the use of relative biological activity as the best parameter for allowing meaningful comparisons between compounds. The parameter for relative biological activity is log 1/C (where C is the molar concentration of an organic compound) producing a standard biological response in a well defined system at a constant time interval.

The TEC_{50} value for 7-cyclohexyl-1-methyltetralin expressed in molar terms is 0.254 mmol kg^{-1}, which equates to a relative biological activity of 0.595. The relative biological activity of 6-cyclohexyltetralin can be calculated as 0.686 and that for 7-cyclohexyl-1-propyltetralin as 0.268. This approach is fine for ranking individual compounds but not possible for complex mixtures such as the isolated monoaromatic UCM. However, Killops & Al-Juboori (1990) suggested that the aromatic UCM consisted of compounds with an average molecular weight of 300. This is a reasonable value when one considers that the model compounds, found by gas chromatography to be within the lower molecular weight end of the hump (Figure 3.22), have molecular weights of 214, 228 and 256. If the calculated TEC_{50} value of 500 μg g^{-1} is used then the relative biological activity of the isolated monoaromatic UCM is 0.85. The effects observed for the synthetic compounds and the isolated monoaromatic UCM are similar to those observed for other hydrocarbons with similar physicochemical properties (reviewed by Donkin *et al.*, 1991) and have been summarised in Table 4.6. A comparison of the data in Table 4.6 suggests that the model UCMs hydrocarbons and the isolated UCM fit well within this data set. 6-cyclohexyltetralin and 7-cyclohexyl-1-methyltetralin have biological activities (0.686 and 0.595) at roughly average values of the data set, being similar to naphthalene (0.619) and 1-phenylheptane (0.698), whilst 7-cyclohexyl-1-propyltetralin has a slightly lower biological activity (0.268). The isolated monoaromatic UCM actually shows a higher biological activity (0.85) similar to chloronapthalene (0.85). This suggests that the isolated monoaromatic UCM behaves in the same way as the resolved monoaromatic compounds, probably by the addition of the small effect of each component. However in terms of TEC_{50} expressed in μmol g^{-1} the TEC_{50} value for the isolated monoaromatic UCM is much larger than that of the other aromatic compounds, perhaps suggesting that only a proportion of the monoaromatic UCM is responsible for the effects.

Table 4.6 The effect of hydrocarbons on the filter feeding rate of mussels (*Mytilus edulis*) – a synthesis of the data of Donkin *et al* (1989, 1991) and Wraige (1997)* with present study+

Compound	Carbon number	$LogK_{ow}$	Aq. Sol. $\mu g\ L^{-1}$	WEC_{50} $\mu g\ L^{-1}$	WEC_{50} $\mu mol\ L^{-1}$	TEC_{50} $\mu g\ g^{-1}$	TEC_{50} $\mu mol\ g^{-1}$	Log 1/C C=molar conc.
Toluene	7	2.65	578600	2347	25.51	16	0.17	0.769
naphthalene	10	3.35	30640	922	7.2	31	0.24	0.619
n-propylbenzene	9	3.69	52170	862	7.18	27	0.23	0.63
chloronaphthalene	10	4.08	22490	270	1.66	22	0.14	0.85
acenaphthalene	12	3.92	3930	382	2.48	29	0.19	0.721
biphenyl	12	3.95	7000	295	1.92	16	0.1	1
phenanthrene	14	4.57	1180	148	0.86	31	0.17	0.769
dibenzothiophene	12	4.42	n.a.	94	0.51	14	0.08	1.09
pyrene	16	5.18	135	>0.4	>0.2	>189	>0.94	> 0.02
fluoranthene	16	5.22	263	80	0.4	627	3.10	-0.491
1-phenylheptane	13	6.11	269	93	0.53	35	0.2	0.698
1-phenyloctane	14	6.73	71	79	0.41	82	0.43	0.366
1-phenyldecane	16	7.55	5	>200e	>0.92	>78	>0.36	<0.44
6-cyclohexyltetralin*	16	6.33, 6.77		24	0.11	44	0.206	0.686
7-cyclohexyl-1-methyltetralin+	17			42	0.18	58	0.254	0.595
7-cyclohexyl-1-propyltetralin*	19	7.91, 8.17		62	0.24	138	0.539	0.268
Isolated monoaromatic UCM+				518		500	1.1666	0.85

Theoretically it can be hypothesised that as narcotic effects occur at a relatively constant body burden of toxicant each molecule of narcotic toxicant is functionally equivalent at the site of toxic action. Therefore when equal numbers of molecules reach the site of toxic action a similar toxicological response will be produced. It is thus expected that mixture toxicity in terms of body burden is likely to be concentration additive (McCarty, 1986). Several studies 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 (*e.g.* Hermens *et al.*, 1984, 1985; Deneer *et al.*, 1988). Information on the joint effects of sub-lethal toxicity is scarce although there is conjecture that as the concentration of toxicants in a mixture are reduced to the level of no-effect the potential for addition is reduced (Hermens *et al.*, 1984). Hermens *et a*l. (1984) found that although joint toxicity at a sub-lethal level was lower than at a lethal level the toxicity of the mixture remained higher than that of the individual chemicals and was still rather near concentration addition. Thus the monoaromatic UCM, consisting of hundreds of compounds is likely to achieve an effect through the addition of all of its constituents.

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. It was suggested that these results were anomalous as the compounds had relatively high aqueous solubility (263 $\mu g\ L^{-1}$ and 135 $\mu g\ L^{-1}$) and that the apparent lack of toxicity despite efficient accumulation was due to sequestration or some other mechanism which reduced concentrations at the active site. 1-Phenylundecane was considered to have too low a solubility at 5 $\mu g\ L^{-1}$ and be indicative of a solubility cut-off. Thus, a cut-off effect was occurring despite very efficient bioaccumulation of all compounds tested which could be indicative of a molecular size related cut-off at the site of toxic action (*e.g.* associated with

binding membrane proteins) or of sequestration into a toxicology unavailable form (*e.g.* crystals or in lysosomes). Donkin *et al.* (1989, 1991) suggested a solubility cut-off of 70 $\mu g\ L^{-1}$ for such compounds on the reduction of mussel feeding rate. It would therefore be useful to study the aqueous solubility of the model compounds along with the monoaromatic UCM to determine if all, or only part, of the monoaromatic UCM is capable of causing the effects observed.

It was suggested herein that a longer exposure time would be suitable for studying monoaromatic UCM hydrocarbons as they do not appear to reach equilibrium within the 24 hour period. Theoretical considerations suggest that the time taken to reach the site of toxic action is irrelevant, therefore the exposure conditions are irrelevant. However, this is related to the concentration of the compound actually at the site of toxic action. When Wraige (1997) exposed mussels to 4-propyloctane, utilising the same approach as the present study, although the total body burden increased linearly with exposure period, no further reduction in mussel feeding rate was found after 24 hours, when the concentration in the gill tissue had reached steady state. This 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. The gill is the major site of uptake of chemicals from the water phase by aquatic animals (Hayton & Barron, 1990). Exchange at the gills is presumed to be as a result of diffusion across the epithelium separating water and blood (Barber *et al.*, 1988) and is relatively rapid. Initial uptake of a pollutant by the gills is followed by a slightly less rapid transfer to the circulatory fluid, followed by a much slower transfer to, and accumulation in, storage lipid reserves, until equilibrium or saturation storage capacity is reached (Farrington, 1991). It was therefore suggested that TEC_{50} values may vary and increase with longer exposure times when the gill tissue has reached saturation and bioaccumulation into the tissue continues. Wraige (1997) suggested that to maintain the sensitivity of the TEC_{50}

value exposure times were kept to 24 hours. The 24 hour exposure used herein indicates that the TEC_{50} value obtained for the model UCM compound 7-cyclohexyltetralin was therefore a meaningful measurement. However the isolated monoaromatic UCM is an extremely complex mixture, and as it accumulated more slowly than the model UCM hydrocarbons into the mussel tissue as a whole, so this might therefore be the case for accumulation into the gill tissue. It would be interesting to study the accumulation of the isolated monoaromatic UCM into the gill over a range of time periods to deduce at what exposure steady state is reached. If indeed the gill is the site of toxic action it would be useful to study pollutants in mussel tissues at impacted sites in the gill tissue rather than the tissue as a whole to determine the compounds that are responsible for the observed effects. It would be interesting to find how the UCM as a whole, and in particular the monoaromatic UCM, is reflected in this measurement.

4.6 Summary

The aim of the present study was to investigate the toxicity of the synthesised model monoaromatic UCM hydrocarbon 7-cyclohexyl-1-methyltetralin and compare the results with a previous study by Wraige (1997) in order to examine the effect of alkyl substitution of the 'base' cyclohexyltetralin structure on toxicity. These compounds are thought to be good models of monoaromatic UCM components according to currently available data and this study aimed to determine how the model compounds fitted in with existing theories on toxicological response. A further aim was to compare the toxicity elicited by the model compounds in terms of nominal aqueous concentrations and total body burden with the response found for the isolated monoaromatic UCM (Chapter 2).

As an initial study a repeat experiment was carried out with 6-cyclohexyltetralin at an aqueous exposure of 50 μg L^{-1}.This was previously shown to have reduced the feeding rate of mussels by 50%. A comparable reduction was observed which was statistically significant meaning that the results were reproducible and thus results from a further model monoaromatic UCM hydrocarbon and the isolated monoaromatic UCM could be compared with previous data. The test procedure was a modification of the procedure of Donkin *et al.* (1989, 1991) by Wraige (1997). This procedure had been standardised with butylcyclohexane meaning results from all the studies were comparable.

7-cyclohexyl-1-methyltetralin, synthesised herein (Chapter 3) in good yield and purity was exposed to mussels for 24 hours and a significant reduction (70 %) in feeding rate was observed. The WEC_{50} values of the three model compounds and the isolated monoaromatic UCM were calculated as 24, 42, 62, and 518 μg L^{-1} respectively. The models suggest an increase in alkyl substitution causes an increase in the WEC_{50} suggesting that the monoaromatic UCM may contain larger structures than the models. Variations in aqueous exposure based expressions of toxicity are generally attributed to differences in the kinetics of uptake of chemicals tested. Therefore, prior to steady state, the toxicity of slower accumulating chemicals increases with increasing exposure time (Donkin *et al.*, 1989). It would appear that the monoaromatic UCM is slowly accumulated within mussel tissue, suggesting that it had not achieved steady state. This would also contribute to its high WEC_{50} value.

A TEC_{50} value of 58 μg g^{-1} was calculated for 7-cyclohexyl-1-methyltetralin, which related to 0.254 mmol kg^{-1}. This was comparable to previous data on various aromatic hydrocarbons as well as two model aromatic hydrocarbons. A TEC_{50} value for the isolated monoaromatic UCM was calculated at 500 μg g^{-1}. This is much larger than for the model compounds, although as mussel feeding rate was not reduced over 50 % in the exposure experiment (Chapter 2), therefore large errors are incurred in the extrapolation of the data.

However, at an accumulation of 75 μg g^{-1} mussel feeding rate was reduced by 60, 53, 28 and 35 % for 6-cyclohexyltetralin, 7-cyclohexyl-1-methyltetralin, 7-cyclohexyl-1-propyltetralin and the isolated monoaromatic UCM, suggesting that if the monoaromatic UCM was accumulated to the same extent as the model UCM compounds a comparable reduction in feeding rate would be observed. This result supported the use of model compounds to understand the UCM, and supports the current theories of a constant internal potency and mixture toxicity. The accumulation into the mussel tissue of the UCM was much slower than for the model compounds, suggesting that it had not reached a steady state and required prolonged exposure to achieve this, or that only part of the monoaromatic UCM was bioavailable towards the mussels. When compared in terms of relative biological activity the isolated monoaromatic UCM has a value similar to those determined for other resolved compounds. Mixture toxicity in terms of body burden is likely to be concentration additive. Thus it might be expected that all of the components of the UCM add to the toxicity of the mixture. The similarity in mussel feeding rate response to total accumulated body burdens of the model aromatic UCM compounds and the isolated monoaromatic UCM, as evidenced in Figure 4.10 certainly supports this contention.

Therefore although the model UCM compounds are capable of reducing the feeding rate of mussels, and so is the isolated monoaromatic UCM, it is not obvious from the results whether sub-components, or all of the monoaromatic UCM hydrocarbons, are responsible for the observed reduction. Although if the models are indeed representative of the monoaromatic UCM then the lower molecular weight proportion of the monoaromatic UCM is certainly important.

Chapter 5. Aqueous solubility studies of model monoaromatic UCM compounds and an isolated monoaromatic UCM.

The aqueous solubility of a compound is an important physicochemical characteristic in determining its fate and behaviour in the marine environment. Aqueous solubility of hydrophobic compounds determined by the classical stirring method is impeded by their intrinsic nature, therefore the aqueous solubilities of the model aromatic UCM hydrocarbons were determined using a generator column method. Aqueous solubility was determined over a range of temperatures and salinities. A generator column containing the monoaromatic UCM isolated from a crude oil was set up to determine whether solutions of a monoaromatic UCM could be generated.

5.1 Introduction

Many ecotoxicological effects assessments are made on the basis of estimation techniques known as Quantitative Structure Activity Relationships (QSARs). These attempt to statistically relate the biological activity of a compound to its physicochemical properties (Cronin and Dearden, 1995). The use of QSARs in the prediction of aquatic toxicity has been reviewed (*e.g.* Nirmalakhandan & Speece, 1988; Donkin & Widdows 1991; Donkin, 1994; Cronin & Dearden, 1995)

The octanol-water partition coefficient (commonly used as log K_{ow}) occupies a key position in the early stages of the environmental assessment of a new chemical. It is often used to assess environmental partitioning and screen for bioconcentration in fish (reviewed by Brooke *et al.*, 1986). Log K_{ow} is generally considered the best measure of hydrophobicity and as such is widely accepted as a characteristic QSAR for unreactive non-ionisable compounds whose mode of toxic action is non-specific narcosis, such as hydrocarbons. A review of QSARs and the role played by partitioning and lipophilicity is provided by Dearden (1985). Although log K_{ow} is the most commonly used parameter to describe narcosis, QSARs using water solubility have also been successfully correlated to observed effects (*e.g.* Abernathy *et al.*, 1986).

For organic chemicals causing toxicity primarily by narcosis, Fergusonian theory suggests that at the site of toxic action, toxicant concentration should be relatively constant for the biological response in question (McCarty, 1986). This means that the toxicity to aquatic organisms of individual compounds is considered to be equal on the basis of their internal molar concentration (Peterson 1994). The difference in measured toxicity amongst hydrocarbons is a result of their equilibrium partitioning behaviour between water and the organism.

Studies on mussels show that some chemicals can be accumulated to considerable concentrations without causing a narcotic effect (Donkin *et al.*, 1989, 1991). Anaesthetic potency increases with lipid solubility, however a point is reached where a further increase in lipid solubility no longer causes a toxic effect to be observed, this is known as the effect cut-off (Seeman 1972). This occurs as a consequence of reduced water solubility such that insufficient amounts enter the organism to elicit a toxic response. Response cut-offs are an established characteristic of narcotic toxicity.

If a chemical is significantly soluble in water it can be transported directly to aquatic organisms which can accumulate it across their body. This is a major route of uptake for gill breathing animals. Wraige (1997) suggested that the gill was the site of toxic action for mussels. Wraige (1997) found that upon exposure to 4-propyloctane mussel feeding rate was related to the concentration in the gill tissue. No further decrease was found once the concentration in the gill had reached steady state, which was estimated to take 24 hours.

In general terms, lower molecular weight compounds are more soluble than higher molecular weight compounds but the higher molecular weight compounds have a higher acute toxicity. In addition, aromatic compounds are more soluble than aliphatic compounds of comparable molecular weight, and this enhanced solubility is likely to be the reason why aromatic fractions are more toxic than aliphatic fractions and have been consistently implicated in laboratory and field studies as the likely cause of observed effects (*e.g.* Widdows *et al.* 1987; Widdows *et al.*, 1995a). This suggests that the aromatic UCM, or components within it, will be more soluble than an aliphatic UCM of comparable molecular weight and a review of the literature in section 1.6 suggests aromatic UCMs may exist in a dissolved form.

A reduction in Scope for Growth in mussels exposed to petroleum has been demonstrated to occur primarily by feeding rate reduction. The responsiveness of mussel ciliary feeding rate to hydrocarbons has been reported by Donkin *et al.* (1989, 1991) who found that a cut-

off in toxicity occurred at 70 μg L^{-1}. Compounds with an aqueous solubility below 70 μg L^{-1} failed to induce a significant response in feeding rate despite greater accumulation into mussels than the more soluble toxic compounds. For the hydrocarbons studied Donkin *et al.* (1989, 1991) suggested that whilst log K_{ow} was a good predictor of toxicity and bioconcentration, aqueous solubility was a better indicator of cut-off point. This cut-off effect has been attributed to the low aqueous solubility of large hydrophobic molecules so that, although they have high partition coefficient, they are not soluble enough to get to the site of toxic action. In terms of molecular size this equated to the C_{11} compound, undecane, for aliphatic compounds, and the four-ring compound, pyrene, for aromatic compounds.

In Chapter 4 the mussel toxicological assay developed by Donkin *et al.* (1989, 1991) with some minor modifications (Wraige, 1997) was applied to the model aromatic UCM hydrocarbon 7-cyclohexyl-1-methlytetralin and this was found to reduce feeding rate by up to 70 %, indicating this compound is likely to have an aqueous solubility greater than 70 μg L^{-1}. In Chapter 2 the same assay was used to assess a monoaromatic UCM isolated from a crude oil, and a reduction in feeding rate was observed (~40%). It is not known where this cut-off affects the toxicity of monoaromatic UCMs. It may be that those components within the UCM which have sufficient solubility were responsible for the observed decrease in mussel feeding rate observed in Chapter 2 and the larger molecular weight components were accumulated without causing a toxic response.

5.1.1. Methods for determination of aqueous solubility

The methods used for the determination of aqueous solubility and log K_{ow} can be divided into two main classes:- estimation methods and direct determination.

5.1.1.1 Estimation methods

The estimation of aqueous solubility and log K_{ow} has been extensively reviewed (Yalkowsky, 1992). A number of methods have been developed for the prediction of these values from chemical structure. These methods are based on a constructionist fragment methodology where the structure is divided into fragments (atoms or larger functional groups), and the value of each fragment (or group) is added together to give an estimated value. The Leo fragment and ClogP methods use a group contribution method and the SRC (Syracuse Research Corporation) software uses multiple regression to quantify the weights of different structures on the principle of least squares deviation. Estimates of the log K_{ow} and aqueous solubility of the model aromatic UCM compounds were calculated using an established computer program (values provided by Prof. John Dearden, Liverpool John Moores University) and are summarised in Table 5.1. Aqueous solubility data suggest that these compounds are not soluble enough (< 70 $\mu g\ L^{-1}$) to induce a response in mussel feeding rate. Previous work with 6-cyclohexyltetralin and 7-cyclohexyl-1-propyltetralin (Wraige, 1997) and 7-cyclohexyl-1-methyltetralin (this study, Chapter 4) has shown these compounds to be toxic as measured by mussel feeding rate activity even though their estimated solubility suggested otherwise. The log K_{ow} estimates suggest that the model UCM hydrocarbons may be described as highly hydrophobic (log K_{ow} > 6) although it is possible that these values are overestimates. The data used to derive fragment contributions for both chemical group and regression equations are derived from experimentally measured values of a limited set of chemicals so the applicability of such models is not universal (Gombar & Enslein, 1996). As these fragments are generally derived from

relatively small molecules their applicability to larger more hydrophobic molecules, such as the model aromatic UCM hydrocarbons, is questionable, especially when it is known there may be considerable errors using estimation methods for compounds with log K_{ow} > 6 (Chessels, 1991). Therefore, although these estimation methods provide a useful guide, it is desirable to directly determine the aqueous solubility of the model aromatic UCM compounds.

Table 5.1 Estimates of log K_{ow} and aqueous solubility for three model monoaromatic UCM hydrocarbons (data provided by Prof. J. Dearden, Liverpool John Moores University)

Compound	Estimated log K_{ow}	Estimated aqueous solubility $\mu g\ L^{-1}$
6-cylohexyltetralin	6.61[a] 6.33[b] 6.77[d]	152[c] 60[d]
7-cyclohexyl-1-methyltetralin	7.27[a] 6.85[b] 7.19[d]	22.4[d]
7-cyclohexyl-1-propyltetralin	7.73[a] 7.91[b] 8.17[d]	3[c] 2[d]

[a] Leo fragment

[b] ClogP

[c] microQSAR

[d] SRC

5.1.1.2 Direct determination

The classic procedure for directly determining aqueous solubility of a compound is the slow stirring method. Saturated solutions are prepared by adding an excess of the solute to be measured to water. This is mechanically stirred for 24 hours and the aqueous concentration determined. However, difficulties are encountered in the direct measurement of highly hydrophobic compounds owing to their inherent nature. Problems encountered include, the incomplete equilibration of hydrocarbons with aqueous media, dispersion rather than true dissolution (accommodation), failure to remove suspended microcrystals, and adsorptive losses from solution. This method is clearly unsuitable for the direct determination of the aqueous solubility of model aromatic UCM hydrocarbons. 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 & Banergee, 1992). A detailed description and critical evaluation of the technique has been presented by May (1978) and May *et al.* (1978). The generator column method is designed to avoid problems with adsorption onto container walls and offers the potential for generating known solubilities of slightly soluble organic compounds. With this technique water can be saturated reasonably quickly by passing it through a temperature controlled generator column packed with inert material on which the test compound is coated. The flow rate is slow enough to avoid colloidal dispersions while the larger interfacial area between the organic and aqueous phase allows rapid equilibration to take place. As the system walls become equilibrated errors due to adsorption are minimised. Generator columns have been used to generate saturated solutions of very sparingly soluble compounds with concentrations reaching as low as 0.000074 μgL^{-1} for octachlorodibenzodioxin (Shui *et al.*, 1988). Modified columns have also been used to measure log K_{ow} values of up to 8.29 (Woodburn *et al.*, 1984).

5.2 Aims

Consideration of the physicochemical properties of the model monoaromatic UCM hydrocarbons would suggest that these compounds are not water soluble enough to be toxic and impair mussel feeding rate. However, previous work (Wraige, 1997) and work in the present study (Chapter 4) indicates otherwise. The WEC_{50} values calculated for 7-cyclohexyl-1-methyltetralin and 7-cyclohexyl-1-propyltetralin (42 and 62 μg L^{-1} respectively) are above the estimated values for their aqueous solubility (22.4 and 2-3 μg L^{-1} respectively). Therefore, the aims of this study were to measure the aqueous solubility of 6-cyclohexyltetralin, 7-cyclohexyl-1-methyltetralin and 7-cyclohexyl-1-propyltetralin. UCMs are considered 'insoluble' and therefore non-toxic, although circumstantial evidence (Chapter 1) suggests that aromatic UCMs may exist in a dissolved form. An isolated monoaromatic UCM reduced mussel feeding rate by ~40 % at the highest nominal aqueous concentration of 200 μg L^{-1} (Chapter 2). It was not obvious whether all or only a proportion of the isolated monoaromatic UCM was responsible for the reduction. Therefore a further aim of this study was to use the generator column method to see if an aqueous solution of the isolated monoaromatic UCM could be generated.

5.3 Experimental Approach

The model monoaromatic UCM hydrocarbons 6-cyclohexyltetralin, 7-cyclohexyl-1-methyltetralin and 7-cyclohexyl-1-propyltetralin were synthesised in good purity and yield as described in Chapter 3 and a monoaromatic UCM was isolated from a crude oil as described in Chapter 2.

The generator column set-up is illustrated in Figure 5.1. Each generator column consisted of a high pressure liquid chromatography steel column 30 cm x 0.46 mm i.d. with 2 μm

stainless steel frits at both ends, dry packed with glass beads 60-80 mesh coated with the test compound. Previous workers have used a variety of column sizes packed with either glass beads or Chromosorb packing (typically 60-80 mesh). The glass beads were added to the test compound dissolved in solvent which was removed by rotary evaporation followed by a gentle stream of nitrogen to give a 0.005% coating. Previous workers have used coatings from 0.003% (Friensen *et al.*, 1990) up to 2 % (Dickhut *et al.*, 1986). Once packed the generator column was attached to a pump by means of Teflon tubing and attachments, and water was supplied from a water reservoir. Temperature control (25 and 15 °C) was by means of a dip chiller unit and a water bath in which the generator column and the water reservoir were immersed. This was covered with insulation balls to minimise any fluctuations in temperature and evaporation of water with time. A 3L beaker containing either MilliQ water or seawater (salinity 35‰), to which mercuric chloride was added for sterilisation, acted as a water reservoir. Temperature and salinity were monitored throughout the experiments.

Water was pumped through the column at a rate of 1 mL min^{-1}. The first 500 mL of solution was discarded to allow the system to equilibrate before measurement, consistent with Dunnivant & Elzermann (1988). Effluent from the column was collected directly into a 100 mL separating funnel containing 25 mL DCM. Once the samples had been collected an internal standard was spiked into the water in 100 μL acetone (for the model UCM hydrocarbons) and the funnel stoppered. Each funnel was shaken for 5 minutes with care so as not to form an emulsion. The DCM fraction was separated off and the water samples further extracted with another 25 mL DCM. The DCM extracts were combined and passed through an anhydrous sodium sulphate column. Analysis was performed using GCMS operated in selected ion monitoring mode (SIM) for model UCM compounds 6-cyclohexyltetralin, 7-cyclohexyl-1-methyltetralin and 7-cyclohexyl-1-propyltetralin, and in full scan mode for the monoaromatic UCM.

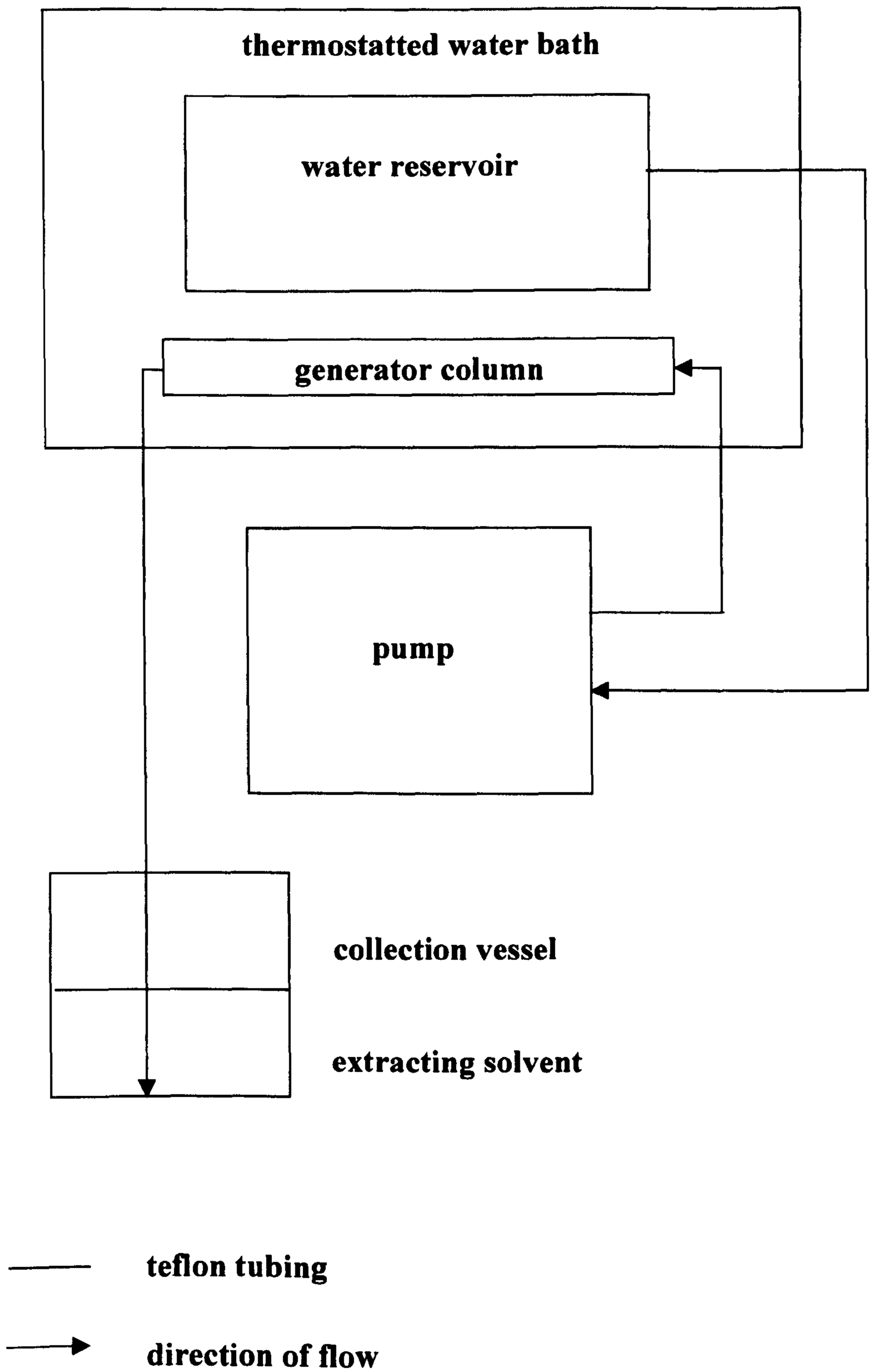


Figure 5.1 Illustration of generator column set-up for aqueous solubility determination.

5.4 Results

5.4.1 Validation of experimental set-up by determination of aqueous solubility of anthracene.

To validate the method anthracene was chosen as a test compound as its aqueous solubility had previously been determined by this and other methods. There are many reports in the literature of the aqueous solubility of anthracene determined using a generator column. These are summarised in Table 5.2. The majority of the authors report an aqueous solubility of approximately 40 μg L^{-1} in distilled water at 25 °C. The disparity of other measurements has been suggested to occur from the use of non-specific analytical techniques such as UV detection, combined with the presence of small quantities of phenanthrene in commercial samples of anthracene, leading to a greater response, and thus increasing the reported aqueous solubility (May *et al.*, 1978).

Table 5.2 Reported aqueous solubility of anthracene as determined by generator column method (distilled water, 25 °C)

Reference	Reported value μg L^{-1}
May *et al.*(1978)	44.6
Mackay & Shui (1975)	73 ± 0.5
Schwarz (1977)	41 ± 0.3
De Maagd *et al.* (1999)	93 ± 12
May & Wasik (1978)	43.4
Billington *et al.* (1988)	44.3
Wasik *et al.* (1983)	44.6
Walters & Luthy (1984)	69.8
Vadas *et al.* (1991)	58
Lu *et al.* (1978)	30

The effect of salinity and temperature on the aqueous solubility of anthracene has also been studied by using a generator column technique (Whitehouse, 1984; May *et al.*, 1978). These values are presented in Table 5.3. Thus, method validation also extended to the effect of temperature and salinity on the measurement of aqueous solubility.

Table 5.3 The effect of temperature and salinity on the reported measurement of aqueous solubility of anthracene determined by the generator column method.

Reference	Reported Value $\mu g\ L^{-1}$	Temperature °C	Salinity ppt
May *et al.* (1978)	42.2 ± 0.6	25	0
	29.1 ± 0.1	18.3	0
	17.5 ± 0.3	14.1	0
Whitehouse (1984)	44.19 ± 0.713	25.3	0
	32.40 ± 0.178	25.3	36.5
	23.70 ± 0.356	17	0
	17.39 ± 0.321	17	36.5
	17.70 ± 0.231	12.9	0
	12.19 ± 0.178	12.9	36.5

The results from the anthracene generator column are summarised in Table 5.4 and can be compared to previously reported values from the literature (Table 5.2). It can be seen that the values obtained in this study compare well with previous measurements. Results are also comparable to measurements carried out by Whitehouse (1984) and May *et al.* (1978) over a salinity and temperature range (Table 5.4). Thus, the method employed herein was judged to be suitable for the aqueous solubility determination of the model aromatic UCM hydrocarbons over a temperature and salinity range.

Table 5.4 Aqueous solubility of anthracene as determined by generator column method

Conditions	Aqueous solubility μg L^{-1} mean ± st. dev.	
	Present study (n =10)	Whitehouse (1984)
Distilled water 25 ° C	45 ± 2	44
Seawater 25 °C	29 ± 1	32
Distilled water 15 °C	22 ± 1	27 (17 °C) 17 (12 °C)

5.4.2 Determination of the aqueous solubility of model aromatic UCM compounds.

The aqueous solubility of the model aromatic UCM hydrocarbons determined at 25 °C in distilled water is summarised in Table 5.5. Values are reported as the mean ± standard deviation (n = 10). 6-cyclohexyltetralin was found to have the greatest water solubility at 109 μg L^{-1} and a decrease in solubility was observed with increasing chain length of the alkyl substituent on the cyclohexyltetralin structure (methyl, 45 μg L^{-1}; propyl, 23 μg L^{-1}). All the values are greater than those predicted by the computer software (Table 5.1). Only 6-cyclohexyltetralin is of sufficient solubility to cause toxicity as described by Donkin *et al.* (1989). However, only 7-cyclohexyl-1-propyltetralin was below its WEC_{50} value (calculated in Chapter 4 to be 62 μg L^{-1}). Although the toxicity tests were not carried out at 25 °C in distilled water but at 15 °C in seawater, the cut-off described by Donkin *et al.* (1989, 1991) of 70 μg L^{-1} was based on solubility data at 25 °C in distilled water.

Table 5.5 Aqueous solubility of model aromatic UCM hydrocarbons, 6-cyclohexyltetralin, 7-cyclohexyl-1-methyltetralin and 7-cyclohexyl-1-propyltetralin as determined by generator column method in distilled water at 25 °C

Compound	Aqueous solubility μg L^{-1} mean ± st. dev. (n = 10)
6-cyclohexyltetralin	109 ± 6
7-cyclohexyl-1-methyltetralin	45 ± 4
7-cyclohexyl-1-propyltetralin	23 ± 3

The aqueous solubility of the model aromatic UCM hydrocarbons determined at 25 °C in seawater is summarised in Table 5.6. Values are reported as the mean ± standard deviation (n = 10). Seawater reduces solubility of the model hydrocarbons as compared to distilled water. This is consistent with salting-out theory. The effect is more pronounced with increasing alkyl substitution on the cyclohexyltetralin base structure with 7-cyclohexyl-1-propyltetralin being reduced by almost half of its aqueous solubility in distilled water.

Table 5.6 Aqueous solubility of model aromatic UCM hydrocarbons, 6-cyclohexyltetralin, 7-cyclohexyl-1-methyltetralin and 7-cyclohexyl-1-propyltetralin as determined by generator column method in seawater at 25 °C

Compound	Aqueous solubility μg L^{-1} mean ± st. dev. (n = 10)
6-cyclohexyltetralin	75 ± 3
7-cyclohexyl-1-methyltetralin	27 ± 3
7-cyclohexyl-1-propyltetralin	13 ± 2

The aqueous solubility of the model aromatic UCM hydrocarbons determined at 15 °C in distilled water is summarised in Table 5.7. Values are reported as the mean ± standard deviation (n = 10). A decrease in temperature caused a decrease in aqueous solubility.

Table 5.7 Aqueous solubility of model aromatic UCM hydrocarbons, 6-cyclohexyltetralin, 7-cyclohexyl-1-methyltetralin and 7-cyclohexyl-1-propyltetralin as determined by generator column method in distilled water at 15 °C

Compound	Aqueous solubility ug L^{-1} mean ± st. dev. (n= 10)
6-cyclohexyltetralin	95 ± 5
7-cyclohexyl-1-methyltetralin	40 ± 2
7-cyclohexyl-1-propyltetralin	17 ± 2

The aqueous solubility of the model aromatic UCM hydrocarbons determined at 15 °C in seawater is summarised in Table 5.8. Values are reported as the mean ± standard deviation (n = 10). The action of increasing salinity and decreasing temperature caused the greatest decrease in aqueous solubility.

Table 5.8 Aqueous solubility of model aromatic UCM hydrocarbons, 6-cyclohexyltetralin, 7-cyclohexyl-1-methyltetralin and 7-cyclohexyl-1-propyltetralin as determined by generator column method in seawater at 15 °C

Compound	Aqueous solubility ug L^{-1} mean ± st. dev. (n= 10)
6-cyclohexyltetralin	56 ± 3
7-cyclohexyl-1-methyltetralin	21 ± 2
7-cyclohexyl-1-propyltetralin	9 ± 2

A summary of the aqueous solubility data generated for the model UCM hydrocarbons, 6-cyclohexyltetralin, 7-cyclohexyl-1-methyltetralin and 7-cyclohexyl-1-propyltetralin is presented in Table 5.9. These data are also shown graphically in Figure 5.2 along with the estimated data from the Syracuse software (Table 5.1). The estimated data from the software package are actually very similar to the values of aqueous solubility determined at 15 °C in seawater for 6-cyclohexyltetralin and 7-cyclohexyl-1-methyltetralin, although still underestimates the aqueous solubility of 7-cyclohexyl-1-propyltetralin.

Table 5.9 Summary of aqueous solubility data for model UCM hydrocarbons 6-cyclohexyltetralin, 7-cyclohexyl-1-methyltetralin and 7-cyclohexyl-1-propyltetralin.

Compound	Aqueous solubility $\mu g\ L^{-1}$ mean ± std. dev. (n = 10)			
	25°C 0 ‰	25°C 35 ‰	15°C 0 ‰	15°C 35 ‰
6-cyclohexyltetralin	109 ± 6	75 ± 3	95 ± 5	56 ± 3
7-cyclohexyl-1-methyltetralin	45 ± 4	27 ± 3	40 ± 2	21 ± 2
7-cyclohexyl-1-propyltetralin	23 ± 3	13 ± 2	17 ± 2	9 ± 2

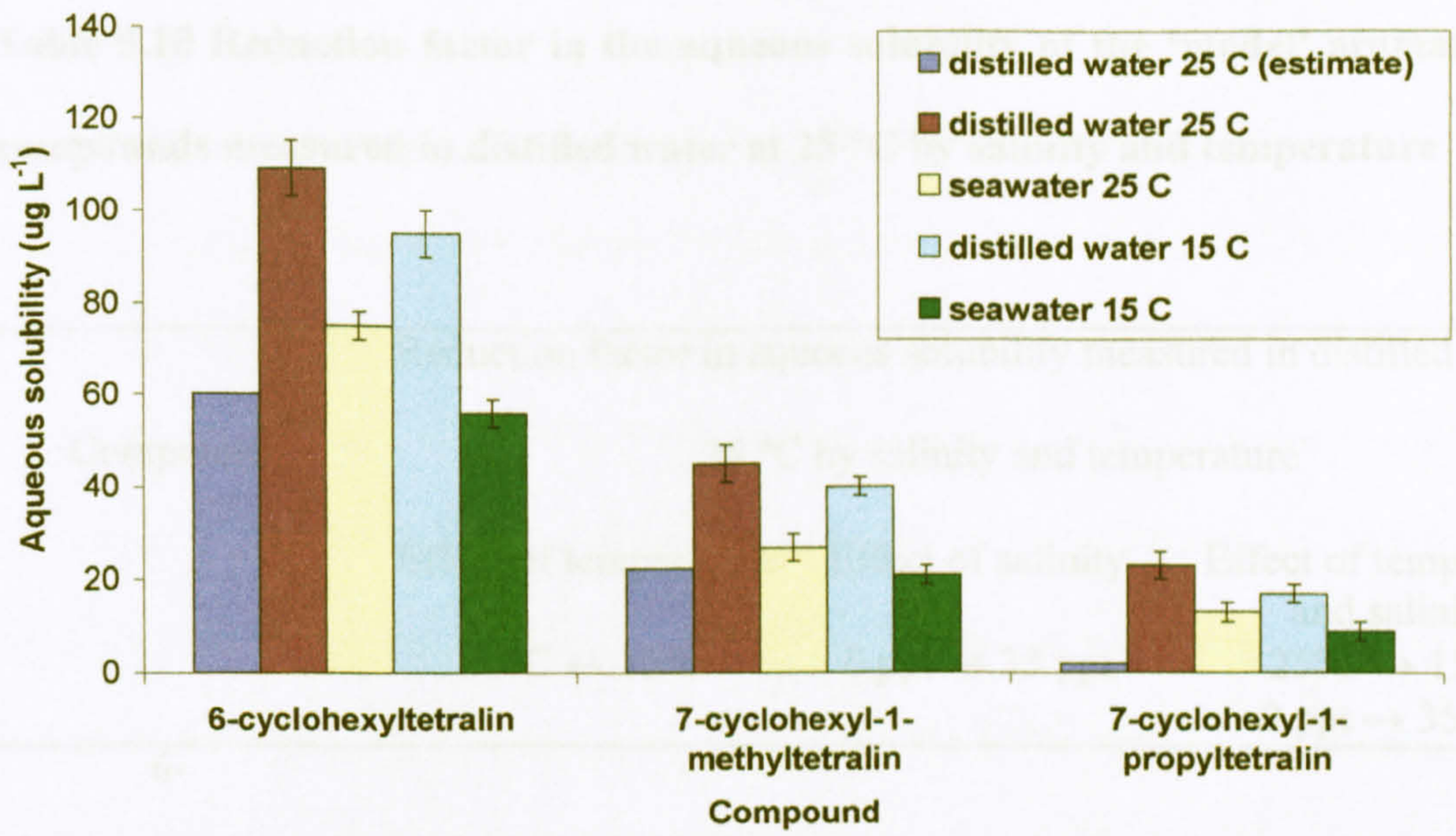


Figure 5.2 Comparison of the aqueous solubility of model aromatic UCM hydrocarbons 6-cyclohexyltetralin, 7-cyclohexyl-1-methyltetralin and 7-cyclohexyl-1-propyltetralin as determined by estimation and direct methods under variable environmental conditions

In order to clarify the effects of salinity, temperature and the length of the alkyl chain substituent on the aqueous solubility of these compounds, Table 5.11 documents the reduction factor in the aqueous solubility of 6-cyclohexyltetralin, 7-cyclohexyl-1-methyltetralin and 7-cyclohexyl-1-propyltetralin measured in distilled water at 25 °C by these processes. The reduction factorl in aqueous solubility measured in distilled water at 25 °C by salinity and temperature was calculated by dividing the aqueous solubility measured at a different temperature or salinity by the aqueous solubility measured in distilled water at 25 °C. This allowed the relative effects of temperature and salinity on the aqueous solubility of these compounds to be determined and highlighted differences between the compounds.

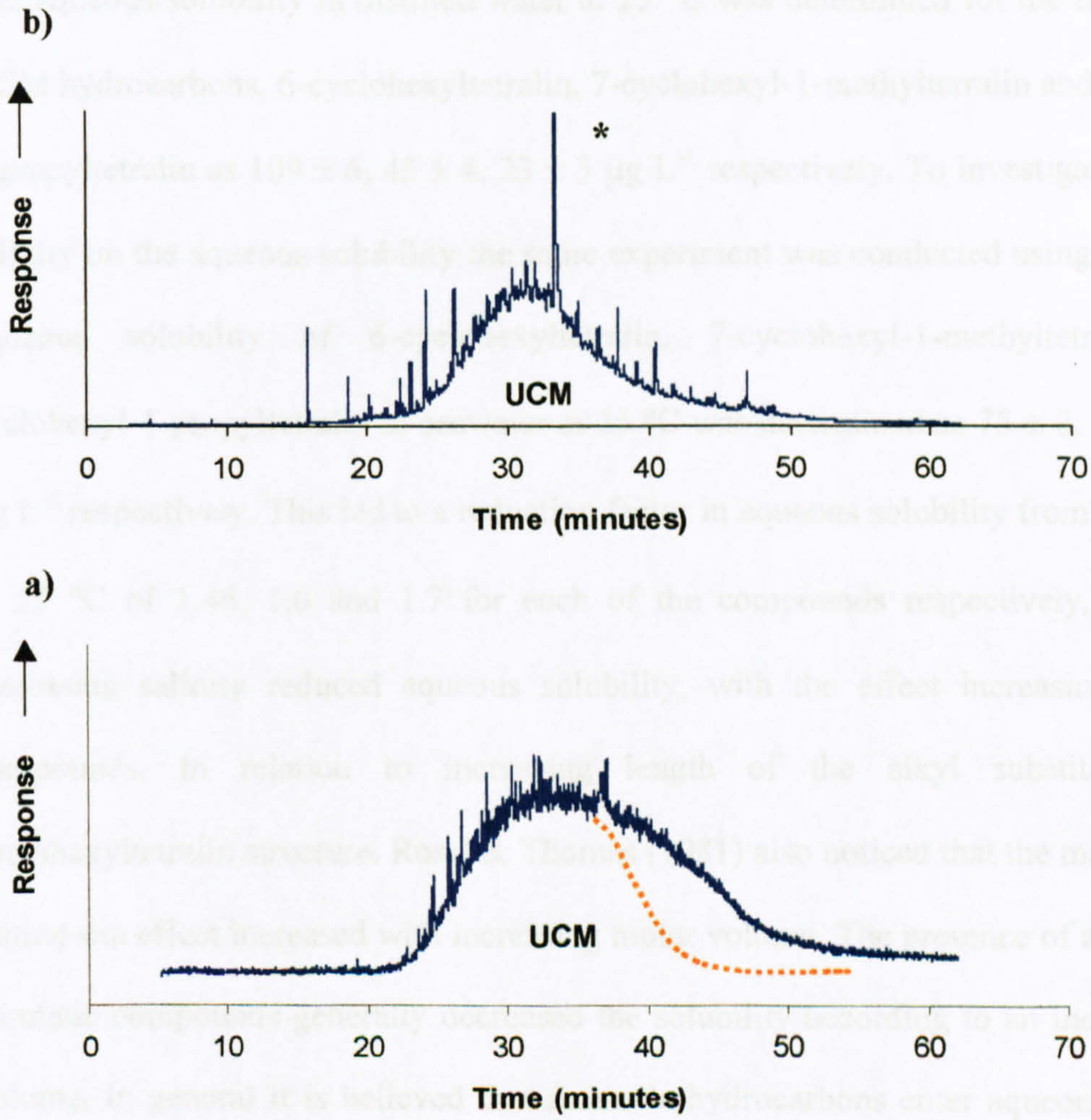
Table 5.10 Reduction factor in the aqueous solubility of the 'model' aromatic UCM compounds measured in distilled water at 25 °C by salinity and temperature

Compound	Reduction factor in aqueous solubility measured in distilled water at 25 °C by salinity and temperature		
	Effect of temperature 25°C → 15°C	Effect of salinity 0 ppt → 35 ppt	Effect of temperature and salinity 25°C → 15°C 0 ppt → 35 ppt
6-cyclohexyltetralin	1.15	1.46	1.96
7-cyclohexyl-1-methyltetralin	1.12	1.6	2.14
7-cyclohexyl-1-propyltetralin	1.3	1.7	2.5

5.4.3 Generation of an aqueous solution of an isolated monoaromatic UCM

The aim of this experiment was to see if it was possible to generate an aqueous solution from the monoaromatic UCM isolated from a crude oil, shown to reduce mussel feeding rate (Chapter 2). Figure 5.3b shows the gas chromatogram of the DCM extract of water passed through a generator column coated with the isolated monoaromatic UCM (shown in Figure 5.3a) after 500 mL had passed through the column and the solution was assumed to have reached equilibrium. The generated solution is dominated by a large unresolved complex mixture. It can be seen that the UCM in both samples is present over a similar retention time range, but in the generated solution the UCM maxima occurs at 31 minutes compared to 35 minutes for the isolated monoaromatic UCM. It would also appear that a proportion of the compounds (shown by the dotted red line) are not present in the generated solution. These are the higher molecular weight compounds, which one might expect to be relatively insoluble. The water sample extract clearly shows that a UCM is present, suggesting that most of the monoaromatic UCM hydrocarbons are indeed soluble. The gas chromatogram also shows some resolved peaks, but these were identified as phthalates, thought to have contaminated the sample in the work up of the aqueous solution. Ali (1994) investigated sources of contamination. Sample contamination may be experienced at any step in a preparation procedure and a number of potential sources have been reported. The most common contaminants were found to be phthalate plasticisers.

The unresolved components were measured and an external calibration graph used to quantify (see chapter 2) the amount of monoaromatic UCM hydrocarbons present in the aqueous solution. This gave a concentration of 560 $\mu g\ L^{-1}$.



* phthalate plasticiser contamination

..... proportion of UCM not present in generated solution

Figure 5.3. Gas chromatograms of a) isolated monoaromatic UCM b) generated solution from isolated monoaromatic UCM

5.5. Discussion

The aqueous solubility in distilled water at 25 °C was determined for the model aromatic UCM hydrocarbons, 6-cyclohexyltetralin, 7-cyclohexyl-1-methyltetralin and 7-cyclohexyl-1-propyltetralin as 109 ± 6, 45 ± 4, 23 ± 3 μg L^{-1} respectively. To investigate the effect of salinity on the aqueous solubility the same experiment was conducted using seawater. The aqueous solubility of 6-cyclohexyltetralin, 7-cyclohexyl-1-methyltetralin and 7-cyclohexyl-1-propyltetralin in seawater at 25 °C was determined as 75 ± 3, 27 ± 3, 13 ± 2 μg L^{-1} respectively. This led to a reduction factor in aqueous solubility from distilled water at 25 °C of 1.46, 1.6 and 1.7 for each of the compounds respectively, meaning that increasing salinity reduced aqueous solubility, with the effect increasing through the compounds, in relation to increasing length of the alkyl substitution on the cyclohexyltetralin structure. Rossi & Thomas (1981) also noticed that the magnitude of the salting-out effect increased with increasing molar volume. The presence of alkyl groups on aromatic compounds generally decreased the solubility according to an increase in molar volume. In general it is believed that aromatic hydrocarbons enter aqueous solution in a manner similar to other non-electrolytes, that is their dissolution is attended by a net decrease in entropy. This has been interpreted as being due to the increasing structuring of water molecules in proximity of the solute. Addition of salt to a non-aqueous non-electrolyte solution results in either a decrease in solubility (salting-out) or an increase (salting-in). Such effects are believed to result from the disruption or enhancement, respectively, of structured water (Eganhouse & Calder, 1976). Therefore salting-out is the process that has taken place in this instance.

To investigate the effect of temperature on the aqueous solubility of the model compounds the same experiment was conducted at 15 °C giving an aqueous solubility of 95 ± 5, 40 ± 2, 17 ± 2 μg L^{-1} for 6-cyclohexyltetralin, 7-cyclohexyl-1-methyltetralin and 7-cyclohexyl-

1-propyltetralin respectively. This resulted in a reduction factor of 1.15, 1.2 and 1.3 for the compounds respectively in relation to their aqueous solubility in distilled water at 25 °C. Thus decreasing temperature also caused a decrease in aqueous solubility which was greater for 7-cyclohexyl-1-propyltetralin than for 6-cyclohexyltetralin. May *et al.* (1978) reported a decrease in the solubility of PAHs with a decrease in temperature, and Dickut *et al.* (1986) reported that the aqueous solubility of six polychlorinated biphenyls (PCBs) increased exponentially with temperature over a 0.4-80 °C range. A decrease in aqueous solubility with increasing salt concentration and decreasing temperature is considered normal behaviour (Whitehouse, 1984). The effect of temperature on aqueous solubility was not as marked as salinity for the model aromatic UCM hydrocarbons within the range examined. This was in contrast to anthracene, examined in this study and by others (Whitehouse, 1984), which showed a greater decrease in aqueous solubility with respect to temperature rather than salinity over this range. Gearing and Gearing (1982) also reported that PAHs are more sensitive to small changes in temperature than salinity. The likely cause of the observed difference between anthracene and the model UCM hydrocarbons is that anthracene is a solid at room temperature whereas the model compounds are oily liquids. Therefore an increase in temperature will have a greater effect on the solubilisation of anthracene.

Aqueous solubility was also determined in seawater at 15 °C resulting in an aqueous solubility of 56 ± 3 μg L^{-1} for 6-cyclohexyltetralin, 21 ± 2 μg L^{-1} for 7-cyclohexyl-1-methyltetralin and 9 ± 2 μg L^{-1} for 7-cyclohexyl-1-propyltetralin. The effect of salinity and temperature together caused the biggest reduction in aqueous solubility but the reduction factor for this was not the sum of the two effects individually.

The aqueous solubility data for the model monoaromatic UCM compounds 6-cyclohexyltetralin, 7-cyclohexyl-1-methyltetralin and 7-cyclohexyl-1-propyltetralin were all found to be below 70 μg L^{-1} in seawater at 15°C. Although the toxicity tests were

carried out at 15 °C in seawater the effect cut-off noted by Donkin *et al.* (1989, 1991) was deduced from data calculated at 25 °C in distilled water. Even then, only 6-cyclohexyltetralin has an aqueous solubility above this level. To examine the relationship between aqueous solubility and the toxicity of the model UCM compounds, the results of this study were plotted (Figure 5.4) along with data from Donkin *et al.* (1989, 1991) and Wraige (1997). It would appear that the graph highlights the cut-off effect in toxicity caused by low aqueous solubility. This observation is consistent with theories of narcotic cut-off, which suggest that it is primarily due to aqueous solubility declining more rapidly with increasing molar volume than solubility in the hydrophobic active site increases. Consequently the aqueous phase contains insufficient compound to deliver a toxic load by partition into the active site (Donkin *et al.*, 1991).

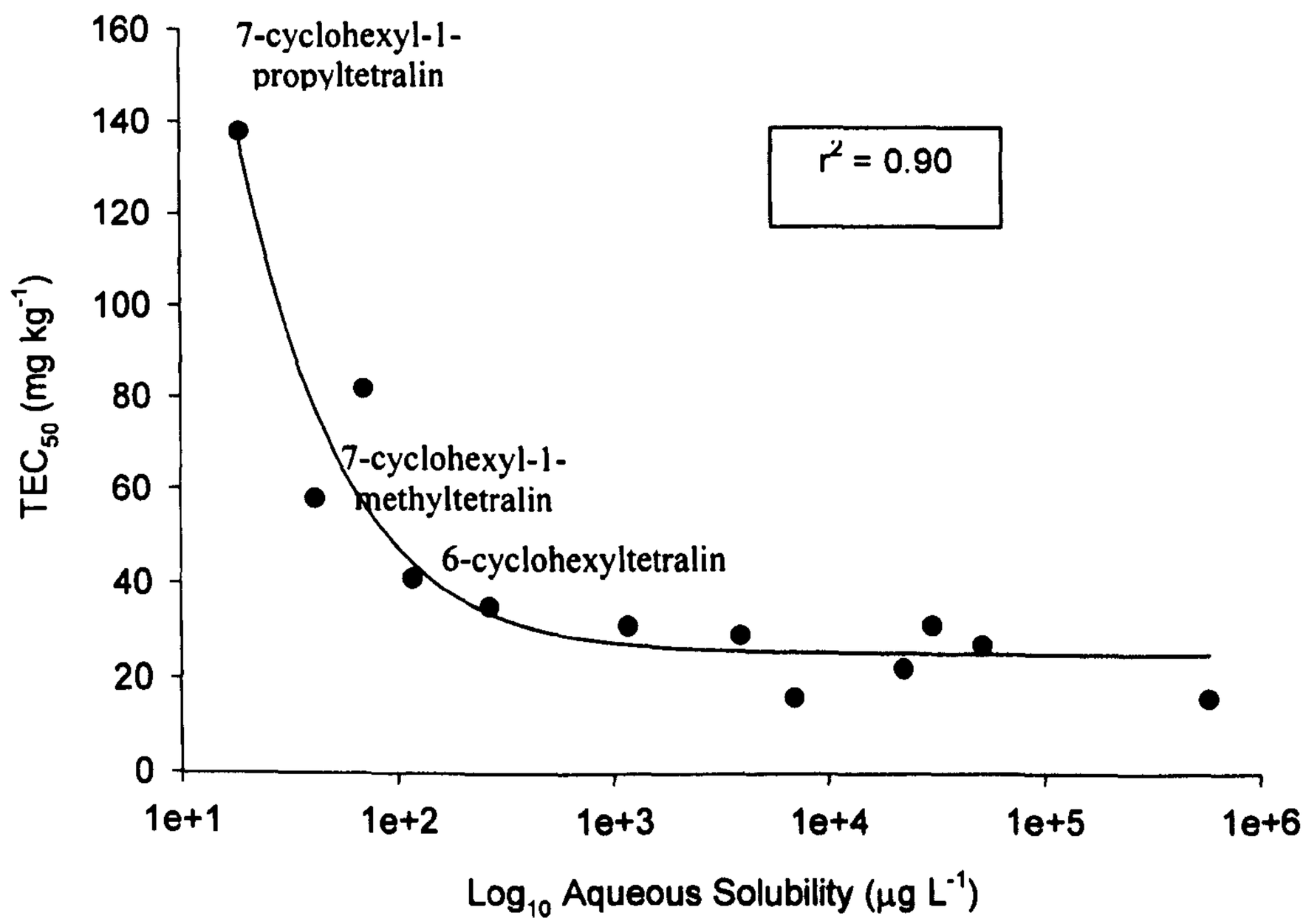


Figure 5.4. The relationship between aqueous solubility and biological effect TEC_{50} – a synthesis of the data of Donkin *et al* (1989, 1991) and Wraige (1997) with the present study

Table 5.11 The effect of hydrocarbons on the filter feeding rate of mussels (*Mytilus edulis*) – a synthesis of the data of Donkin *et al* (1989, 1991) and Wraige (1997)* with present study+

Compound	Carbon number	$LogK_{ow}$	Aq. Sol. $\mu g\ L^{-1}$	WEC_{50} $\mu g\ L^{-1}$	WEC_{50} $\mu mol\ L^{-1}$	TEC_{50} $\mu g\ g^{-1}$	TEC_{50} $\mu mol\ g^{-1}$	Log 1/C C=molar conc.
Toluene	7	2.65	578600	2347	25.51	16	0.17	0.769
naphthalene	10	3.35	30640	922	7.2	31	0.24	0.619
n-propylbenzene	9	3.69	52170	862	7.18	27	0.23	0.63
chloronaphthalene	10	4.08	22490	270	1.66	22	0.14	0.85
acenaphthalene	12	3.92	3930	382	2.48	29	0.19	0.721
biphenyl	12	3.95	7000	295	1.92	16	0.1	1
phenanthrene	14	4.57	1180	148	0.86	31	0.17	0.769
dibenzothiophene	12	4.42	n.a.	94	0.51	14	0.08	1.09
pyrene	16	5.18	135	>0.4	>0.2	>189	>0.94	> 0.02
fluoranthene	16	5.22	263	80	0.4	627	3.10	-0.491
1-phenylheptane	13	6.11	269	93	0.53	35	0.2	0.698
1-phenyloctane	14	6.73	71	79	0.41	82	0.43	0.366
1-phenyldecane	16	7.55	5	>200e	>0.92	>78	>0.36	<0.44
6-cyclohexyltetralin*	16	6.33 - 6.77	109	24	0.11	44	0.206	0.686
7-cyclohexyl-1-methyltetralin+	17	6.85 – 7.27	45	42	0.18	58	0.254	0.595
7-cyclohexyl-1-propyltetralin*	19	7.73 - 8.17	23	62	0.24	138	0.539	0.268
Isolated monoaromatic UCM+			560	518	1.866	500	1.1666	0.85

This suggests that increasing branching on the cyclohexyltetralin base structure will mean that the compound will not have sufficient solubility to reach the site of toxic action and will accumulate in the tissues without inducing a further response. In Chapter 4 it was seen that 7-cyclohexyl-1-propyltetralin was accumulated to a far greater extent by mussels than 6-cyclohexyltetralin and 7-cyclohexyl-1-methyltetralin, and also far more than the isolated monoaromatic UCM. So although exposure to 7-cyclohexyl-1-propyltetralin reduces mussel feeding rate it would appear that the solubility limit is being reached whereby more of the compound is accumulated without causing a response. This suggests that the compound accumulates in areas other than at the site of toxic action. If indeed the gill is the site of toxic action then one would expect to see a concentration in the gill tissue comparable to the other model compounds. The cut-off of 70 $\mu g\ L^{-1}$ suggested by Donkin *et al.* (1989, 1991) has effectively been extended by the model monoaromatic UCM compounds, as 7-cyclohexyl-1-propyltetralin, with an aqueous solubility of $23 \pm 3\ \mu g\ L^{-1}$ was able to elicit a response, although it would appear a proportion of this was accumulated without causing an effect. This would account for the higher TEC_{50} value observed for this compound.

This also suggests that compounds within the monoaromatic UCM with a solubility less than 7-cyclohexyl-1-propyltetralin will become increasingly limited in their ability to reach the site of toxic action. However some of the components, at least, must be soluble enough, in order to have reduced mussel feeding rate (Chapter 2).

A generator column was set up in a similar manner to that used for determining the aqueous solubility of the model compounds but containing glass beads coated with the isolated monoaromatic UCM (Chapter 2). Generator columns are usually used for the determination of the aqueous solubility of single compounds, however, Ghosh *et al.*, (1998) used the generator column method to study an industrial mixture of polychlorinated biphenyls (PCBs) as a novel method for generating media for toxicological testing. Their

study showed that all congeners of the PCB mixture were to some degree water soluble at each measured concentration.

The effluent from the generator column contained an aqueous solution that was characterised by a large UCM (Figure 5.3). It would appear that not all of the original isolated monoaromatic UCM that was coated on the beads was found in the solution. A proportion of the higher molecular weight components were not present suggesting they are relatively insoluble. The amount of monoaromatic UCM hydrocarbons present in solution was calculated at 560 $\mu g\ L^{-1}$. This would appear high in comparison to the model compounds. However, when Barron *et al.* (1999) prepared WAFs of weathered distillate oils, which included a predominant UCM, the concentration over a similar range, being 600-900 $\mu g\ L^{-1}$, was considered indicative of dissolved and not particulate or emulsified oil.

According to Raoult's Law the solubility of a compound present in an ideal mixture is equal to the solubility of the pure compound multiplied by its mole fraction in the mixture. However mixture solubility may deviate from ideal behaviour. Previous investigations of aqueous concentrations of mixtures have found that interaction between components in a mixture can result in complex deviations from ideal solution behaviour. Reported deviations from ideal behaviour have resulted in enhanced, reduced and ideal aqueous concentrations for different components in the mixtures studied (Sherblom *et al.*, 1992). In studying the multicomponent solubility of hydrocarbons in water Leinonen & Mackay (1973) found that the solubility of each hydrocarbon component was greater by 6-35 % than the value expected by prediction on the basis of the solubility of the hydrocarbon component being proportional to its mole fraction in the hydrocarbon phase. Further to this Burris and MacIntyre (1985) suggested that the aqueous phase of a contaminant resulting from the equilibration of an organic mixture (such as petroleum) of dissimilar components with water could be 2-3 times larger than predicted by ideal behaviour. Therefore the

monoaromatic UCM may not behave like an ideal mixture and enhancement of the components may have occurred.

The exact structure of the components in the monoaromatic UCM may also be extremely important. Sutton & Calder (1975) investigated the solubility of several alkylbenzenes and observed that solubility was a function of molar volume and solute-solvent interactions. This has been observed for the model monoaromatic UCM hydrocarbons. For monosubstituted alkylbenzenes, such interactions were related to the size of the alkyl substituent. For polysubstituted alkylbenzenes, such interactions were also influenced by the proportions of the substituents on the ring. For alkylnaphthalenes a similar effect was observed, however 2-ethylnaphthalene was more soluble than 2-dimethylnaphthalene, although they have similar molar volumes. It was suggested that the ethyl group may fit more easily into the water structure than the two methyl groups or, was more soluble due to the extra flexibility of the ethyl side chain.

5.6. Summary

The aqueous solubility of anthracene, 6-cyclohexyltetralin, 7-cyclohexyl-1-methyltetralin and 7-cyclohexyl-1-propyltetralin has been determined in distilled water (25 °C and 15°C) and in seawater at the same temperatures (except anthracene 25 °C only).

Anthracene was found to have an aqueous solubility of 45 ± 2 μg L^{-1} in distilled water at 25 °C, 29 ± 1 μg L^{-1} in seawater at 25 °C, and 22 ± 1 μg L^{-1} in distilled water at 15 °C. These results compared well with previously reported data and validated the system for the determination of the aqueous solubility of the three model aromatic UCM compounds at different temperature and salinity.

6-cyclohexyltetralin was found to have an aqueous solubility of 109 ± 6 μg L^{-1} in distilled water at 25 °C, 75 ± 3 μg L^{-1} in seawater at 25 °C, 95 ± 5 μg L^{-1} in distilled water at 15 °C, and 56 ± 3 μg L^{-1} in seawater at 15 °C.

7-cyclohexyl-1-methyltetralin was found to have an aqueous solubility of 45 ± 4 μg L^{-1} in distilled water at 25 °C, 27 ± 3 μg L^{-1} in seawater at 25 °C, 40 ± 2 μg L^{-1} in distilled water at 15 °C, and 21 ± 2 μg L^{-1} in seawater at 15 °C.

7-cyclohexyl-1-propyltetralin was found to have an aqueous solubility of 23 ± 3 μg L^{-1} in distilled water at 25 °C, 13 ± 2 μg L^{-1} in seawater at 25 °C, 17 ± 2 μg L^{-1} in distilled water at 15 °C, and 9 ± 2 μg L^{-1} in seawater at 15 °C.

The results are consistent with normal behaviour in that a decrease in aqueous solubility was observed with an increase in salinity and decrease in temperature. These effects were largest for 7-cyclohexyl-1-propyltetralin, which is consistent with a larger molar volume. Salinity exerted the largest effect on the aqueous solubility of the model aromatic UCM hydrocarbons compared to temperature for anthracene. This was thought to occur owing to anthracene being in solid form at room temperature compared to the model compounds which are oily liquids.

These solubility values extend the measured effects of soluble hydrocarbons on mussel feeding rate according to previously established QSAR for *Mytilus edulis* (Donkin *et al.*, 1991). When the data were compared to the data of Donkin *et al.*, (1989, 1991) it was found that a cut-off effect could be visualised in the form of a plot of water solubility against the body burden when a 50 % reduction in mussel feeding rate occurred. 7-Cyclohexyl-1-propyltetralin has a relatively low aqueous solubility in water and appears to represent the start of the cut-off. This was supported from the observation in Chapter 2 that 7-cyclohexyl-1-propyltetralin was accumulated to the greatest extent in the mussel tissue without inducing more of a response.

The isolated monoaromatic UCM appeared to be appreciably soluble (compared to the model UCM hydrocarbons); however, it has been reported that equilibration of liquid organic mixtures with water may result in concentrations in the aqueous phase that differ significantly from those predicted on the basis of assumptions of ideal solution behaviour (Burris & MacIntyre, 1985). The UCM present in the generated solution did not contain all of the compounds present in the isolated monoaromatic UCM as evidenced by Figure 5.3. The higher molecular weight compounds were not present, suggesting they are not capable of being soluble enough to reach the site of toxic action. This result indicates that the reduction in feeding rate by the isolated monoaromatic UCM (Chapter 2) was caused by only a proportion of the compounds present, i.e. those compounds that are soluble enough to reach the site of toxic action.

Chapter 6. Photodegradation studies.

Previous studies have shown that some aromatic hydrocarbons are photodegraded in the marine environment. Recently, the photoenhancement of the toxicity of water-accommodated fractions of weathered oil to marine organisms has been reported. However, the potential for unresolved complex mixtures of aromatic hydrocarbons to photodegrade does not appear to have been previously considered. As a preliminary study the photodegradation kinetics of 6-cyclohexyltetralin, 7-cyclohexyl-1-methyltetralin and 7-cyclohexyl-1-propyltetralin were therefore determined herein under exposure to simulated sunlight from a xenon arc lamp, using solutions from a generator column. The reactivities suggest that phototransformation of real aromatic UCMs should now be studied.

6.1 Introduction

Petroleum compounds may degrade by various weathering mechanisms in the marine environment. Photooxidation is one of the major causes of compositional changes in crude oil spilled onto seawater, yet the phenomenon has been poorly characterised (Dutta *et al.*, 2000). Environmental photolysis (or photodegradation) is defined as a chemical reaction that occurs only in the presence of light. These reactions, which may occur in surface waters, on soil, and in the atmosphere, are driven by sunlight, which has a significant photon flux only above 290 nm in the near UV (Mill, 1999). Compounds that absorb UV/visible light can react photochemically by reaching an excited state through the direct absorption of light (direct photolysis) or by accepting energy from an excited donor molecule (sensitised photolysis). Reactions can also occur with reactive oxygen species, such as singlet oxygen and hydroxyl radicals, formed from photochemical reactions with other organic molecules such as humic acids (Kim *et al.*, 2000).For direct photooxidation to take place there must be spectral overlap between radiation from the light sources (*i.e.* the sun in environmental terms) and the light absorption by the chemical in question. Mononuclear aromatics being inactive in the UV region above 290 nm mostly undergo indirect photolysis. However, some monoaromatic compounds may undergo direct photolysis due to the formation of charge transfer complexes (Belt *et al.*, 1998) between the aromatic hydrocarbons and oxygen, leading to a shift in the absorption band towards longer wavelengths.

Many environmental pollutants interact with solar radiation in the near UV region in a manner which greatly increases their toxic effects (Davenport *et al.*, 1994). Photomediated toxicity of environmental contaminants may occur by two mechanisms. *In vitro* photomodification of a contaminant in environmental media may occur to produce a photoproduct that is more toxic than the parent compound. This may happen when oxygen

is introduced, which often causes the product to be more polar and more water-soluble. *In vivo* sensitisation (indirect photosensitised oxidation) can occur where an organism bioaccumulates photoactive chemicals and the toxic effects are manifested upon exposure of the organism to solar radiation (Cleveland *et al.*, 2000).

Photooxidation mainly affects the aromatic components of crude oil (Ali, 1994). They are particularly sensitive to this process compared to the saturated compounds which are resistant (Garret *et al.*, 1998). Photooxidation rates of aromatic hydrocarbons in the marine environment have been shown to be significant, and to normally result in oxygenated products (reviewed by Ali, 1994). Studies have shown that photoproducts of petroleum hydrocarbons are toxic to marine animals (Jaquot, 1996 and references therein). In fact, it has long been established that the oxidation of aromatic components of oil increases its toxicity (Larson *et al.*, 1977, 1979), with the formation of carboxylic acids, aromatic ketones, alcohols and aldehydes. Polar oxidation products of hydrocarbons have also been shown to accumulate in tropical mussels (Burns, 1993) and it has been suggested that these products may contribute to decreasing physiological performance.

In contrast, the postulated mechanism of photosensitisation is absorption of UV energy by the chemical within the organism causing subsequent tissue injury, with no change in the chemical structure taking place. Light energy excites the photosensitising chemical to a triplet energy state; this energy is then transferred to molecules within the cell or cell membrane, rapidly generating reactive oxygen species and causing tissue damage. The potential for photoenhanced toxicity by this mechanism has been reviewed by Barron & Ka'aihue (2001). These phototoxic components of oil appear to be restricted to specific polycyclic aromatic hydrocarbons (Mekenyan *et al.*, 1994). Photoenhanced toxicity of other compounds may occur but appears to be limited to the photomodification of chemicals in water (*i.e* formation of more toxic and reactive chemicals) rather than activation of tissue residues.

Chemical oxidation of an aliphatic UCM caused an increase in toxicity and reduction in mussel feeding activity (Thomas *et al.*, 1995). The relatively non-toxic nature of the aliphatic UCM was attributed to low aqueous solubility. Chemical oxidation was thought to form products with greater aqueous solubility but of sufficient hydrophobicity to be narcotic toxicants. Therefore, it might reasonably be assumed that some or all of the aromatic UCM has the potential to photodegrade, with the possible generation of toxic products, but to date this has not been investigated. This suggests it would be pertinent to study the possible oxidation of aromatic UCM compounds under environmental conditions, *i.e.* by photodegradation.

Although there are many reports in the literature of the photodegradation behaviour of organic compounds in solution (*e.g.* Belt *et al.*, 1998), very few have adequately simulated environmental conditions. One of the main limitations has been the use of inappropriate light sources which do not provide light of a suitable spectral quality and stable light intensity or maintain a constant temperature for the illumination period, thus preventing kinetic studies. Natural sunlight comprises a broad range spectrum of radiation. Radiation of wavelengths below 290 nm, which are the most energetic, are largely absorbed by ozone in the upper atmosphere. Therefore, for a photochemical reaction taking place at the earth's surface, wavelengths greater than 290 nm are most important.

Ideally, solar radiation should be used for experiments in environmental photochemistry. However, because of restrictions arising from the changes in intensity, both on a daily and a seasonal basis, and variation in wavelength distribution depending on latitude and general weather conditions, use of natural sunlight is inconvenient (Kirk 1986). Artificial radiation sources are preferred as they offer greater stability and reproducibility than outdoor studies utilising natural light. Artificial lights can be filtered to simulate solar radiation, and in this respect suitably filtered xenon lamps have been widely advocated (reviewed by Ali, 1994). Lamps produce a lot of heat during operation and require a good

cooling system (Zepp, 1982). Miller & Zepp (1983) noted that an increase in sample temperature can lead to an increased rate of photolysis. Therefore experiments should be ideally conducted under isothermal conditions during the entire irradiation period. Other important factors that need to be taken into consideration are the concentration of the initial substrates, the light intensity and the reaction times, because these may affect the yields and types of products.

6.2 Aims

Although photooxidation is now recognised as an important factor of oil weathering its possible influences on UCM components have yet to be studied (Killops, 1986). The main aim of the work in this chapter was to assess the photodegradation potential of synthetic model aromatic UCM hydrocarbons under environmentally realistic conditions. A further aim was to define the kinetics of photolysis of the model aromatic UCM compounds 6-cyclohexyltetralin, 7-cyclohexyl-1-methyltetralin and 7-cyclohexyl-1-propyltetralin using a previously verified irradiation system.

6.3 Experimental Approach

Model aromatic UCM hydrocarbons 6-cyclohexyltetralin, 7-cyclohexyl-1-methyltetralin and 7-cyclohexyl-1-propyltetralin were synthesised in good yield and purity as described in Chapter 3 (details Chapter 7). The initial aqueous concentration of a test substrate is an important factor which may affect the percentage yield and type of products formed following photoxidation. Concentrations should be environmentally realistic and not exceed the solubility limit of the test compound. The use of polar co-solvents may also affect photodegradation behaviour. Therefore, aqueous solutions of the synthetic model

aromatic UCM hydrocarbons for the present study were produced using a generator column, as described earlier in Chapter 5 (details Chapter 7). This allowed the formation of stable solutions, not exceeding the aqueous solubility of the compound of interest. Samples were generated directly into quartz photolysis tubes and alternate samples used for exposure and dark controls. Samples were exposed to a Heraeus Suntest CPS xenon arc lamp for 5, 10, 15 and 20 hour exposure periods. This lamp accurately simulates the spectrum of radiation that reaches the Earth's surface from the sun as it filters out energetic wavelengths below 290 nm (Figure 6.1). The use of a xenon lamp is advantageous as it can shorten irradiation time and maximise product yields. For each time period five samples were placed under the lamp and a further five samples were covered in foil to act as dark controls. To maintain a constant temperature during irradiation the quartz tubes were held in a specially designed stainless steel tank (Parker & Leahey, 1988) partially filled with glycerol, in a metal holder. The tank was cooled by circulating thermostatted water. Glycerol was used as a coolant instead of water to avoid losses due to evaporation. The tank holding the tubes was positioned under the light source, with the aid of a laboratory jack, at a distance of approximately 20 cm from the lamp. The position of the tank on the jack and the jack underneath the lamp were carefully delineated and were consistent between different exposure times. Temperature was controlled using a Conair chiller unit (25 °C ± 1 °C) and monitored using a squirrel data logger at the 'in' and 'out' flow terminals of the chiller unit. Before sample irradiation, the lamp and cooling system were switched on for an hour to allow the lamp to reach its maximum radiation intensity and for the glycerol to adjust to the required temperature. It was found that the chiller unit needed to be set at 20°C to give a stable temperature in the tank of 25°C. This temperature was the lowest achievable with the experimental set-up. Solutions could be easily generated at this temperature which is also the average temperature of the Arabian Gulf (Ali, 1994), thus mimicking realistic environmental conditions. The experimental set-up is shown in Figure

6.2. After irradiation, samples were removed from the stainless steel holder, covered in foil and stored in the fridge (4 °C) until work-up. Upon opening the photolysis vessels, they were immediately spiked with an internal standard. Internal standards were spiked at concentrations equivalent to half of the aqueous solubility of the compound under study. For example, after exposure, samples containing 7-cyclohexyl-1-methyltetralin at an initial concentration of 40 μg L^{-1} were spiked with 6-cyclohexyltetralin at a concentration of 20 μg L^{-1}. All aqueous solutions were extracted in a separatory funnel three times with DCM after irradiation. The combined organic phase was passed over anhydrous sodium sulphate to remove water. The extracted samples were concentrated by careful rotary evaporation followed by evaporation under nitrogen. All extraction and concentration steps were performed with minimum exposure to light. To study the photodegradation kinetics the DCM extracts were examined by SIM GCMS.

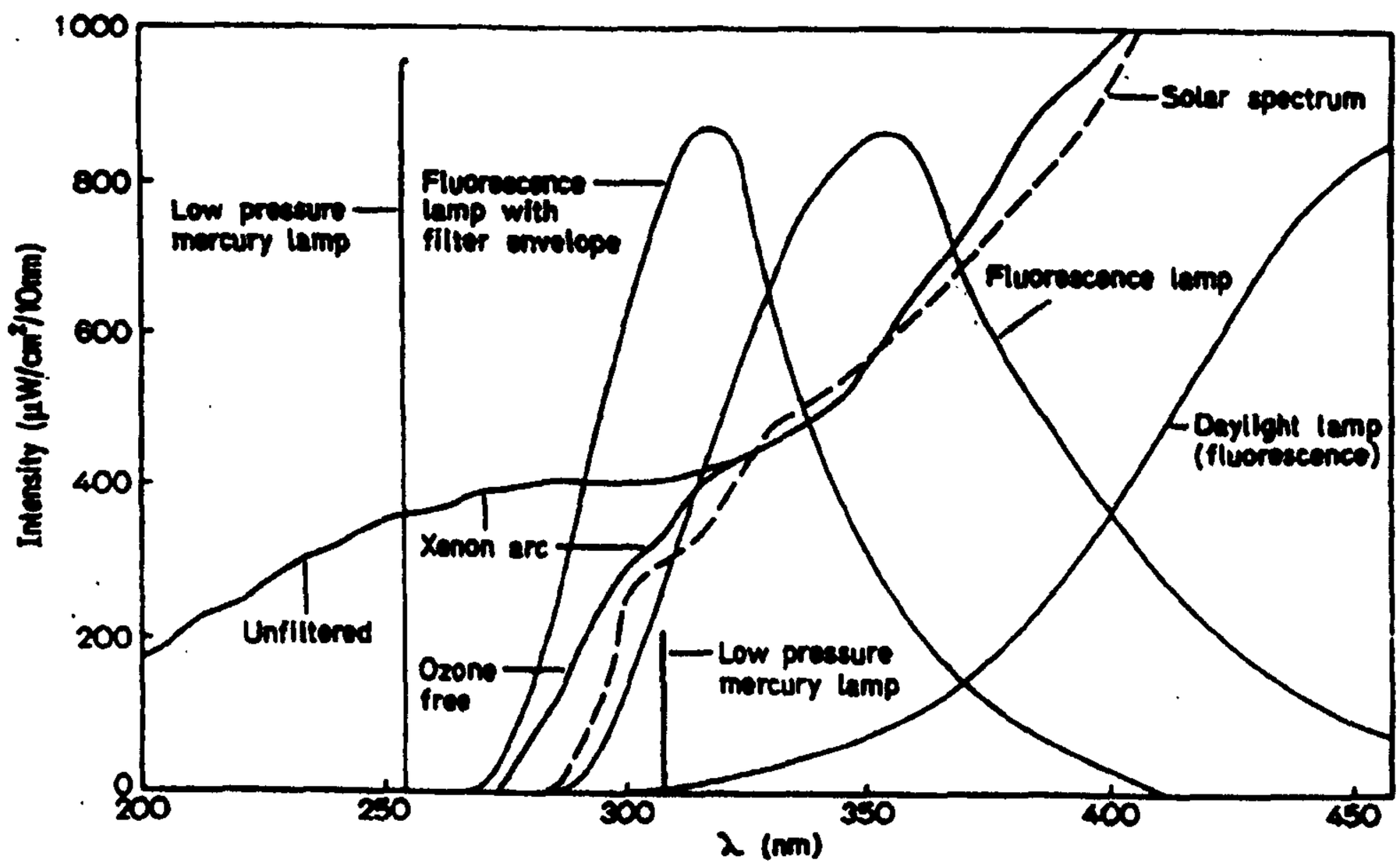
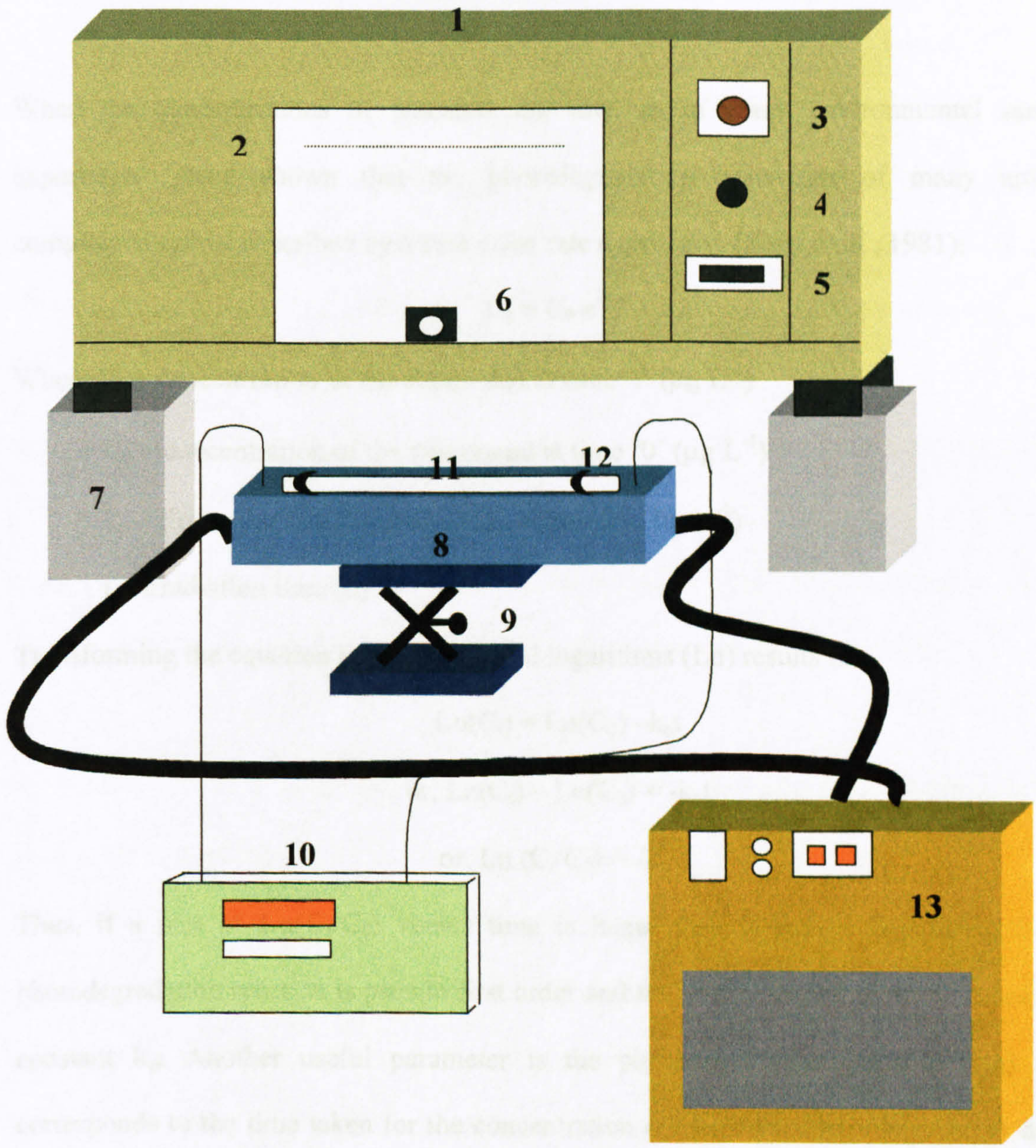


Figure 6.1 Spectral distribution of the emission spectra of the most important artificial radiation sources (Roof, 1982)



1	Heraeus Suntest exposure machine	2	Xenon lamp
3	Lamp potentiometer setting	4	Lamp switch
5	Lamp meter	6	Lamp compartment
7	Breeze block	8	Stainless steel tank
9	Jack to support tank	10	Temperature datalogger
11	Quartz tubes	12	Tube holder
13	Chiller unit		

Figure 6.2 Schematic arrangement of photolysis experimental apparatus

6.4 Results

When the concentrations of reactants are low, as in many environmental samples, experiments have shown that the photodegradation behaviour of many aromatic compounds can be described by a first order rate expression (Zepp *et al.*, 1981):

$$\mathbf{C_t = C_0\, e^{-kpt}}$$

Where C_t = concentration of the compound at time 't' ($\mu g\ L^{-1}$)

C_0 = concentration of the compound at time '0' ($\mu g\ L^{-1}$)

k_p = first order rate constant of the compound ($time^{-1}$)

t = irradiation time (h)

Transforming the equation by taking natural logarithms (Ln) results in:

$$Ln(C_t) = Ln(C_0) - k_p t$$

$$\text{or, } Ln(C_t) - Ln(C_0) = -k_p t$$

$$\text{or, } Ln\,(C_t/C_0) = -k_p t$$

Thus, if a plot of $\mathbf{Ln(C_t/C_0)}$ versus time is linear then it can be concluded that the photodegradation reaction is pseudo first order and the slope is equal to the first order rate constant $\mathbf{k_p}$. Another useful parameter is the photodegradation half-life ($\mathbf{t_{1/2}}$) which corresponds to the time taken for the concentration of photoreactive compound to decline to half its original concentration ($\mathbf{C_0}$)

$$i.e.\ C_t = 0.5\ C_0$$

therefore;

$$Ln\ 0.5C_0/C_0 = -k_p t_{1/2}$$

$$-0.693 = -k_p t_{1/2}$$

$$\mathbf{t_{1/2} = 0.693/k_p}$$

As the samples in this study were well mixed and contained the chemicals of interest at very low concentrations their behaviour was assumed to comply with previous

observations for aromatic hydrocarbons and the above calculations performed on the data. Photolysis rate constants were calculated using first order kinetics by plotting Ln Ct/C_0 against time. Thus, the rate constant $\mathbf{k_p}$ of each compound was derived from the experimentally determined concentration data. A graphical plot of $Ln(C_t/C_0)$ *versus* irradiation time yielded a linear regression line, the slope of which was equivalent to $\mathbf{k_p}$. The time taken for a 50 % reduction in the initial concentration of the compound, *i.e.* the half-life ($\mathbf{t_{1/2}}$) was calculated from the equation $\mathbf{t_{1/2} = 0.693/k_p}$

Data were tested for normality using standardised skewness and standardised kurtosis. All data were found to be parametric, thus a t-test was used to compare means and ANOVA used to test the distribution of the data. In all statistical analyses differences at the <5% level were considered significant. Statgraphics 4 was used for all data analysis.

6.4.1 Experiment 1. Photodegradation of 6-cyclohexyltetralin (distilled water, 25°C, 0-20 hour exposure)

The results of the exposure of 6-cyclohexyltetralin to simulated solar radiation over the range of exposure periods are given in Table 6.1. Results are given as the mean ± standard deviation of the five samples at each exposure period and expressed as a percentage relative to 0 h (*i.e.* dark controls, 20 samples). Dark controls carried out at each exposure time were not statistically different and the data were pooled. The data were normally distributed and data from the 20 hour exposure were statistically different from dark controls. The concentration of 6-cyclohexyltetralin decreased exponentially with time from 100% to 27% in 20 hours (*i.e.* $C_t = C_0\ e^{-kpt}$; Figure 6.3). Values of $Ln(C_t/C_0)$ were calculated and plotted against the time of irradiation (Figure 6.4). A good fit of regression line was obtained (r^2=0.9872) and the value of the photodegradation pseudo first order rate constant ($\mathbf{k_p}$) of 6-cyclohexyltetralin was determined as 0.068 h^{-1}. This gave a value for the half-life of 6-cyclohexyltetralin as 10.3 h under the xenon lamp.

Table 6.1 Photodegradation of generated aqueous solutions of 6-cyclohexyltetralin in distilled water irradiated under xenon lamp at 25 °C.

Irradiation time (h)	Concentration ($\mu g\ L^{-1}$) Mean ± std. dev. (n = 5)	Concentration relative to 0 h (%)
0	109.7 ± 8.5 (n=20)	100
5	75.1 ± 5.5	68
10	50.9 ± 5.5	46
15	42.3 ± 7.0	39
20	26.9 ± 6.1	25

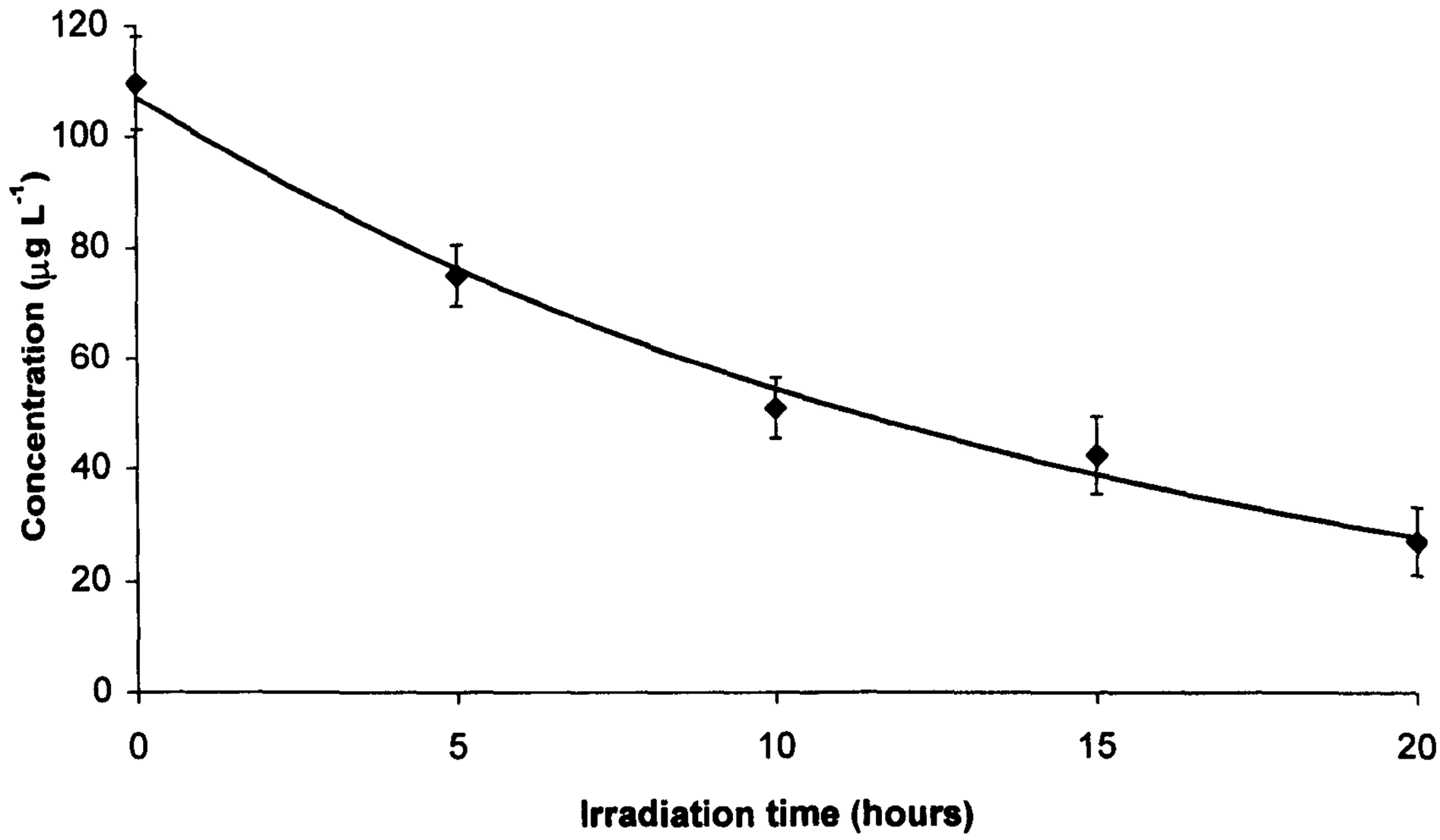


Figure 6.3 Photodegradation of generated solutions of 6-cyclohexyltetralin irradiated under xenon lamp (mean ± std. dev., n = 5; except at 0 h, n = 20)

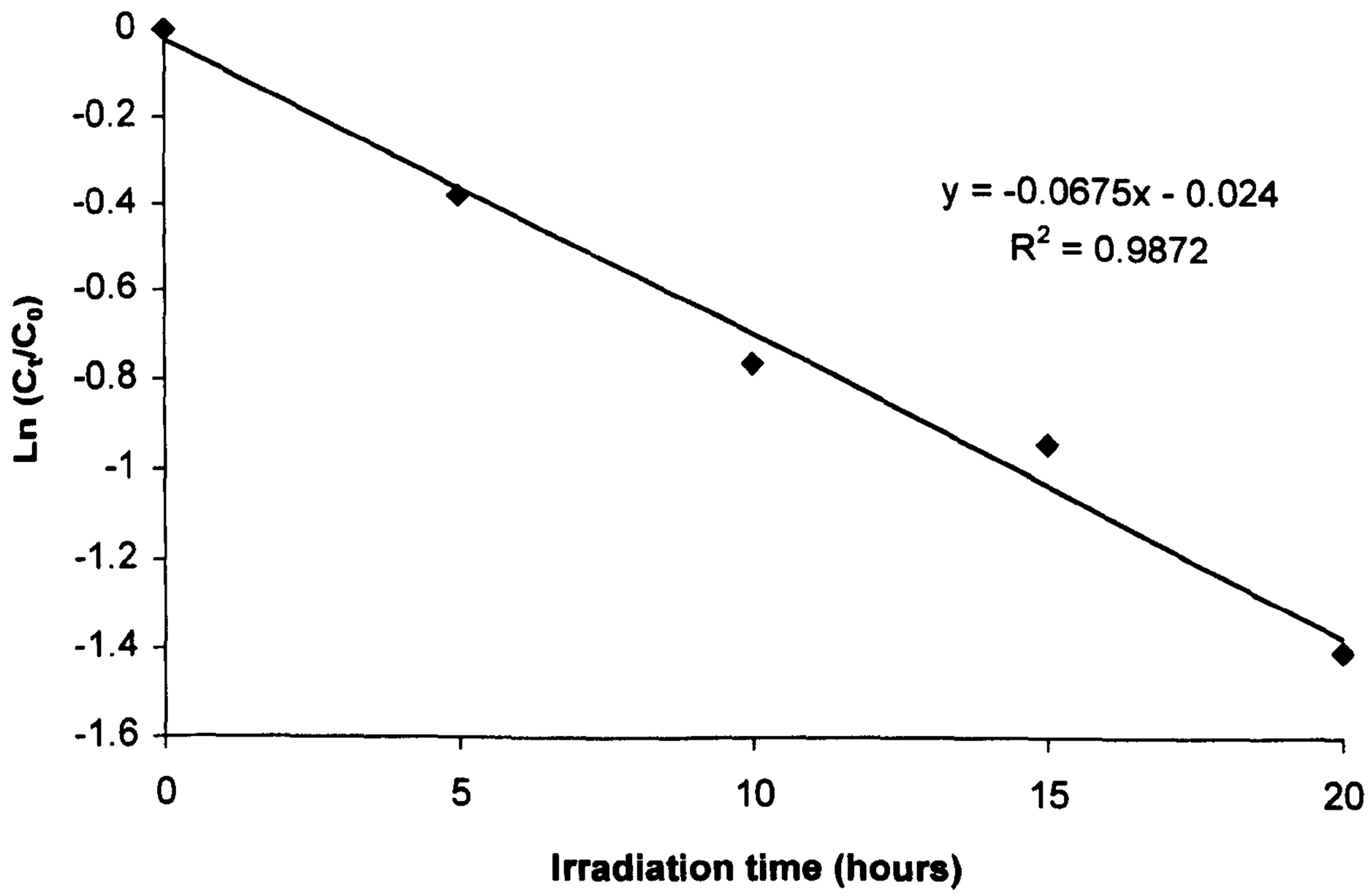


Figure 6.4 Photodegradation rate constant (k_p) of 6-cyclohexyltetralin irradiated under xenon lamp.

6.4.2 Experiment 2. Photodegradation of 7-cyclohexyl-1-methyltetralin (distilled water, 25°C, 0-20 hour exposure)

A summary of the photodegradation data obtained for 7-cyclohexyl-1methyltetralin is shown in Table 6.2. Results are given as mean ± standard deviation of the five samples at each exposure period and expressed as a percentage relative to 0 hour. Results from the photodecay of 7-cyclohexyl-1-methyltetralin are shown in Figure 6.5. The concentration of 7-cyclohexyl-1-methyltetralin decreased from 100% to 17% after 20 hours of irradiation. The dark controls at each exposure time were not statistically different and therefore the data were pooled. The data were normally distributed and data from the 20 hour exposure were statistically different from dark controls (0 h). The photodegradation was found to follow first order reaction kinetics (Figure 6.6) and a good fit of regression ($r^2 = 0.9829$) was obtained. The photodegradation rate constant ($\mathbf{k_p}$) was determined as 0.09 h^{-1} with a half-life ($t_{½}$) of 7.7 hours.

Table 6.2 Photodegradation of generated solutions of 7-cyclohexyl-1-methyltetralin in distilled water irradiated under xenon lamp at 25°C.

Irradiation time (h)	Concentration ($\mu g\ L^{-1}$) Mean ± std. dev. (n=5)	Concentration relative to 0 h (%)
0	43.6 ± 5.3 (n=20)	100
5	25.6 ± 4.5	59
10	17.2 ± 5.0	39
15	9.3 ± 3.5	21
20	7.6 ± 1.7	17

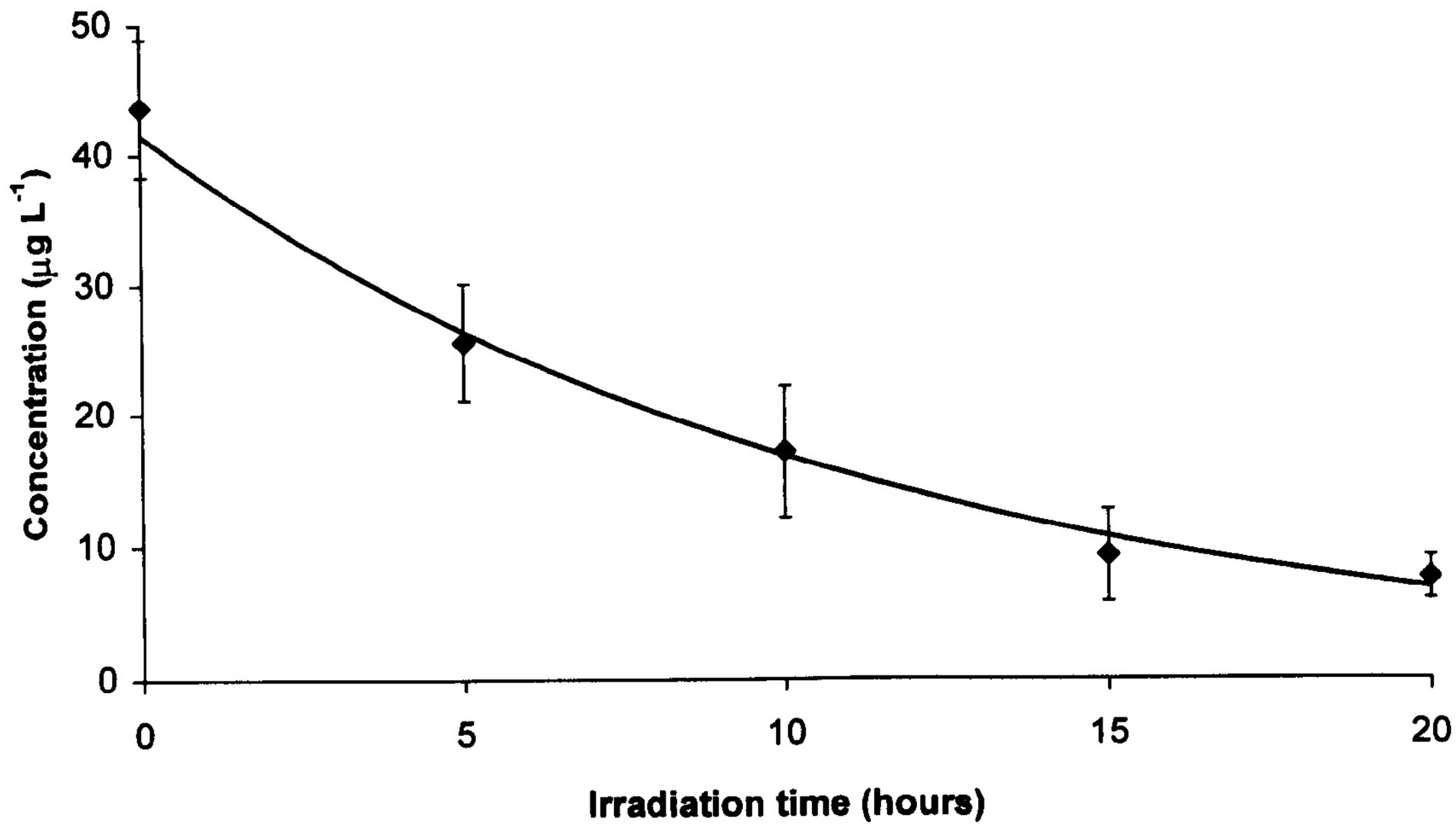


Figure 6.5 Photodegradation of generated solutions of 7-cyclohexyl-1-methyltetralin irradiated under xenon lamp (mean ± std. dev., n = 5; except at 0 h, n = 20)

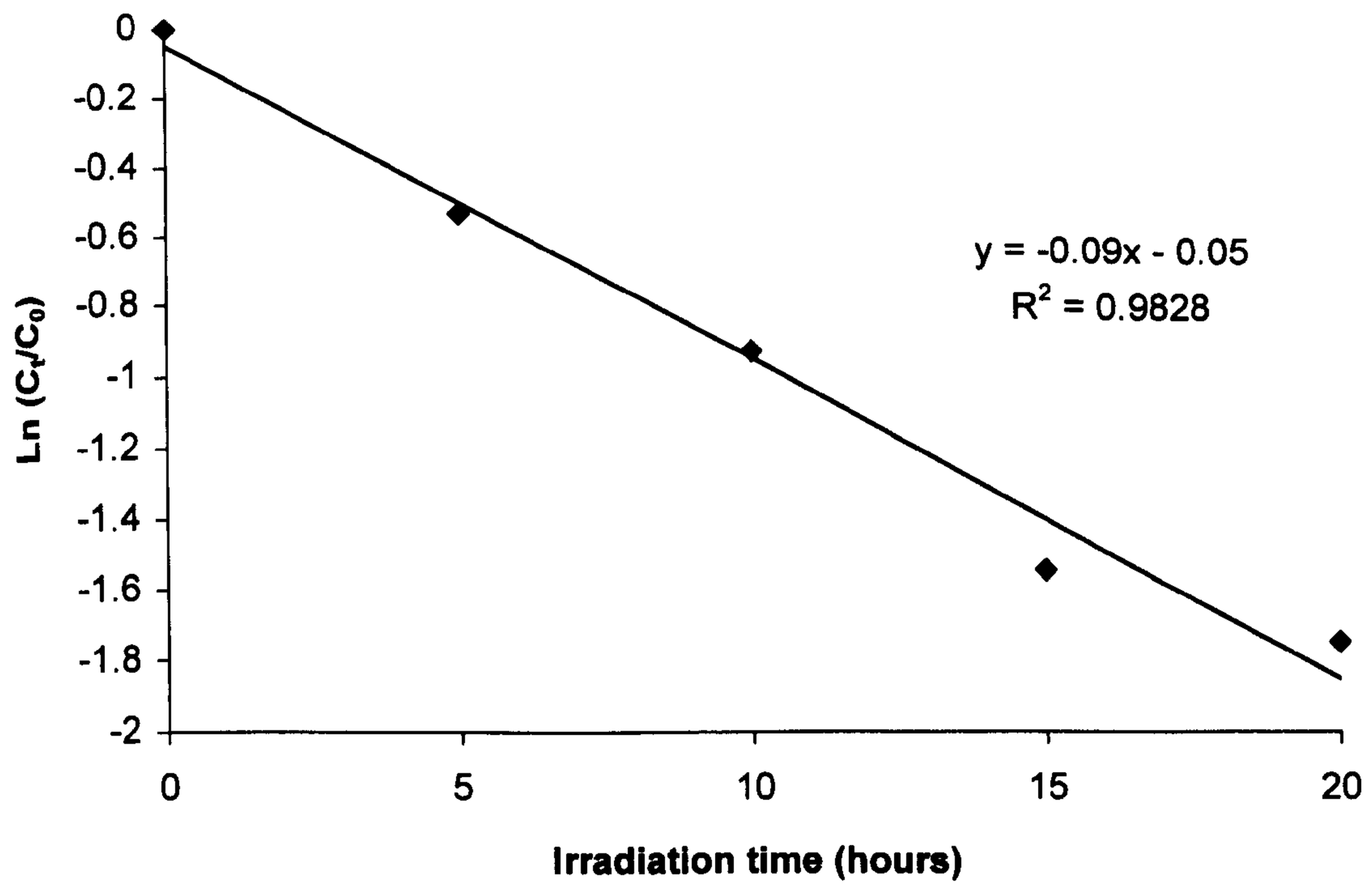


Figure 6.6 Photodegradation rate constant (k_p) of 7-cyclohexyl-1-methyltetralin irradiated under xenon lamp.

6.4.3 Experiment 3. Photodegradation of 7-cyclohexyl-1-propyltetralin (distilled water, 25°C, 0-20 hour exposure)

Table 6.3 summarises the effect of simulated solar radiation on 7-cyclohexyl-1-propyltetralin over time. Results are expressed as the mean of 5 samples ± standard deviation and as a percentage relative to 0 hour. The exponential decay of 7-cyclohexyl-1-propyltetralin is shown in Figure 6.7. This model aromatic UCM hydrocarbon was degraded by over 50 % in 5 hours. Dark controls were not statistically different and therefore the data were pooled. The sample data was normally distributed and data from the 20 hour exposure were statistically different from dark controls (0 h). From the slope of the graph (r^2 = 0.9877) in Figure 6.8 the photodegradation rate constant ($\mathbf{k_p}$) of 7-cyclohexyl-1-propyltetralin was 0.14 h^{-1} giving a half life ($\mathbf{t_{½}}$) of 4.9 hours.

Table 6.3 Photodegradation of generated solutions of 7-cyclohexyl-1-propyltetralin in distilled water irradiated under xenon lamp at 25°C.

Irradiation time (h)	Concentration ($\mu g\ L^{-1}$) Mean ± std. dev. (n=5)	Concentration relative to 0 h (%)
0	24.2 ± 5.7 (n=20)	100
5	10.5 ± 2.5	43
10	5.9 ± 1.6	24
15	2.3 ± 1.3	9
20	1.5 ± 0.7	6

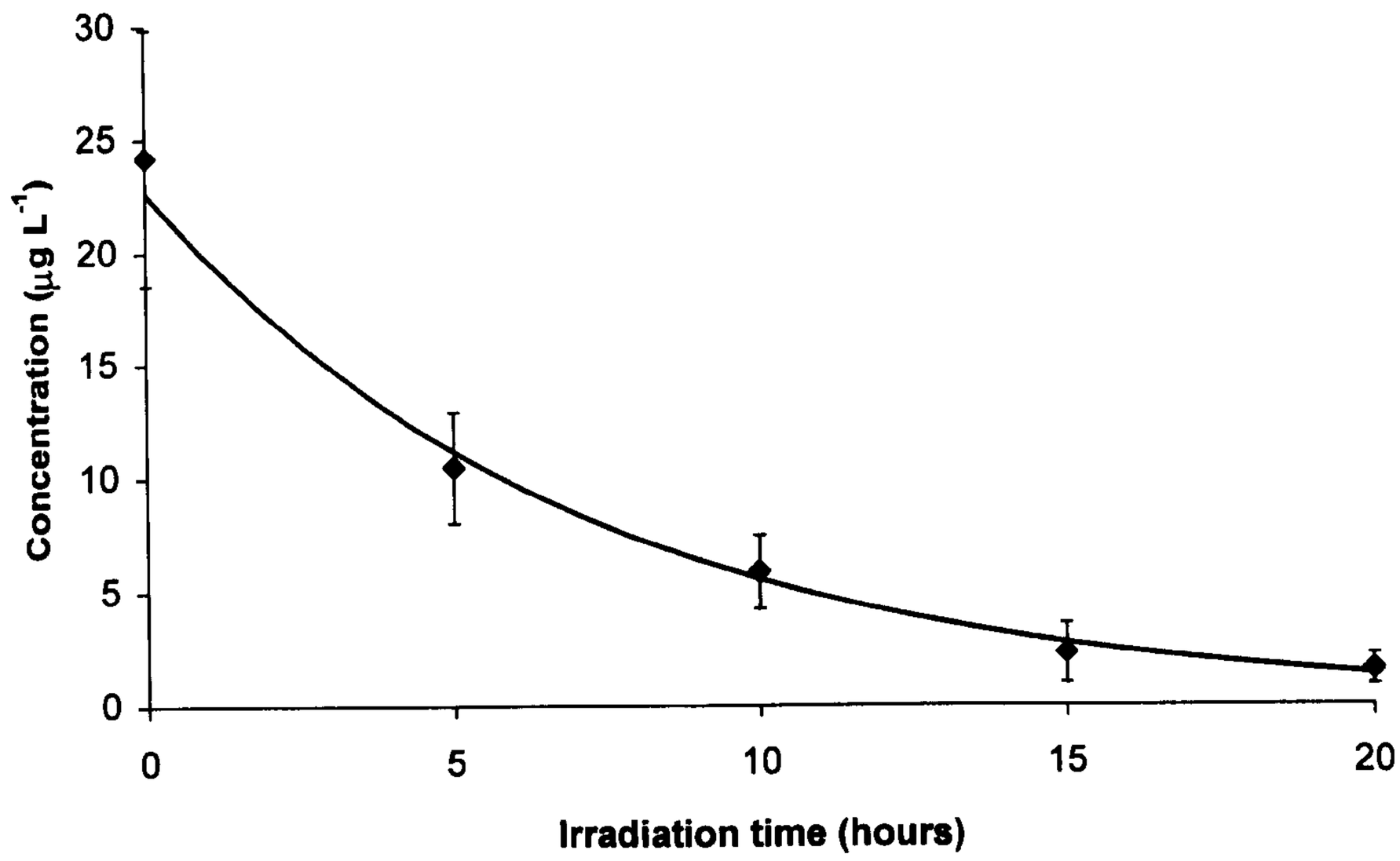


Figure 6.7 Photodegradation of generated solutions of 7-cyclohexyl-1-propyltetralin irradiated under xenon lamp (mean ± std. dev., n = 5; except at 0 h, n = 20)

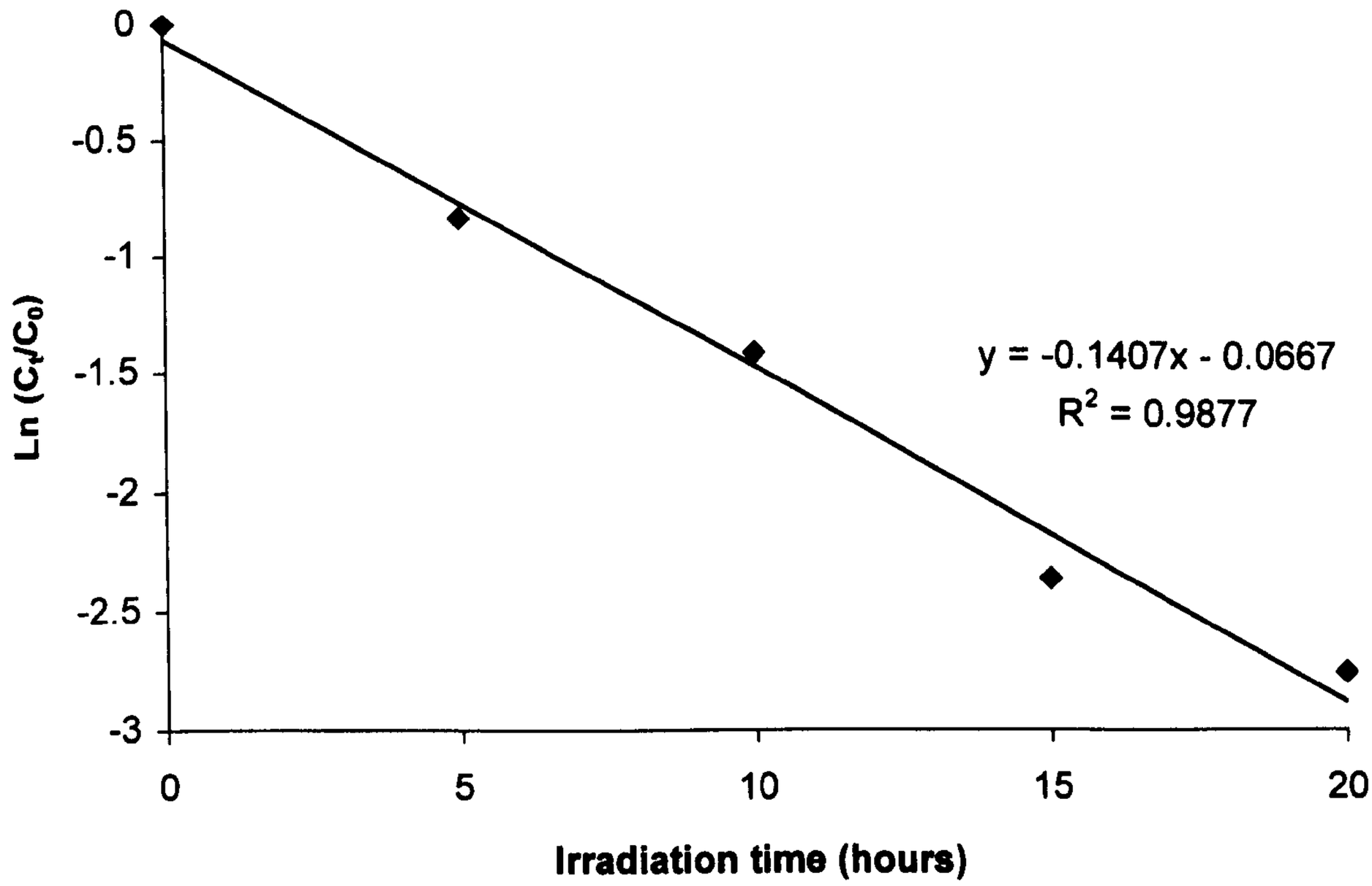


Figure 6.8 Photodegradation rate constant (k_p) of 7-cyclohexyl-1-propyltetralin irradiated under xenon lamp.

6.4.4 Comparison of results from the photodegradation of model monoaromatic UCM compounds

The photodegradation of the three model aromatic UCM hydrocarbons was compared by plotting the percentage of the compound undegraded against irradiation time (Figure 6.9). 6-cyclohexyltetralin degraded the slowest and 7-cyclohexyl-1-propyltetralin degraded the fastest. The kinetic treatment described in Section 6.4 was applied to all the model UCM compounds and Figure 6.10 shows a comparison of $Ln(C_t/C_0)$ plotted against time of irradiation. Photodegradation rate constants and half-lives are summarised in Table 6.4. 6-cyclohexyltetralin degraded the slowest with a half-life of 10.6 hours under the xenon lamp whilst 7-cyclohexyl-1-propyltetralin degraded the fastest with a half-life of 4.9 hours. This indicates that an increase in alkyl substitution on these 'model' aromatic compounds may be responsible for an increase in their photodegradation rate.

Table 6.4 Photodegradation rate constants (k_p) and half-lives of model aromatic UCM compounds in distilled water under xenon lamp at 25°C

Compound	Photodegradation Rate Constant (k_p) (h^{-1})	Half Life ($t_{½}$) (h)
6-cyclohexyltetralin	-0.068	10.26
7-cyclohexyl-1-methyletralin	-0.090	7.70
7-cyclohexyl-1-propyltetralin	-0.141	4.9

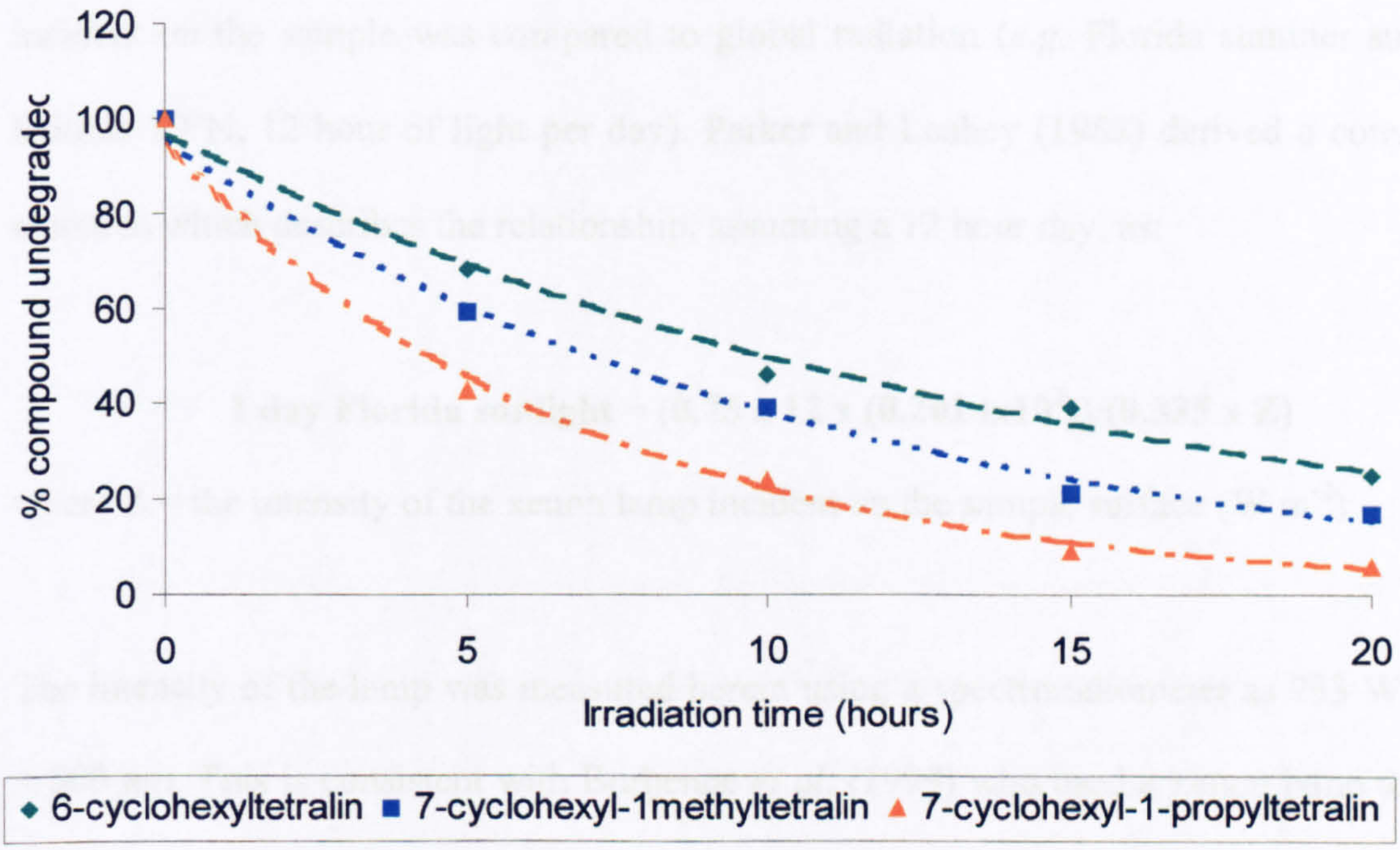


Figure 6.9 Photodecay of generated solutions of three model aromatic UCM hydrocarbons irradiated under a xenon lamp

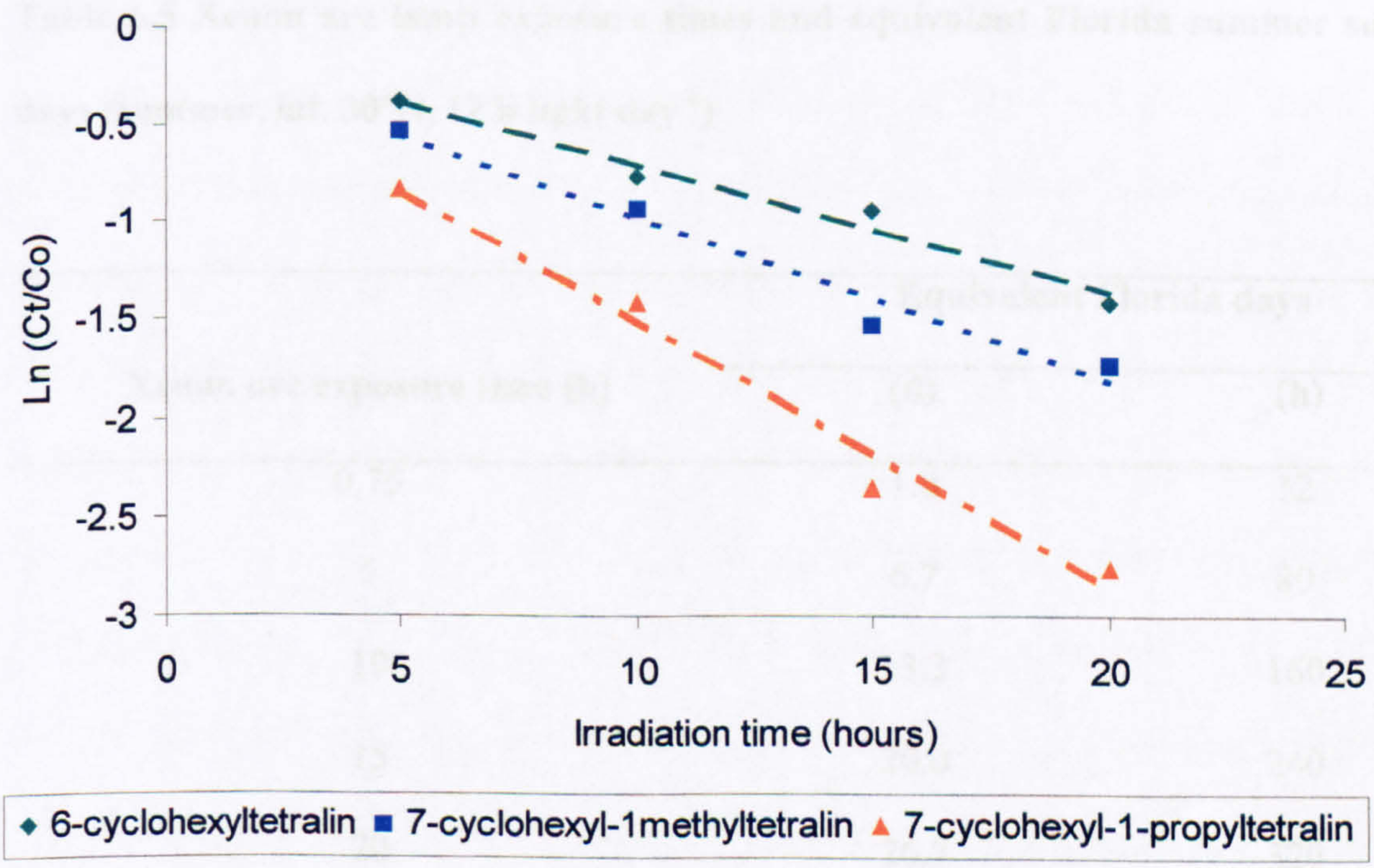


Figure 6.10 Photodegradation rate constants of three model aromatic UCM hydrocarbon irradiated under a xenon lamp

In order to relate the rate of degradation caused by the xenon lamp to the rate likely to occur in natural sunlight the light intensity from the calibrated Heraeus xenon lamp incident on the sample was compared to global radiation (*e.g.* Florida summer sunlight, latitude 30°N, 12 hour of light per day). Parker and Leahey (1988) derived a conversion equation which describes the relationship, assuming a 12 hour day, as:

$$\textbf{1 day Florida sunlight} = (0.75 \times 12 \times (0.2014 \times 10^{2}))/(0.335 \times Z)$$

where Z = the intensity of the xenon lamp incident on the sample surface (W m^{-2})

The intensity of the lamp was measured herein using a spectroradiometer as 733 W m^{-2} at < 800 nm. This is consistent with Burhenne *et al.* (1999) who used a xenon lamp to study the photodegradation of quinolones and found the intensity to be 800 Wm^{-2}. The equivalent Florida days and hours, determined using the above equation are shown in Table 6.5.

Table 6.5 Xenon arc lamp exposure times and equivalent Florida summer sunlight days (summer, lat. 30°N, 12 h light day^{-1})

	Equivalent Florida days	
Xenon arc exposure time (h)	**(d)**	**(h)**
0.75	1.0	12
5	6.7	80
10	13.3	160
15	20.0	240
20	26.7	320

From Table 6.5 it can be seen that an exposure time of 15 hours under the xenon arc lamp is equivalent to 20 days of Florida sunlight. This implies that 6-cyclohexyltetralin would take 13 days to degrade under natural conditions to half its original value, whilst 50 % degradation of 7-cyclohexyl-1-methyltetralin would occur in 10 days and 7-cyclohexyl-1-propyltetralin would have a half-life of almost 7 days under these conditions.

6.5 Discussion

It has been reported that the xenon lamp experimental system used herein to determine the photodegradation rates of 'model' aromatic UCM hydrocarbons, 6-cyclohexyltetralin, 7-cyclohexyl-1-methyltetralin and 7-cyclohexyl-1-propyltetralin, realistically simulates environmental conditions and provides a good model for the fate of pesticides (Parker & Leahey, 1988; de Betrand & Barcelo, 1991) and petroleum (Ali, 1994). Where $\mathbf{C_t}$ and $\mathbf{C_o}$ are the concentrations of the compound at initial time (0 h) and at time **t**, $\mathbf{k_p}$ is the first order photodegradation constant, and therefore, the photodegradation of 6-cyclohexyltetralin, 7-cyclohexyl-1-methyletralin and 7-cyclohexyl-1-propyltetralin can be described as pseudo first order rate, *i.e.* $\mathbf{Ln\ (C_t/C_o) = -k_p t}$. The rate constants and half-lives for 6-cyclohexyltetralin, 7-cyclohexyl-1-methyltetralin and 7-cyclohexyl-1-propyltetralin are the first reported for such compounds and were found to be –0.068 h^{-1} ($t_{1/2}$ = 10.26 h), -0.090 h^{-1} ($t_{1/2}$ = 7.70 h), and –0.141 h^{-1} ($t_{1/2}$ = 4.9 h), respectively. 6-Cyclohexyltetralin degraded the most slowly out of the three compounds whilst 7-cyclohexyl-1-propyltetralin degraded the fastest. It would appear from these three examples that alkyl group substitution benzylic to the aromatic ring of the tetralin structure affects photodegradation rate with the larger alkyl group leading to an increase in the photodegradation rate constant ($\mathbf{k_p}$) and a decrease in the half life ($\mathbf{t_{1/2}}$) of the compound. This is consistent with the observation by Garret *et al.* (1998) that greater size and increasing alkyl substitution

increase the sensitivity of aromatic compounds to photochemical oxidation. Light induced fragmentation and oxidation at the benzyl position of aromatic compounds has also previously been demonstrated (Ehrhardt & Petrick, 1985; Rontani, 1985).

A survey of the literature also revealed that other workers found that similar types of compounds (*i.e* monoaromatic hydrocarbons) underwent photodegradation in their studies. For example, Ehrhardt & Petrick (1985) studied the photodegradation of alkylbenzenes as a homogenous solution and as a surface film in purified seawater and MilliQ. Ehrhardt (1992) later showed that as a group of compounds, alkyl substituted benzenes in the seawater soluble fractions of crude oil could be photooxidised in sunlight. Using the same experimental system as used herein, Ali (1997) demonstrated that in the absence of any sensitisers, certain compounds, such as C_4 alkylbenzenes, of the water-soluble fraction of crude oil were photodegraded effectively.

Low aqueous solubility can often make photolysis experiments practically difficult. Many studies use aqueous solutions of acetonitrile and water to overcome this problem (e.g. Friesian *et al.*, 1996). The procedure used herein overcame this problem by using solutions from a generator column previously set up to measure the aqueous solubility of the compounds of interest, thus using saturated aqueous solutions and more accurately mimicking environmental conditions. The only other report of incorporating the generator column into a further experiment as a means of creating test solutions has been by Kim & O'Keefe (2000) who studied the photodegradation of polychlorinated dibenzodioxins (PCDDs). However, their study was carried out at 39-42 °C limiting its use as an environmental study.

The results of the current study could be further related to environmental conditions by extrapolating the irradiation time under the xenon lamp to that of Florida midday sun. The half lives of 6-cyclohexyltetralin, 7-cyclohexyl-1-methyltetralin and 7-cyclohexyl-1-propyltetralin under the xenon lamp equated to 13, 10, and 7 days of Florida midday sun

and compare well with a previous study by Ali (1994) who found that the half lives of alkylbenzenes were 4-12 days Florida midday sun.

However, in the marine environment other conditions may affect the photodegradation of these compounds. For example, the use of seawater has been reported to increase the photodegradation of phenanthrene several times over that in distilled water (Fukuda *et al.*, 1988). Certain dissolved organic compounds in natural water such as humic substances may also affect photooxidation reactions (reviewed by Zepp, 1981). They can photosensitise a variety of reactions and thus become important determinants of the photochemical pathway of some compounds (Zepp *et al.*, 1985). There can also be light absorbing material present which slows the photolysis rate for compounds which undergo direct photooxidation (Mill *et al.*, 1981).

The UV spectrum of the model compounds (Figure 6.11) shows no significant absorption above 290nm, which is the limit of radiation reaching the Earth's surface and the cut off in radiation emitted from the xenon lamp. This suggests that the compounds would not be directly photooxidised. However, direct photolysis is the only mechanism for photolysis of organic chemicals in pure water and in saturated hydrocarbon solutions (Kim *et al.*, 2000). Sydnes *et al.* (1985) demonstrated that when toluene and dimethylbenzene were deoxygenated no photodegradation products were found. It was therefore suggested that the original photodegradation was found to occur over a charge transfer complex of toluene (or dimethylbenzene) with molecular oxygen. The formation of charge transfer complexes shifts the light absorption to higher wavelengths thus facilitating the photodegradation process. Ranby & Rabek (1975) found that the charge transfer complex of benzene and oxygen increases the absorption of benzene up to 340 nm.

It is possible that this process occurred in the photodegradation of the model UCM aromatic hydrocarbons. However, the photoirradiation of toluene and dimethylbenzene as neat samples carried out by Sydnes *et al.* (1985) deviated from that observed in the natural

environment. Ali (1994) found that C_4 alkylbenzenes irradiated within the seawater soluble fraction of Kuwait crude oil showed evidence of first order degradation, and suggested that the absorption of the compounds just within the wavelength emitted by the xenon lamp may have facilitated their degradation. Upon further inspection of the UV spectrum of the 'model' compounds (Figure 6.11) it can be seen that the UV/Vis absorption for 7-cycloheyxl-1-propyltetralin (the compound with the fastest photodegradation rate) tails further into the wavelength range above 290 nm than that of 6-cyclohexyltetralin (the compound with the slowest degradation rate). Therefore, the mechanism may have been direct photodegradation with the faster degradation rate being due to the greater absorbance above 290 nm.

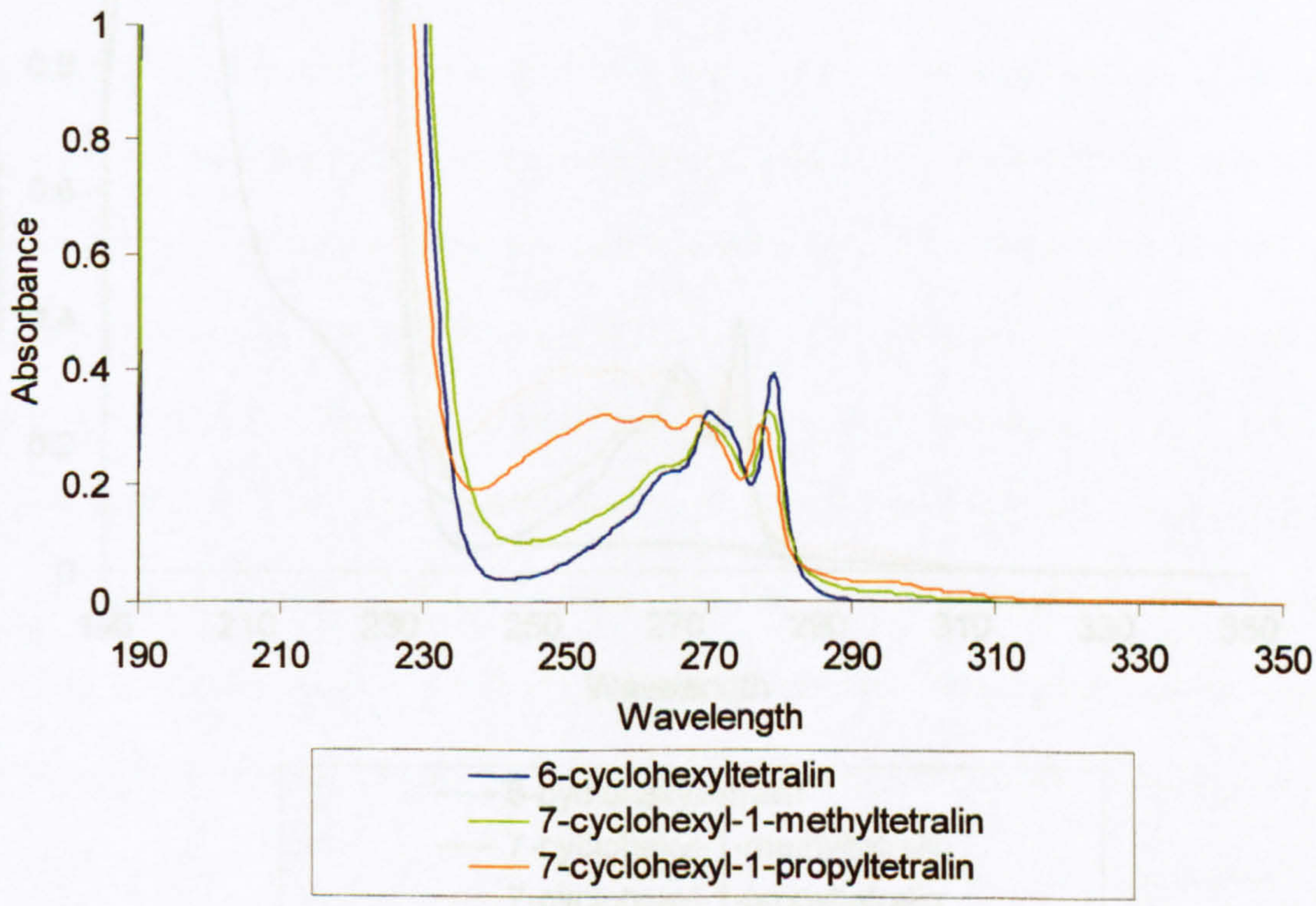


Figure 6.11 UV/Visible spectra (190-500 nm) of model monoaromatic UCM compounds (1 cm path length quartz cell).

Owing to low aqueous solubility samples were analysed in acetonitrile:water (60:40, v/v) solution.

As the 'model' aromatic UCM compounds photodegraded then it is possible that a 'real' monoaromatic UCM may photodegrade. Dutta & Harayama (2000) found that photooxidation of crude oil increased the concentration of alkanes in the samples and suggested these were formed by the cleavage of alkyl side chains from aromatic compounds found in the UCM. Therefore, a UV/Visible spectrum of the isolated monoaromatic UCM (Chapter 2) was taken and compared to the model compounds (Figure 6.12). The absorbance of the isolated monoaromatic UCM tails into the higher wavelengths (*i.e.* above 290 nm), in a similar manner to the model compounds, indicating that it too will be likely to undergo direct photooxidation in the marine environment, possibly at a similar rate.

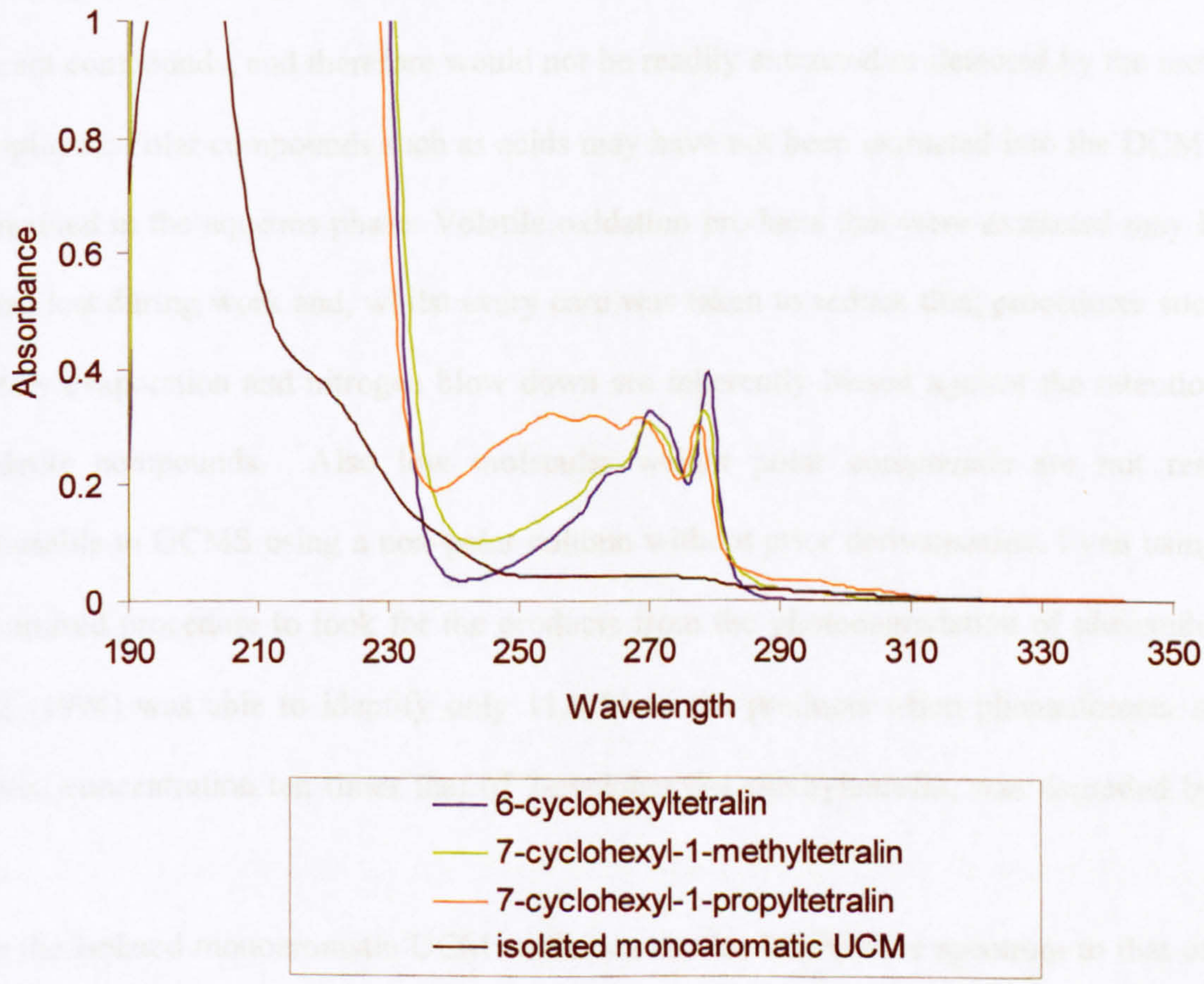


Figure 6.12 Comparison between the UV/Visible spectra (190-500 nm) of model monoaromatic UCM compounds and an isolated monoaromatic UCM (1 cm path length quartz cell).

Owing to low aqueous solubility samples were analysed in acetonitrile:water (60:40, v/v) solution

Whilst one might not expect compounds that accumulate in the marine environment, such as UCM hydrocarbons, to photodegrade, compounds such as polychlorinated dibenzofurans (PCDFs) and polychlorinated dibenzodioxins (PCDDs), which are persistent environmental contaminants, have been shown to undergo aqueous photodegradation under environmental conditions (reviewed by Friesian *et al.*, 1996). These compounds have low quantum yields for direct photolysis at 313 nm and low molecular absorptivities above the solar cut-off at 290 nm, much like the 'model' UCM compounds.

Whist it was not the aim of the experiment to determine the photo-products from the photodegradation of the 'model' aromatic UCM hydrocarbons, the DCM extracts were also run in full scan mode GCMS but this did not reveal any photodegradation products. This is not surprising since photooxidation products are likely to be far more polar than their parent compounds, and therefore would not be readily extracted or detected by the method employed. Polar compounds such as acids may have not been extracted into the DCM and remained in the aqueous phase. Volatile oxidation products that were extracted may have been lost during work and, whilst every care was taken to reduce this, procedures such as rotary evaporation and nitrogen blow down are inherently biased against the retention of volatile compounds. Also low molecular weight polar compounds are not readily amenable to GCMS using a non-polar column without prior derivatisation. Even using an optimised procedure to look for the products from the photodegradation of phenanthrene Ali (1994) was able to identify only 11.6 % of the products when phenanthrene, at an initial concentration ten times that of 7-cyclohexyl-1-methyltetralin, was degraded by 94 %.

As the isolated monoaromatic UCM exhibits a similar UV/Visible spectrum to that of the model UCM hydrocarbons that photodegraded, it would be interesting to see if this process also occurred upon irradiation of solutions of the isolated monoaromatic UCM; however the practicalities of carrying this out may be far more difficult than for the model

compounds. For example, subtle changes in the UCM distribution may not be detected by GCMS. Although, the use of a prior fractionation step such as normal phase HPLC would separate, for example, the original isolated monoaromatic 'hump' from a possible oxidised 'hump'.

Chemical oxidation of an aliphatic UCM was found to increase its toxicity as measured by a reduction in mussel feeding rate (Thomas *et al*, 1995). Ecological effects due to the interactions of natural solar radiation and environmental contaminants may be exacerbated by recent increases in the levels of UV radiation reaching the earth (Cleveland *et al.*, 2000). Therefore, it would be interesting to expose a solution of an isolated monoaromatic UCM to the xenon lamp and then expose mussels to this solution to see if an isolated monoaromatic UCM exhibits photoenhanced toxicity. The toxicological assay used to study the effect of the isolated monoaromatic UCM was conducted using 1.4 L of toxicant solution in duplicate at each exposure concentration taken from toxicant solution of 10 L. The current set-up with the xenon lamp only permits 5 x 50 mL samples to be irradiated at one time. Therefore methodology used herein would need modifying for this to be achievable.

6.6 Summary

Photooxidation is a potentially significant process in the degradation of spilled oil at sea. Moreover, a fundamental understanding of the effect of photochemical degradation on crude oil is a prerequisite for providing an accurate description of the recent history and potential fate of oil spilled within the marine environment (Garret *et al.*, 1998).

The degradation kinetics of the 6-cyclohexyltetralin, 7-cyclohexyl-1-methyltetralin and 7-cyclohexyl-1-propyltetralin as 'model' aromatic UCM compounds were studied using a xenon lamp. The aqueous solutions used were provided by using the generator column

previously set up for the determination of the aqueous solubility of these compounds (Chapter 5). The use of aqueous solutions from a generator column as a test substrate is novel in that only one other report of this was found in the literature (Kim *et al.*, 2000).

The rate constants and half-lives for 6-cyclohexyltetralin, 7-cyclohexyl-1-methyltetralin and 7-cyclohexyl-1-propyltetralin are the first reported for such compounds and were found to be –0.068 h^{-1} ($t_{1/2}$ = 10.26 h), -0.090 h^{-1} ($t_{1/2}$ = 7.70 h), and –0.141 h^{-1} ($t_{1/2}$ = 4.9 h), respectively. These were related to environmental conditions (*i.e.* Florida summer sunlight days) to give half lives of 13, 10 and 7 days which is similar to those found by Ali (1994) using the same experimental set up for alkylbenzenes (4-12 days) suggesting that these compounds may undergo photodegradation within the marine environment.

The length of the alkyl chain substituent appears to be an important factor in the rate of degradation, with the compound with the longest chain length (7-cyclohexyl-1-propyltetralin) degrading the fastest. This is consistent with the observation by Garret *et al.*, (1998) that greater size and increasing alkyl substitution increase the sensitivity of aromatic compounds to photochemical oxidation. The mechanism of photodegradation is uncertain, but may have been by the formation of a charge transfer complex with oxygen, or by direct photodegradation, with the compounds absorbing radiation just within the wavelength cut-off of the xenon lamp.

Degradation products could not be identified using GCMS (apolar phase) indicating that if they were indeed recovered they were too polar for this technique, not inconsistent with the formation of oxygenated compounds *e.g.* acids.

Comparison of the UV/Visible spectra of the 'model' UCM compounds with that of an isolated monoaromatic UCM were comparable in the >290 nm range suggesting that the monoaromatic UCM may behave in a similar manner to the model compounds, and supports their use as models.

Chapter 7. Experimental Details.

This chapter provides the details of the experimental work carried out in this study.

7.1 General laboratory procedures

All glassware was soaked in Decon 90™, rinsed thoroughly with hot tap water, then rinsed with distilled water, and oven dried at 120 °C overnight. Glassware was covered with aluminium foil during storage, and solvent rinsed (x3) with clean solvent immediately prior to use.

All solvents were HPLC or glass distilled grade (Rathburn Chemical Ltd, Walkerburn, U.K.). Solvent purity was routinely monitored by analysing a 100 mL sample which was rotary evaporated (Buchi, 40°C), transferred to a vial, evaporated under nitrogen to approximately 0.5 mL, and analysed (0.5 μL aliquot) using gas chromatography (GC).

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). Adsorbents were activated (silica 24 h, 120 °C; aluminium oxide 12 h, 450 °C), cooled in a desiccator and deactivated by the addition of water (MilliQ). Typically, silica was deactivated to 5 % w/w and aluminium oxide was deactivated to 1.5 % w/w. These were mechanically mixed for 2 h to ensure complete homogenisation, and stored in a desiccator until use within 24 h. When fully activated silica was required the silica was removed from the oven, cooled in a desiccator and used immediately.

Anhydrous sodium sulphate, cotton wool, antibumping granules, sand and Soxhlet thimbles were all Soxhlet-extracted (DCM, 24 h) prior to use. Hydrochloric acid and MilliQ water were extracted with DCM (x 3). Sodium hydroxide pellets were sonicated (10 min x 3) in DCM.

7.2 Instrumental details

7.2.1 Gas chromatography (GC)

Instrument: Carlo Erba 5300 Mega series gas chromatograph

Column: DB-5 fused silica capillary column, 25m x 0.32mm i.d. (J & W Inc.)

Injector: On-column

Carrier gas: Hydrogen, flow rate 2 mL min^{-1}

All chromatograms were recorded using a Shimadzu C-R4A Chromatopac integrator. The typical oven temperature programme was 40 – 300 °C at 5 °C min^{-1}, held at 300 °C for 10 minutes. Column performance was monitored by injection of an alkane mixture.

7.2.2 Gas chromatography-mass spectrometry (GCMS)

GC details:

Instrument: Hewlett Packard MSD GC(5890) – MSD(5970)

Column: HP-1 Ultra, fused silica column, 12 m x 0.2 mm i.d. (Hewlett Packard)

Injector: Autosplitless injection (250 °C)

Carrier gas: Helium (40 kPa head pressure)

The typical oven temperature programme was 40 – 300 °C at 5 °Cmin^{-1}, held at 300 °C for 10 minutes.

Mass spectrometer operating conditions:

Ion source temperature: 280 °C

Ionisation energy: 70 eV

Full Scan: mass range 50 - 550 Daltons

Selected ion monitoring (SIM):

Compound	Ions *m/z*
Anthracene	178
6-cyclohexyltetralin	145, 171
7-cyclohexyl-1-methyltetralin	145, 171
7-cyclohexyl-1-propyltetralin	171, 213, 214

When selected ion monitoring was employed the mass spectrometer was tuned to maximise response at *m/z* 219 to give maximum sensitivity in the region of the ions being monitored.

7.2.3 Nuclear magnetic resonance spectroscopy (NMR)

A Jeol EX270 MHz high resolution (Fourier Transform) FT-NMR spectrometer was used to record ^{13}C and ^{1}H-NMR spectra of samples in deuterated chloroform (trimethylsilane reference).

7.2.4 Infrared spectroscopy (IR)

A Perkin Elmer FT-IR spectrometer operating at a resolution of 4 cm^{-1}, 32 sample scans, 4000 - 400 wave numbers, was used to record infra-red spectra of compounds as liquid films (NaCl discs) or KBr discs.

7.3 Determination of aromatic UCM hydrocarbon concentrations in mussels from Whitby 1990

7.3.1 Sample collection

Mussels were previously collected from Whitby as part of a monitoring programme in 1991 (Widdows *et al.*, 1995b) and stored at –20 °C until analysed, ten years later. A repeat analysis of the mussel tissue was carried out using the original HPLC method of Widdows *et al.* (1991) to ensure no degradation had occurred during the storage period.

7.3.2 Two phase extraction method

The method used was a modification of that described by Rhead (1971). Mussel tissue was spiked with internal standard (deuterated phenanthrene 50 μg in 100 μL acetone) immediately prior to analysis. Phenanthrene (d_{10}) was obtained from Aldrich Ltd and purity was greater than 99% as determined by GC. Wet mussel tissue (~30 g) was acidified to pH 1 using concentrated hydrochloric acid, and 15 mL of a mixture of *n*-pentane:2-propanol (1:4 v/v) was added. This mixture was sonicated (40 min). *n*-Pentane (120 mL) and MilliQ water (117 mL) were added and this mixture was shaken (5 min), followed by centrifugation at 2000 rpm (20 min). The upper pentane layer was decanted and retained. The procedure was repeated twice and the resulting pentane fractions combined, dried over anhydrous sodium sulphate overnight, concentrated to 1 mL by rotary evaporation and analysed by GC.

7.3.3 Fractionation of mussel tissue by open column chromatography

Mussel total organic extract (TOE) was fractionated into aliphatic and aromatic fractions using a modification of the method of Wraige (1997). A glass column (350 mm x 10 mm) was packed with a pentane slurry of silica (60-100 mesh, fully activated, 10g) under

aluminium oxide (grade 1, neutral, 1.5 % deactivated, 10 g). The sample was applied to the top of the column with a sample:adsorbent ratio of 1:200. Sequential elution of the column with solvents of increasing polarity yielded the following fractions:

Fraction 1	'aliphatic'	1.5 column volumes *n*-pentane (45mL)
Fraction 2	'aromatic'	2 column volumes *n*-pentane:DCM (1:1, v/v) (60 mL)

These fractions were concentrated by rotary evaporation to 1 mL, followed by evaporation under a gently stream of nitrogen, and analysed using GCMS.

7.3.4 Quantification of total unresolved and resolved hydrocarbons

Quantification was performed using Chemstation™ (Hewlett Packard) software. The total area of the chromatogram was calculated using AREASUM parameters. Resolved components were integrated manually as no one set of parameters adequately quantified the peaks present, and the unresolved component was then calculated from total hydrocarbons minus the resolved hydrocarbons. The concentration of the unresolved component was calculated relative to the internal standard assuming a response factor of 1. Risebrough *et al.* (1983) found that values for the concentration of the UCM obtained using the response of n-C_{23} did not differ significantly from those obtained using the mean responses for *n*-alkanes within the range of the UCM.

7.4 Isolation of a monoaromatic hydrocarbon unresolved complex mixture

7.4.1 Rotary evaporation of crude oil

Gullfaks crude oil (5 g) was placed in a 50 mL round bottomed flask and rotary evaporated under water pump vacuum at 50 °C (10 min).

7.4.2 Fractionation by open column chromatography

Fractionation of 100 mg subsamples into aliphatic and aromatic fractions was performed using open column chromatography, with a sample:adsorbent ratio of 1:200. A 350 x 10 mm column containing 10 g fully activated silica over 10 g alumina (0.15 % deactivated) was eluted with 1.5 column volumes (45 mL) *n*-pentane to yield the aliphatic fraction, followed by 2 column volumes (60 mL) of *n*-pentane:DCM (1:1, v/v) to remove the aromatic fraction.

7.4.3 Normal-phase HPLC fractionation

Normal-phase HPLC (Dionex GP40 gradient pump, Dionex Corp) was performed utilising 3 Hypersil Hyperprep HSAPS-2 columns (250mm x 10mm x 8 μm) in series with a guard column (ThermoHypersil). The mobile phase/solvent gradient was 0-40 minutes (100 % hexane), 40-41 minutes (gradient to100% DCM), at a flow rate of 1 mL min^{-1}. UV-Vis detection was performed at 254 nm (Dionex AD20 UV absorbance detector). It was determined that 15 mg on column was the maximal amount per run that allowed good separation and recovery of fractions. Fractions were collected corresponding to UV absorption minima and analysed by GCMS. This process was repeated to allow isolation of a sufficient quantity (50 mg) of the desired material (fraction 2; 22-29 minutes).

7.5 Investigations into the effect of an isolated aromatic unresolved complex mixture 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).

7.5.1 Test substrate

The monoaromatic unresolved complex mixture was isolated from Gullfaks produced crude oil as described in Section 7.4

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

Mussels of size 10-20 mm were collected from the intertidal zone at Whitsand Bay, Cornwall, U.K. This site is known to be relatively uncontaminated by petroleum hydrocarbons (Wraige, 1997). Mussels were cleaned of epibionts and held in open flow polythene tanks in recirculating seawater at 33 ‰ salinity, with an artificially produced tidal regime which aerially exposed the mussels for 2.5 h twice each day. The temperature of the system was maintained at 15 °C and mussels were continuously fed an algal culture of *Isochrysis galbana*. Mussels were acclimated to laboratory conditions for 7 days before use in any experiments.

7.5.3 Preparation of toxicant solutions

Test solutions were prepared using Eddystone Filtered Seawater (EFSW) which had been filtered to remove particles greater than 45 μm. 10 L glass aspirators were filled with EFSW and magnetically stirred to create a vortex. The test compound was dissolved in acetone and added by means of a syringe, discharging the solution directly into the seawater, ensuring that any emulsion was held in the vortex until it had dissolved. Acetone

was added to the control aspirators in the same manner. Previous studies by Donkin *et al.* (1989) and Wraige (1997) have demonstrated that at the concentration used (0.001 % v/v) acetone has no effect upon mussel feeding rate. The aspirators were stoppered and solutions stirred for 2 h before use.

7.5.4 Exposure of mussels to toxicant

A modification of the method of Donkin *et al.* (1991) by Wraige (1997) was used. Groups of 7 mussels (12 mm ± 2 mm) were placed in 2 L tall glass beakers. 1.4 L of toxicant solution (prepared as described in Section 7.4.4.) was added to each beaker. Water movement was maintained by a magnetic stirrer constrained within a glass dish to prevent contact with the mussels. The top of each beaker was covered with perforated aluminium foil through which the mussels were continuously fed with an algal culture of *Isochrysis galbana*, controlled by means of a peristaltic pump. For each exposure concentration two separate experiments were conducted along with controls for a 24 hour exposure period.

7.5.5 Measurement of mussel feeding rate

After the 24 h exposure period mussels were transferred individually to 300 mL beakers each containing 200 mL of toxicant solution (for each experiment a control vessel containing no mussel was also set up). A 30 minute acclimatisation period was allowed for the mussels to open their valves and resume pumping, after which time a predetermined volume of algal culture was added to each beaker giving a concentration of 24000-30000 cells mL^{-1}. Each beaker was carefully stirred with a glass rod to ensure an even distribution of the algal culture. After 5 minutes a 20 mL aliquot was removed using a syringe and placed in a 20 mL glass vial. After 15 minutes a further 20 mL aliquot was removed and the cell numbers of these samples determined using a model D Coulter counter set to measure particles greater than 3 μm.

7.5.6 Tissue analysis

Mussel tissue was dissected and stored in solvent rinsed vials at –20 °C until required for analysis. The samples were thawed to room temperature and mussel tissue (typically 0.5 g) was placed in a stoppered centrifuge tube with 5 mL of 3M sodium hydroxide. These tubes were placed in a heated water bath at 60 °C for 20 minutes. Once cooled, 10 mL of *n*-hexane was added and the tubes vigorously shaken for 5 minutes. The samples were then centrifuged at 2000 rpm for 10 minutes and the upper *n*-hexane layer transferred to a conical flask. The hexane extract was dried over anhydrous sodium sulphate overnight and rotary evaporated to 1 mL, followed by evaporation under a gentle stream of nitrogen. The samples were then analysed by GCMS using external calibration.

7.5.7 External calibration of aromatic unresolved complex mixture hydrocarbons

The amount of the aromatic UCM accumulated by the animals and observable in the gas chromatograms was quantified using an external calibration graph. The monoaromatic UCM isolated from Gullfaks oil was run on the GCMS over a range of concentrations in triplicate to give a calibration graph. Quantification was performed using Chemstation™ (Hewlett Packard) software. The total area of the chromatogram was calculated using AREASUM parameters. Resolved components were integrated manually as no one set of parameters adequately quantified the peaks present and the unresolved component was then calculated from total hydrocarbons minus the resolved hydrocarbons. The unresolved component present in extracted mussel tissue was calculated using the calibration graph from the calibrated weight of isolated monoaromatic UCM.

7.6 Synthesis of a model monoaromatic UCM hydrocarbon

7-cyclohexyl-1-methyltetralin was synthesised essentially using the methods of Wraige (1997) and Sturt (2000).

7.6.1 Synthesis of 3-benzoyl(4'-cyclohexyl)propanoic acid

Succinic anhydride (12 g, 0.12 mol), phenylcyclohexane (16 mL, 0.1 mol) and 1,1,2,2-tetrachloroethane (100 mL) were placed in a three-neck 250 mL round bottomed flask (rbf). The mixture was heated gently using an electric heating mantle (80 °C), with stirring by mechanical stirrer to dissolve the succinic anhydride, giving a clear yellow liquid. On cooling, 32 g (0.24 mol) of finely ground aluminium chloride was added in small portions over a 30 minute period. This was left stirring for 30 minutes during which time the mixture turned dark red and became more viscous. After this time the contents of the flask were poured into a 400 mL beaker half full of ice and rinsed with distilled water. Concentrated HCl (~5 mL) was added until no more dark red material was apparent and the mixture was a milky yellow colour. This mixture was then transferred to a 500 mL round bottom flask (rbf) ready for steam distillation.

The mixture was distilled (2 h) with addition of water as necessary to keep the volume in the flask at around 300 mL. At this time the majority of the tetrachloroethane (~95 %) had been collected. Once cooled 1M $NaCO_3$ was added until all the HCl was neutralised, indicated by the cessation of effervescence and pH paper measurements. The alkaline mixture was steam distilled again until all the tetrachloroethane was recovered.

The distilled mixture was placed in a beaker on a hotplate, further 1M $NaCO_3$ was added to dissolve the product, and 1 g decolourising carbon was added. Precipitated alumina was removed by filtration using a heated filter funnel and the filtrate left to cool. This was slowly acidified with concentrated HCl which resulted in the formation of a creamy precipitate upon neutralisation. The product was obtained by vacuum filtration, washing

with water to remove any HCl, and left to dry in a drying cabinet (40 °C) overnight. 3-Benzoyl(4'-cyclohexyl)propanoic acid was synthesised in good yield (65 %) and purity (>98 % by GC) and fully characterised using GC, GCMS, IR and NMR.

MS: *m/z* 332 ($M^{+\cdot}$, 4 %); *m/z* 317 ($M^{+\cdot}$-CH_3, 29 %); *m/z* 187 (benzylic cleavage, 100 %)

IR: 3423 cm^{-1} (νO-H); 3039 cm^{-1} (νCH aro); 2853 cm^{-1}, 2924 cm^{-1} (νC-H); 1712cm^{-1}, 1679 cm^{-1} (νC=O)

^{13}C-NMR: (ppm) 197.5, 179.1, 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* 3.3, *t* 2.8, *br m* 2.6, *m* 1.8, *m* 1.4

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

KOH (6 g, 0.1 mol) was dissolved in diethylene glycol (40 mL) by gentle heating. Hydrazine hydrate (3.2 mL, 0.0663 mol) and 3-benzoyl(4'-cyclohexyl)propanoic acid (11.5 g, 0.044 mol) were added and the mixture refluxed (1 h, 130 °C) to form the hydrazone. Once the hydrazone was formed the aqueous material was distilled off and collected in order to reach a high enough temperature for the decomposition of the hydrazone. This was achieved by reflux (4 h, 230 °C). The resulting material was left overnight to cool and then poured into a 200 mL beaker half full with ice and acidified with concentrated HCl to Congo Red whilst stirring with a glass rod. Upon neutralisation a brown sticky lump was formed which was extracted with diethyl ether (100 mL), water washed (3 x 50 mL) and dried over anhydrous Na_2SO_4 (~5 g). The resultant extract was treated with ~1 g decolourising carbon, followed by hot filtration and rotary evaporation to yield 4-phenyl(4'-cyclohexyl)butanoic acid in good yield (83 %) and purity (>97 % by GC). The acid was characterised using GC, GCMS, IR and NMR.

MS: *m/z* 318 ($M^{+\cdot}$, 28%), 303 ($M^{+\cdot}$-CH_3, 41%), 186 (benzylic cleavage, 100%)

IR: 3416 cm^{-1} (νOH); 1708 cm^{-1} (νC=O); 2922 cm^{-1}, 2850 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, 26.0.

^{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.

7.6.3 Synthesis of 7-cyclohexyl-1-tetralone

Polyphosphoric acid (36 g) was heated in a beaker to 90 °C on a hotplate whilst 4-phenyl(4'-cyclohexyl)butanoic acid (9 g, 0.346 mol) was warmed to 70 °C. These were mixed together and stirred vigorously using a glass rod at temperature (30 min). A further portion of polyphosphoric acid (12 g) was added and the mixture stirred for a further 10 minutes to ensure the complete conversion to the tetralone. Once the mixture had cooled it was poured into a 100 mL beaker half full of ice. This was left to stand overnight to separate. The crude tetralone was extracted with diethyl ether, water washed (2 x 50 mL), then base extracted (2 x 20 mL; 5 % NaOH) to remove any unreacted acid, and water washed until washings were neutral. The organic extract was then dried over Na_2SO_4 and solvent removed by rotary evaporation to give 7-cyclohexyl-1-tetralone in good yield (80 %) and purity (>97 % by GC). This was characterised using GC, GCMS, IR and NMR.

MS: *m/z* 228 ($M^{+\cdot}$, 100 %), 200 ($M^{+\cdot}$-CO, 28 %), 185 (48 %) 172 (58 %).

IR: 2925cm^{-1}, 2852cm^{-1} (νC-H); 1685cm^{-1} (νC=O).

^{13}C-NMR (ppm) 198.7, 146.7, 142.0, 132.3, 128.7, 125.0, 44.1, 39.2, 34.3, 29.3, 26.7, 26.0, 23.3.

^{1}H-NMR (ppm) *s* 7.9, *d* 7.3, *d* 7.1, *t* 2.9, *t* 2.7, *br m* 2.5, *q* 2.1, *m* 1.8, *m* 1.4.

7.6.4 Synthesis of 7-cyclohexyl-1-hydroxy-1-methyltetralin

Mg turnings (0.90 g, 3.7 mol) dried at 120 °C overnight were added to sodium dried diethyl ether, and iodomethane (3.92 mL, 0.063 mol) in sodium dried diethyl ether (7.5 mL) was added dropwise with gentle heating until a cloudy precipitate was formed. This indicated the formation of the alkylmagnesium iodide *i.e.* the Grignard reagent, to which 7-

cyclohexyltetralone (3.32 g, 1.45 mol) dissolved in diethyl ether (7.5 mL) was carefully added over a period of 30 minutes, followed by heating under reflux (1 h). Once the mixture was cooled it was poured into a beaker containing approximately 100 mL of ice with 20 mL saturated ammonium chloride. This was extracted with ether, water washed (3 x 50 mL), dried over anhydrous Na_2SO_4, and the solvent removed by rotary evaporation. The tertiary alcohol was obtained in good yield (78 %) and purity (94 %) and the product was characterised using GC, GCMS, IR and NMR.

MS: *m/z* 301 ($M^{+\cdot}$-CH_3, 100 %), 226 ($M^{+\cdot}$-HO^+TMS, 55 %), 75 (35 %), 301 (26 %), 143 (18 %).

IR: 3366 cm^{-1} (νOH); 2925 cm^{-1}, 2850 cm^{-1} (νC-H).

^{13}C-NMR: 146.2, 142.6, 133.6, 128.7, 125.6, 124.6, 70.7, 40.4, 39.9, 34.5, 30.6, 29.5, 26.9, 26.1, 20.5

^1H-NMR: *s* 7.4, *m* 7.0, *m* 2.8, 2.5, 1.9, 1.4, *s* 1.5.

7.6.5 Synthesis of 7-cyclohexyl-1-methenyltetralin and 7-cyclohexyl-1-methyl-3,4-dihydronaphthalene

The alcohol 7-cyclohexyl-1-hydroxy-1-methyltetralin was dehydrated by reflux (1.5 h) with orthophosphoric acid (10mL). Once the mixture was cooled it was transferred to a separating funnel and saturated NaCl (5mL) added. The mixture was then extracted with diethyl ether (2 x 20 mL), and the extracts combined and the solvent removed to yield an isomeric mixture of alkenes.

Crude reaction products (a thick brown oil) were cleaned using open column chromatography employing fully activated silica and elution with hexane to give a clear oily liquid.

GC Purity: >98%

MS: *m/z* 226 ($M^{+\cdot}$, 100 %), 224 ($M^{+\cdot}$-2, 39%) 143, (64 %), 129 (64 %)

IR: 3021 cm^{-1} (νC=C), 2925 cm^{-1}, 2851 cm^{-1} (νC-H).

^{13}C-NMR: 158.1, 146.1, 136.7, 135.0, 134.8, 132.3, 44.0, 37.2, 36.5, 36.1, 34.5, 28.6

^{1}H-NMR: *m* 7.0, *m* 5.8, *t* 2.8, *m* 2.5, 2.2, *s* 2.0, *m* 1.8, 1.4

7.6.6 Synthesis of 7-cyclohexyl-1-methyltetralin

Hydrogen was bubbled through 100 mg of the purified alkene mixture dissolved in hexane (~7 mL) using palladium (50 mg) as a catalyst (3 h). After this time the catalyst was removed by filtration through a cotton wool plug and the solvent removed under a stream of nitrogen. This gave a yield of 75 %. Removal of trace amounts of unconverted alkenes was accomplished by elution through an Ag^+ silica column with *n*-pentane.

GC purity: >99 %

IR: 3001 cm^{-1} (νCH aro), 2924 cm^{-1}, 2850 cm^{-1} (νC-H alip).

MS: *m/z* 228 ($M^{+\cdot}$, 61 %), 213 ($M^{+\cdot}$-CH_3, 51 %), 145 (100 %), 129 (64 %), 128 (26 %)

^{13}C-NMR (ppm): 143.6, 142.1, 134.3, 129.4, 127.4, 125.2, 44.3, 34.9, 32.9, 32.5, 30.1, 26.7, 25.6, 22.5, 20.1

^{1}H-NMR (ppm): *s* 7.1, *m* 7.0, *m* 2.9, *m* 2.7, *m* 2.5, *m* 1.9, 1.4, *d* 1.3

7.6.7 Isolation of 6-cyclohexyltetralin

6-cyclohexyltetralin was purified from crude reaction products by elution through an Ag^+ silica column with *n*-pentane in good yield (78 %).

GC purity: >98 %

MS: *m/z* 214 ($M^{+\cdot}$, 100 %), 171 (99 %), 145 (46 %), 131 (49 %), 129 (68 %)

IR: 3001 cm^{-1} (νCH aro), 2924 cm^{-1}, 2850 cm^{-1} (νC-H alip).

^{13}C-NMR (ppm) 145.2, 136.8, 134.5, 129.0, 127.4, 124.0, 44.2, 34.6, 29.5, 28.9, 26.9, 26.2, 23.3.

^{1}H-NMR (ppm) *m* 7.1, *m* 2.9, *br m* 2.5, *m* 1.9, *m* 1.5

7.6.8 Isolation of 7-cyclohexyl-1-propyltetralin

A crude mixture of the 7-cyclohexyl-1-propenyltetralin and 7-cyclohexyl-1-propyl-3,4-dihydronapthalene (Wraige, 1997) was subjected to silica column chromatography, further purified by Ag^+ chromatography and hydrogenated (70.5 mg), to give 7-cyclohexyl-1-propyltetralin (56.6 mg) in good yield (80 %).

GC purity: >98 %.

MS: *m/z* 256 ($M^{+\cdot}$, 14 %), 213 (100 %), 131 (29 %), 129 (16 %).

IR: 3001 cm^{-1} (νCH aro), 2924 cm^{-1}, 2850 cm^{-1} (νC-H alip).

^{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) *q* 6.9, *br m* 2.7, *br m* 2.4, *m* 1.3-1.7, *t* 0.9.

7.7 Investigations into the effect of 7-cyclohexyl-1-methyltetralin upon mussel feeding rate

The same procedure was followed as for investigating the effect of an isolated monoaromatic UCM described in section 7.5 with some minor alterations. Exposure concentrations were 0, 12.5, 25, 50 and 100 μg L^{-1}. Immediately prior to analysis mussel tissue was thawed to room temperature and spiked with an internal standard. 6-Cyclohexyltetralin was used for 7-cyclohexyl-1-methyltetralin and *vice versa*. The extracts were analysed by GCMS and the accumulated compound quantified using integration software and an internal standard calibration.

7.8 Investigations into the aqueous solubility behaviour of model aromatic UCM hydrocarbons

7.8.1 Test materials

Anthracene was obtained from Aldrich with a purity of > 99 % as determined by GC. The model aromatic UCM hydrocarbons, 6-cyclohexyltetralin, 7-cyclohexyl-1-methylteltralin and 7-cyclohexyl-1-propyltetralin were synthesised in good yield and purity (> 98 %) as described in section 7.6. An aromatic unresolved complex mixture was isolated from a crude oil as described in section 7.4.

7.8.2 Experimental set-up

Saturated solutions were prepared using a generator column set-up based on the original method of May *et al.* (1978). Each generator column consisted of a HPLC stainless steel column 30 cm x 0.46 mm i.d. with 2 μm stainless steel frits at either end, dry packed with glass beads 60-80 mesh (Alltech Associates) coated with test compound. Prior to coating with the test compound the beads were cleaned in a Soxhlet thimble with 100 mL each of distilled water, acetone and hexane respectively, placed in an oven at 120 °C overnight, then left in a dessicator until required. The size of the beads was measured using a microscope and were sized at 0.17 - 0.25 mm diameter corresponding to 60 - 80 mesh size. Test compounds (35 mg) were dissolved in 50 mL of hexane to which 7 g of beads were added. The solvent was removed by rotary evaporation followed by a gentle stream of nitrogen to give a 0.005 % coating. Once packed the generator column was attached to a HPLC pump (Waters Associates Chromatography Pump, 6000A Solvent Delivery System) by means of Teflon tubing and attachments, supplied by a water reservoir.

Temperature control (25 °C) was by means of a water bath in which the generator column and water reservoir were immersed. Temperature was monitored using a Squirrel datalogger (SQ32-4U, Grant Instruments) equipped with two temperature probes and set to

record every 10 minutes. A 3 L beaker acted as a water reservoir. This contained MilliQ water to which was added mercuric chloride (20 μg L^{-1}) for sterilisation and covered in foil for protection.

7.8.3 Generation of solutions

The column was flushed with 500 mL water to allow equilibration of the system before measurement. This is consistent with previous studies (*e.g.* Dunnivant & Elzermann, 1988). Water was pumped through the generator column at a rate of 1 mL min^{-1}. The effluent from the column was collected directly into a 100 mL separating funnel containing 25 mL DCM. Samples were collected every 50 minutes and 10 samples were collected per measurement.

7.8.4 Extraction of solutions

Once the sample had been collected an internal standard was spiked into the water in 100 μL acetone and the funnel stoppered. Internal standards would preferably have been deuterated analogues of the analyte to be measured; however these compounds were not available commercially. The next choice was a compound of similar chemical structure and GC retention time, therefore the 'model' compounds served as internal standards for one another. Each funnel was shaken for 5 minutes with care not to form an emulsion. The DCM fraction was separated off and the water sample extracted with a further 25 mL DCM. The DCM extracts were combined and passed through an anhydrous sodium sulphate column. Analysis was performed using GCMS operated in selected ion monitoring mode (SIM) (see Section 7.2.2).

7.8.5 Effect of salinity on aqueous solubility

The effect of salinity was determined by carrying out the above procedure but replacing MilliQ water with filtered seawater at 35 ‰. Salinity was monitored using a conductivity meter (WTW, Germany) utilising a conductivity cell TetroCon 325 calibrated with 0.01mol L^{-1} KCl.

7.8.6 Effect of temperature on aqueous solubility

The effect of temperature was determined by carrying out the above procedure at 15 °C by using a dip chiller unit (HetoFrig, Denmark) along with the water bath to achieve the required temperature.

7.8.7 Modifications for generator column using isolated monoaromatic UCM

The generator column method was employed in exactly the same way as for the model compounds. Distilled water was used at a temperature of 25 °C. Extracted samples were analysed in full scan and quantified using an external calibration graph (as in Chapter 2)

7.9 Investigations into the photodegradation of model aromatic UCM hydrocarbons

7.9.1 Test compounds

6-Cyclohexyltetralin and 7-cyclohexyl-1propyltetralin were purified from the crude reaction products from earlier studies (Wraige, 1997) as described in sections 7.6.7 and 7.6.8. 7-Cyclohexyl-1-methyltetralin was synthesised using the procedures described in Sections 7.6.1. – 7.6.6.

7.9.2 Preparation of model compound solutions

Saturated solutions of the model UCM hydrocarbons, 6-cyclohexyltetralin, 7-cyclohexyl-1-methyltetralin and 7-cyclohexyl-1-propyltetralin were prepared using a generator column, as described in section 7.8.3. Solutions were generated directly into photolysis vessels, which were covered in foil until exposed. Solutions were generated using distilled water at 25°C therefore the temperature of the samples did not need to be adjusted before exposure. The photolysis vessels were 25 cm long quartz tubes with an internal diameter of 1.8 cm and 19/26 mm socket ground glass joints with quartz stoppers. Each tube held 50 ml of sample to be irradiated or act as a control. Alternate samples were used as exposure and control vessels.

7.9.3 Method of exposure

Samples were exposed to a Suntest CPS xenon lamp which emitted radiation at environmentally realistic wavelengths (confirmed by measurement of its output spectrum). Radiation was emitted from a 1.8 kW xenon arc installed horizontally in a parabolic reflector and fitted with an UV filter with a radiation limit of 290 nm in the UV range, corresponding to natural sunlight.

An arrangement was made so that 5 solutions in individual stoppered quartz tubes could be placed beneath the xenon lamp. After being filled with the generated solution of 'model' UCM hydrocarbon, these tubes were tightly capped and sealed with PTFE tape to keep the tube tightly sealed and help minimise any volatilisation that might occur during irradiation. To maintain a constant temperature during irradiation the tubes were held in a specially designed stainless steel tank partially filled with glycerol in a metal holder. The tank was cooled by circulating thermostatted water (chiller thermo circulator model 05/CTCHG, Conair) through the tank. Glycerol was used instead of water to avoid loss of coolant due to evaporation. The tank holding the tubes was positioned under the light source with the

aid of a laboratory jack at a distance of approximately 20cm from the lamp. The position of the jack and tank were marked to maintain consistency between following exposures. The temperature of the glycerol during irradiation was monitored using a Squirrel datalogger (SQ32-4U, Grant Instruments) equipped with two temperature probes (positioned at the inlet and outlet of the water chiller unit) and set to record every 10 minutes.

Before sample irradiation, the lamp and the cooling of the glycerol tank were switched on for an hour to allow the lamp to reach its maximum radiation intensity and to allow the glycerol to adjust to the temperature required.

The radiation intensity of the xenon lamp incident on the reaction tubes was measured and the quartz tubes were subjected to an average radiation intensity of 733 W m^{-2}.

Samples were irradiated for 5, 10, 15 and 20 hours. 5 samples were carried out at each exposure period and 5 dark controls were also carried out in parallel for each exposure period. After the specified irradiation periods photolysis tubes were cooled in a refridgerator prior to the work-up procedure.

7.9.4 Work-up of irradiated solutions

Upon opening, each photolysis/control vessel was spiked with an internal standard. 6-cyclohexyltetralin was the internal standard for 7-cyclohexyl-1-methyltetralin and 7-cyclohexyl-1-propyltetralin; and for 6–cyclohexyltetralin, 7-cyclohexyl-1-methyltetralin was used. The concentration of internal standard used was half the expected concentration before photooxidation (*i.e.* half the aqueous solubility value). The spiked samples were carefully decanted into separating funnels (100 mL), each tube was rinsed with DCM (3 x 5 mL), and rinsings added to the separating funnel. The aqueous sample was then solvent extracted (3 x 15 mL DCM). After phase separation the DCM extracts were combined and excess water removed using a large (5 x 2 cm) anhydrous sodium sulphate column. The solvent was reduced by careful rotary evaporation to ~5 mL, followed by evaporation

under nitrogen to ~1 mL. Samples were analysed by GCMS operating in selected ion monitoring mode (see Section 7.2.2).

Chapter 8. Summary and further work

This chapter summarises the main conclusions of the work presented herein and suggests directions for further work

8.1 Summary

Considerable quantities (an estimated 2.5 million tonnes per year) of petroleum and petroleum fractions are introduced into the marine environment. Most research has concentrated on those hydrocarbon components which are resolved by GC and thus identifiable by GCMS. However, unresolved complex mixtures of hydrocarbons accumulate when petroleum is weathered. UCMs are therefore widespread in the marine environment occurring, for example, in molluscs, fish, sediment and water-borne particles. As such, the presence of a UCM or 'hump' in the gas chromatogram of the aliphatic fraction of a sample is considered a marker for petroleum contamination. Although these unresolved aliphatic compounds are bioaccumulated to a large extent they do not appear to pose a threat to marine organisms. In contrast aromatic UCMs have not been extensively studied. Little is known of the composition of UCMs particularly regarding the aromatic component, the presence of which, in polluted samples, is often ignored and seldom reported. However, these compounds are present in samples at a high level and a survey of the literature indicates that they may be of environmental concern. The overall aim of this study was to therefore assess the possible impact of these compounds accumulating within the marine environment.

A previously verified approach was used to determine the aromatic UCM concentration in mussel tissue from Whitby harbour collected as part of a monitoring programme in 1990 and found to have reduced Scope for Growth. The mussel tissue was also analysed by HPLC as in the original monitoring scheme and found to have comparable levels of 2-3 ring aromatic hydrocarbons, showing that no degradation of the sample had occurred during storage. Analysis by GCMS revealed that the sample was dominated by aromatic UCM hydrocarbons, the concentration of which was comparable to the amount found in the mussels in 1995. The amount of aromatic UCM found in the mussels correlated with

the amount of 'total toxic hydrocarbons' at this and two other sites suggesting that the aromatic UCM may be responsible for the observed effects. This suggested the need to investigate the environmental behaviour of these compounds.

The overall objectives of the current study were therefore

- to isolate a monoaromatic unresolved complex mixture from a crude oil for toxicological testing
- synthesise a 'model' aromatic UCM hydrocarbon for toxicological testing
- measure the aqueous solubility of three 'model' aromatic UCM hydrocarbons
- generate an aqueous solution from the isolated unresolved complex mixture
- test the photodegradation potential of the model compounds under environmentally realistic conditions.

The experimental data from the whole of this study are summarised in Table 8.1

In order to investigate the toxicity of aromatic UCM hydrocarbons a monoaromatic UCM was isolated from a crude oil. Gullfaks (North Sea) produced crude oil was chosen as most of the resolved components had already been removed by in-reservoir biodegradation. This crude oil was also the cargo of the *Braer* which was spilled off Shetland in 1993. The isolated monoaromatic UCM (representative of a monoaromatic UCM entering the environment) was exposed to mussels to determine its effect on mussel feeding rate (a sensitive parameter in the Scope for Growth measurement). At the highest nominal aqueous concentration tested (200 $\mu g\ L^{-1}$) the monoaromatic UCM hydrocarbons were accumulated and filtering rate was significantly reduced (~40%) in 24 hours. It is assumed that exposure of mussels to a lower concentration over a greater exposure time would achieve a similar total body burden and thus exhibit a similar effect. The accumulated hydrocarbons were present in the same retention time range of the gas chromatogram as

those to which they were exposed indicating that the accumulation of monoaromatic UCM hydrocarbons was responsible for the reduction in mussel feeding rate. Mussels accumulated up to 90 $\mu g\ g^{-1}$ wet weight (~400 $\mu g\ g^{-1}$ dry weight) of the monoaromatic UCM hydrocarbons, which is comparable to the amounts found at petroleum impacted sites with reduced Scope for Growth. This suggests that monoaromatic UCM hydrocarbons are not only a quantitatively important environmental burden but are also of some toxicological significance. Recent reports on the toxicity of weathered oils have not been able to identify all of the components responsible for the effects observed and the toxicity of the monoaromatic UCM hydrocarbons may be one of the factors overlooked.

A model UCM hydrocarbon was synthesised in order to more conveniently study the behaviour of monoaromatic UCMs. 7-cyclohexyl-1-methyltetralin was synthesised in good purity and yield by a seven stage synthetic pathway, each stage being fully characterised by GC, GCMS, IR and NMR. Two further model monoaromatic UCM hydrocarbons, 6-cyclohexyltetralin and 7-cyclohexyl-1-propyltetralin, were isolated from the crude reaction products from a previous study. These compounds, unavailable from commercial sources were synthesised/isolated in quantities large enough to allow thorough structural characterisation and pure enough for subsequent studies to help increase the understanding of the fate, effects and reactions of monoaromatic UCMs within the marine environment. 7-cyclohexyl-1-methyltetralin had a demonstrable narcotic effect causing a reduction in mussel feeding activity. At a nominal aqueous exposure of 100 $\mu g\ L^{-1}$ mussels accumulated up to 145 $\mu g\ g^{-1}$ wet weight of the compound which reduced mussel feeding rate by up to 70 % in the 24 hour exposure period. The result herein was compared to those of Wraige (1997) who used the same assay to assess the toxicity of 6-cyclohexyltetralin and 7-cyclohexyl-1-propyltetralin, to determine the generality of the toxicity of these alkyltetralins, and possibly indicate which subcomponents of the monoaromatic UCM were responsible for the observed reduction in feeding rate.

The WEC_{50} values (Table 8.1) of the model monoaromatic UCM hydrocarbons indicated that increasing substitution on the 'base' cyclohexyltetralin structure increased the WEC_{50} value. The larger WEC_{50} value for the isolated monoaromatic UCM was probably due to its slow accumulation into the mussel tissue suggesting it had not reached steady state within the tissues in the 24 hour exposure period. The TEC_{50} value for 7-cyclohexyl-1-methyltetralin was comparable to 6-cyclohexyltetralin and 7-cyclohexyl-1-propyltetralin studied by Wraige (1997) and other resolved aromatic hydrocarbons studied by Donkin *et al.*, (1989, 1991). At similar accumulated body burdens, *e.g.* 75 $\mu g\ g^{-1}$ wet weight, the response in mussels followed a similar trend for the model monoaromatic UCM hydrocarbons and the isolated monoaromatic UCM, suggesting the joint toxicity of all the components of the monoaromatic UCM. However the larger TEC_{50} value calculated for the isolated monoaromatic UCM possibly indicated that not all of the UCM accumulated was responsible for the toxic effect observed. In theory, mixture toxicity, in terms of body burden is likely to be concentration-additive, thus the effect of the monoaromatic UCM is the effect of the addition of all its constituents. However a cut off in toxicity was noted by Donkin *et al.*, (1989, 1991) whereby compounds were accumulated but no response was observed. Thereby some of the monoaromatic UCM components could have been accumulated without causing a toxic response. This cut-off was associated with limited aqueous solubility, therefore the aqueous solubility of the model UCM hydrocarbons was determined using a generator column in distilled water and seawater at 25 °C and 15 °C (Table 8.1). An increase in salinity and decrease in temperature was found to reduce aqueous solubility. This is considered normal behaviour and was consistent with other reports of such effects on aromatic compounds. The aqueous solubility of the model compounds was lower than the cut-off suggested by Donkin *et al.*, (1989, 1991) of 70 $\mu g\ L^{-1}$, suggesting these compounds should not elicit a toxic response. When aqueous solubility was plotted against TEC_{50} a clear indication of the solubility cut-off was

observed. So although 7-cyclohexyl-1-propyltetralin induced a response in mussel feeding rate not all the accumulated compound was responsible for this effect, resulting in a higher TEC_{50} value, indicating a similar possibility for the monoaromatic UCM. A generated solution of the isolated monoaromatic UCM showed that these compounds were indeed soluble enough to be responsible for the toxic response, but also that not all of the compounds present in the isolated monoaromatic UCM were present in the generated solution. The higher molecular weight compounds were absent from the generated solution suggesting that these could be accumulated by mussels without inducing a toxic response, as they are not soluble enough to reach the site of toxic action. Therefore the use of the model UCM compounds has allowed a greater understanding of the mechanisms behind the toxicological response of the isolated monoaromatic UCM and have shown that these compounds as a group actually fit into the present theories on critical body burden, mixture toxicity and aqueous solubility cut-off. The results herein suggest that consideration should be given to monoaromatic UCM hydrocarbon burdens when toxicological examination of the effects of oil in the environment are made.

In the environment photodegradation is a potentially significant process for aromatic compounds, which can also influence the toxicity of such aromatic compounds. The degradation kinetics of 6-cyclohexyltetralin, 7-cyclohexyl-1-methyltetralin and 7-cyclohexyl-1-propyltetralin were studied using a xenon lamp (Table 8.1). Comparison of the UV/visible spectra of the model monoaromatic UCM hydrocarbons with the isolated monoaromatic UCM suggests that the monoaromatic UCM may behave in a similar manner to the model compounds and be capable of photodegrading in the marine environment.

In conclusion this study has reported on the behaviour of monoaromatic UCM hydrocarbons in the marine environment in terms of toxicity, aqueous solubility and photodegradation by the study of model monoaromatic hydrocarbons and an isolated

monoaromatic UCM. This has provided the first demonstrable evidence for the direct toxicity of isolated monoaromatic UCM hydrocarbons to marine organisms and suggests these compounds should be considered in the long term impacts of oil in the environment, and also warrant further study.

Table 8.1 Summary of experimental data pertaining to model monoaromatic UCM hydrocarbons and an isolated monoaromatic UCM

Compound	Toxicology					Aqueous solubility				Photodegradation		
	WEC_{50} ($\mu g\ L^{-1}$)	WEC_{50} ($\mu mol\ L^{-1}$)	TEC_{50} ($\mu g\ g^{-1}$)	TEC_{50} ($\mu mol\ g^{-1}$)	Log 1/C	25 °C 0 ppt ($\mu g\ L^{-1}$)	25 °C 35 ppt ($\mu g\ L^{-1}$)	15 °C 0 ppt ($\mu g\ L^{-1}$)	15 °C 35 ppt ($\mu g\ L^{-1}$)	Rate constant k_p (h^{-1})	Half life $t_{1/2}$ (h)	Half life (equivalent Florida days)
6-cyclohexyltetralin	24	0.11	41	0.206	0.686	109	75	95	56	-0.068	10.26	13
7-cyclohexyl-1-methyltetralin	42*	0.18*	58+	0.254+	0.595*	45	27	40	21	-0.090	7.70	10
7-cycloheyxl-1-propyltetralin	62*	0.24*	138+	0.539+	0.268*	23	13	17	9	-0.141	4.90	7
Isolated monoaromatic UCM	518	1.87	500	1.167	0.851	560						

+ Wraige (1997) * calculated from Wraige (1997)

8.2. Further Work

The re-examination of mussel tissue at a petroleum impacted site where reduced Scope for Growth was correlated with 2-3 ring aromatic hydrocarbons, as measured by HPLC, demonstrated that the aromatic fraction was dominated by aromatic UCMs. A further correlation between 'total toxic hydrocarbons' and the levels of aromatic UCMs was found at 3 sites where measurements had been taken. In light of the result herein, that an isolated monoaromatic UCM reduced feeding rate when accumulated to levels similar to those found in mussels at impacted sites, a fruitful area of further work would be the analysis of aromatic UCMs, especially the monoaromatic fraction, at further sites where effects have been correlated to the concentration of aromatic hydrocarbons, measured by non-specific methods, to strengthen the evidence presented herein. As the monoaromatic UCM was isolated from Gullfaks crude oil which was similar, if not identical, to the crude spilt by the *Braer* around the Sheltand Islands, U.K. in 1993, it would certainly be prudent to investigate this area in relation to the levels of monoaromatic UCMs accumulated in organisms in conjuction with an assessment of biological status.

It would also be interesting to conduct a Toxicity Identification Evaluation (TIE) approach at such sites. This involves not only the analysis of tissue extracts from polluted sites that exhibit an effect (e.g. SFG) to link with observed effects in the field, but also the fractionation of such extracts, followed by toxicological assessment, to determine the exact cause of the effect noted in the field. While TIE type procedures have been mainly applied to sediments, it would be useful if these procedures could be applied to mussel tissue, and the fractionation include separation of the UCM by methods similar to those used to isolate the monoaromatic fraction, to determine if the monoaromatic UCMs present in mussels elicits an effect.

Wraige (1997) suggested that the gill was the site of toxic action and once compounds had reached steady state in the gill they were bioaccumulated into the tissue without causing any further reduction in mussel feeding rate. If indeed the gill is the site of toxic action, analysis of contaminants should focus on the quantities present in the gill rather than total body burdens. If the mussel feeding rate assay for the isolated monoaromatic UCM were to be repeated, it would be useful to use either larger mussels or a larger population to provide enough tissue to study the accumulation in the gill. It would be interesting to find out whether or not the monoaromatic UCM hydrocarbons achieve saturation in the gill within 24 hours. It was assumed that a similar response would be observed if the mussels bioaccumulated a similar level of the monoaromatic UCM upon exposure to a lower aqueous concentration over a longer time period. Therefore once the time to reach saturation in the gill had been established an exposure for this time period would allow a more meaningful measurement of the TEC_{50} value. It would also be beneficial to use a fractionation step such as normal phase HPLC to remove any biogenic compounds extracted from the mussel tissue that might interfere with the analysis.

A standardised approach to the fractionation and measurement of aliphatic and aromatic UCMs would be extremely valuable as it would allow the comparison of data between different researchers and allow a more meaningful assessment of the possible detrimental effects of the accumulation of such compounds.

Other further work with monoaromatic UCM hydrocarbons should include the assessment of these compounds using different organisms and different biological endpoints to determine the sensitivity of organisms to these compounds, and the extent of their impact within the marine environment. It would also be worthwhile to isolate monoaromatic UCMs from different crude oils, using a similar procedure as that presented in this study, to find out whether the observed effect is universal for oils from different sources.

6-cyclohexyltetralin, 7-cyclohexyl-1-methyltetralin and 7-cyclohexyl-1-propyltetralin were used as 'model' monoaromatic UCM compounds to further understand the toxicity and solubility of the monoaromatic UCM. These compounds were also found to photodegrade under a xenon lamp indicating that the monoaromatic UCM may also photodegrade. Compounds may photodegrade to produce more toxic products, therefore a worthwhile experiment would be to expose a solution of the isolated monoaromatic UCM to a xenon lamp followed by exposure of mussels to the solution, to ascertain whether or not the monoaromatic UCM exhibits photoenhanced toxicity.

The model UCM compounds used herein were considered 'good' models of the monoaromatic UCM based on current available data, and ions present in the mass spectra of these synthetic compounds were found in the mass spectral analysis of the isolated monoaromatic UCM, further lending support to the suggestion of these compounds as surrogates for the monoaromatic UCM. Additional studies of the aromatic UCM composition of a wide range of oils and oil residues would be worthwhile to determine whether such alkylated compounds or their analogues are widespread in oil, for example by preparative GC. This works by diverting the gas flow whilst a peak emerges at the detector allowing an analyte of interest to be trapped and collected, for example to 'prep' out compounds from the unresolved complex mixture. Material collection in this manner can be subsequently analysed using GCMS techniques. If these compounds prove to be important then toxicological assays using other biological endpoints should be conducted.

Abernathy, S., Bobra, A.M., Shui, W.Y., Wells, P.G. and Mackay D. (1986). Acute lethal toxicity testing of hydrocarbons and chlorinated hydrocarbons to two planktonic crustaceans: the key role of organism-water partitioning. *Aquatic Toxicology.* **8**, 163-174.

Aceves, M. and Grimalt, J.O. (1993). Gas chromatographic screening or organic compounds in urban aerosols.II. Changes in hydrocarbon composition during storage. *Journal of Chromatography A.* **655**, 133-140.

Al-Saad, H.T., Shamishoam, S.M. and Abaychi, J.K. (1998). Seasonal distribution of dissolved and particulate hydrocarbons in Shatt Al-Arab Estuary and the north-west Arabian Gulf. *Marine Pollution Bulletin.* **36**, 850-855.

Ali (1994) *The dissolution and photodegradation of Kuwait crude oil in seawater.* PhD Thesis. University of Plymouth, Plymouth, U.K.

Altgelt, K.H. and Boduszynski, M.M. (1994) *Composition and Analysis of Heavy Petroleum Fractions.* Marcel Dekker, New York, U.S.A.

Atlas, R.M. (1995). Petroleum biodegradation and oil spill bioremediation. *Marine Pollution Bulletin.* **31**, 178-182.

Axiak, V. (1991) Sublethal toxicity test: physiological responses. In, *Ecotoxicology and the Marine Environment.* (Eds, P.D. Abel and Axiak, V.), pp 132-144. Ellis Horwood.

Axiak, V. and George, J.J. (1987). Effects of exposure to petroleum hydrocarbons on the gill functions and ciliary activities of a marine bivalve. *Marine Biology.* **94**, 241-249.

Barber, M.C., Suarez, L.A. and Lassiter, R.R. (1988) Modelling bioconcentration of nonpolar organic pollutants by fish. *Environmental Toxicology and Chemistry.* **7**, 545-558.

Barron, M.G., Poorabsky, T. and Ogle, S. (1999). Are aromatic hydrocarbons the primary determinant of petroleum toxicity to aquatic organisms? *Aquatic Toxicology.* **46**, 253-268.

Barron, M.G. and Ka'Aihue, L. (2001). Potential for Photoenhanced Toxicity of Spilled Oil in Prince William Sound and Gulf of Alaska Waters. *Marine Pollution Bulletin.* **43**, 86-92.

Baumard, P., Budzinski, H., Garrigues, P., Dizor, H. and Hansen, P.D. (1999). Polycyclic aromatic hydrocarbons in recent sediments and mussels (*Mytilus edulis*) from the Western Baltic Sea: occurrence, bioavailability and seasonal variations. *Marine Environmental Research.* **47**, 17-47.

Bayne, B.L., Widdows, J., Moore, M.N., Salkeld, P., Worral, C.M. and Donkin, P. (1982). Some ecological consequences of the physiological and biochemical effects of petroleum compounds on marine molluscs. *Phil. Trans. R. Soc. Lond. B.* **297**, 219-239.

Bayona, J.M., Fernandez, P., Porte, C., Tolosa, I., Valls, M., Albaiges, J. (1991). Partitioning of urban wastewater organic microcontaminants among coastal compartments. *Chemosphere.* **23**, 313-326.

Belt, S., Rowland, S. and Scaiano, J.C. (1998). Rate constants for the abstraction of hydrogen from alkyl aromatics by alkoxyl radicals and excited state ketones: relevance to the

photodegradation of the water soluble fraction of crude oil. *Marine Chemistry.* **61**, 157-162.

Betton, C. (1994). Oils and Hydrocarbons. In, *Handbook of Ecotoxicology Volume 2.* (Ed., P. Calow), pp. 244-263. Blackwell Scientific Publications, Oxford.

Billington, J.W., Huang, G.L., Szeto, F., Shiu, W.Y. and Mackay, D. (1988). Preparation of aqueous solutions of sparingly soluble organic substances. I. Single component systems. *Environmental Toxicology and Chemistry.* **7**, 117-124

Bobra, A.M., Shui, W.Y., and Mackay, D. (1983). Acute toxicity of fresh and weathered crude oils to *Daphnia magna. Chemosphere.* **12**, 1137-1149.

Boehm, P.D., (1980). Evidence for the decoupling of dissolved, particulate and surface microlayer hydrocarbons in northwestern Atlantic continental shelf waters. *Marine Chemistry.* **9**, 255-281.

Boehm, P.D. and Fiest, D. (1982). Subsurface distributions of petroleum from an offshore well blowout. The Ixtoc blow out, Bay of Campeche. *Environmental Science and Technology.* **16**, 67-74.

Bomboi, M.T., and Hernandez, A. (1991). Hydrocarbons in urban runoff: their contribution to the wastewaters. *Water Research.* **25**, 557-565.

Bouloubassi, I., Fillaux, J. and Saliot, A. (2001) Hydrocarbons in surface sediments from the Changjiang (Yangtze River) Estuary, East China Sea. *Marine Pollution Bulletin.* **42**, 1335-1346.

Boylan, D.B., and Tripp, B.W. (1971). Determination of hydrocarbons in seawater extracts of crude oils and crude oil fractions. *Nature.* **230**, 44-47.

Breitmaier, E. (1993) *Structure Elucidation by NMR in Organic Chemistry.* John Wiley and Sons Ltd, Chichester, U.K.

Brooke, D.N., Dobbs, A.J. and Williams, N. (1986). Octanol-water partition coefficients (P); measurement, estimation and interpretation, particularly for chemicals with $P>10^{5}$. *Ecotoxicology and Environmental Safety.* **11**, 251-260.

Burgess, R.M., and McKinny, R.A. (1999). Importance of interstitial, overlying water and whole sediment exposures to bioaccumulation by marine bivalves. *Environmental Pollution.* **104**, 373-382.

Burhenne, J., Ludwig, M. and Spiteller, M. (1999). Polar photodegradation products of quinolones determined by HPLC/MS/MS. *Chemosphere.* **38**, 1279-1286.

Burns, K.A. and Smith, J.L. (1981). Biological monitoring of ambient water quality: the case for using bivalves as sentinel organisms for monitoring petroleum pollution in coastal waters. *Estuarine, Coastal and Shelf Science.* **13**, 433-443.

Burns, K.A., (1993). Evidence for the importance of including hydrocarbon oxidation products in environmental assessment studies. *Marine Pollution Bulletin.* **26**, 77-85.

Burris, D.R., and MacIntyre, W.G. (1985). Water solubility behaviour of binary hydrocarbon mixtures. *Environmental Toxicology and Chemistry.* **4**, 371-377.

Call, D.J., Brooke, M.L., Knuth, M.L., Poirer, S.H. and Hoglund, M.D. (1985) Fish subchronic toxicity prediction model for industrial organic chemicals that produce narcosis. *Environmental Toxicology and Chemistry.* **4**, 335-341

Capuzzo, J.M. (1985). Biological effects of petroleum hydrocarbons on marine organisms: integration of experimental results and predictions of impacts. *Marine Environmental Research.* **17**, 272-276.

Chessels, M., Hawker, D.W., and Connel, D.W. (1991). Critical evaluation of the measurement of the 1-octanol/water partition coefficient of hydrophobic compounds. *Chemosphere.* **22**, 1175-1190.

Clark, R.B. (1992) Oil Pollution. In, *Marine Pollution*, pp 28-49. Clarendon Press, Oxford.

Cleveland, L., Little, E.E., Calfee, R.D. and Barron, M.G. (2000). Photoenhanced toxicity of weathered oil to Mysidopsis bahia. *Aquatic Toxicology.* **49**, 63-76.

Connell D.W. and Miller, G.J. (1981) Petroleum hydrocarbons in aquatic ecosystems – behaviour and effects of sublethal concentrations-Part 1. *CRC Critical Reviews in Environmental Control.* **11**, 37-104.

Connell, D.W., Wu, R.S.S., Richardson, B.J., Leung, K., Lam, P.S.K. and Connell, P.A. (1998). Fate and risk evaluation of persistent organic contaminants and related compounds in Victoria Harbour, Hong Kong. *Chemosphere.* **36**, 2019-2030.

Cronin, M.T.D. and Dearden, J.C. (1995). Review: QSAR in toxicology 1.Prediction of aquatic toxicity. *Quant. Struct.-Act. Relat.* **14**, 1-7.

Davenport, R., Johnson, L.R., Schaeffer, D.J. and Balbach, H. (1994). Light enhanced toxicity of TNT and related compounds to Daphnia magna and Lytechinus variagalus embryos. *Ecotoxicology and Environmental Safety*. **27**, 14-22.

De Bertrand, N., and Barcelo, D. (1991). Photodegradation of the carbamate pesticides aldicarb, carbaryl and carbofuran in water. *Analytica Chimica Acta*. **254**, 235-244.

De Maagd, P.G., Ten Hulscher, D.T.E.M., Van den Heuvel, H., Opperhuizen, A. and Sijm, D.T.H.M. (1999). Physicochemical properties of polycyclic aromatic hydrocarbons: aqueous solubilities, n-octanol/water partition coefficients, and Henry's law constant. *Environmental Toxicology and Chemistry*. **17**, 251-257.

Dearden, J.C. (1985). Partitioning and lipophilicity in quantitative structure-activity relationships. *Environmental Health Perspectives*. **61**, 203-228.

Deneer, J.W., Sinnige, T.L., Seinen, W., and Hermens J.L.M. (1988). The joint acute toxicity to *Daphnia magna* of industrial organic chemicals at low concentrations. *Aquatic Toxicology*. **12**, 33-38.

Dickhut, R.M., Adren, A.W., and Armstrong, D.E. (1986). Aqueous solubilities of six polychlorinated biphenyl congeners at four temperatures. *Environmental Science and Technology*. **20**, 807-810.

Donkin, P., and Widdows, J. (1986). Ecotoxicology-effects of chemicals on the aquatic environment. *Chemistry and Industry*. **21**, 732-736.

Donkin, P., Widdows, J., Evans, S.V., Worral, C.M. and Carr, M. (1989). Quantitative structure-activity relationships for the effect of hydrophobic organic chemicals on rate of feeding by mussels (*Mytilus edulis*). *Aquatic Toxicology*. **14**, 277-294.

Donkin, P. and Widdow, J. (1990). Quantitative structure activity relationships in aquatic invertebrate toxicology *Reviews in Aquatic Sciences.* **2**, 375-398.

Donkin, P., Widdows, J., Evans, S.V., and Brinsley, M.D. (1991). QSARs for the sublethal responses of marine mussels (*Mytilus edulis*). *The Science of the Total Environment.* **109/110**, 461-476.

Donkin, P. (1994) *Quantitative structure activity relationships*. In, Handbook of Environmental Toxicology II. (Ed., P. Calow), pp 321-347. Blackwell Scientific, Oxford.

Doubal, A. and Al-Shiwafi, N.A. (1998). Dissolved/dispersed hydrocarbons in the Arabian region. *Marine Pollution Bulletin.* **36**, 844-850.

Dunnivant, F.M. and Elzerman, A.W. (1988). Aqueous solubility and Henry's law constant data for PCB congeners for evaluation of QSPRs. *Chemosphere.* **17**, 525-541.

Dutta, T.K. and Harayama, S. (2000) Fate of crude oil by combination of photooxidation and biodegradation. *Environmental Science and Technology.* **34**, 1500-1505.

Eganhouse, R.P., and Calder J.A. (1976). The solubility of medium molecular weight aromatic hydrocarbons and the effects of hydrocarbons, co-solutes and salinity. *Geochimica et Cosmochimica Acta.* **40**, 555-561.

EGOSS (1994) *The Environmental Impact of the Wreck of the Braer*. Scottish Office: Edinburgh, 1994.

Ehrhardt, M., and Petrick, G. (1985). The sensitized photo-oxidation of n-pentadecane as a model for abiotic decomposition of aliphatic hydrocarbons in seawater. *Marine Chemistry.* **16**, 227-238.

Ehrhardt, M., Burns, K.A. and Bicego, M. (1992). Sunlight-induced compositional alterations in the seawater-soluble fraction of crude oil. *Marine Chemistry.* **37**, 53-64.

Engelhardt, F.R., Gilfillan, E.S., Boehm, P.D. and Mageau, C. (1985). Metabolic effects and hydrocarbons fate in Arctic bivalves exposed to dispersed petroleum. *Marine Environmental Research.* **17**, 245-249.

Farrington, J.W. (1991). Biogeochemical processes governing exposure and uptake of organic pollutant compounds in aquatic organisms. *Environmental Health Perspectives.* **90**, 75-84

Ferguson, J. (1939). The use of chemical potentials as indices of toxicity. *Proceedings of the Royal Society of London B.* **127**, 387-404.

Fowler, S.W., Readman, J.W., Oregiono, B., Villeneuve, J.P. and McKay, K. (1993). Petroleum hydrocarbons and trace metals in nearshore gulf sediments and biota before and after the 1991 war: an assessment of temporal and spatial trends. *Marine Pollution Bulletin.* **27**, 171-182.

Franke, C., Studinger, G., Berger, G., Bohling, S. and Bruckmann, U. (1994). The assessment of bioaccumulation. *Chemosphere.* **29**, 1501-1514.

Friesen, K.J., Vilk, J. and Muir, D.C.G. (1990). Aqueous solubilities of selected 2,3,7,8-substitued polychlorinated dibenzofurans (PCDFs). *Chemosphere.* **20**, 27-32.

Friesen, K.J., Foga, M.M. and Loewen, M.D. (1996). Aquatic photodegradation of polychlorinated dibenzofurans: rates and photoproduct analysis. *Environmental Science and Technology.* **30**, 2054-2510.

Fukada, K., Inagaki, Y., Maruyama, T., Kojima, H.I. and Yoshida, T. (1988). On the photolysis of alkylated naphthalenes in aquatic systems. *Chemosphere.* **17**, 651-659.

Garrett, R.M., Pickering, I.J., Haith, C.E. and Prince, R.C. (1998). Photooxidation of crude oils. *Environmental Science and Technology.* **32**, 3719-3723.

Gearing, P.J. and Gearing, J.N. (1982). Behaviour of No.2 fuel oil in the water column of controlled ecosystems. *Marine Environmental Research.* **6**, 115-132.

GESAMP (IMO/FAO/UNESCO/WMO/WHO/IAEA/UN/UNEP joint Group of Experts on the Scientific Aspects of Marine Pollution) (1993) *Impact of oil and related chemicals and wastes on the marine environment.* Reports and Studies (50).

Ghosh, U., Weber, A.S., Jensen, J.N. and Smith, J.R. (1998). Dissolved PCB congener distribution in generator column solutions. *Water Research.* **32**, 1373-1382.

Gilfillan, E.S., Mayo, D.W., Page, D.S., Donovan, D. and Hanson, S. (1977). Effects of varying concentrations of petroleum hydrocarbons in sediments on carbon flux in *Mya arenaria*. In, *Physiological Responses of Marine Biota to Pollutants* (Eds., F.J. Vernberg and *et al.*). pp. 299-314. Academic Press.

Glegg, G.A. and Rowland, S.J., (1996). The Braer Oil Spill - hydrocarbon concentrations in intertidal organisms. *Marine Pollution Bulletin.* **32**, 486-492

Gold-Bouchot, G., Sima-Alverez, R., Zapata-Perez, O. and Guemez-Ricalde, J., (1995). Histopathological effects of petroleum hydrocarbons and heavy metals on the American oyster (*Crassostrea virginica*) from Tabasco, Mexico. *Marine Pollution Bulletin.* **31**, 439-445.

Goldberg, E.D., Bowne, V.T., Farrington, J.W., Harvery, G.R., Marint, J.H., Parker, P.L., Riseborough, R.W., Robertson, W., Scheider, E. and Gamble, E. The Mussel Watch. *Environmental Conservation.* **5**:101-125.

Gombar, V.K. and Enslein, K. (1996). Assessment of n-octanol/water partition coefficient: when is the assessment reliable? *Journal of Chemical Information and Computer Sciences.* **36**, 1127-1134.

Gough, M.A. (1989). *Characterisation of Unresolved Mixtures of Hydrocarbons.* PhD Thesis, Polytechnic South West, Plymouth, U.K.

Gray, J.S. (1992). Biological and ecological effects of marine pollutants and their detection. *Marine Pollution Bulletin.* **25**, 48-50.

Hansch, C., Kim, D., Leo, A.J., Novellino, E., Silipo, C. and Vittoria, A. (1989). Toward a quantitative comparative toxicology of organic compounds. *Critical Reviews in Toxicology.* **19**, 185-226.

Hayton, W.L. and Barron, M.G. (1990) Rate-limiting barriers to xenobiotic uptake by the gill. *Environmental Toxicology and Chemistry.* **9**, 151-157

Heintz, R.A., Short, J.W. and Rice, S.B. (1999). Sensitivity of fish embryos to weathered crude oil: Part II. Increased mortality of pink salmon (*Oncorhynchus gorbuscha*)

embryos incubating downstream from weathered Exxon Valdez crude oil. *Environmental Toxicology and Chemistry.* **18**, 494-503.

Hermens, J.L.M., Canton, H., Janssen, P. and Dejong, R. (1984). Quantitative structure-activity relationships and toxicity studies of mixtures of chemicals with anaesthetic potency: acute lethal and sublethal toxicity to *Daphnia magna*. *Aquatic Toxicology.* **5**, 143-154.

Hermens, J.L.M., Koneman, H., Leeuwangh, P. and Musch, A. (1985). Quantitative structure-activity relationships in aquatic toxicity studies of chemicals and complex mixtures of chemicals. *Environmental Toxicology and Chemistry.* **4**, 279

Horstad, I., Larter, S.R., Dypvik, H., Aagard, P., Bjornvik, A.M., Johamsen, P.E. and Eriksen, S. (1990) Degradation and maturity controls on oil field petroleum column heterogeneity in the Gullfaks field, Norwegian North Sea. *Organic Geochemistry.* **16**, 497-510.

Huang-Minlon (1946) A simple modification of the Wolff-Kischner reduction. *Journal of the American Chemical Society.* **68**, 2487-2488.

Jaquot, F., Guiliano, M., Doumenq, P., Munoz, D. and Mille, G. (1996) In vitro photooxidation of crude maltenic fractions: evolution of fossil biomarkers and polycyclic aromatic hydrocarbons. *Chemosphere.* **33**, 671-681.

Jones, D.M., Douglas, A.G., Parkes, R.J., Taylor, J., Giger, W. and Schaffner, C. (1983). The recognition of biodegraded petroleum derived aromatic hydrocarbons in recent sediments. *Marine Pollution Bulletin.* **14**, 103-108.

Killops, S.D. and Readman J.W. (1985). HPLC fractionation and GC-MS determination of aromatic hydrocarbons from oils and sediments. *Organic Geochemistry.* **8**, 247-257.

Killops, S.D. (1986). Identification of petrogenic contamination in recent sediments by HPLC monitoring of aromatic components: a preliminary investigation. *Chemosphere.* **15**, 229-241.

Killops, S.D. and Al-Juboori, M.A.H. (1990). Characterisation of the unresolved complex mixture (UCM) in the gas chromatograms of biodegraded petroleum. *Organic Geochemistry.* **15**, 147-160.

Kim, M. and O'Keefe, P.W. (2000). Photodegradation of polychlorinated dibenzo-p-dioxins and dibenzofurans in aqueous solutions and in organic solvents. *Chemosphere.* **41**, 793-800.

Kirk, J.T.O. (1986) Incident solar radiation. In, *Light and Photosynthesis in Aquatic Ecosystems.* Cambridge University Press UK, pp 24-68.

Könnemann, H. (1981). Quantitative structure activity relationships in fish toxicity studies. *Toxicology.* **19**, 209-221.

Krank, M.H.S., Wijnands, P., Govers, H.A.J., Admiral, W. and de Voogt, P. (1997) Structural based differences in ecotoxicity of benzoquinoline isomers to the zebra mussel (*Dreissena polymorpha). Environmental Toxicology and Chemistry.* **16**, 2158-2163

Larson, R.A., Hunt, L.L. and Blankenship, D.W. (1977). Formation of toxic products from a #2 fuel oil by photooxidation. *Environmental Science and Technology.* **11**, 492-496.

Larson, R.A., Bott, T.L., Hunt, L.L. and Rogenmuser, K. (1979). Photooxidation products of a fuel oil and their antimicrobial activity. *Environmental Science and Technology.* **13**, 965-969.

LeDreau, Y., Jaquot, F., Doumenq, P. and Guilieno, M. (1997). Hydrocarbons balance of a site which had been highly and chronically contaminated by petroleum wastes of refinery (from 1956-1992). *Marine Pollution Bulletin.* **34**, 456-468.

Leinonen, P.J. and Mackay, D. (1973) The multicomponent solubility of hydrocarbons in water. *The Canadian Journal of Chemical Engineering.* **51**, 230-233

Lu, P.Y., Metcalf, R.L. and Carlson, E.M. (1978) Environmental fate of five radiolabelled coal conversion by-products evaluated in a laboratory model ecosystem. *Environmental Health Perspectives.* **24,** 201-208.

Macias-Zamora, J.V. (1996). Distribution of hydrocarbons in recent marine sediments off the coast of Baja California. *Environmental Pollution.* **92**, 45-53.

Mackay, D. and Shui, W.Y. (1975). The determination of the solubility of hydrocarbons in aqueous sodium chloride solutions. *The Canadian Journal of Chemical Engineering.* **53**, 239-243.

Mackenzie, A.S., Lamb, N.A. and Maxwell, J.R. (1982). Steroid hydrocarbons and the thermal history of sediments. *Nature.* **295**, 223-226.

March, J. (1985) *Advanced Organic Chemistry*, p 486 and 1096. 3rd Edition. John Wiley and Sons, U.S.A.

Martin, M., Ichikawa, G., Goetzl, J., De Los Reyes, M. and Stephenson, M. (1984). Relationships between physiological stress adn trace toxic substances in the bay mussel, *Mytilus edulis*, from San Francisco Bay, California. *Marine Environmental Research.* **11**, 91-110.

Mason, R.P. (1988). Hydrocarbons in mussels around the Cape peninsula, South Africa. *South African Journal of Marine Science.* **7**, 139-151.

May, W.E. and Wasik, S.P. (1978). Determination of the solubility behaviour of some polycyclic aromatic hydrocarbons in water. *Analytical Chemistry.* **50**, 997-1000.

May, W.E., Wasik, S.P. and Freeman, D.H. (1978). Determination of the aqueous solubility of polynuclear aromatic hydrocarbons by coupled column chromatographic technique. *Analytical Chemistry.* **50**, 175-179.

McAuliffe, C. (1966) Solubility in water of paraffin, cycloparaffin, olefin, acetylene, cycloolefin and aromatic hydrocarbons. *Journal of Physical Chemistry.* **70**, 1267-1275.

McCarty, L.S. (1986) The relationship between aquatic toxicity QSARs and bioconcentration for some organic chemicals. *Environmental Toxicology and Chemistry.* **5**, 1071-1080.

McCarty, L.S. (1987) Relationship between toxicity and bioconcentration for some organic chemicals. I. Examination of the relationship. In, *QSAR in Environmental Toxicology – II*, (Ed., K.L. Kaiser), pp 207-219. D Reidel Publishing, Dordrecht, The Netherlands.

Mekenyan, O.G., Ankley, G.T., Vieth, G.D. and Call, D.J. (1994). QSARs for the photoinduced toxicity I. Acute lethality of polycyclic aromatic hydrocarbons to *Daphnia magna. Chemosphere.* **28**, 567-585.

Meylan, W.A., Howard, P.H., Boethling, R.S., Aronson, D., Printup, H. and Gouchie, S. (1999). Improved method for estimating bioconcentration/bioaccumulation factor from octanol/water partition coefficient. *Environmental Toxicology and Chemistry.* **18**, 664-672.

Mill, T., Mabey, W.A., Lan, B.Y. and Baraze, A. (1981). Photolysis of polycyclic aromatic hydrocarbons in water. *Chemosphere.* **10**, 1281-1290.

Mill, T. (1999). Predicting photoreaction rates in surface water. *Chemosphere.* **38**, 1379-1390.

Miller, G.C. and Zepp, R.G. (1983). Extrapolating Photolysis rates from the laboratory to the environment. *Residue Reviews.* **85**: 89-110.

Moore, S.F., and Dwyer, R.L. (1974). Effects of oil on marine organisms: a critical assessment of published data. *Water Research.* **8**, 819-827.

Mortimer, M. and Connell, D.W. (1995) Effect of exposure to chlorobenzenes on growth rates of the crab *Portunas pelagicus*. *Environmental Science and Technology.* **29**, 1881-1886.

Murray, A.P., Gibbs, C.F. and Kavanagh, P.E. (1983). Estimation of total aromatic hydrocarbons in environmental samples by high pressure liquid chromatography. *International Journal of Environmental Analytical Chemistry.* **16**, 167-195.

NAS (1980) *The International Mussel Watch.* National Academy of Sciences. Washington, D.C.

Neff, J.M., Cox, B.A., Dixet, D. and Anderson, J.W. (1976) Accumulation and release of petroleum-derived aromatic hydrocarbons by four species of marine animals. *Marine Biology*. **38**, 279-289.

Neff, J.M., Ostazeski, S., Gardiner, W. and Stejskal, I. (2000) Effects of weathering on the toxicity of three marine offshore Australian crude oils and a diesel fuel to marine animals. *Environmental Toxicology and Chemistry* .**19**, 1809-1821.

Nirmalakhandan, N. and Speece, R.E. (1988) Structure-activity relationships. *Environmental Science and Technology*. **22**, 606-615.

Nirmalalhandan, N. and Speece, R.E. (1989). Prediction of aqueous solubility of organic chemicals based on molecular structure 2. Application to PNAs, PCBs, PCDDs, etc. *Environmental Science and Technology*. **23**, 708-713.

Oudot, J., Fusey, P., Van Praet, M., Feral, J.P. and Gaill, F. (1981). Hydrocarbons weathering in seashore invertebrates and sediments over a two year period following the Amoco Cadiz oil spill: influence of microbial metabolism. *Environmental Pollution (Series A)*. **26**, 93-110.

Paparo, A. (1972). Innervation of the lateral cilia in the mussel *Mytilus edulis*. *Biological Bulletin*. **143**, 592-604.

Parker, S. and Leahey, J.P. (1988) Development of a method to investigate the photodegradation of pesticides. *Brighton Crop Protection Conference-Pests and Diseases-1988*, pp 663-668.

Pawlisz, A.V. and Peters, R.H. (1993). A test of the equipotency of internal burdens of nine narcotic chemicals using *Daphnia magna*. *Environmental Science and Technology.* **27**, 2801-2806.

Payne, J.F., Fancey, L.L., Hellou, J., King, M.J. and Fletcher, G.L. (1995). Aliphatic hydrocarbons in sediments; a chronic toxicity study with winter flounder (*Pleuronectes americanus*) exposed to oil well drill cuttings. *Canadian Journal of Fisheries and Aquatic Sciences.* **52**, 2724-2735.

Pelletier, M.C., Burgess, R.M., Ho, K.T., Kuhn, A., McKinney, R.A. and Ryba, S.A. (1997) Phototoxicity of individual polycyclic aromatic hydrocarbons and petroleum to marine invertebrate larvae and juveniles. *Environmental Toxicology and Chemistry.* **16**, 2190-2199

Peters, K.E. and Moldowan, J.M. (1993) *The Biomarker Guide. Interpreting Molecular Fossils in Petroleum and Ancient Sediments.* Prentice Hall Inc. New Jersey, U.S.A.

Peterson, D.R. (1994). Calculating the toxicity of hydrocarbon mixtures. *Chemosphere.* **29**, 2493-2506.

Ranby, B. and Rabek, J.F. (1975) *Photodegradation, Photo-oxidation and Photostabilisation of Polymers*. John Wiley and Sons, London.

Readman, J.W., Preston, M.R. and Mantoura, F.C. (1986). An integrated technique to quantify sewage, oil and PAH pollution in estuarine and coastal environments. *Marine Pollution Bulletin.* **17**, 298-308.

Readman, J.W., Bartocci, J., Tolosa, I., Fowler, S.W., Oregioni, B. and Abdulraheem, M.Y. (1996). Recovery of the coastal marine environment in the Gulf following the 1991 war-related oil spills. *Marine Pollution Bulletin.* **32**, 493-498.

Revill, A.T. (1992) *Characterisation of Unresolved Complex Mixtures of Hydrocarbons by Degradative Methods.* PhD Thesis. University of Plymouth, Plymouth, U.K.

Revill, A.T., Carr, M. and Rowland, S.J. (1992). Use of oxidative degradation followed by capillary gas chromatography-mass spectrometry and multidimensional scaling analysis to fingerprint unresolved complex mixtures of hydrocarbons. *Journal of Chromatography A.* **589**, 281-286.

Rhead, M.M., Eglinton, G., Draffan, G.H. and England, P.J. (1971) Conversion of oleic acid to saturated fatty acids in Severn estuary sediment. *Nature.* **232**, 327-330.

Risebrough, R.W., DeLappe, B.W., Walker, W., Simoneit, B.R.T., Grimalt, J., Albaiges, J., Reguero, J.A.G., Noll, A.B.T. and Fernandez, M.M. (1983). Application of the mussel watch concept in studies of the distribution of hydrocarbons in the coastal zone of the Ebro Delta. *Marine Pollution Bulletin.* **14**, 181-187.

Rontani, J.F. (1991). Production of acyclic isoprenoid compounds during the photodegradation of chlorophyll-a in seawater. *J. Photochem. Photobiol. A. Chem.* **59**, 369-377.

Roof, A.A.M. (1982). Aquatic photochemistry. In, *The Handbook of Environmental Chemistry, Reactions and Processes Volume 2 Part B.* (Ed., O. Hutzinger). pp. 43-72. Berlin: Springer-Verlag.

Rossi, S.S. and Thomas, W.H. (1981). Solubility behaviour of three aromatic hydrocarbons in distilled water and natural seawater. *Environmental Science and Technology.* **15**, 715-716.

Schwarz, F. (1977) Determination of temperature dependence of solubilities of polycyclic aromatic hydrocarbons in aqueous solution by a fluorescence method. *Journal of Chemical Engineering Data* **22,** 273

Seeman, P. (1972). The membrane actions of anaesthetics and tranquillisers. *Pharmacological Reviews.* **24**, 583-633.

Shaw, D.G. and Reidy, S.K. (1979). Chemical and size fractionation of aqueous petroleum dispersions. *Environmental Science and Technology.* **13**, 1259-1263.

Sherblom, P.M., Gschwend, P.M. and Eganhouse, R.P. (1992) Aqueous solubilities, vapour pressures, and 1-octanol-water partition coefficients for C_9-C_{14} linear alkylbenzenes. *Journal of Chemical Engineering Data.* **37**, 394-399

Shui, W.Y., Doucette, W., Gobas, F.A.P.C., Andres, A. and Mackay, D. (1988). Physical chemical properties of chlorinated dibenzo-p-dioxins. *Environmental Science and Technology.* **22**, 651-658.

Shui, W.Y., Bobra, M., Bobra, A.M., Maijanen, L.S. and Mackay, D. (1990). The water solubility of crude oils and petroleum products. *Oil and Chemical Pollution* 7, 57-84.

Smaal, A.C. and Widdows, J. (1994) The scope for growth of bivalves as an integrated response parameter in biological monitoring. In, *Biomonitoring of coastal waters* (Ed., K.J.M. Kramer), pp 247-268. CRC Press.

Smith, R.M. and Busch, K.L. (1999) *Understanding Mass Spectra – A Basic Approach.* John Wiley and Sons Inc, Chichester, U.K.

Soler, M., Grimalt, J.O. and Albaiges, J., (1989). Vertical distribution of aliphatic and aromatic hydrocarbons in mussels from the Amposta offshore oil production platform (Western Mediterranean). *Chemosphere.* **18**, 1809-1819.

Soto, C., Hellebust, J.A., Hutchinson, T.C. and Sawa, T. (1974) Effect of naphthalene and aqueous crude oil extracts on the green flagellate *Chlamydomonas angulosa. Canadian Journal of Botany.* **53**, 109-117.

Stuart, B. (1996) *Modern Infrared Spectroscopy.* John Wiley and Sons, Chichester, U.K.

Sturt, H. (2000). *Molecular Characterisation of Fluidised Catalytic Cracker Feedstocks using Ruthenium Tetroxide Oxidation: a Study of Model Hydrocarbons.* PhD Thesis, University of Plymouth, Plymouth, U.K.

Sutton, C. and Calder, J.A. (1975) Solubility of alkylbenzenes in distilled water and seawater at 25 °C. *Journal of Chemical Engineering Data.* **20**, 320-322.

Sydnes, L.K., Hemmingsen, T.H., Skare, S., Hansen, S.H., Flka-Peterson, I., Lonning, S. and Ostgaard, K. (1985) Seasonal variations in weathering and toxicity of crude oil on seawater under Arctic conditions. *Environmental Science and Technology.* **19**, 076-1081.

Thomas, K.V. (1995). *Characterisation and Environmental Effects of Unresolved Complex Mixtures of Hydrocarbons.* PhD Thesis. University of Plymouth, Plymouth. U.K.

Thomas, K.V., Donkin, P., and Rowland, S.J. (1995). Toxicity enhancement of an aliphatic petrogenic unresolved complex mixture (UCM) by chemical oxidation. *Water Research.* **29**, 379-382.

Thompson, S., and Eglinton, G. (1978). Composition and sources of pollutant hydrocarbons in the Severn Estuary. *Marine Pollution Bulletin.* **9**, 133-136.

Vadas, G.G., MacIntyre, W.G. and Burris, D.R. (1991) Aqueous solubility of liquid hydrocarbon mixtures containing dissolved solid components. *Environmental Toxicology and Chemistry.* **10**, 633-639.

Van Den Heuvel, M.R., McCarty, L.S., Lanno, R.P., Hickie, B.E. and Dixon, D.G. (1991). Effect of total body lipid on the toxicity and toxicokinetics of pentachlorophenol in rainbow trout (*Oncorhynchus mykiss*). *Aquatic Toxicology.* **20**, 235-251.

Van Haelst, A.G., Heesen, P.F., van der Wielen, F.W.M. and Govers, H.A.J. (1994). Determination of n-octanol/water partition coefficients of tetrachlorobenzyltoluenes individually and in a mixture by the slow stirring method. *Chemosphere.* **29**, 1651-1660.

van Wezel, A.P., de Vries, D.A.M., Kostense, S., Sijm, D.T.H.M. and Opperhuizen, A. (1995). Intraspecies variation in lethal body burdens of narcotic compounds. *Aquatic Toxicology.* **33**, 325-342.

Veith, G.D., Call, D.J. and Brooke, L.T. (1983). Structure-toxicity relationships for the fathead minnow, Pimephales promelas: narcotic industrial chemicals. *Canadian Journal of Fisheries and Aquatic Sciences.* **40**, 743-748.

Venkatesan, M.I. and Kaplan, I.R. (1982). Distribution and transport of hydrocarbons in surface sediments of the Alaskan outer continental shelf. *Geochimica et Cosmochimica Acta.* **46**, 2135-2149.

Verhaar, H.J.M., Van Leeuwen, C.J. and Hermens, J.L.M. (1992). Classifying environmental pollutants.1.Structure-activity relationships for prediction of aquatic toxicity. *Chemosphere.* **25**, 471-491.

Verscheuren, K. (1983) *Handbook of Environmental Data on Organic Chemicals,* 10. 2nd Edition. Van Nostrand Reinhold. New York.

Vogel, A.I. (1989) *Vogels Textbook of Practical Organic Chemistry,* 5th Edition. Eds. Furniss B.S., Hannaford A.J., Smith P.W.G. and Tatchell A.R. Longman Scientific and Technical.

Volkman, J.K., Holdsworth, D.G., Neill, G.P. and Bavor, H.J. (1992). Identification of natural, anthropogenic and petroleum hydrocarbons in aquatic sediments. *The Science of the Total Environment.* **112**, 203-219.

Wade, T.L., Sericano, J.L., Gardinali, P.R., Wolff, G. and Chambers, L. (1998). NOAAs 'mussel watch' project: Current use organic compounds in bivalves. *Marine Pollution Bulletin.* **37**, 20-26.

Walters, R.W. and Luthy, R.G. (1984) Equilibrium adsorption of polycyclic aromatic hydrocarbons from water onto activated carbon. *Environmental Science and Technology.* **18,** 395-403.

Wang, Z. and Fingas, M. (1997). Developments in the analysis of petroleum hydrocarbons in oils, petroleum products and oil-spill related environmental samples by gas chromatography. *Journal of Chromatography A.* **774**, 51-78

Warton, B. (1999) *Studies of the saturate and aromatic hydrocarbon unresolved complex mixtures in petroleum.* PhD Thesis, Curtin University of Technology, Perth, Australia.

Warton, B., Alexander, R. and Kagi, R.I. (1999) Characterisation of the ruthenium tetroxide oxidation products from the aromatic unresolved complex mixture of a biodegraded crude oil. *Organic Geochemistry.* **30**, 1255-1272.

Wasik, S.P., Miller, M.M., Tewari, Y.B., May, W.E., Sonnefeld, W.J., Devooe, H. and Zoller, W.H. (1983). Determination of the vapour pressure, aqueous solubility and octanol/water partition coefficient of hydrophobic substances by coupled generator column/liquid chromatography. *Residue Reviews.* **85**, 29-42.

Whitehouse, B.G. (1984). The effects of temperature and salinity on the aqueous solubility of PAHs. *Marine Chemistry.* **14**, 319-332.

Widdows, J., Bayne, B.L., Donkin, P., Livingstone, D.R., Lowe, D.M., Moore, M.N. Evans, S.V. and Moore, S.F., (1982). Responses to *Mytilus edulis* on exposure to the water accommodated fraction of North Sea oil. *Marine Biology.* **67**, 15-31.

Widdows, J. (1985). Physiological responses to pollution. *Marine Pollution Bulletin* **16**, 129-134.

Widdows, J., Donkin, P. and Evans, S.V., (1985). Recovery of *Mytilus edulis L.* from chronic oil exposure. *Marine Environmental Research.* **17**, 250-253.

Widdows, J., Donkin, P., Salkeld, P.N. and Evans S.V. (1987). Measurement of scope for growth and tissue hydrocarbon concentrations of mussels (*Mytilus edulis)* at sites in the vicinity of the Sullom Voe terminal - a case study. In, *Fate and effects of oil in marine ecosystems* (Eds., J. Kuiper and W.J. van der Brink). pp. 269-275. Dordrecht: Martinus Nijhoff Publishers.

Widdows, J., and Donkin, P. (1989). The application of combined tissue residue chemistry and physiological measurements of mussels (*Mytilus edulis*) for the assessment of environmental pollution. *Hydrobiologia.* **188-189**, 455-461.

Widdows, J., Burns, K.A., Menon, N.R., Page, D.S. and Soria, S. (1990). Measurement of physiological energetics (scope for growth) and chemical contaminants in mussels (*Arca zebra)* transplanted along a contamination gradient in Bermuda. *J. Exp. Mar. Biol. Ecol.* **138**, 99-117.

Widdows J. and Donkin, P. (1992) Mussels and environmental contaminants: Bioaccumulation and physiological aspects. In, *The Mussel Mytilus edulis; Ecology, Physiology, Genetics and Culture,* pp 383-425. Elsevier, Amsterdam.

Widdows, J. (1994) Marine and estuarine invertebrate toxicity tests. In, *Handbook of Ecotoxicology*, Volume 1 (Ed., P. Calow), pp 145-166. Blackwell Scientific, Oxford.

Widdows, J., Donkin, P., Evans, S.V., Page, D.S. and Salkeld, P.N., (1995a). Sublethal biological effects and chemical contaminant monitoring of Sullom Voe (Shetland) using mussels (*Mytilus edulis*). *Proceedings of the Royal Society of Edinburgh.* **103B**, 99-112.

Widdows, J., Donkin, P., Brinsley, M.D., Evans, S.V., Salkeld, P.N., Franklin, A., Law, R.J. and Waldock, M.J. (1995b). Scope for growth and contaminant levels in North Sea mussels *Mytilus edulis*. *Marine Ecology Progress Series*. **127**, 131-148.

Woodburn, K.B., Doucette, W.J. and Andren, A.W. (1984). Generator column determination of octanol/water partition coefficients for selected polychlorinated biphenyl congeners. *Environmental Science and Technology*. **18**, 457-459.

Wraige, E.J. (1997). *Studies on the synthesis, environmental occurrence and toxicity of unresolved complex mixtures (UCMs) of hydrocarbons*. PhD Thesis. University of Plymouth, Plymouth, U.K.

Yalkowsky, S.H. and Banerjee, S. (1992). *Aqueous Solubility, Methods of Estimation for Organic Compounds*. Marcel Dekker Inc. New York.

Zepp, R.G., Baughman, G.L. and Schlotzhauer, P.F. (1981). Comparison of photochemical behaviour of various humic substances in water I. Sunlight induced reactions of aquatic pollutants. *Chemosphere*. **10**, 109-117.

Zepp, R.G., (1982). Experimental approaches to environmental photochemistry. In, *The Handbook of Environmental Chemistry, Reactions and Processes, Volume 2 part B* (Ed., O. Hutzinger). pp. 19-21. Berlin: Springer-Verlag.

Zepp, R.G., Schlotzhauer, F. and Merritt Sink, R. (1985). Photosensitized transformations involving electronic energy transfer in natural waters: role of humic substances. *Environmental Science and Technology*. **19**, 74-81.