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Biodegradation, water solubility and characterisation studies of unresolved complex mixtures (UCMs) of aromatic hydrocarbons

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**BIODEGRADATION, WATER SOLUBILITY AND CHARACTERISATION
STUDIES OF UNRESOLVED COMPLEX MIXTURES (UCMs) OF AROMATIC
HYDROCARBONS**

by

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BIODEGRADATION, WATER SOLUBILITY AND CHARACTERISATION STUDIES OF UNRESOLVED COMPLEX MIXTURES (UCMs) OF AROMATIC HYDROCARBONS

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ABSTRACT

Following release to the environment, crude oil becomes subject to weathering processes which remove the simple, volatile hydrocarbons leaving an unresolved complex mixture (UCM) comprising the more environmentally persistent components. Aromatic UCMs have been reported in a wide variety of environmental matrices. Recent studies indicate such material, particularly monoaromatic hydrocarbons, is sufficiently bioavailable to marine organisms (*e.g.* mussels) to elicit toxicological responses. However, little else is known about the environmental fate and composition of petroleum-derived aromatic UCMs. The overall aim of this work was to investigate aromatic UCMs with particular emphasis on their biodegradability, water solubility and composition.

To assess persistence in the environment, an 'aromatic' UCM was isolated from Tia Juana Pesado crude oil (Venezuela) and this UCM exposed to the hydrocarbon degrading bacterium *Pseudomonas fluorescens* (Texaco). Five synthesised alkylcyclohexyltetralins and an alkylcyclohexylnaphthalene, proposed previously as 'average' structures for some aromatic UCM components, were also exposed under the same conditions. After 50 days, biodegradation of the compounds had not exceeded 20%, the most resistant (~2% biodegradation) being those with C₃-C₅ alkyl chains. These latter results were comparable to those observed for the 'aromatic' UCM (~2%). Using North Sea oil or *n*-hexadecane as co-substrates, the synthetic compounds were also exposed to a natural consortium of bacteria to provide more environmentally realistic conditions. After 119 days the branched chain C₅ homologue (~60%) and the naphthalene (~12%) still remained, yet under these severe conditions the components of the North Sea crude oil co-substrate were extensively degraded with even the highly bioresistant pentacyclic hopanes exhibiting biodegradation.

Aqueous solutions of an 'aromatic' and 'monoaromatic' UCM were produced (at 25 °C) using a generator column technique. These 'solubilised' UCM fractions may represent UCM hydrocarbons which are bioavailable, and which are most toxic to aquatic organisms. An altered UCM was clearly evident in the aqueous phase extracts, suggesting that many of the compounds in the original aromatic and monoaromatic UCMs exhibited similar physicochemical properties and mole fractions. However, higher molecular weight hydrocarbons were absent from the generated 'solutions', indicating a solubility cut-off point based predominantly on molecular weight. The molecular weight distribution differed significantly for the 'solubilised' aromatic and monoaromatic UCMs, indicating that Raoult's Law may describe a critical control in the dissolution of complex mixtures.

Comprehensive two-dimensional gas chromatography-time of flight-mass spectrometry (GCxGC-ToF-MS) analysis was used to provide vastly increased separation power and characterisation of a water 'soluble' monoaromatic UCM. Over 1200 compounds were separated by the chromatography, of which about 500 had distinct mass spectra from the ToF-MS analysis. A detailed characterisation of some of these monoaromatic UCM hydrocarbons *via* comparison to mass spectra registered in the NIST library permitted the identities of over 100 monoaromatic UCM components to be inferred. Compounds identified include novel alkylated homologues of benzene, indene, indan, tetralin, biphenyl, diphenylmethane and tetrahydrophenanthrene.

This study has shown that a UCM appeared to be comprised of the geochemically minor isomers and analogues of known major crude oil constituents. This finding is extremely important given that the narcotic toxicity of petroleum hydrocarbons is additive. As UCMs often account for a large proportion of crude oil mass, these persistent residues may also contribute significantly to the observed narcotic toxicity of crude oil.

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LIST OF COMMON ABBREVIATIONS

BTEX	Benzene, toluene, ethylbenzene and xylene
EPA	Environment Protection Agency
FID	Flame ionisation detector
GC	Gas chromatography
GC-MS	Gas chromatography – mass spectrometry
GCxGC	Gas chromatography x gas chromatography
GCxGC-ToF-MS	Gas chromatography x gas chromatography – time-of-flight – mass spectrometry
HPLC	High performance liquid chromatography
K _{ow}	Octanol-water partition coefficient
MITI	Ministry of International Trade and Industry
MS	Mass spectrometry
LC50	Concentration of toxicant which results in 50% mortality
PAH	Polycyclic aromatic hydrocarbon
SAR	Structure-activity relationships
SFG	Scope for Growth
SIM	Selected ion monitoring
TOE	Total organic extract
ToF-MS	Time-of-flight – mass spectrometry
TPH	Total petroleum hydrocarbons
UCM	Unresolved complex mixture
UV	Ultra-violet
WSF	Water soluble fraction

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Signed

Dated

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Chapter 1

Introduction

A review of the current knowledge concerning unresolved complex mixtures (UCMs) of hydrocarbons in the environment, in particular those comprised of aromatic hydrocarbons, is presented. The available literature indicates that these hydrocarbon UCMs are ubiquitous in environmental samples contaminated with crude oil and with some refined petroleum fractions. Whilst UCMs are generally associated with weathered crude oil residues they are also present in fresh crude oils. Weathering processes, particularly biodegradation, serve to enhance UCM concentrations in oils through removal of the chromatographically resolvable components. Studies of the molecular composition of these resistant UCM compounds are reviewed. Due to the persistent nature of UCM hydrocarbons, their behaviour and impacts, particularly on the marine environment, are discussed, and the underlying principles for the current study are presented.

1.1 Introduction

Today, the world's oceans receive a wide range of contaminants, many of which enter the marine environment *via* isolated or chronic discharges. One of the pollutant classes with the highest media profile is crude oil, perhaps due to the effects associated with large isolated spillages frequently resulting from oil tanker accidents. Crude oil has become an intrinsic part of global society, providing energy for power, heating and powering motor vehicles and as a raw material. This global dependence has led to the continued rise in the transportation of crude oil and petroleum derived products, estimated to be in the region of 150,000 tonnes per annum (National Research Council, 2003). This in turn, has resulted in the continued release of petroleum hydrocarbons to the marine environment. Current estimates (National Research Council, 2003) suggest in excess of 1.3m tonnes reach the marine environment annually. However, the ecological impact of this is difficult to assess as this represents thousands of individual releases. Furthermore, the effect of each spill is dependent upon the volume and type of crude oil spilled, weather conditions and the environment into which it enters (National Research Council, 2003; Heubeck *et al.*, 2003).

Although they do not necessarily have the most environmentally profound effects, large oil spills gain most media attention (Table 1.1). Such accidents are exemplified by the grounding of the *T/V Exxon Valdez* in Prince William Sound, Alaska in March, 1989, where approximately 36,000 tonnes of Alaskan North Slope crude oil was released into one of the most fragile ecosystems on the planet. The immediate result of major accidents such as these are the powerful visual effects of crude oil in the environment, such as oil covered animals and their subsequent high mortality rates (*e.g.* Heubeck *et al.*, 2003; Figure 1.1). In 1999 the tanker *Erika* sank in the Bay of Biscay, releasing 20,000 tonnes of heavy fuel oil which polluted 400 km of the French Atlantic coast, killing 44,000 seabirds. In addition to animals, detrimental effects to the health of oil spill clean-up workers have also been reported in the immediate aftermath of major spillages (*e.g.* Bosch, 2003). Whilst

pollution effects of this magnitude are not everyday occurrences and inputs of oil to the sea have been significantly reduced in recent years (*e.g.* ITOPF, 2002; National Research Council, 2003), tanker accidents still occur with a worryingly regularity (Clark, 2001). Most recently, the *Prestige* sank off the coast of NW Spain causing another major oil spill.

Table 1.1. Summary of some of the largest, most environmentally significant and most recent oil tanker spills, edited from Clark (2002).

Date	Tanker	Location	Spillage (t)
19/07/1971	<i>Atlantic Empress</i>	Off Tobago	287 000
16/03/1978	<i>Amoco Cadiz</i>	Brittany, France	223 000
11/03/1991	<i>Haven</i>	Genoa, Italy	144 000
18/03/1967	<i>Torrey Canyon</i>	Scilly Isles, UK	119 000
03/01/1993	<i>Braer</i>	Shetland Isles, UK	85 000
15/02/1996	<i>Sea Empress</i>	Milford Haven, UK	72 000
16/04/1992	<i>Katina P</i>	Off Maputo, Mozambique	72 000
04/03/1989	<i>Exxon Valdez</i>	Prince William Sound, Alaska	37 000
13/11/2002	<i>Prestige</i>	Off NW coast of Spain	> 30 000 ^a
12/12/1999	<i>Erika</i>	Off Brittany, France	20 000 ^b
16/01/2001	<i>Jessica</i>	San Cristobal Island, Galapagos	1000 ^c

^a*Prestige* oil spill data added from Bohannon and Bosch (2003), Serret *et al.* (2003). ^b*Erika* oil spill data added from Le Hir and Hily (2002) and Le Moigne (2003). ^c*Jessica* spill data added from Kingston *et al.* (2003).



Photo by US Environmental Protection Agency

Figure 1.1. Photograph of an oiled seabird, typical of the media images associated with crude oil spills in the environment, yet contamination of the marine environment with unresolved complex mixtures of oil derived hydrocarbons has received comparatively little media attention (but see Jones, 2003).

Of course, petroleum hydrocarbon releases to the environment are not simply limited to major oil tanker accidents. In fact the latter only account for an estimated 7.5% of annual crude oil inputs to the marine environment (calculated from National Research Council, 2003). In addition, considerable amounts of petroleum products are discharged into the marine environment through runoff, industrial and sewage effluents, stormwater drains, general shipping activities, and natural oil seeps (Zheng and Richardson 1999; National Research Council, 2003; Readman *et al.*, 2002).

1.2 Unresolved Complex Mixtures (UCMs) of hydrocarbons

The long-term ecological impacts of crude oil releases are less obvious and less well understood. Crude oil is an exceedingly complex amalgamation of thousands of individual compounds each with distinct physico-chemical characteristics (Readman *et al.*, 1992; Schwarzenbach *et al.*, 2003). Long-term impacts are thought to depend largely on the fate and behaviour of these complex hydrocarbon mixtures in the environment. Resolved peaks corresponding to abundant *n*-alkane constituents (*e.g.* Figure 1.2a) typically dominate gas chromatograms of fresh (undegraded) crude oils. Whilst many compounds differ significantly from each other in terms of their physico-chemical properties, the sheer number of compounds in crude oil means it is inevitable that many, homologous (or pseudohomologous) and isomeric compounds will exhibit very similar properties. This includes a continuum of compounds with closely similar boiling points. When examined by gas chromatography such components have similar retention characteristics and are effectively unresolved. These have become known as Unresolved Complex Mixtures (UCMs; Thompson and Eglinton, 1978; Frysinger *et al.*, 2003). UCMs are present in the gas chromatograms of all oils but are especially apparent in chromatograms of weathered oils (*e.g.* Gill and Robotham, 1989; Gough and Rowland, 1990; Button *et al.*, 1992; Readman *et al.*, 1996; Gogou *et al.*, 2000; Ziolli and Jardim 2003; Figure 1.2b).

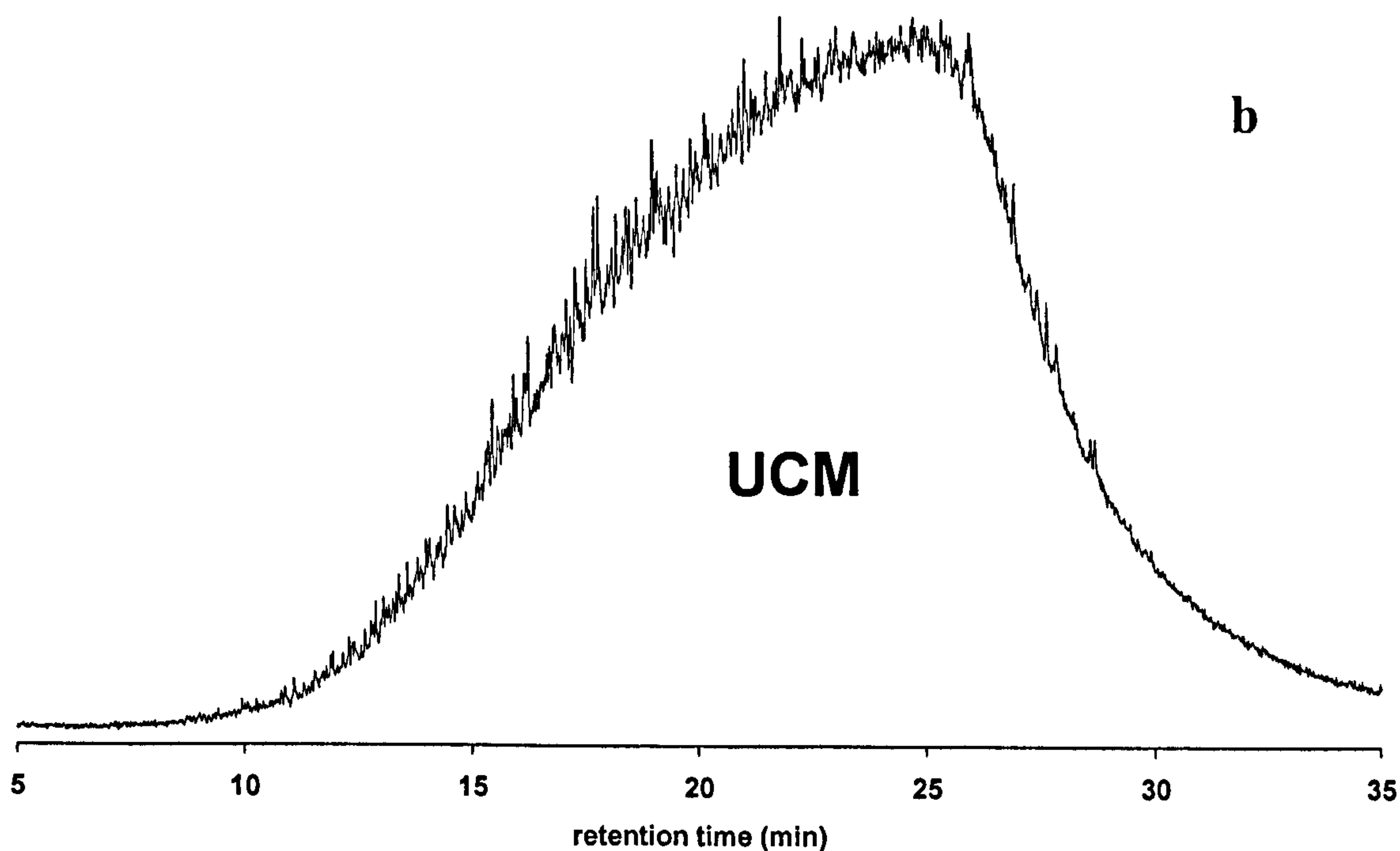
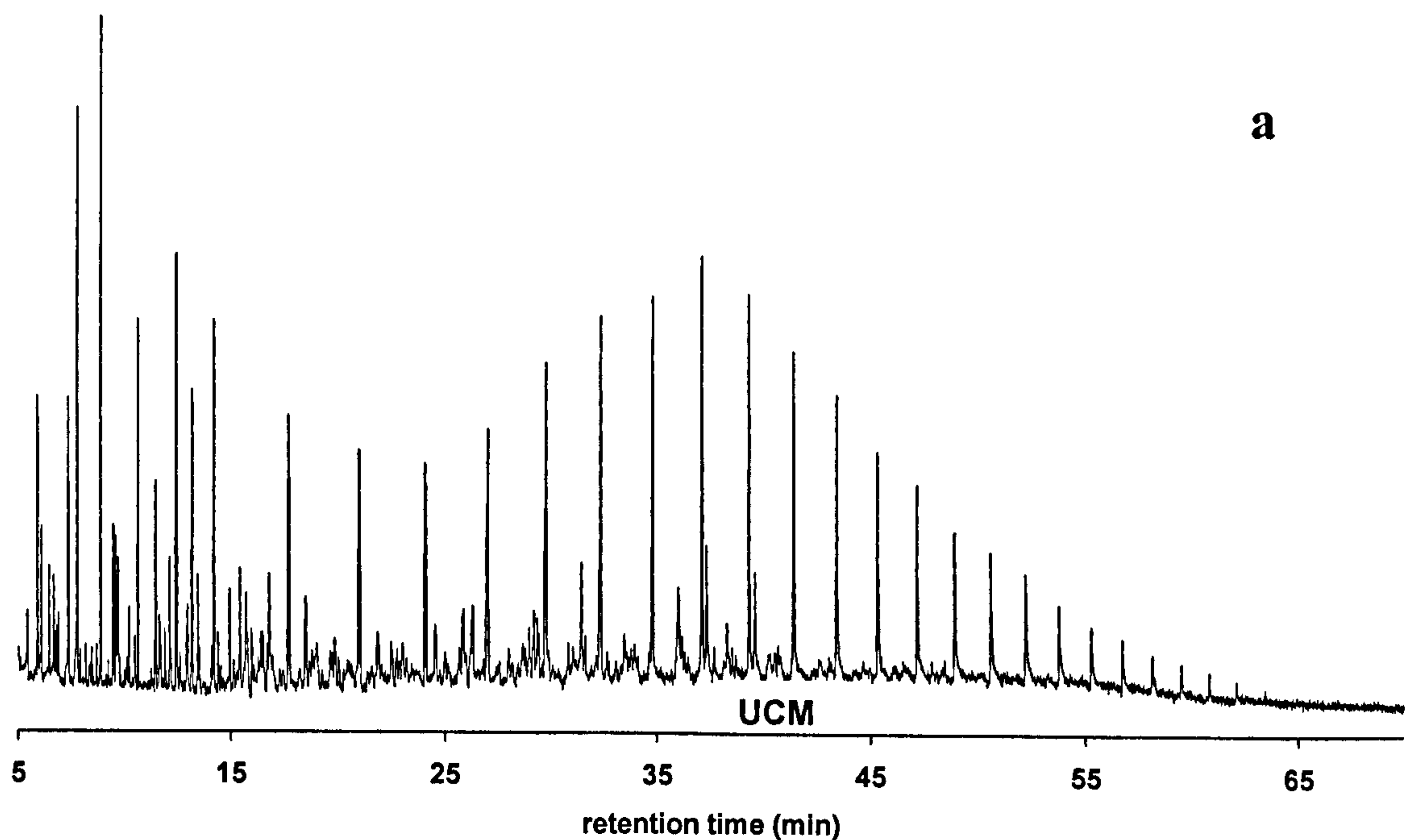


Figure 1.2. Gas chromatograms of (a) fresh Forties crude oil; (b) biodegraded Tia Juana Pesado crude oil. The former chromatogram is normalised on the largest resolved component peaks, effectively masking the large unresolved complex mixture of hydrocarbons under the baseline. In the chromatogram of the biodegraded oil the removal of the resolved compounds by bacteria has left a residue, which is normalised on the apex of the unresolved feature (UCM), making it much more pronounced.

UCMs produce a raised baseline in the gas chromatogram of the hydrocarbon fraction (Readman *et al.*, 2002) and are colloquially known as a ‘hump’ (*e.g.* Gough and Rowland, 1990; Rowland *et al.*, 2001; Smith *et al.*, 2001). These ‘humps’ are clearly visible in gas chromatograms of weathered crude. Although fresh oils contain UCMs, the latter are generally suppressed by the high abundance of a few individual compounds such as *n*-alkanes (*e.g.* Button *et al.*, 1992; Figure 1.2a). It is only when these resolved compounds are removed *via* weathering processes that the UCM becomes the predominant feature in the gas chromatograms.

The weathering of complex mixtures such as crude oil is complicated and the dynamics inter-linking such processes, their dependence and effects upon each other, is convoluted and not fully understood (Jezequel *et al.*, 2003). However, it is known that these processes are most effective in the removal of crude oil hydrocarbons when they are acting in unison. Owing to their different physico-chemical properties, once released into the environment crude oil hydrocarbons weather at different rates and behave in different ways (Readman *et al.*, 1992; Gogou *et al.*, 2000; Schwarzenbach *et al.*, 2003). Those most susceptible to weathering processes are removed rapidly leaving the more resistant hydrocarbons to persist in the environment for longer periods of time. Initially the most significant process is volatilisation (Readman *et al.*, 1992). The weathering of remaining components then proceeds *via* processes such as dissolution, biodegradation and photooxidation, (Readman *et al.*, 1992; Readman *et al.*, 1996; Gogou *et al.*, 2000; Chaîneau *et al.*, 2003; Jezequel *et al.*, 2003). Volatile hydrocarbons of low molecular weight and those that are readily biodegradable are thus the most susceptible to weathering processes. Examples include the *n*-alkanes (up to C₄₄) and low molecular weight aromatic compounds such benzene, toluene, ethylbenzene, xylene (BTEX) and naphthalene (*e.g.* Atlas, 1981; Leahy and Colwell, 1990; Atlas and Bartha, 1992; Alexander, 1999).

Weathering of crude oil generally coincides with an increase in the UCM contribution to the hydrocarbon burden of environmental samples (*e.g.* Seymour and Geyer, 1992; Chaîneau *et al.*, 2003). The remaining UCM hydrocarbons are therefore the most persistent in the environment, sharing similar physical and chemical characteristics (*e.g.* Fung *et al.*, 2004). This has led to the presence of UCMs in environmental samples being widely used as an indication of petrogenic hydrocarbon inputs (*e.g.* Gill and Robotham, 1989; Readman *et al.*, 1996; Wakeham, 1996; Ahmed *et al.*, 1998; Gonzalez-Barros *et al.*, 1998; Zheng and Richardson 1999; Gogou *et al.*, 2000; Doskey 2001; Yunker and Macdonald, 2003; Ziolli and Jardim 2003). For example, Kingston *et al.* (2003) used UCMs in sediment samples from the Galapagos Islands to distinguish sites contaminated by fresh crude oil from the *Jessica* cargo in 2001 from those which were contaminated by previous pollution events (*e.g.* general port activities in the area during the years preceding the tanker spill).

1.2.1 Origins of UCMs

UCMs are known to be present in what are termed “fresh” as well as weathered crude oils, accounting for up to 30% of the mass of some fresh oils (Revill, 1992; Table 1.2). Therefore, (although the subject has been rarely addressed), it is assumed that UCM generation occurs at some time during the genesis of the other (resolved) hydrocarbons of crude oil. Killips and Al-Juboori (1990), Revill (1992) and Warton (1999) have provided possibly the most comprehensive reviews of the origins of UCM hydrocarbons available to date.

In an effort to verify the origin of UCM hydrocarbons experimentally, Revill (1992) subjected polythene to hydrous pyrolysis (350°C, 24 and 48 hr), which is a technique widely advocated for laboratory simulation of the catagenic stage of oil formation (*e.g.* Lewan *et al.*, 1979; Lewan *et al.*, 1986; Lewan and Williams, 1987). GC analysis of the

urea non adduct fraction of the organic extracts of the pyrolysates revealed the presence of quite significant UCMs, with longer pyrolysis times increasing the amount of unresolvable material. Natural catagenesis of kerogen (an ill-defined natural macromolecular precursor of crude oil) was suggested to produce at least a proportion of the UCM hydrocarbons found in crude oils. Killops and Al-Juboori (1990) also suggested that the UCM hydrocarbons are derived from kerogen during petroleum generation in the same way as the resolved components (*e.g.* *n*-alkanes). Warton *et al.* (1999) suggest that saturated compounds and the alkyl substituents attached to aromatic rings may have a common origin. They proposed that saturated hydrocarbons are formed by defunctionalisation of natural products containing the same carbon skeleton, such as carboxylic acids and alcohols. Alkylaromatic hydrocarbons were suggested to form *via* alkylation of the aromatic system by such functionalised compounds followed by defunctionalisation to form hydrocarbons (Warton, 1999; Warton *et al.*, 2002). Wilkes *et al.* (1998) also propose that alkylaromatic hydrocarbons arise from the addition of acylium carbocations (from carboxylic acids) onto aromatic rings. Hydrocarbon UCMs may even be generated during different steps of catagenesis. For example, the decarboxylation of UCMs of carboxylic acids in biodegraded oils following their palaeosterilisation at high temperatures (>80 °C) (*cf.* Wilhelms *et al.*, 2001; Watson *et al.* 2002).

Table 1.2. Gravimetric composition of six crude oils from various depositional environments (taken from Revill, 1992; Rowland and Revill, 1995).

Oil	% Aliphatics	% Aromatics	% Asphaltenes and polars*	Aliphatic urea adduct % of aliphatics	Aliphatic urea non adduct % of aliphatics ⁺ (aliphatic UCM)	Urea non adduct % of oil ⁺ (Total 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 sourced
2 Carbonate sourced
3 Marine sourced

1.3 Environmental occurrence of UCMs

Petroleum hydrocarbon contamination is often highest in environmental samples collected near sites of concentrated anthropogenic activity *e.g.* cities and ports (Bomboi and Hernandez, 1991; Connell *et al.*, 1998; Gogou *et al.*, 2000; Tsapakis *et al.*, 2002; Wetzel and Van Vleet, 2003; Yunker and Macdonald, 2003). Other places include point sources of pollution such as river discharges and off shore production (Mason 1988; Maldonado *et al.*, 1999). Despite the persistence of UCM hydrocarbons in crude oil residues, these recalcitrant mixtures are presumably still subject to processes such as slow dissolution, flocculation and adsorption to particulate matter. This can lead to their transport across large distances, resulting in their occurrence in regions far away from the source of pollution. Burns *et al.* (2001) suggest that ocean currents are able to transport organic material, including discrete tar balls, very long distances. Gogou *et al.* (2000) and Doskey (2001) suggest atmospheric transport to be a significant source of hydrocarbons to deep-sea areas. Ternois *et al.* (1998) observed large UCMs in the hydrocarbon extracts of a sediment trap survey which was conducted in the remote Indian Ocean sector of the Southern Ocean. Consequently petrogenic hydrocarbons appear ubiquitous in their nature being present in virtually all environmental samples.

Initially, UCMs documented in the literature were reported to be predominantly associated with the aliphatic fractions of crude oil. This was perhaps due in part to the use of GC-flame ionisation detection (FID) and GC-mass spectrometry (MS) to analyse the saturated (aliphatic) fractions of environmental samples, which then frequently exhibited ‘aliphatic’ UCMs. Also, initial attempts to characterise UCMs focused on the aliphatic fractions (*e.g.* Gough, 1989; Gough and Rowland, 1990; Revill, 1992; Revill *et al.*, 1992). As a result, aliphatic UCMs have been widely reported in the saturated fraction of environmental samples including sediments (*e.g.* Wade *et al.*, 1994; Wakeham, 1996; Zheng and Richardson 1999; Gogou *et al.*, 2000; Lopez *et al.*, 2000; Doskey 2001; Meniconi *et al.*,

2002; Reddy *et al.*, 2002; Wetzel and Van Vleet 2003), aerosols (*e.g.* Preston and Merrett, 1991; Preston *et al.*, 1992; Mendez *et al.*, 1993; Bi *et al.*, 2002; Tsapakis *et al.*, 2002), sediment traps (*e.g.* Ternois *et al.*, 1998; Burns *et al.*, 2001; Burns *et al.*, 2003) urban runoff (*e.g.* Wade *et al.*, 1994) and the water column (*e.g.* Requejo and Boehm 1985; Maldonado *et al.*, 1999). They have also been reported in mussels (*e.g.* Farrington *et al.*, 1988; Fung *et al.*, 2004), oysters (*e.g.* Gold-Bouchot *et al.*, 1995), fish (*e.g.* Ahmed *et al.*, 1998) and even terrestrial mammals such as wolves (Gonzalez-Barros *et al.*, 1998).

1.3.1 Aromatic UCMs

More recent studies have revealed that aromatic UCM hydrocarbons are also common in crude oil residues and environmental samples. One of the main reasons for the previous lack of reports was the techniques employed for analysis of aromatics (Wang and Fingas, 1997; Smith, 2002). Aromatic compounds are commonly monitored using non-chromatographic fluorescence techniques. When chromatographic techniques are used, selected ion monitoring (SIM) methods, designed to measure the molecular ions of priority pollutant aromatic hydrocarbons (*e.g.* PAH), are most often utilised due to regulatory requirements for PAH determination (*e.g.* Boxall and Maltby, 1995; Shailaja and D'Silva 2003). These methods essentially 'filter' the total aromatic hydrocarbon data, leaving the aromatic UCM undetected. In many cases, when GC analysis exhibits an aromatic UCM in the chromatograms of environmental samples, these are not mentioned when the data is discussed (*e.g.* Wakeham 1996; Lopez *et al.*, 2000) or if the data are discussed they are not quantified (*e.g.* Bomboi and Hernandez, 1991). Recent indications that the aromatic UCM is the most toxic fraction of weathered crude oil residues (*e.g.* Smith 2002; Smith *et al.*, 2001; Rowland *et al.*, 2001; Wraige, 1997) are perhaps responsible for an increasing number of reports of the presence of aromatic UCMs in environmental samples.

However, before this recent and growing interest, several earlier studies reported aromatic UCMs in a broad array of environmental matrices worldwide (Table 1.3). For example, in sediments (*e.g.* Atlas, 1981; Jones *et al.*, 1983; Readman *et al.*, 1986; Fowler *et al.*, 1993; McMillen *et al.*, 1995; Readman *et al.*, 1996; Le Dreau *et al.*, 1997; Reddy *et al.*, 2002), in the water column (Requejo and Boehm 1985; Douabul and Al-Shiwafi, 1998), in urban runoff (*e.g.* Bomboi and Hernandez, 1991), in soils (*e.g.* Chaineau *et al.*, 2003), and in urban aerosols (*e.g.* Aceves and Grimalt, 1993a and b). Aromatic UCMs have also been reported in bivalves (*e.g.* Farrington *et al.*, 1982; Soler *et al.*, 1989; Fowler *et al.*, 1993; Wraige, 1997; Rowland *et al.*, 2001; Smith, 2002) and fish (*e.g.* Fowler *et al.*, 1993). The range of concentrations of aromatic UCMs found in some environmental samples is summarised in Table 1.3. Though this is a limited summary of a data-deficient research area, the available information suggests aromatic UCMs are quantitatively important in environmental samples. In fact aromatic UCMs frequently account for the majority (up to 98%) of the aromatic hydrocarbon burden in such samples, far outweighing the PAH.

1.4 Environmental impacts of petroleum derived hydrocarbons

1.4.1 The bioaccumulation of petroleum derived hydrocarbons in aquatic organisms

The impacts of crude oil and some of its known constituents (individual hydrocarbons or simple mixtures) on marine organisms have been studied for many years. These studies have generally found that petroleum hydrocarbons, once released into the environment, can accumulate to varying degrees in aquatic organisms. Certain chemicals can accumulate in organisms *via* direct uptake from the surrounding medium (*e.g.* seawater) by gills or skin (bioconcentration) or by ingestion of particle-bound chemicals (bioaccumulation) as well as *via* the food chain (biomagnification) (Franke *et al.*, 1994; Ziolli and Jardim, 2002). Even when standard ecotoxicity tests indicate no detectable acute or chronic effects, bioaccumulation and biomagnification are regarded as hazard criteria in themselves. Some effects are only recognised in the later phases of life. Multi-generation effects may manifest themselves only in higher trophic levels of a food web (Franke *et al.*, 1994; Clarke *et al.*, 2000; Ziolli and Jardim, 2002). Factors which are thought to influence bioaccumulation in organisms include diffusion behaviour through cell membranes, metabolism in organisms, accumulation behaviour of the metabolites, accumulation in specific organs and tissues, uptake and depuration kinetics (Franke *et al.*, 1994). Although many studies focus on the effects of individual or simple mixtures of hydrocarbons on aquatic organisms, these are not necessarily environmentally realistic scenarios. Investigations have highlighted significant differences in the uptake rate of an individual compound from the rates observed for the same compound present in a complex mixture (Dauble *et al.*, 1986). Dauble *et al.* (1986) suggest that competitive interactions for biotic transport may occur among the mixture constituents, particularly if compounds possess similar physical or chemical properties. The presence of chemicals of similar size and form are suggested to result in saturation of the hydrophobic binding sites.

Typical organisms used for monitoring the bioaccumulation of petroleum hydrocarbons include fish (e.g. Neff *et al.*, 1985; Readman *et al.*, 1992) and bivalves (mussels and oysters) (e.g. Donkin *et al.*, 1991; Widdows *et al.*, 1995; Donkin *et al.*, 2003). However, aquatic organisms such as bivalves are most susceptible to oil pollution events, as they are unable to avoid oil spills and possibly because they are unable to rapidly depurate or metabolise hydrocarbons. For example, Readman *et al.* (1992) observed much lower concentrations of petroleum hydrocarbons in fish compared with bivalves in the Arabian Gulf following the crude oil releases during the 1991 Gulf War. Neff *et al.* (1985) also observed much lower concentrations of petroleum hydrocarbons in plaice (*Pleuronectes platessa*) than in oysters (*Crassostrea gigas*) collected from the same impacted site. This was attributed to the ability of plaice to rapidly metabolise and excrete these compounds. The persistent nature of some crude oil component hydrocarbons in the environment has led to concerns regarding their bioavailability to aquatic organisms. Ahmed *et al.* (1998) suggested that, due to their stability, petroleum and petroleum hydrocarbons in the marine environment, when ingested by marine organisms, might be retained for long periods. This observation is supported by Neff *et al.* (1985) who found that oysters accumulated petroleum hydrocarbons to high concentrations and were slow to depurate them over a 27-month period. Furthermore, Soler *et al.* (1989) have shown that aromatic hydrocarbons bioaccumulate to higher concentrations in older mussels. This indicates that rates of elimination or metabolism are much slower than those of accumulation.

1.4.2 Toxicity of petroleum derived hydrocarbons in aquatic organisms

The bioaccumulation of petroleum hydrocarbons in aquatic organisms following a spill or from low level chronic discharges has been shown to elicit significant sublethal and lethal responses. Rudolph *et al.* (2001) found that long term exposure of sublethal concentrations were as deleterious to organisms as periodic exposures to higher concentrations. Thus,

evaluation of contaminant profiles in shellfish can provide advantages over measurements in water and bottom sediment samples. For example, mussel-based measurements are a reflection of pollutant bioavailability to successive consumers in the food chain. These allow an assessment of the sublethal or lethal effects of pollutants on organisms at higher trophic levels (Clarke *et al.*, 2000). Bioaccumulation of petroleum hydrocarbons has been frequently reported to trigger ecotoxicological effects in aquatic organisms such as fish (*e.g.* Neff *et al.*, 1985; Khan, 1998; Neff *et al.*, 2000; Rudolph *et al.*, 2001; Shailaja and D'Silva, 2003; Bhattacharyya *et al.*, 2003), mussels (*e.g.* Wraige, 1997; Rowland *et al.*, 2001; Smith *et al.*, 2001; Smith, 2002; Erben *et al.*, 2003), crustaceans (*e.g.* Neff *et al.*, 2000; Erben *et al.*, 2003), mysids (*e.g.* Barron *et al.*, 1999a and b; Neff *et al.*, 2000), amphipods (*e.g.* Boxall and Maltby, 1995; Page *et al.*, 2002), octopus (Long and Holdway, 2002), and daphnia (Bhattacharyya *et al.*, 2003). Other organisms associated with aquatic environments, have also been studied, such as amphibians (*e.g.* Huang *et al.*, 2003) and birds (*e.g.* Andres, 1999). It should be noted that sublethal responses affected by exposure to petroleum hydrocarbons might be species dependent or more significant in particular aquatic organisms (Long and Holdway, 2002). For example, Neff *et al.* (2000) monitored the toxicity of water soluble fractions (WSFs) of fresh and weathered crude oils to a variety of aquatic organisms including clownfish (*Amphiprion clarkii*), tropical penaeid shrimp (*Penaeus vannamei*), sea urchin larvae (*Arbacia punctulata*), silverside minnows (*Menidia beryllina*), and mysids (*Americamysis bahia*). The acute toxicities of the generated WSFs varied for the different species, reflecting the different sensitivities to the hydrocarbons in crude oil WSFs. However, the relative sensitivities of the organisms were very similar for a particular WSF, indicating that the relative sensitivities to different components of the WSFs are similar. The life stage of a specific organism may also determine how susceptible it is to impairment by petroleum hydrocarbons (Barron *et al.*, 1999b; Long and Holdway, 2002). Furthermore, the sublethal responses of organisms at early life stages may lead to mortality later in life by increasing vulnerability to disease,

parasitism, or predation (Heintz *et al.*, 1999). The WSFs of crude oils, especially fresh oils, are generally assumed to contain abundant low molecular weight hydrocarbons such as BTEX. However, for many years large UCMs have also been clearly evident in the WSFs of both fresh and weathered crude oils (*e.g.* Boylan and Tripp, 1971; Boehm and Fiest, 1982; Sydnes *et al.*, 1985; Ali, 1994). Although these soluble UCMs are rarely mentioned or discussed, their toxicological impacts should be considered.

Petroleum hydrocarbons have been frequently shown to bioaccumulate in specific or target organs of the body. This 'storage' can render them harmless and in other cases can lead to problems associated with a particular organ. For example, Neff *et al.* (1985) observed that plaice contaminated with petroleum concentrated the hydrocarbons in liver and muscle tissue. Although the hydrocarbons were bioaccumulated to relatively low concentrations, a variety of sublethal histopathologic (microscopic anatomical changes in diseased tissue) and biochemical alterations were evident. These included severe lesions of the digestive tract and liver, which would have led to impaired digestion and absorption of food. Khan (1998) reported very similar effects in winter flounder (*Pleuronectes americanus*) following exposure to petroleum hydrocarbons. A wide range of adverse effects have been observed in organisms as a result of petroleum hydrocarbon contamination and accumulation. Whilst these are not general to all organisms, some of the most common sublethal conditions include external lesions, cell damage, lack of food in the digestive tract, neurosensory disruption, behavioural and developmental abnormalities, reduced fertility, physical deformities and the generally poor condition of the organism (Green and Trett, 1989; Khan, 1998; Carls *et al.*, 1999; Heintz *et al.*, 1999; Shailaja and D'Silva, 2003). Bhattacharyya *et al.* (2003) suggest that oil spilled on the surface of water may also limit oxygen exchange and coat the gills of aquatic organisms. This can cause problems for aquatic organisms with their oxygen-supply and respiration. Benthic organisms may be affected by oil settling on the sediment surface and accumulating in the sediment.

Whilst bioaccumulation of crude oil hydrocarbons is known to cause detrimental effects in aquatic organisms, the composition of the ingested oil will have a considerable effect on the severity of these responses. There have been a large number of investigations into determining just which components of crude oil are responsible for the majority of the toxicological effects. These studies have highlighted particular compounds and groups of compounds which are thought to elicit many of the observed problems. The components which have attracted the most interest are those readily identifiable by GC-MS analysis of environmental samples and tissue extracts. In particular, ecotoxicological studies have indicated that polycyclic aromatic hydrocarbons (PAHs) can accumulate in organisms and cause toxicological (carcinogenic or mutagenic) responses (*e.g.* Eickhoff *et al.*, 2003; Hutchinson *et al.*, 2003). This observation has led to the inclusion of 16 PAHs on the Environmental Protection Agency (EPA) priority pollutants list. More recently, attention has shifted to lower molecular weight aromatic hydrocarbons such as benzene and naphthalene and their alkylated derivatives, which have also been found to accumulate in aquatic organisms to toxic levels (*e.g.* Page *et al.*, 2002; Erben *et al.*, 2003; Huang *et al.*, 2003). Some of these studies have indicated that oils with lower concentrations of resolved aromatic hydrocarbons can be highly toxic and that PAHs are not necessarily the major toxicants (Barron *et al.*, 1999a; Rudolph *et al.*, 2001). Neff *et al.* (2000) observed that monoaromatic hydrocarbons accounted for 86-95% of the acute toxicity of the WSF of fresh oils. Although the concentrations of monoaromatic hydrocarbons were reduced in weathered crude oils, the WSF still accounted for 34-86% of the total toxicity. Whilst spilled oil is known rapidly to lose the acutely toxic hydrocarbon fractions *via* weathering, sufficient amounts of low molecular weight aromatic hydrocarbons appear to be resistant to weathering and capable of eliciting toxic responses.

The toxicity of monoaromatic and other low molecular weight aromatic hydrocarbons is unsurprising considering the processes controlling the bioavailability of hydrocarbons to

aquatic organisms. The fraction of crude oil that dissolves into natural waters is predominantly responsible for the acute toxic effects observed in marine organisms. The lipophilic nature of the dissolved hydrocarbons allows them to partition across cell membranes in the organisms into fatty tissue and cell centres (Zioli and Jardim, 2002). Lower molecular weight hydrocarbons (monoaromatics) are generally more soluble in an aqueous medium than those of higher molecular weight (*e.g.* PAHs). Eickhoff *et al.* (2003) observed that the water solubility¹ and chemical structure of a suite of PAHs appeared to be important factors in the affect of PAH exposure and bioconcentration in Dungeness crabs (*Cancer magister*). An increase in PAH molecular weight results in an accompanying decrease in solubility which may prevent its bioaccumulation to toxicological levels. Monoaromatic hydrocarbons are much more water soluble, and therefore make a greater contribution to the overall toxicity of a complex mixture such as crude oil (Neff *et al.*, 2000). Bioaccumulation and toxicological effects are primarily controlled by the bioavailability of the substance of concern, and studies should therefore only test within the range of water solubility of the substance (Dauble *et al.*, 1986; Franke *et al.*, 1994). As a result most toxicity studies use generated water soluble fractions (WSFs) of the test substrate to provide a more environmentally realistic assessment of toxicological impacts (*e.g.* Carls *et al.*, 1999; Heintz *et al.*, 1999).

1.4.3 Bioaccumulation and toxicity of UCMs in aquatic organisms

Previous studies (*e.g.* Neff *et al.*, 2000; Page *et al.*, 2002) have shown that the toxicity of crude oil is greater than the contribution made by the GC resolvable (saturate and aromatic) constituents. The cause of this toxicity has been attributed to other components, with a number of cases suggesting that hydrocarbons present in the UCM were responsible (*e.g.* Neff *et al.*, 2000; Page *et al.*, 2002). In particular, soluble hydrocarbons of a low molecular weight are considered good candidates for a contribution to this toxicity. Despite

¹ A review of the factors affecting the water solubility of hydrocarbons is presented in Chapter 2

the reduction in crude oil toxicity with increased weathering (due to the removal of the most soluble but often the most toxic compounds) residual hydrocarbons were still found to exhibit a substantial toxicity (Chaineau *et al.*, 2003). This is evidence for the toxicity of UCMs which are the dominant feature of weathered crude oils.

This concept has led to a number of studies focusing on the environmental fate and impacts of unresolved complex mixtures of hydrocarbons. If UCMs are to be held accountable for this observed toxicity, they need to be shown to bioaccumulate in marine organisms. Investigations have shown that UCMs are indeed present in the organic extracts of tissue taken from a wide variety of organisms. For example, Farrington *et al.* (1982) found that the aromatic UCM concentration in the mussel, *Mytilus edulis*, decreased slowly over time following exposure to an oil spill. Extracts quickly became dominated by the aromatic UCM as resolved components were rapidly depurated. The authors suggested that compounds contributing to the UCM appeared to be among the most persistent indicators of oil contamination. Most frequently aliphatic UCMs are reported in organisms, (e.g. Fung *et al.*, 2004). Recently, aromatic UCMs have been observed on an increasing basis in aquatic organisms such as mussels, *Mytilus edulis* (e.g. Farrington *et al.*, 1982; Gold-Bouchot *et al.*, 1995; Wraige, 1997; Smith *et al.*, 2001; Rowland *et al.*, 2001; Smith, 2002). Soler *et al.* (1989) report the presence of significant aromatic and monoaromatic UCMs in mussels collected from the legs of an oil production platform in Amposta (W. Mediterranean). Monoaromatic UCMs have also been reported in studies by Smith (2002) and Rowland *et al.* (2001). These occurrences of UCMs imply that their bioaccumulation may lead to biomagnification through food chains or webs to higher trophic levels. Significantly, Farrington *et al.* (1982) found that long-term exposure of bivalves to chronic oil inputs resulted in longer persistence of petroleum hydrocarbons when moved to a clean environment to depurate.

Until very recently, no specific research had focused on the environmental and toxicological impacts of UCMs, perhaps due to the complex nature of such mixtures. Owing to the continued release of crude oils and petroleum-derived materials into the environment, it is essential that the toxicological effects of UCMs are understood. Previous work by Thomas (1995) indicated that the aliphatic UCM isolated from a lubricating oil feedstock was non-toxic. This was attributed to the low water solubility of the saturated hydrocarbon UCM components. It is not known how general these findings are for other aliphatic UCMs. Studies by Wraige (1997), Barron *et al.* (1999a), Rowland *et al.* (2001) and Smith (2002) have addressed the toxicity of aromatic UCMs to aquatic organisms. Having studied a suite of oils, Barron *et al.* (1999a) found the most toxic was that with the lowest aromatic content, but with the largest UCM. An environmentally weathered oil containing a large UCM was found to exhibit sublethal and lethal responses in the mysid shrimp (*Mysidopsis bahia*) (Barron *et al.*, 1999b). Gold-Bouchot *et al.* (1995) observed toxic effects in oysters containing a UCM, with damage to the digestive and gills system increasing linearly with UCM accumulation. The authors concluded that it is difficult to assign toxic effects to individual compounds, due to the complex nature of UCMs.

A few studies have indicated the presence of contaminant UCMs in aquatic organisms exhibiting poor health. However, the first study directly associating the toxicological impacts of aromatic UCMs was conducted by Wraige (1997). Mussels (*Mytilus edulis*) used in Scope for Growth (SfG) studies by Widdows *et al.* (1995) were found to have concentrations of aromatic hydrocarbons in the body tissue which were inversely related to the SfG measurements. Gas chromatographic analysis of the aromatic fractions of the mussel tissue by Wraige (1997) indicated they were dominated by UCMs (Smith 2002; Figure 1.3).

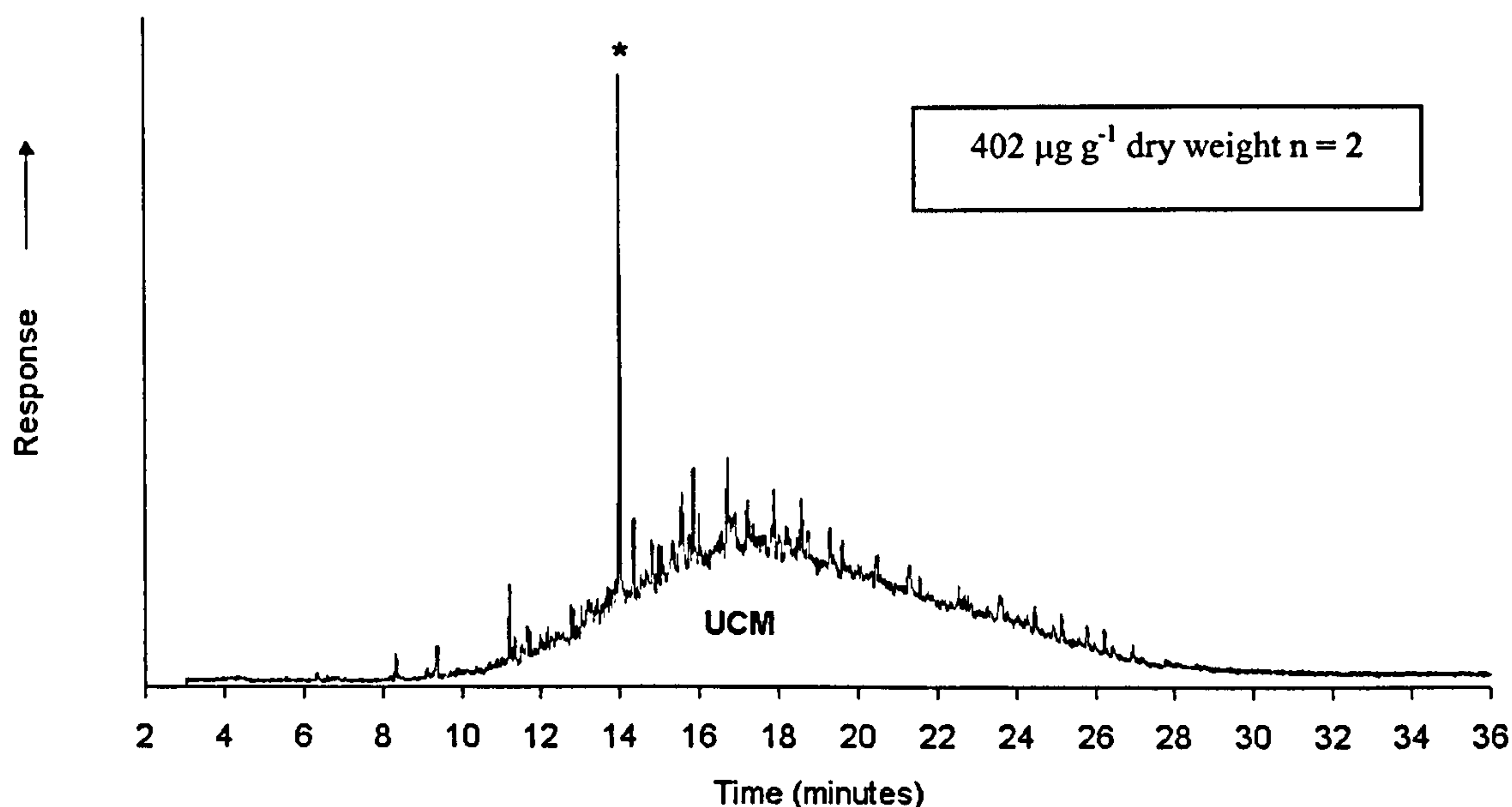


Figure 1.3. Gas chromatogram of the aromatic hydrocarbon fraction of mussels (*Mytilus edulis*) from Whitby, UK, 1990 (Smith, 2002). * *internal standard d₁₀ phenanthrene*.

The collection of fresh mussels from the impacted sample sites allowed a comparison between the concentration of total toxic hydrocarbons present in the mussel tissue (Widdows *et al.*, 1995) and the aromatic UCM burden determined by Wraige (1997). Table 1.4 summarises the data, which indicates a strong correlation ($r^2 = 0.99$, $n = 3$) between aromatic UCM concentration and total toxic hydrocarbons; albeit for a very small dataset.

Table 1.4. Concentrations of 'total toxic aromatic hydrocarbons' (Widdows *et al.*, 1995) determined by HPLC-UV analysis compared to 'aromatic UCM hydrocarbons' (Wraige, 1997) determined by GC-MS in mussels (*Mytilus edulis*) from impacted sites in the UK.

Sample site	Total toxic hydrocarbons ($\mu\text{g g}^{-1}$)	Aromatic UCM hydrocarbons ($\mu\text{g g}^{-1}$)
Teesmouth (UK)	77.7	83-94
Whitby (UK)	390	365-496
Cleethorpes (UK)	124.9	102-136

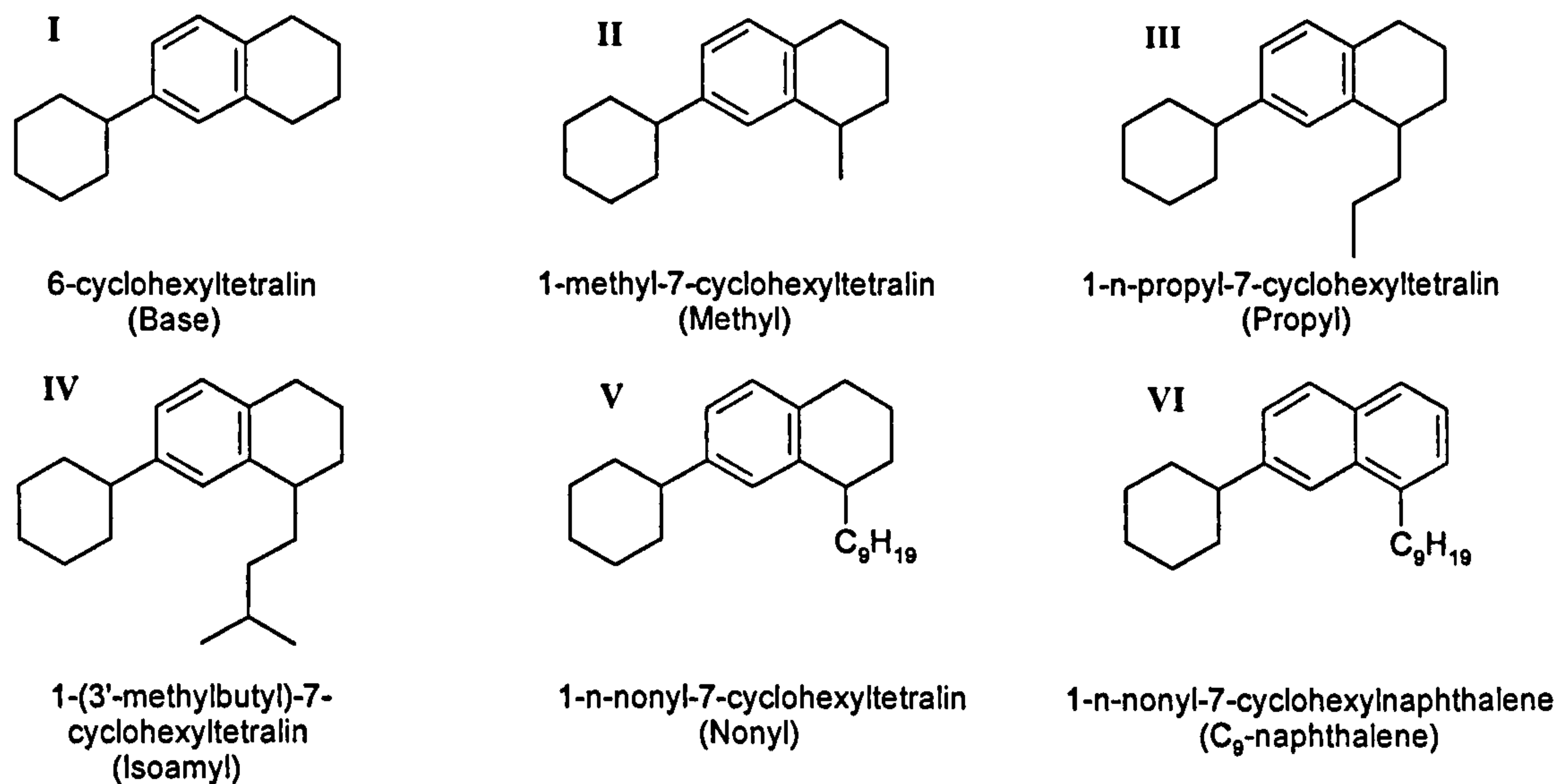


Figure 1.4. Synthetic alkyltetralins (I-V) and alkylnaphthalene (VI) suggested to contain the structural features of some aromatic UCM components. Compounds I-III were synthesised by Smith (2002) and compounds IV-VI by Sturt (2000). All were purified by HPLC by the present author as described in Chapter 5.

Rowland *et al.* (2001) and Smith (2002) possibly provided the first studies that directly monitored the toxic responses elicited by aromatic UCMs. Mussels (*M. edulis*) were exposed to a monoaromatic UCM, isolated from Gullfaks (North Sea) crude oil. An increase in monoaromatic UCM body burden was accompanied by a decrease in the mussel feeding rate. Mussels accumulated $90 \mu\text{g g}^{-1}$ wet weight tissue (equivalent to $350 \mu\text{g g}^{-1}$ dry weight) of the monoaromatic UCM, which caused a 40% decrease in the feeding rate compared with control populations. Similar studies were conducted using synthetic dialkyltetralins thought to be representative components of monoaromatic UCMs based on structures for 'average' monoaromatic UCM hydrocarbons proposed by Thomas (1995)² (Figure 1.4; Wraige, 1997; Smith *et al.*, 2001; Smith, 2002). Of these, 6-cyclohexyltetralin (I) produced a 50% reduction in feeding rate and 7-cyclohexyl-1-methyltetralin caused a 70% reduction in feeding rate in 24 hours. These hydrocarbons (Figure 1.4) appeared to be at least as toxic to mussels as the monoaromatic UCM isolated from Gullfaks crude oil.

² The reasons behind the proposition of these structures are reviewed in Section 1.5.

Recently, Donkin *et al.* (2003) extracted the toxicants from contaminated mussels (*Mytilus edulis*) collected from impacted sites around the UK. The mussels chosen for study had low SfG (Widdows *et al.*, 1995). The extracts were then fractionated using open column chromatography and HPLC. The use of a mini-mussel assay allowed the effects of each of the isolates on mussels to be monitored for toxicological impacts. The greatest reduction in feeding rate was observed in mussels exposed to a HPLC fraction dominated by 'monoaromatic' hydrocarbons, appearing as a UCM when examined by GC-MS.

Hydrocarbons often exert their toxicity to mussels and similar organisms through a common mode of action, usually referred to as non-specific narcosis (McCarty, 1987a; Verhaar *et al.*, 1992). Thus, the toxicity of a mixture of compounds acting by the same mechanism is that of concentration addition (Deneer *et al.*, 1988; Peterson, 1994). Compounds present below their individual 'no-toxic-effect' concentrations contribute to the joint toxicity of the mixture when the compounds act through the same toxic mechanism. This 'additive' effect helps to explain the observed ecotoxicological effects of aromatic and monoaromatic UCMs, which contain thousands of hydrocarbons each probably at a very low concentration within the mixture.

1.5 Composition of UCMs and characterisation of their components

If some of the components of UCMs could be identified, such information might allow a partial explanation of the additive toxic effects. Gas chromatography (GC) combined with mass spectrometry (MS) is the most widely used analytical technique employed in the structural determination of crude oil hydrocarbons. Fractionation of crude oils and their subsequent analysis by GC-MS have led to many compounds (both aromatic and aliphatic) being characterised. Initially, UCMs observed in the gas chromatograms were considered

rather a hindrance to accurately measuring the resolved components, particularly if the resolved component was small relative to the UCM (Killops and Readman, 1985; Farrington *et al.*, 1988). The complexity of UCMs has, until the recent availability of certified reference material, rendered them difficult to quantify and virtually impossible to resolve chromatographically. This has prevented identification of a substantial proportion of UCM petroleum hydrocarbons (Gough and Rowland, 1990; Ziolli and Jardim, 2003). Analyses of aromatic UCMs using standard analytical techniques are usually only capable of confirming the 'aromatic' nature of the components (Jones *et al.*, 1983). However, scientists have begun attempts to determine the detailed composition of UCMs, with some success. Initial studies, focusing on aliphatic UCMs isolated from crude oils, indicated that they contained large proportions of alkyl substituted 'T-branched' alkanes (Gough and Rowland, 1990). The techniques used to study aliphatic UCMs have been documented and reviewed extensively in other work (*e.g.* Gough, 1989; Gough and Rowland, 1990; Killops and Al-Juboori, 1990; Revil, 1992; Thomas, 1995; Wraige, 1997; Warton, 1999; Smith, 2002) and will not be discussed herein.

The composition of aromatic UCMs has only come under scrutiny in more recent years, and is possibly even more important owing to the observed toxicity of aromatic, and in particular monoaromatic, UCMs (*e.g.* Rowland *et al.*, 2001; Donkin *et al.*, 2003). UCMs have been fractionated to generate class specific isolates (*e.g.* 'aromatic' or 'monoaromatic'), all still exhibiting a UCM when analysed by standard GC methods. The sub-fractions have often appeared almost as complex as the 'parent' UCM (*e.g.* Killops and Readman 1985; Wraige, 1997; Smith 2002). Smith (2002) and Wraige (1997) both provide comprehensive reviews of the attempts and results of much of the work regarding characterisation of aromatic UCMs. Briefly, Killops and Readman (1985) and Wraige (1997) isolated 'aromatic' UCMs using high performance liquid chromatography (HPLC). In both cases the UCMs appear to be principally 'monoaromatic' in nature (~80% as

determined by GC and HPLC). The identification of branched chain alkanes as possible aliphatic UCM components, led to the suggestion that branched chain alkylated aromatics might also be abundant components of aromatic 'humps' (Jones *et al.*, 1983). Killops and Al-Juboori (1990) studied the bulk composition of an 'aromatic' UCM using proton (^1H) and carbon (^{13}C) NMR spectroscopy, infra-red (IR) spectroscopy, ultra-violet (UV) spectroscopy, elemental analysis, electron impact (EI)-MS and chemical ionisation (CI)-MS. The data indicated the UCM was composed of aromatic hydrocarbons containing large proportions of alkyl substituents. Other studies of 'aromatic' UCMs (Farrington *et al.*, 1982; Douabul and Al-Shiwafi, 1998; Revill, 1992) also found evidence for large proportions of mono- and di-aromatic hydrocarbons. These were in fact also highly aliphatic in nature, possibly containing cyclo-aliphatic constituents (*e.g.* Warton, 1999). Revill *et al.* (1992) and Warton (1999) suggest that the data were consistent with the presence of alkylated naphthenoaromatic hydrocarbons. As both branched alkanes and an alkyl aromatic hydrocarbon exhibited significant resistance to biodegradation, it was suggested that they were sufficiently resistant to weathering processes to be considered as candidate UCM components (Gough, 1989; present study).

Warton *et al.* (1999) and Thomas (1995) also used chemical oxidation techniques in conjunction with the bulk analytical techniques described above, to try to further characterise 'aromatic' and 'monoaromatic' UCMs. This allowed partial structural elucidation of some UCM hydrocarbons by '*retro*-structural analysis' back to the unoxidised UCM compounds. Thomas (1995) used ruthenium tetroxide to oxidise a hydrotreated heavy cycle oil. GC-MS analysis revealed the presence of α,ω -dicarboxylic acids and branched dicarboxylic acids as well as monocarboxylic acids in the products, indicating that substituted tetralins (tetrahydronaphthalenes) might be UCM components. The study allowed the proposition of an 'average' aromatic UCM hydrocarbon structure (Figure 1.5). The observations reported by Warton (1999) and Warton *et al.* (1999)

indicated that components of a 'monoaromatic' UCM isolated from Leatherjacket-1 crude oil (Gippsland Basin, Australia) contained straight, branched and isoprenoidal side chains as well as cyclohexyl groups and bicyclic components attached directly to the aromatic ring system. These compounds were suggested to include alkylbenzenes, toluenes and xylenes, as well as alkyltetralins and alkylindans, thus confirming the findings of Thomas (1995). Further support for the proposal of these compounds as 'monoaromatic' UCM components was provided by previous reports. These highlighted the occurrence of alkylated aromatic hydrocarbons (*e.g.* Dutta and Harayama, 2001) and in particular highly alkylated tetralins in crude oils (Williams *et al.*, 1988; Forster *et al.*, 1989; Alexander *et al.*, 1992).

To better resolve UCMs however, it became clear that a more powerful analytical technique capable of resolving individual components and compound classes was required. Gas chromatography x gas chromatography (GCxGC) permits the chromatographic separation of mixtures of compounds simultaneously on two different stationary phases. The system provided a method of resolving a mixture in two dimensions, but was initially used in 'fingerprinting' studies (*e.g.* Frysinger *et al.*, 2003) until a suitably specific detector was available. In the last few years, time-of-flight mass spectrometers (ToF-MS), have proven sufficiently fast to be coupled to GCxGC systems and presently GCxGC-ToF-MS provides a powerful technique for the separation of complex mixtures combined with a method for characterising the resolved compounds. A detailed review of GCxGC and GCxGC-ToF-MS analysis and its application to UCMs is presented in Chapter 4.

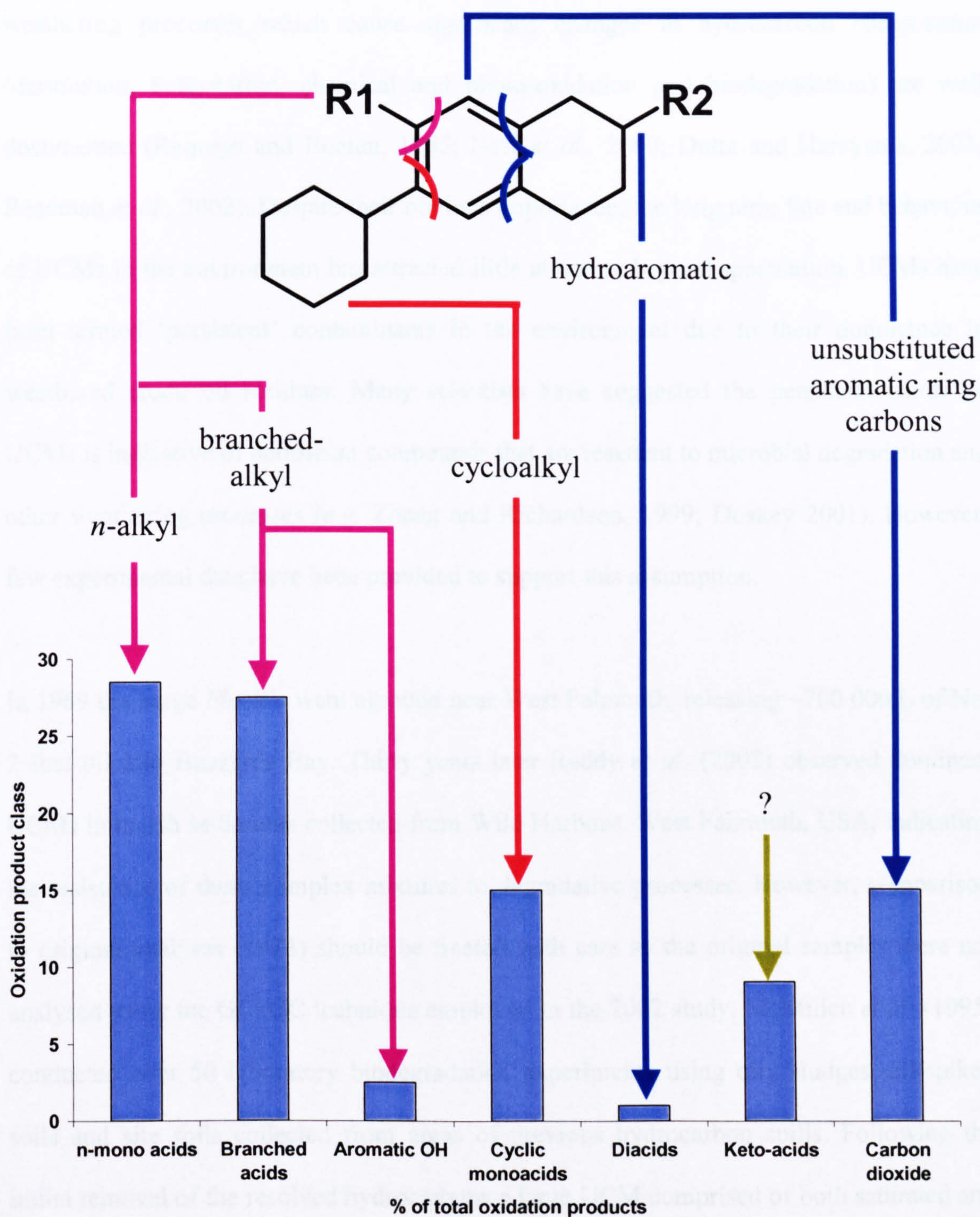


Figure 1.5. An 'average' aromatic UCM hydrocarbon as proposed by Thomas (1995) on the basis of oxidative characterisation of a heavy cycle oil.

1.6 Environmental fate and transport of UCMs

The environmental fate of fresh crude oils has been studied extensively. Many of the weathering processes which cause significant changes in hydrocarbon composition (dissolution, evaporation, chemical and photo-oxidation and biodegradation) are well documented (Requejo and Boehm, 1985; Neff *et al.*, 2000; Dutta and Harayama, 2001; Readman *et al.*, 2002). Despite their obvious importance, the long term fate and behaviour of UCMs in the environment has attracted little attention beyond speculation. UCMs have been termed 'persistent' contaminants in the environment due to their dominance in weathered crude oil residues. Many scientists have suggested the persistent nature of UCMs is indicative of petroleum compounds that are resistant to microbial degradation and other weathering processes (*e.g.* Zheng and Richardson, 1999; Doskey 2001). However, few experimental data have been provided to support this assumption.

In 1969 the barge *Florida* went aground near West Falmouth, releasing ~700 000 L of No. 2 fuel oil into Buzzards Bay. Thirty years later Reddy *et al.* (2002) observed dominant UCMs in marsh sediments collected from Wild Harbour, West Falmouth, USA, indicating the resistance of these complex mixtures to degradative processes. However, comparison to original analyses (1973) should be treated with care as the original samples were not analysed using the GCxGC technique employed in the 2002 study. McMillen *et al.* (1995) conducted over 50 laboratory biodegradation experiments using oily sludges, oil-spiked soils and site soils collected from areas of previous hydrocarbon spills. Following the initial removal of the resolved hydrocarbons a large UCM comprised of both saturated and aromatic compounds remained. Continued exposure resulted in much greater biodegradation of the saturated UCM fraction (73%) compared with the aromatic UCM fraction (36%). Supporting this, Aceves and Grimalt (1993a) report degradation of the aliphatic UCM in aerosol samples stored in a refrigerator for a period of 2 years, but observed no degradation of the aromatic UCM in that time. The sources and physical-

chemical properties of petroleum hydrocarbons are known largely to control their transport and fate in the marine environment (Gogou *et al.*, 2000). However, the complex nature of UCMs makes determining these properties extremely challenging, hence an understanding of their fate in the environment is difficult to establish. The most important weathering processes include 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). The effects of these processes on aromatic UCMs must be studied to achieve a comprehensive understanding of their environmental impact.

1.7 This study

Owing to their persistent nature and widespread occurrence in the environment and the apparent toxicity of monoaromatic UCMs towards certain aquatic organisms, UCMs have sparked considerable interest in the scientific and public community, as exemplified in a recent article in *New Scientist* magazine (Jones, 2003). In addition, a number of legislative and governmental organisations have begun to acknowledge the occurrence and potential hazardous effects of petroleum derived UCMs in the environment and even to human health (*e.g.* Environment Agency, 2003)

Thus far however, little attention has been paid to the environmental fate of these compounds, and no one has been fully able to analytically characterise the component hydrocarbons. The present study aims to address these omissions in the knowledge of unresolved complex mixtures of petroleum derived hydrocarbons in the environment.

Chapter 1 has reviewed the existing literature for the origins and occurrence of aromatic UCMs. The attempts to resolve and characterise component hydrocarbons from UCMs

have been documented. Evidence for the environmental impacts of aromatic and monoaromatic UCMs was presented and discussed, and the lack of knowledge regarding their environmental fate highlighted.

Chapter 2 aims to provide information about the behaviour and fate of UCMs in the environment. The persistence of aromatic UCMs in the environment was determined by measuring the resistance of an aromatic UCM isolated from biodegraded Tia Juana Pesado (Venezuela) crude oil to biodegradation by the known hydrocarbon degrading bacterium *Pseudomonas fluorescens*. A suite of alkylcyclohexyltetralins (Figure 1.4., I-V) and an alkylcyclohexylnaphthalene (Figure 1.4, VI) were exposed to the same micro-organisms to determine the resistance of these hydrocarbons to biodegradation. This provided compound specific comparisons to the aromatic UCM data and further evidence for the candidacy of compounds I-V as 'model' monoaromatic UCM components. The 'model' monoaromatic compounds were additionally exposed to a natural consortium of bacteria collected from an impacted site. This was intended to provide a more environmentally realistic view of the behaviour of these hydrocarbons. These studies also included the use of single compound (hexadecane) and complex mixture (crude oil) co-substrates allowing the resistance of the alkylcyclohexyltetralins to be measured relative to known resistant crude oil components.

Chapter 3 investigates the water solubility characteristics of a pure hydrocarbon and of both an 'aromatic' and a 'monoaromatic' UCM, isolated from Tia Juana Pesado crude oil, by generating aqueous solutions of the test substrates. The water solubility of a compound or mixture is directly related to the physico-chemical properties of the constituent hydrocarbons. Solubility is a dominant parameter in determining the bioavailability of hydrocarbons to aquatic organisms and therefore of ecotoxicological effects. The water solubility of 6-cyclohexyltetralin (Figure 1.4, I) was determined, so that the dissolution behaviour of a single compound, having structural characteristics similar to those

tentatively assigned to the molecular composition of aromatic UCM components, could also be studied in relation to the isolated UCMs. Study of this compound also provided calibration of the method, based upon data reported by Smith (2002).

Chapter 4 outlines a potential method for the increased resolution and characterisation of UCMs. The aqueous solution of 'monoaromatic' UCM generated by methods described in Chapter 3, was analysed using GCxGC-ToF-MS, employing apolar 1st dimension and polar 2nd dimension GC columns. Mass spectra of hundreds of the now resolved UCM compounds were compared with published library spectra and Mass Frontier™ software was used to help validate the proposed structures by generating fragmentation pathways from the parent compound to the major ions observed in the mass spectra. As a result 16 compounds were characterised in detail, with the identification of hundreds of other components as isomers and homologues being inferred from the data.

Chapter 5 presents the experimental details relevant to Chapters 2 – 4

Chapter 6 provides a summary of the major findings of this study, and suggests possible areas for future scientific research.

Chapter 2

Biodegradation experiments

An *n*-alkane (*n*-pentacosane, *n*-C₂₅), an 'aromatic' unresolved complex mixture (UCM) of hydrocarbons from a biodegraded crude oil, and a series of alkylcyclohexyltetralins previously proposed to be structurally representative of some UCM compounds, were exposed to the common aerobic bacterium, *Pseudomonas fluorescens* (Texaco), in the laboratory at room temperature for up to 50 days. These conditions led to the rapid oxidation of the *n*-alkane (9 days) whereas the synthetic aromatic compounds proved to be significantly resistant to biotransformation, as did the aromatic UCM hydrocarbons from the crude oil.

In the second part of the study, a series of the alkylcyclohexyltetralins with a fresh North Sea crude oil or excess *n*-hexadecane as co-substrates were exposed to a natural microbial population in seawater. Rapid oxidation of *n*-alkanes and the other major resolved components of North Sea crude oil was observed. Some of the alkylcyclohexyltetralins proved to be quite resistant to biotransformation, suggesting they might accumulate as biodegraded oil residues in the environment and may indeed be suitable candidates for UCM compounds.

2.1 Introduction

Hydrocarbons originally from crude oil are found in many environmental compartments, including seawater, soils, ground water, rivers and sediments (Environment Agency, 1998, National Research Council, 2003). Ultimately this material accumulates in the world's oceans and marine sediments, which can be thought of as the major sinks. Nevertheless, the absence of gross pollution on the sea surface and shores indicates that considerable destruction of oil takes place. This means that there must be processes at work in the environment, which facilitate the removal of pollutant petroleum hydrocarbons. Immediately after an oil spill, physical weathering (such as evaporation, dispersion and dissolution) alter the chemical, physical and toxicological properties of the spilled oil (*e.g.* Wang *et al.*, 1994; Clark, 2001; National Research Council, 2003). After initial weathering, microbial degradation is the principal route for removal of oil from the environment (Cerniglia, 1992; Wang *et al.*, 1995). An understanding of this process is essential if the fate of crude oil in the environment is to be determined.

Persistence in the environment is an important consideration in assessing the toxicity of a compound or mixture of compounds. The persistence of hydrocarbons in the environment is dependent upon their resistance to the common removal processes that occur in these ecosystems. However, the particular conditions and characteristics of an environment into which hydrocarbons are introduced will also affect the degree of efficiency of the removal processes (Alexander, 1999). The biodegradation of both individual hydrocarbons and hydrocarbon mixtures (*e.g.* crude oil) in the laboratory and in the field has been studied since the 1940's (*e.g.* ZoBell, 1945; McKenzie and Hughes, 1976; Atlas, 1981; Fedorak and Westlake, 1981; Jones *et al.*, 1983; Connan, 1984; Bayona *et al.*, 1986; Rowland *et al.*, 1986; Haigler *et al.*, 1992; Peters and Moldowan, 1993; Bragg *et al.*, 1994; Fayad and Overton, 1995; Heath *et al.*, 1997; Wang *et al.*, 1998; Olson *et al.*, 1999; Kanaly and Harayama, 2000; Leblond *et al.*, 2001). In the case of extensive or prolonged exposure to

microbial activity, most hydrocarbons eventually undergo some degree of biodegradation. The complete or ultimate degradation of hydrocarbons results in the formation of CO₂ – so called mineralisation (Alexander, 1999). However, complete microbial degradation does not always occur. In these cases compounds are transformed, rather than metabolised, into compounds such as acids and ketones.

2.1.1 Biodegradation of crude oils

Oil-contaminated environmental samples commonly contain mixtures of hydrocarbons. These mixtures, which can vary in their complexity, may contain hydrocarbons from a single (*e.g.* petroleum) or multiple sources. Although numerous studies have demonstrated the biodegradation of individual hydrocarbons, Haigler *et al.* (1992) suggest that a comprehensive understanding of the microbial degradation of complex mixtures of hydrocarbons has not been reached. The degradation of mixtures of hydrocarbons often proceeds in a different way to that of the individual component hydrocarbons (Raymond *et al.*, 1971; Atlas, 1981; Atlas and Bartha, 1992; Beckles *et al.*, 1998; Olson *et al.*, 1999).

Olson *et al.* (1999) investigated the biodegradability of a suite of aliphatic and aromatic compounds both individually, and as composite mixtures. It was observed that the degradability of the compounds increased when they were present as mixtures rather than as individual substrates. This phenomenon is known as co-oxidation or co-metabolism, and can facilitate the oxidation and degradation of non-growth substrates by microbes. Co-oxidation/co-metabolism is defined as actively growing microbes oxidising a compound, but not utilising either material (*e.g.* carbon) or energy derived from the oxidation. Crude oils are possibly the most complex mixtures of organic chemicals on the planet, comprising hundreds of thousands of hydrocarbons (Environment Agency, 1998; Alexander, 1999; OSPAR Commission, 2000; Clark, 2001; National Research Council, 2003). Therefore, the

biodegradation of crude oils may be expected to differ significantly from that of individual hydrocarbons due to the occurrence of co-oxidation processes. Co-oxidation processes appear to play an important role in natural environments where mixed groups of bacteria and mixed substrates, such as petroleum, co-exist (*e.g.* Volkman *et al.*, 1984; Bayona *et al.*, 1986; Atlas and Bartha, 1992; Alexander, 1999). Thus, the petroleum degrading potential of an ecosystem should be estimated using a complex mixture of hydrocarbons, typical of those found in nature, as some of the hydrocarbons may influence the metabolism of others (*e.g.* Cooney *et al.*, 1985).

The biodegradation of crude oils is not a phenomenon restricted to hydrocarbons released into the environment. The process occurs naturally in oil wells and reservoirs and can influence the compositional differences observed in crude oils drilled from different oil fields (Volkman *et al.*, 1984; Rowland *et al.*, 1986). However, the biodegradation of petroleum hydrocarbons in such conditions is now thought to be the result of anaerobic metabolism (*e.g.* Connan *et al.*, 1997; Taylor *et al.*, 2001; Larter *et al.*, 2003). This is a major contrast to oil biodegradation in the wider environment which is usually aerobic.

Any crude oils released into the environment will exhibit unique characteristics; thus, crude oil spills have to be assessed on an individual basis, as the composition will vary in each case. The biodegradation of fresh crude oil will be considerably different to that of a previously weathered crude oil. A fresh crude oil will undergo significant alteration in a relatively short period of time following release into the environment. The relative distributions of saturated, aromatic and polar compounds change as biodegradation progresses (*e.g.* McMillen *et al.*, 1995). Although metabolism of all hydrocarbon types is generally simultaneous, the rates of degradation can vary significantly (Fedorak and Westlake, 1981; Jones *et al.*, 1983; Alexander, 1999; Olson *et al.*, 1999). In contrast, weathered crude oils, which have previously been exposed to degradative processes, are

dominated by recalcitrant hydrocarbons which exhibit greater resistance to further physical, chemical and biological processes. Thus, the fate of the component hydrocarbons in a mixture of the complexity of petroleum is extremely complicated and its degradation requires a diverse range of bacterial taxa (Atlas, 1981).

The degradation characteristics of crude oil constituents have been investigated in detail both in the field and in the laboratory (*e.g.* McKenzie and Hughes, 1976; Atlas, 1981; Fedorak and Westlake, 1981; Jones *et al.*, 1983; Connan, 1984; Bayona *et al.*, 1986; Rowland *et al.*, 1986; Atlas and Bartha, 1992; Gough *et al.*, 1992; Peters and Moldowan, 1993; Bragg *et al.*, 1994; Wang *et al.*, 1994; Geerdink *et al.*, 1996; Wang *et al.*, 1998; Olson *et al.*, 1999; Jensen *et al.*, 2000; Leblond *et al.*, 2001; Watson *et al.*, 2002). The general pattern of susceptibility to aerobic biodegradation is: *n*-alkanes degraded before branched alkanes (*e.g.* isoprenoids), smaller aromatic molecules degraded before larger ones, and alkylated polycyclic aromatic hydrocarbons (PAHs) degraded more slowly than the parent compounds (Huesemann, 1995; Garrett *et al.*, 1999). Alkyl branching or substitution within each structural class of hydrocarbons generally increases resistance to microbial attack. The degradation of alkylated components of crude oil (*e.g.* alkyl PAHs) also strongly depends on the number, position, and type of the substituents (*e.g.* Bayona *et al.*, 1986; Garrett *et al.*, 1999). Such relationships have been observed in many studies investigating crude oil, giving a characteristic pattern of biodegradation (*e.g.* Solanas *et al.*, 1984; Novak *et al.*, 1995; McMillen *et al.*, 1995; Leblond *et al.*, 2001).

Biodegradation of hydrocarbons decreases with an increasing number of substituents (*e.g.* Rowland *et al.*, 1986; Prince *et al.*, 1999; Holder *et al.*, 1999; Leblond *et al.*, 2001). For example, Solanas *et al.* (1984) observed a clear preferential degradation of the less substituted aromatics in the C₁-C₄ alkylnaphthalenes. The order of resistance was C₀ < C₁ < C₂ < C_n (where *n* = carbon number of alkyl substituents on an aromatic nucleus). The

presence of one or more methyl groups on an aromatic ring decreases the number of ring positions which can be oxidised (Budzinski *et al.*, 1998; Leblond *et al.*, 2001).

The susceptibility of hydrocarbons to biodegradation also depends on the position of any alkyl substituents (*e.g.* Prince *et al.*, 1999; Holder *et al.*, 1999; Leblond *et al.*, 2001). For example, biodegradation of methyl substituted PAH was found to depend on the position of the methyl substituent, with compounds containing unsubstituted α and β adjacent positions being more readily degraded (Alexander, 1999). For compounds with long alkyl chains (*e.g.* linear alkyl benzenes), biodegradation usually proceeds more rapidly if the alkyl group is joined to the aromatic ring at the last position (ω position) and not an α carbon of the hydrocarbon chain (Figure 2.1; Alexander, 1999; Swisher, 1987).

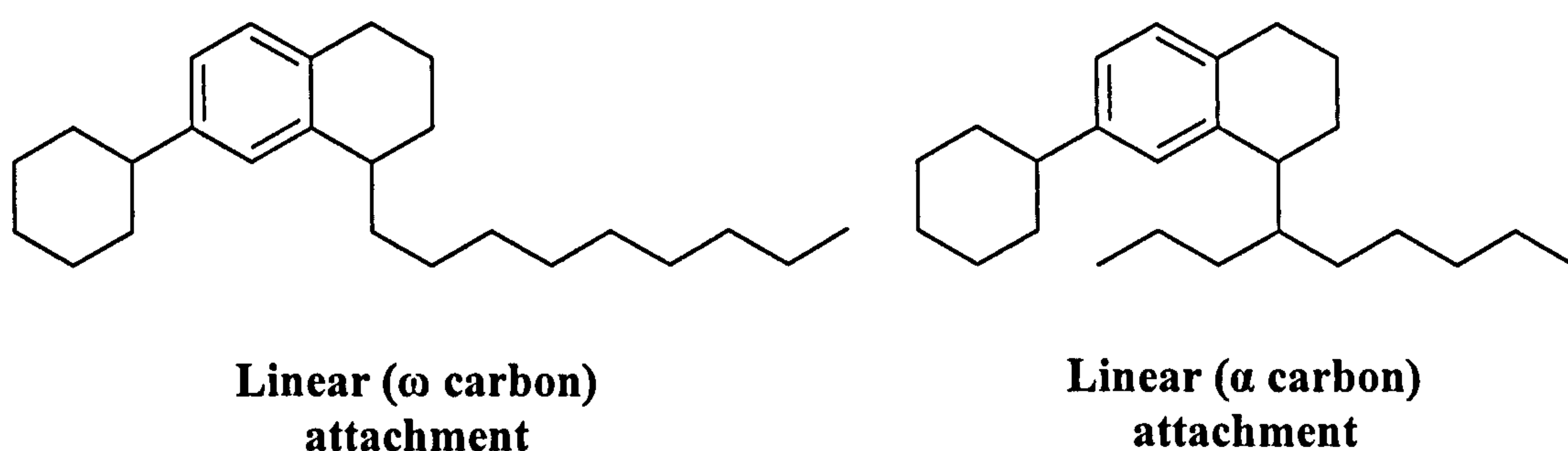


Figure 2.1. Comparison of different C₉ alkyl substituents on the same compound. It is likely that the branched compound would be more resistant to biodegradation because the alkyl moiety is linked near the centre of the chain rather than at the end like the linear compound.

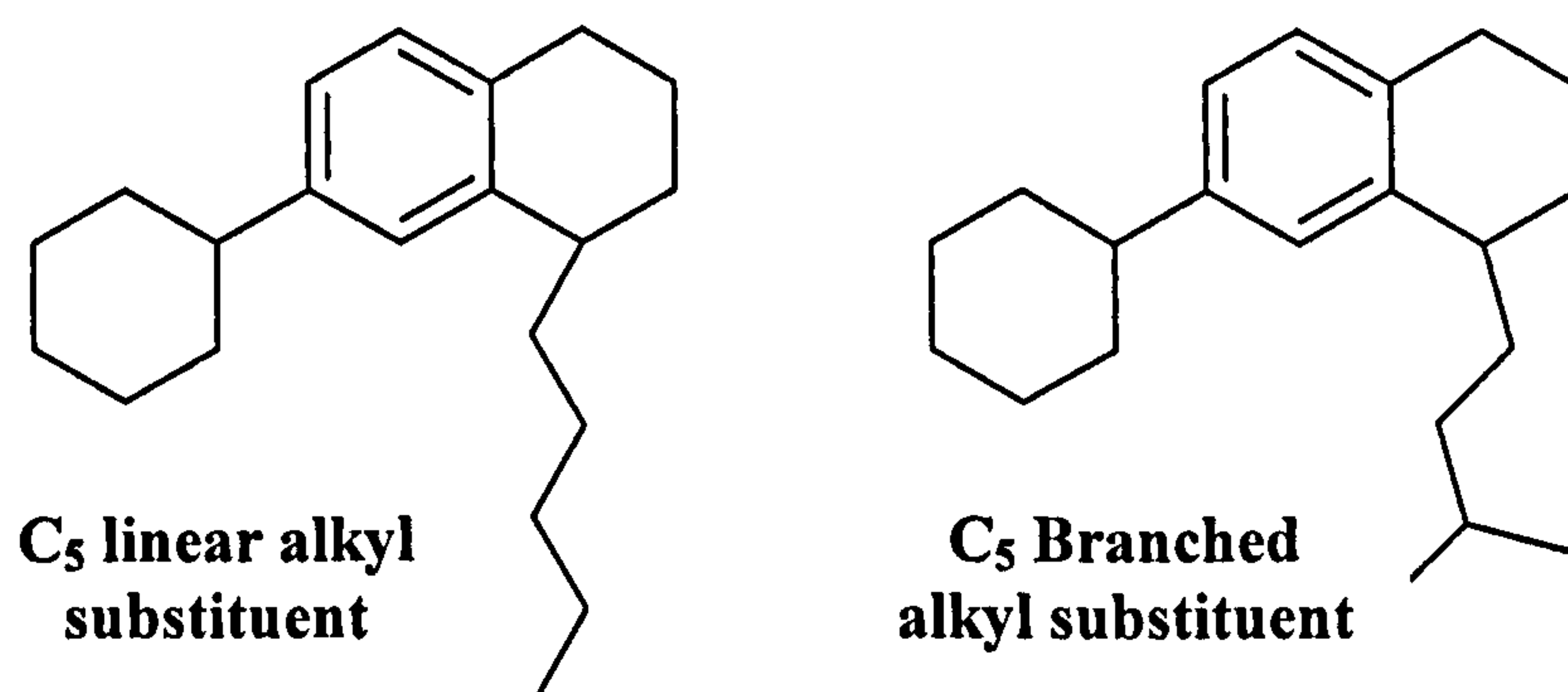


Figure 2.2. Comparison of different C₅ alkyl substituents on the same compound. It is likely that the branched compound would be more resistant to biodegradation as β -oxidation of the alkyl moiety, which removes two carbon atoms at a time, cannot proceed because of the isopropyl group.

The type of substituents present on a hydrocarbon can also significantly affect biodegradability. For example, Alexander (1999) reports that aromatic compounds with branched alkyl substituents (*e.g.* alkylbenzene sulfonates, ABS used as surfactants), are likely to be more resistant to microbial degradation than those with linear substituents (*e.g.* Figure 2.2). The presence of branching on an alkyl substituent will prevent β -oxidation occurring as a C_2 unit cannot be removed.

Other studies investigating the biodegradation characteristics of crude oil hydrocarbons have highlighted the preferential degradation of particular hydrocarbon isomers (*e.g.* Volkman *et al.*, 1984; Rowland *et al.*, 1986; Peters and Moldowan, 1993; Fayad and Overton, 1995; Bost *et al.* 2001; Watson *et al.*, 2002). For example, Volkman *et al.* (1984), observed that biodegradation of alkylnaphthalenes was dependent on the position of the alkyl substituents. Those alkylnaphthalenes containing adjacent substituents were the most resistant. Furthermore, isomers exhibiting β -substituents were more rapidly degraded than those with α -substituents.

2.1.2 Formation and biodegradability of unresolved complex mixtures (UCMs)

When crude oil is subjected to severe biodegradation, most GC resolvable compounds are removed. Gas chromatograms of biodegraded crude oil are dominated by a 'hump' or unresolved complex mixture (UCM) of hydrocarbons containing few identifiable compounds (*e.g.* Killops and Al-Juboori, 1990; Gough *et al.*, 1992; Button *et al.*, 1992; Wang *et al.*, 1994; McMillen *et al.*, 1995; Dutta and Harayama, 2000; Frysinger *et al.*, 2003). This enrichment of the unresolved complex mixture (UCM) through microbial degradation has been noted in recent sediments affected by oil spills (*e.g.* Button *et al.*, 1992; Wang *et al.*, 1994; McMillen *et al.*, 1995) and in laboratory degraded crude oils (*e.g.* Jones *et al.*, 1983; Killops and Al-Juboori, 1990). Analysis indicated that both aliphatic

and aromatic UCMs were enriched during this process (Volkman *et al.*, 1984), with the aromatic UCM becoming pronounced more slowly than the aliphatic UCM (McMillen *et al.*, 1995). Weathered petroleum has been observed to undergo further biodegradation, with more aliphatic and aromatic compounds being removed. However, a mixture of recalcitrant aliphatic and aromatic material (UCM) was still present at the end of the study (Novak *et al.*, 1995). Gough *et al.* (1992) observed an ~20% removal of an aliphatic UCM (isolated from a lubricating oil) by *Pseudomonas fluorescens* over a period of 25 days. Hence, crude oil UCMs are thought to comprise both aliphatic and aromatic compounds, which are relatively inert to microbial degradation.

Meredith *et al.* (2000), reported the presence of *n*-alkanoic and hopanoic acids in oils and an unresolved complex mixture of branched and cyclic components in the gas chromatogram of carboxylic acid fractions from reservoir degraded oils. It was found that this acidic UCM appeared to increase in concentration with an increase in the extent of hydrocarbon biodegradation. This indicates that the products of microbial degradation of crude oil hydrocarbons may include unresolved complex mixtures of acids, which appear to be much less readily degradable than their parent compounds, thus contributing to the observed increase in the acidic UCM. This degradation may result in the production of the observed hopanoic acids in the acidic UCM. Whilst aromatic steroids are thought to be extremely resistant to microbial degradation, analysis has shown that even some of these compounds may be susceptible to biodegradation (Volkman *et al.*, 1984; Peters and Moldowan, 1993).

2.1.3 Environmental factors affecting biodegradation

The rate and extent of petroleum hydrocarbon biodegradation is significantly determined by environmental conditions. Petroleum hydrocarbons released to one environment may

persist indefinitely, whereas under another set of conditions the same hydrocarbons can be completely biodegraded within a few hours or days (Atlas, 1991; ESGOSS, 1994; Environment Agency, 1998; Clark, 2001; National Research Council, 2003). Thus, environmental factors can have a large influence on the fate of released oil. A brief review of the main factors affecting biodegradation of hydrocarbons in the environment is made below.

The persistence of petroleum pollutants will initially depend on the quantity and composition of the hydrocarbon mixture (Atlas, 1991; ESGOSS, 1994; Clark, 2001; National Research Council, 2003). The quantity of oil released into an environment will determine how large the impact will be on the ecosystem, with small releases being removed quite rapidly (Fayad and Overton 1995; Environment Agency, 1998). The composition of the hydrocarbon mixture will also affect the rate of biodegradation. In a fresh crude oil simple aliphatic and aromatic compounds will undergo a rapid rate of removal by bacteria. However, severely degraded crude oils, where the majority of the compounds will be significantly resistant to biodegradation, will remain in the environment for much greater lengths of time. For example, in January 1993 the *MV Braer* was wrecked off Shetland and shed its load of 84 700 t of naturally (in-reservoir) biodegraded Norwegian Gullfaks crude oil (ESGOSS, 1994). The weathered Gullfaks crude oil cargo was characterised by a low saturated and a high aromatic (especially PAH) hydrocarbon content: material typically exhibiting a significant degree of resistance to degradation processes. High concentrations of oil have been found in fine deepwater sediments, indicating little degradation had occurred within the three years following the spill (Environment Agency, 1998). This is as expected given the in-reservoir degraded nature of the crude oil. The rough weather conditions may have led to high levels of emulsification and adsorption of crude oil to particulate material mixed into the water column.

The input of petroleum hydrocarbons to an ecosystem generally results in a rise in the population of hydrocarbon degrading microorganisms (*e.g.* Atlas, 1981; Leahy and Colwell, 1990). However, previous hydrocarbon inputs to an area or body of water can effect the rate and extent of biodegradation of further crude oil inputs to a system (Cooney *et al.*, 1985; Atlas and Bartha, 1992; Alexander, 1999; National Research Council, 2003). In environments subject to frequent or continuous inputs of petroleum hydrocarbons, the microbial population will be dominated by high numbers of hydrocarbon-utilising bacteria acclimatised to growth on crude oil substrates. Further inputs of petroleum hydrocarbons to previously contaminated environments will be met with increased removal rates compared with pristine environments.

The physical conditions or energy level of an impacted site will affect the biodegradation of hydrocarbons. Physical processes, such as wind and wave action, increase the mixing of hydrocarbons within a water column, leading to the formation of water-in-oil and oil-in-water mixtures and emulsions. These processes generally increase the surface area available for colonisation by hydrocarbon-degrading microorganisms, thereby facilitating biodegradation (*e.g.* Atlas, 1991; Atlas and Bartha, 1992; Stewart *et al.*, 1993; Clark, 2001; National Research Council, 2003). For example, following the *Nakhodka* oil spill in Japan (1997), some oil washed up on beaches above the high tide line and, being out of reach of wave action, was protected under rocks. GC-MS analysis of material collected one year after the spill found it was mostly undegraded (Ohashi *et al.*, 1999).

The temperature of an environment can significantly affect the rate at which microorganisms are able to degrade petroleum hydrocarbons. The rate of microbial degradation generally decreases with decreasing temperature (*e.g.* Leahy and Colwell, 1990; Atlas, 1991; Atlas and Bartha, 1992; Garrett *et al.*, 1999). This is sometimes known as the Q_{10} effect, where the rate of activity falls by half for every 10°C drop in temperature

(Atlas, 1991; Stewart *et al.*, 1993; Garrett *et al.*, 1999; National Research Council, 2003). However, the biodegradation of crude oils is found to follow a similar pattern from tropical to arctic environments, and thus temperature might limit the rate, but not the final extent of biodegradation (Garrett *et al.*, 1999). Temperature will also effect the physical state of the petroleum hydrocarbons. At low temperatures the viscosity of crude oil increases which reduces biodegradation and evaporation of toxic low molecular weight hydrocarbons (Leahy and Colwell, 1990; Atlas and Bartha, 1992; National Research Council, 2003). These compounds then remain in high concentrations at the site of the spill and tend to exhibit increased levels of water solubility compared to other crude oil components. Once dissolved in water these compounds are potentially toxic to the natural microbial community (Atlas and Bartha, 1992; National Research Council, 2003).

The availability of oxygen is essential for the aerobic biodegradation of petroleum hydrocarbons by microorganisms (Cooney *et al.*, 1985; Leahy and Colwell, 1990; Atlas and Bartha, 1992; Alexander, 1999; Wackett and Hershberger, 2001; Clark, 2001; National Research Council, 2003). However, in certain environments such as soils, sediments and groundwaters it can be the limiting factor, with oxygen concentrations in such systems varying according to the soil or sediment type and whether water logging has occurred (Atlas, 1991). For example, Novak *et al.* (1995) observed an increase in the degradation rate of weathered crude oils when oxygen availability was increased. Bacterial activity can also reduce oxygen levels in a system to such a level that further degradation is either extremely slow or does not occur at all. This scenario is characteristic of a large input of substrate (*e.g.* an oil spill) to a system, where microbial activity can increase rapidly, thereby utilising available oxygen faster than it can be replaced by diffusion from the atmosphere (ESGOSS, 1994; Alexander, 1999; Clark, 2001). Whilst anaerobic biodegradation of a wide range of petroleum hydrocarbons does occur (*e.g.* Rueter *et al.*, 1994; Coates *et al.*, 1997; Caldwell *et al.*, 1998; Elshahed *et al.*, 2001; Annweiler *et al.*,

2002), this process proceeds at a much slower rate than aerobic biodegradation (*e.g.* Atlas and Bartha, 1992; Chang *et al.*, 1997). However, Peters and Moldowan (1993) suggest in terms of geological time the difference in rate may not be critical, with the extent of biodegradation being comparable between the two processes and this has recently received considerable support (Wilhelms *et al.*, 2001).

Nutrient availability can also play a key role in the rate of hydrocarbon biodegradation. In most aqueous environments hydrocarbon degradation is limited by sub-optimal levels of bioavailable nutrients such as nitrogen and phosphorus (Atlas and Bartha, 1992; Alexander, 1999; Prince *et al.*, 1999; Head and Swannell, 1999; National Research Council, 2003). The addition of fertilisers to nutrient limited systems significantly increases the degradation rate of hydrocarbons (*e.g.* Bragg *et al.*, 1994; Hoff *et al.*, 1995; Abbott *et al.*, 1999; Ohashi *et al.*, 1999; Swannell *et al.*, 1999). For example, Prince *et al.* (1999) observed that nutrient addition to an impacted arctic shoreline (Spitzbergen) was followed by an increase in microbial activity (oxygen consumption, carbon dioxide evolution, and an increase in biomass), which resulted in the biodegradation of oil. Watson *et al.* (2002) concluded that biodegradation of crude oil in seawater, without nutrient addition, leads only to limited biodegradation.

A comparative study on the biodegradation of petroleum hydrocarbons found that a consortium of marine bacteria was more efficient at degrading branched alkanes and aromatic compounds than a freshwater consortium under the same conditions (Holder *et al.*, 1999). Shiaris (1989) and Mille *et al.* (1991) studied the effect of salinity on the biodegradation of petroleum hydrocarbons. Maximum levels of biodegradation occurred at salt concentrations equivalent to seawater (35 g L⁻¹ NaCl). Salt concentrations either higher or lower than this inhibited degradation. More recently two hydrocarbon degrading consortia isolated from crude oil and mangrove sediments were found to be capable of

degrading petroleum hydrocarbons over a wide range of salinity (0 – 100 g L⁻¹). However, salinities over twice that of normal seawater (35 g L⁻¹) decreased the biodegradation rates (Diaz *et al.*, 2000). Alexander (1999) suggests that high salinity values can affect microbial processes. However, there appears to be no strong argument that typical salinity levels of estuaries and oceans are a major deterrent to biodegradation.

A slower rate of petroleum hydrocarbon biodegradation is generally observed in soil and sedimentary environments. Microbial activity is thought to be constrained by adsorption of hydrocarbons to particulate material, which reduces bioavailability (*e.g.* Young, 1984; Manilal and Alexander, 1991; Atlas and Bartha, 1992; Drake *et al.*, 1995; Alexander, 1999). It has also been suggested that adsorption of hydrocarbons onto soil surfaces and/or their diffusion into soil micropores can cause differences in the patterns of degradation observed in aqueous systems (Drake *et al.*, 1995). Atlas and Bartha (1992) suggest that petroleum spilled on soils is largely adsorbed to particulate material, decreasing its toxicity, but contributing to its persistence.

2.1.4 Mechanisms of hydrocarbon biodegradation

The bacterial oxidation of crude oils requires enzymatic transformation of the hydrocarbon components. Individual bacteria contain thousands of enzymes, many of which are involved in the catabolism of wide range of hydrocarbons (Wackett and Hershberger, 2001). Although particular strains of bacteria use limited numbers and types of organic compounds as growth substrates, different species of bacteria are able to biodegrade different hydrocarbons, and many are able to develop modified enzymes capable of degrading 'new' compounds as substrates. The metabolic pathways of hydrocarbon biodegradation have been reviewed in detail (*e.g.* Gibson and Subramanian, 1984; Swisher,

1987; Alexander, 1999; Wackett and Hershberger, 2001) and are only summarised briefly herein.

The initial oxidative bacterial attack on *n*-alkanes is generally at one end of the hydrocarbon chain (terminus), commonly known as ω -oxidation (Britton, 1984; Swisher, 1987; Atlas and Bartha, 1992; Alexander, 1999). Several pathways may be used (e.g. Figure 2.3, a-c) depending on (i) the particular microorganisms involved, (ii) hydrocarbon chain length, (iii) other structural features of the hydrocarbon, and (iv) the operating conditions. A major pathway proceeds *via* the primary alcohol, yielding the corresponding aldehyde, and the carboxylic acid (Figure 2.3; Swisher, 1987; Atlas and Bartha, 1992; Setti *et al.*, 1993; Alexander, 1999). Although less common, initial attack on an alkane may also occur at a central carbon atom, commonly known as sub-terminal oxidation (Britton, 1984; Swisher, 1987; Atlas and Bartha, 1992). Following the formation of a carboxyl group, β -oxidation continues the biodegradation process (Britton, 1984; Swisher, 1987), shortening the chain by two carbons at a time.

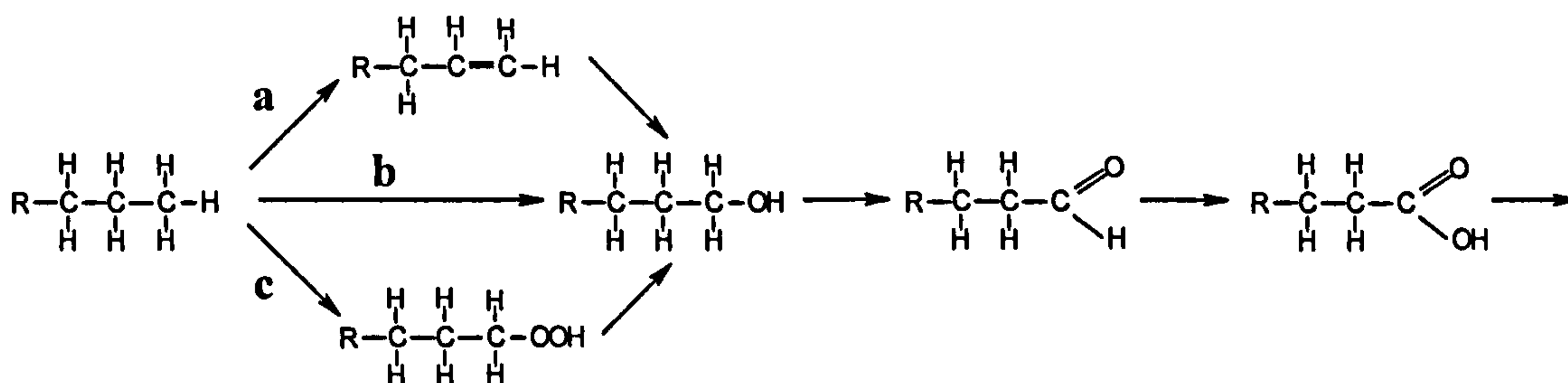


Figure 2.3. ω -oxidation via three pathways (a-c), reproduced from Swisher (1987).

Branching at chain ends can severely hinder initial ω -oxidation and in some cases prevents or hinders normal β -oxidation (Britton 1984; Swisher, 1987). In this case the β -oxidation process can be adapted to nonlinear substrates. When the branching is on the β carbon it becomes blocked to normal β -oxidation and α -oxidation may proceed. β -oxidation resumes

when the next normal β position is unsubstituted. Details of the mechanism of normal α -oxidation have been reviewed by Britton (1984) and Swisher (1987).

Cycloalkanes may also be open to mid-chain attack analogous to the sub-terminal oxidation of linear alkanes (Figure 2.4). For example, a significant number of microbial species can oxidise cyclohexane to cyclohexanol (using monooxygenase) and, *via* dehydrogenation, to cyclohexanone (Trudgill, 1984; Atlas and Bartha, 1992; Wackett and Hershberger, 2001). Complete degradation proceeds with the insertion of an oxygen atom between the carbon bearing the carbonyl oxygen and the adjacent carbon (Baeyer-Villiger-type). After generation of the lactone, the ring is cleaved by hydrolysis of the ester and the open chain carboxylic acid is then mineralised (Figure 2.4; Atlas and Bartha, 1992; Wackett and Hershberger, 2001).

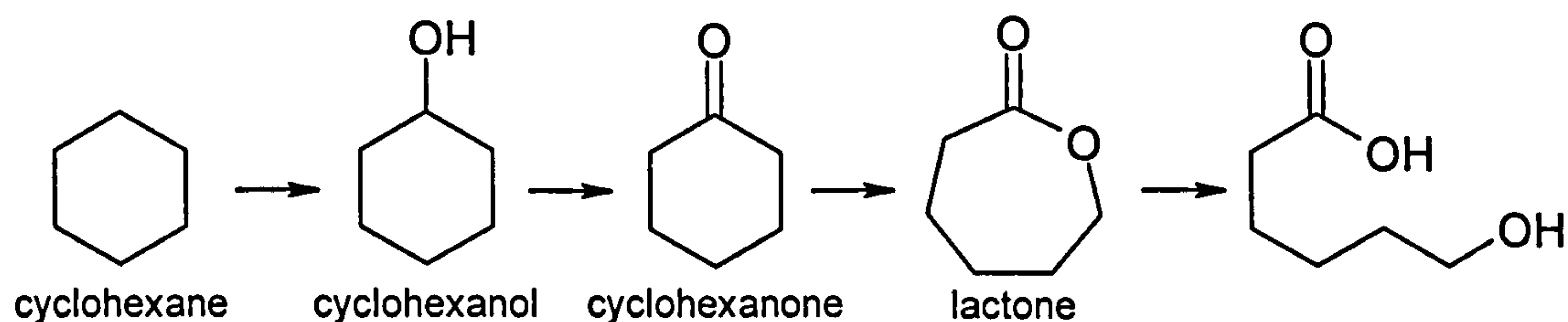
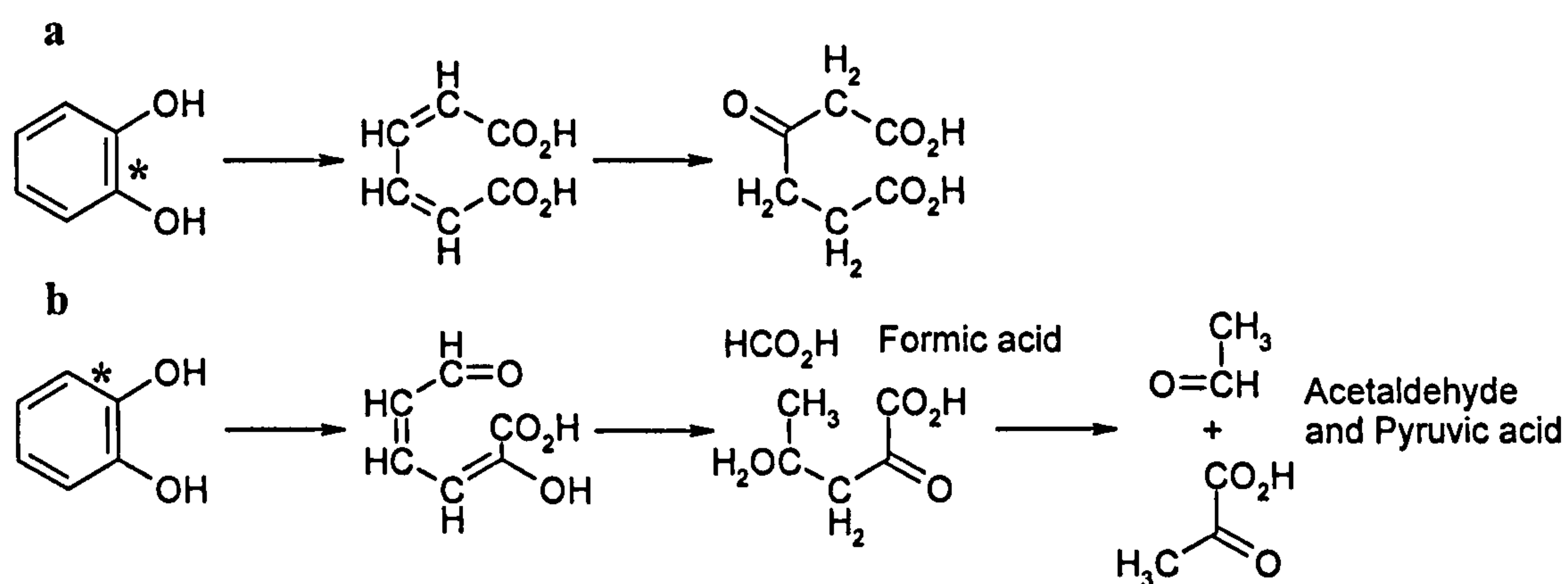


Figure 2.4. Cycloaliphatic compounds are metabolised aerobically *via* hydroxylation and Baeyer-Villiger-type oxygen insertion reactions. Taken from Wackett and Hershberger (2001).

The resonance stability of aromatic ring structures requires metabolic action to overcome higher activation energies than occur during the hydration of non-aromatic double bonds. (Atlas and Bartha, 1992; Wackett and Hershberger, 2001). Two of the common routes for benzene ring degradation are outlined below (Gibson and Subramanian, 1984; Swisher, 1987; Atlas and Bartha, 1992; Iqbal and Mason, 1999). Initially, catechol or a substituted catechol is formed in an enzyme catalysed oxidation with molecular oxygen. The ring is then opened between or adjacent to the two hydroxyl groups. In the first case (*ortho*

cleavage) a dicarboxylic acid is formed which is converted by three successive molecular rearrangements into β -ketoadipic acid. This can then be split by the normal β -oxidation process. In the second, or *meta* pathway, the initial rupture of the ring occurs adjacent to the two hydroxyl groups, leading to the formation of formic acid, acetaldehyde, and pyruvic acid, all of which are common cell metabolites. The *meta* pathway is frequently involved in the biodegradation of substituted catechols (Figure 2.5).

Alkylaromatics can undergo initial oxidation at the end of the alkyl group, with the chain being degraded by the β -oxidation process. Once the oxidation reaches the ring, degradation then proceeds by one of the catechol pathways described in Figure 2.5. However, the initial attack may also be on the ring, particularly when the alkyl chain is



short (e.g. Swisher 1987; Atlas and Bartha, 1992; Cerniglia, 1992).

Figure 2.5. Two of the common routes of aerobic biodegradation of aromatic rings. (a) *ortho* cleavage pathway, (b) *meta* cleavage pathway. Diagram reproduced from Swisher (1987). * Indicates the bond on the aromatic ring where initial microbial attack occurs.

The metabolism of PAHs is similar to that of single-ring benzenoid aromatic hydrocarbons. The degradation of one ring leads to the formation of a catechol on the next ring, where the process is repeated (Wackett and Hershberger, 2001). Microbial attack can occur simultaneously on any of the PAH rings (Alexander, 1999). In the case of

compounds containing alicyclic rings fused to aromatic rings (e.g. tetralin or indan), initial degradation is suggested to proceed *via* the alicyclic moiety, with subsequent degradation of the aromatic ring (Wackett and Hershberger, 2001).

In environments where there is no oxygen present, the anaerobic degradation of hydrocarbons may occur. Instead of using O₂ in the degradation of organic compounds, anaerobes use other electron acceptors such as nitrate, sulphate, ferric iron or CO₂ (Alexander, 1999) often with the production of methane (Elshahed *et al.*, 2001). A review of the mechanisms involved in the anaerobic biodegradation of hydrocarbons is beyond the scope of this study. However, information about this method of microbial degradation is available (e.g. Swisher, 1987; Rueter *et al.*, 1994; Schmitt *et al.*, 1996; Coates *et al.*, 1997; Caldwell *et al.*, 1998; Gieg *et al.*, 1999; Grishchenkov *et al.*, 1999; Yerushalmi *et al.*, 1999; Kropp *et al.*, 2000; Wilkes *et al.*, 2000; Elshahed *et al.*, 2001).

2.1.5 Mono culture - *Pseudomonas fluorescens*

Bacteria capable of degrading petroleum hydrocarbons are widely distributed in marine, freshwater, sedimentary, and soil habitats (Atlas, 1981; Atlas and Bartha, 1992; Alexander, 1999; Clark, 2001; Wackett and Hershberger, 2001; National Research Council, 2003). Taxonomic studies (e.g. Fedorak and Westlake, 1981; Atlas and Bartha, 1992) have shown that many microbial populations isolated from both natural and oil impacted environments contain *Pseudomonas* species. Furthermore, Bartha and Atlas (1977) identified *Pseudomonas* spp. as one of the most important (based on frequency of isolation) genera of hydrocarbon utilisers in aquatic environments. Thus, *Pseudomonas* spp. have frequently been isolated and used in studies investigating the biodegradation of individual or mixtures of petroleum hydrocarbons (e.g. Solanas *et al.*, 1984; Bayona *et al.*, 1986; Robson and Rowland, 1987; Button *et al.*, 1992; Rocha *et al.*, 1992; Haigler *et al.*, 1992; Setti *et al.*,

1993; Al-Hadhrami *et al.*, 1995; Shim and Yang, 1999; Leblond *et al.*, 2001). Many other studies have used the *Pseudomonas fluorescens* strain in particular (e.g. Beech and Gaylarde, 1989; Gough, 1989; Caldini *et al.*, 1995; Sepic *et al.*, 1996; Wilson and Bradley, 1996 a and b; Chang *et al.*, 1997; Heath *et al.*, 1997; Yuan *et al.*, 2000; Barathi and Vasudevan, 2001). These studies have reported that *Pseudomonas fluorescens* is an efficient degrader of both aliphatic and aromatic hydrocarbons, and can be used to study the fate of hydrocarbons in the environment.

2.1.6 Natural bacterial consortium

The use of single strains (mono-cultures) of bacteria in the study of hydrocarbon degradation can provide useful information on the general susceptibility of hydrocarbons to microbial degradation. However, they are not necessarily indicative of environmental conditions where bacteria are present in mixed populations (Atlas and Bartha, 1992). Individual organisms generally metabolise only a limited range of substrates, therefore it is likely that assemblages of different bacterial species with broader enzymatic capabilities have a greater capacity to degrade complex hydrocarbon mixtures (Atlas and Bartha, 1992; Sugiura *et al.*, 1997; Alexander, 1999; Yuan *et al.*, 2000; National Research Council, 2003). The use of mixed cultures of bacteria to study the biodegradation of individual and mixtures of hydrocarbons has been frequently reported (e.g. Fedorak and Westlake, 1981; Cooney *et al.*, 1985; Maue and Dott, 1995; Sugiura *et al.*, 1997; Holder *et al.*, 1999; Marquez-Rocha *et al.*, 1999; Olson *et al.*, 1999; Diaz *et al.*, 2000; Yuan *et al.*, 2000). Sugiura *et al.* (1997) observed that a microbial consortium exhibited higher activity than a mono-culture for crude oil biodegradation. The mono-culture could not degrade certain PAH compounds yet the consortium was able to degrade the same PAH to varying degrees. It appears that mixed cultures degrade crude oil hydrocarbons more comprehensively. Therefore an accurate assessment of their fate in the environment should be measured using natural microbial communities.

2.1.7 Review of the biodegradation legislative tests

The persistence of a compound or mixture within the environment is generally defined in terms of its resistance to biodegradation. The persistence of a substance reflects not only the potential for long-term exposure of organisms but also the potential for the substance to reach the marine environment and to be transported to remote areas. Whilst there is much legislation regarding crude oil and petroleum products in the environment (*e.g.* OSPAR, European Community), there is, as yet, none specifically detailing or governing unresolved complex mixtures of hydrocarbons. For example mineral oils and hydrocarbons of petroleum origin are Black List substances under the EC Directive on Dangerous Substances (76/464/EEC).

To determine the persistence of substances in the environment a suite of standard biodegradation tests have been developed and proposed by organisations such as OECD (Organization for Economic Cooperation and Development), ISO (International Organization for Standardization) and MITI (Ministry of International Trade and Industry, Japan). The OECD biodegradability testing methods are classified into groups, *i.e.*, ready biodegradability tests (OECD 301A-F), inherent biodegradability tests (OECD 302A-C), and seawater tests (OECD 306 series). Ready biodegradability is the complete degradation of organic chemicals to natural inorganic components and biomass without formation of persistent and toxic metabolites. Inherent biodegradation refers to test conditions that are designed to promote biodegradation, and which provide information on the potential for a substance to be biodegraded (Battersby *et al.*, 1999; OSPAR Commission, 2002). Biodegradation can also be classed as either 'primary' where microbial attack causes a change in the chemical structure resulting in the loss of some specific property, or 'ultimate' biodegradation which is the complete mineralisation of a substance (Battersby *et al.*, 1999). The Coordinating European Council (CEC) has developed a test (CEC-L-33-A-93) which was originally intended to measure the biodegradability of hydrocarbons,

specifically two-stroke motor oils, in water. This method measures 'primary' biodegradation and can lead to an overestimate of the role of biodegradation. The 'ready' biodegradability tests (e.g. OECD 301 series) measure 'ultimate' biodegradation and can lead to an underestimate of the biodegradability of an oil product (Battersby *et al.*, 1999). These interpretations should strictly only be applied to pure substances, whilst petroleum products are in fact complex mixtures of many different organic compounds. A summary of the most commonly used biodegradation tests is presented in Table 2.1.

Table 2.1. A review of the available techniques used for testing biodegradability has been summarised by Shell Global Solutions (Biodegradability testing at Shell Global Solutions, http://www.shellglobalsolutions.com/analytical/techniques/bio_test.htm, 2003). The table below summarises some of the biodegradation tests, which have relevance to the studies here.

Test	Guideline
DOC die away (OECD 301A)	This 'ready' biodegradability test is suitable for water-soluble substances - biodegradation is measured as loss of dissolved organic carbon (DOC) over 28 days.
Carbon dioxide evolution (OECD 301B and 301E; ISO14593)	A variety of 'ready' biodegradability tests chosen as being suitable for water soluble and insoluble substances and volatile materials - biodegradation is measured as mineralisation to carbon dioxide over 28 days.
Closed bottle (OECD 301D)	This 'ready' biodegradability test is suitable for water soluble and insoluble substances - particularly suitable for volatile or inhibitory substances. Biodegradation is measured as oxygen uptake in closed bottles over 28 days.
Manometric respirometry (OECD 301F)	This 'ready' biodegradability test is suitable for water soluble and insoluble substances and can also be used for volatile materials. Biodegradation is measured continuously as oxygen uptake over 28 days. The oxygen is replenished automatically. It is particularly suitable for measuring the rate of biodegradation (kinetics).
Semi-continuous, activated-sludge test (OECD 302A)	This 'inherent' biodegradability test is suitable for non-volatile substances soluble in water at concentrations greater than 20 mg L ⁻¹ as dissolved organic carbon (DOC). Units are fed on daily basis with sewage, with and without test substance. Biodegradation is measured as removal of DOC over a 2 to 6 week period.
Biodegradability in sea water (OECD 306)	Test methods are similar to OECD guidelines 301A and 301D, but use seawater as the test medium.
Oil biodegradability (CEC L-33-A-93)	This test measures the primary biodegradability (loss of parent material) of oil products in an aqueous medium. Biodegradation is measured as the loss of infrared (IR) absorbance in solvent extracts over 21 days.

The Office of Prevention, Pesticides and Toxic Substances (OPPTS 835.3110, 1998), of the United States Environmental Protection Agency, states that normally these tests last for 28 days. The pass levels for 'ready' biodegradability are 70 percent removal of DOC and 60 percent of ThOD (theoretical oxygen consumption) or ThCO₂ (theoretical carbon dioxide production) for respirometric methods. Specific chemical analysis can also be used to assess 'primary' degradation of the test substance. The pass levels are lower in the respirometric methods because some of the carbon from the test chemical is incorporated into new cells; thus, the percentage of CO₂ produced is lower than the percentage of carbon being used. OPPTS (1998) states that chemicals reaching a pass level after the 28-day period are deemed not readily biodegradable. OECD guidelines for the testing of chemicals recommend that biodegradation of more than 20% is evidence for 'inherent primary biodegradability', and biodegradation above 70% is evidence for 'inherent ultimate biodegradability'.

Whilst organisations such as OECD and ISO propose detailed methods for the measurement of biodegradability of substances, it is organisations such as the OSPAR Commission which have the power to adopt these tests for regulatory data generation. OSPAR lists oils and hydrocarbons of petroleum origin as substances that shall be subject to 'programmes and measures', and individual hydrocarbons and certain compounds classes (*e.g.* PAHs, PCBs) are listed on either the current OSPAR 'List of Chemicals for Priority Action' or the 'List of Substances of Possible Concern'.

The OSPAR selection procedure identifies certain hazardous substances on the basis of their intrinsic hazardous properties of persistence, liability to bioaccumulate and toxicity (P, B and T). The P, B and T criteria used for the selection of the Substances of Possible Concern by OSPAR are described in DYNAMEC (OSPAR Commission, 2002).

The cut-off values for each of these criteria are as follows:

Persistency (P): Half-life ($t_{1/2}$) of 50 days and

Liability to Bioaccumulate (B): $\log K_{ow} \geq 4$ or $BCF \geq 500$ and

Toxicity (T): T_{aq} : acute $L(E)C_{50} \leq 1 \text{ mg L}^{-1}$, long-term
 $NOEC \leq 0.1 \text{ mg L}^{-1}$.

Where BCF is the 'bioconcentration factor', $L(E)C_{50}$ is the acute toxicity lethal concentration for 50% fatality of a population, and NOEC is the 'no observed effect concentration'.

For persistence, the principal criterion is that the substance has a half-life in the freshwater or marine environment of 50 days or more. OSPAR state that the half-life should be used as the first and main criterion for determining whether substances should be regarded as persistent in the context of the Hazardous Substances Strategy.

OSPAR concludes that many of the ready and inherent biodegradability tests accepted as standard methods of monitoring persistence do not provide half-life data on the substances studied. In contrast the biodegradation experiments carried out as part of the present study provide half-life data on the individual hydrocarbons and hydrocarbon mixtures.

The OSPAR Commission also acknowledges the importance and benefits in using models to estimate the biodegradability or persistence of substances in the environment. For many chemicals no experimental data are available at all, which makes their inclusion onto lists of priority action or possible concern difficult to justify. Fortunately, models are available such as the SYRACUSE BIOWIN used herein (Section 2.3.4). Such models can be used to estimate the potential for biodegradation in the environment.

2.1.8 Aims of the present study

Gough (1989) investigated the biodegradability of proposed aliphatic UCM components (“T-branched” alkanes) with the bacterium *Pseudomonas fluorescens* (strain Texaco). The studies used C₂₅ hydrocarbons so that any variations in biodegradability could be ascribed solely to molecular structure rather than differences in molecular weight. The results indicated that different hydrocarbon structural types underwent different rates and extents of degradation. In particular, an increased resistance to biodegradation was noted for more highly branched hydrocarbons, suggesting that steric factors may be a controlling factor in hydrocarbon sedimentary distributions. It was also found that an aliphatic UCM was partially degraded, initially at a rate comparable to that observed for candidate aliphatic UCM compounds, and that the UCM appeared degraded “as a whole”, *i.e.* no significant reduction in the proportion of resolved compounds vs. unresolved compounds was observed (Figure 2.6).

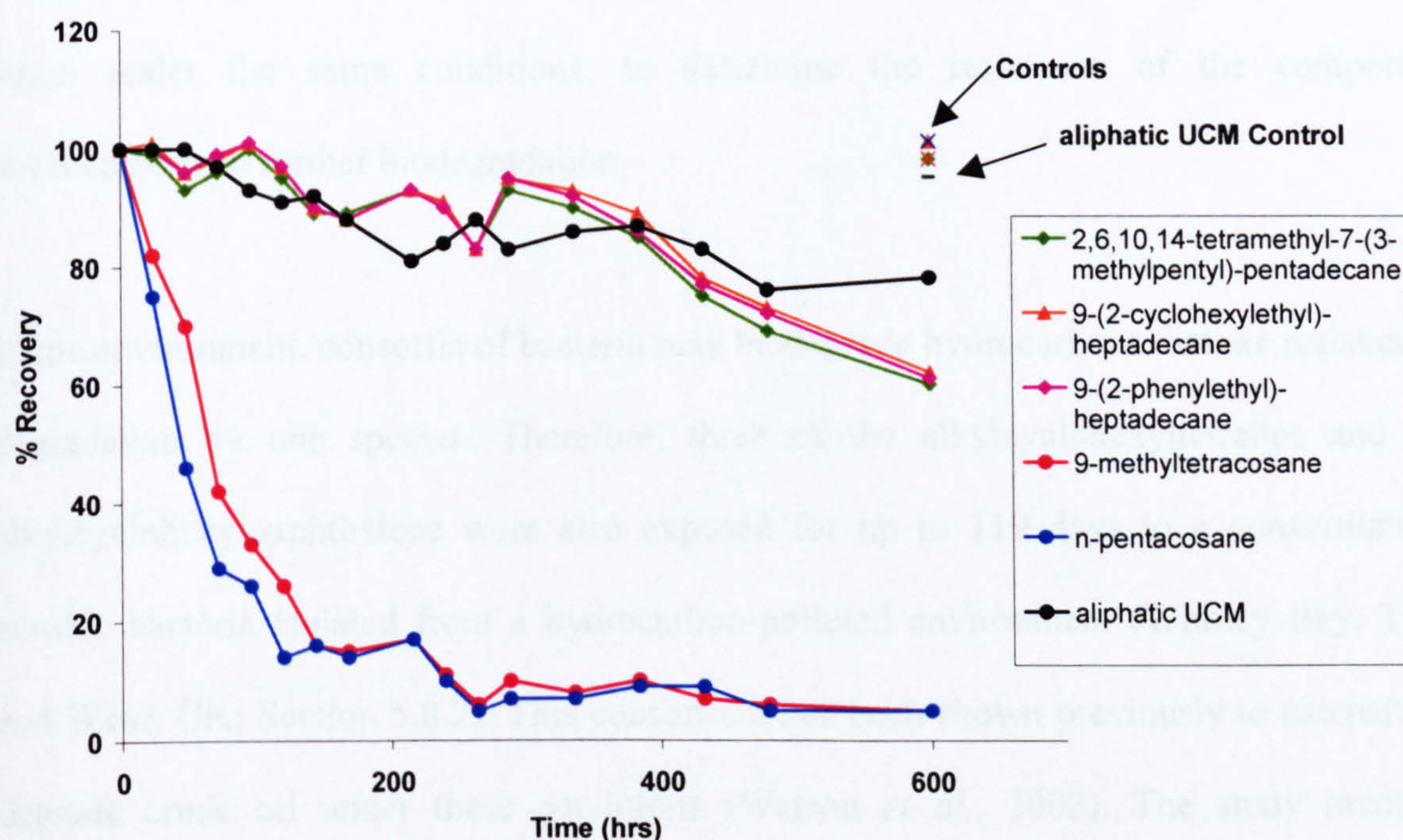


Figure 2.6. Summary of data obtained by Gough (1989) from biodegradation studies using *P. fluorescens*. More highly branched hydrocarbons (candidate aliphatic UCM compounds) and the aliphatic UCM appear to have increased resistance to biodegradation than straight and simply branched alkanes.

In the present study, a series of synthetic alkylcyclohexyltetralins (Wraige, 1997; Sturt, 2000; Smith *et al.*, 2001; Section 5.6 and 5.7) an isolated aromatic UCM (Section 5.4), and for reference *n*-pentacosane, were exposed to aerobic bacteria in well-defined laboratory tests (Section 5.8). Firstly, 6-cyclohexyltetralin (I, Figure 2.7), four 7-cyclohexyl-1-alkyltetralins (II-V, Figure 2.7), 7-cyclohexyl-1-nonylnaphthalene (VI, Figure 2.7) and *n*-pentacosane, were exposed for 50 days to a mono-culture (Section 5.8.1). The bacterial strain used was *Pseudomonas fluorescens* (Texaco) which was isolated from a contaminated metal working fluid by Beech and Gaylarde (1989). The aim here was not to carefully select an unusual bacterial species capable of degrading the target compounds as is the case in bioremediation or biomimetic studies (*e.g.* certain *Sphingomonas* spp; Hernaez *et al.*, 1999). Rather the aim was to investigate whether a common environmental aerobe was capable of degrading the monoaromatic tetralins and diaromatic naphthalene (I-VI, Figure 2.7) as easily as it degrades some other hydrocarbons (*c.f.* Gough *et al.*, 1992). Secondly, an isolated aromatic UCM (Figure 2.8) was exposed to the same bacterial strain under the same conditions, to determine the resistance of the component hydrocarbons to further biodegradation.

In the environment, consortia of bacteria may biodegrade hydrocarbons that are resistant to degradation by one species. Therefore, three of the alkylcyclohexyltetralins and the alkylcyclohexylnaphthalene were also exposed for up to 119 days to a consortium of aerobic bacteria isolated from a hydrocarbon-polluted environment (Whitley Bay, Tyne and Wear, UK; Section 5.8.2). This consortium has been shown previously to extensively degrade crude oil under these conditions (Watson *et al.*, 2002). The study involved incubations with two co-substrates (i) *n*-hexadecane (*n*-C₁₆) and (ii) fresh North Sea crude oil. These studies using the consortium of bacteria were conducted at the University of Newcastle by C. Aitken. All data analysis and processing was completed by the author (A. Booth) at the University of Plymouth.

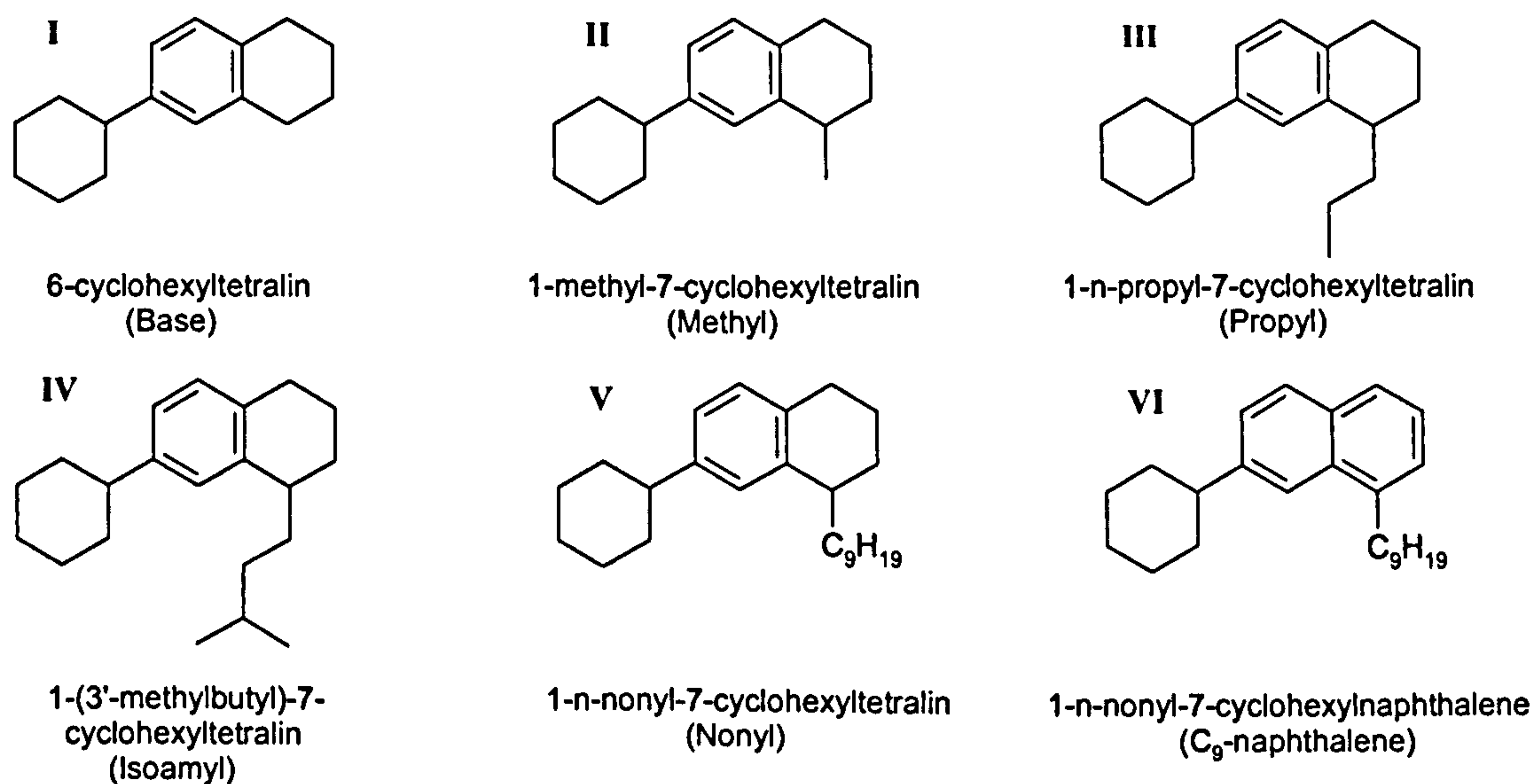


Figure 2.7. Synthetic alkyltetralins (I-VI) suggested as 'average' aromatic UCM components. Compounds I-III were synthesised by Smith (2002) and compounds IV-VI by Sturt (2000) and purified with recovery by HPLC by the present author as described in Chapter 5.

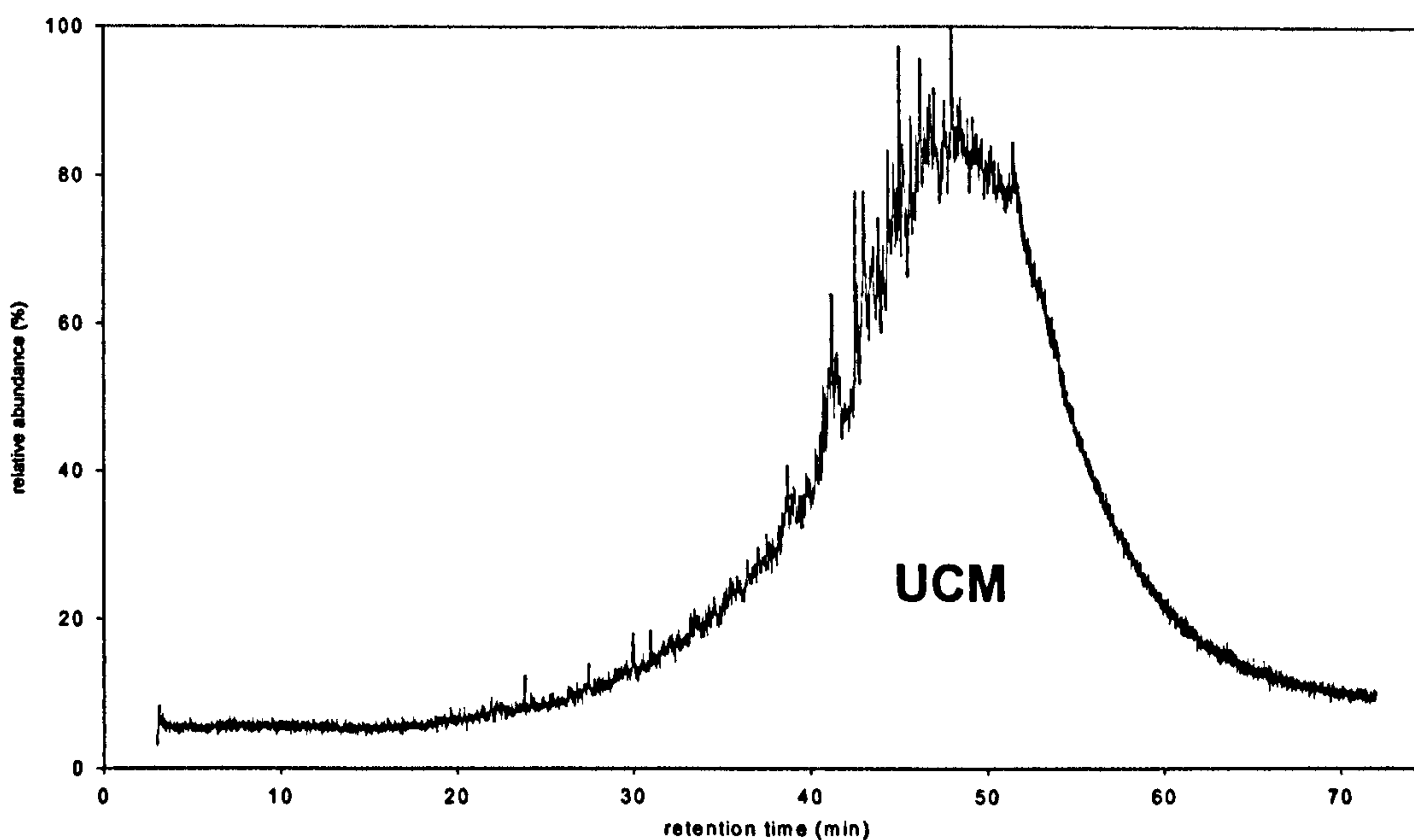


Figure 2.8. Gas chromatogram of the combined 'aromatic' fractions isolated from in-reservoir biodegraded Tia Juana Pesado crude oil (Venezuela) by open column chromatography (Section 5.4).

2.2 Results

2.2.1 Biodegradation studies with *Pseudomonas fluorescens*

2.2.1.1 Biodegradation of *n*-pentacosane (*n*-C₂₅)

The biodegradation of *n*-pentacosane (*n*-C₂₅) by *P. fluorescens* (Texaco) at 22-25 °C for 0-9 days is illustrated in Figure 2.9, along with the data for abiotic controls. The study used *n*-C₂₆ as an internal standard so the data could be adjusted for sample loss during work-up (Section 5.8.1). The aim of the present investigation was to illustrate the well-known capability of *Pseudomonas fluorescens* for rapid biodegradation of *n*-alkanes and to confirm that the experimental conditions were comparable to previous studies (*e.g.* Gough *et al.*, 1992). The rapid decrease in *n*-C₂₅ concentration due to biodegradation was indeed observed during the first few days of exposure. Furthermore, comparison of the data from the current study to that of Gough *et al.* (1992) indicates analogous results and therefore experimental conditions were achieved (Figure 2.9). The viability of the bacteria used was monitored throughout the experiment (Section 5.8.1.2). At every stage, samples were taken and incubated over night. In each case bacterial growth was visually evident, confirming that the bacteria were viable throughout the experiment (Figure 2.10).

2.2.1.2 Biodegradation of the synthetic alkylcyclohexyl-tetralins and naphthalene

Figure 2.11 shows the mean (\pm standard deviation) proportion of the alkylcyclohexyltetralins and alkylcyclohexylnaphthalene (I-VI) recovered in triplicate experiments after exposure to *Pseudomonas fluorescens* for a 50-day period (Section 5.8.1). Compounds I and II appear to have suffered the greatest losses (67% and 56% degradation respectively), whereas compound V and the C₉ cyclohexylnaphthalene (VI) have hardly been affected (2% and 1% degradation respectively). Also shown for comparison are the data for the degradation of *n*-C₂₅. However, comparison of individual compounds (I-VI) at day 50 to the data for the respective abiotic controls indicates only

slight differences. Thus, the removal of compounds I-VI, unlike $n\text{-C}_{25}$ appear to be dominated by abiotic losses, probably due to evaporation.

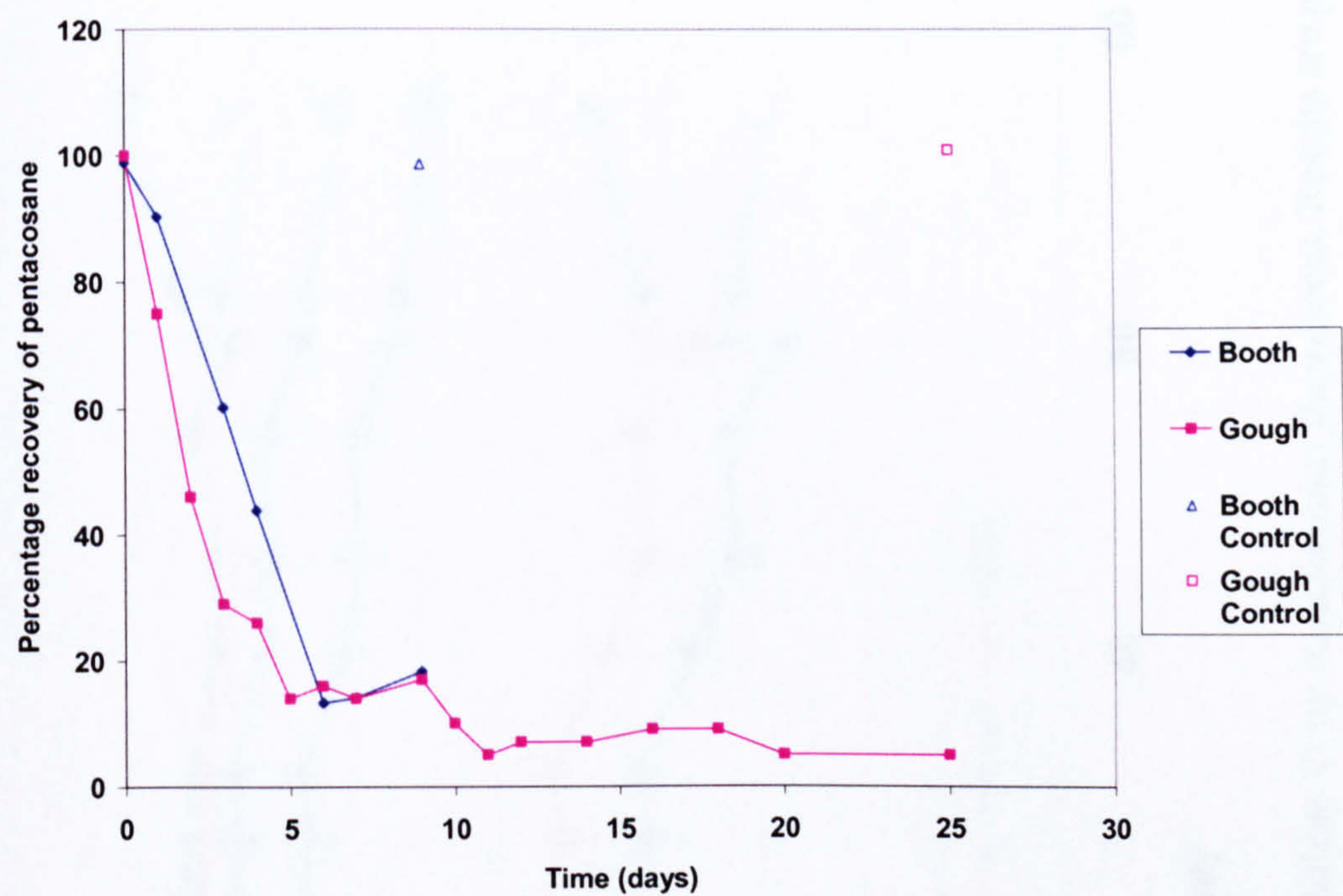


Figure 2.9. Comparison of the degradation rate of n -pentacosane ($n\text{-C}_{25}$) using *P. fluorescens* determined in this study with those observed in a similar study by Gough *et al.* (1992).

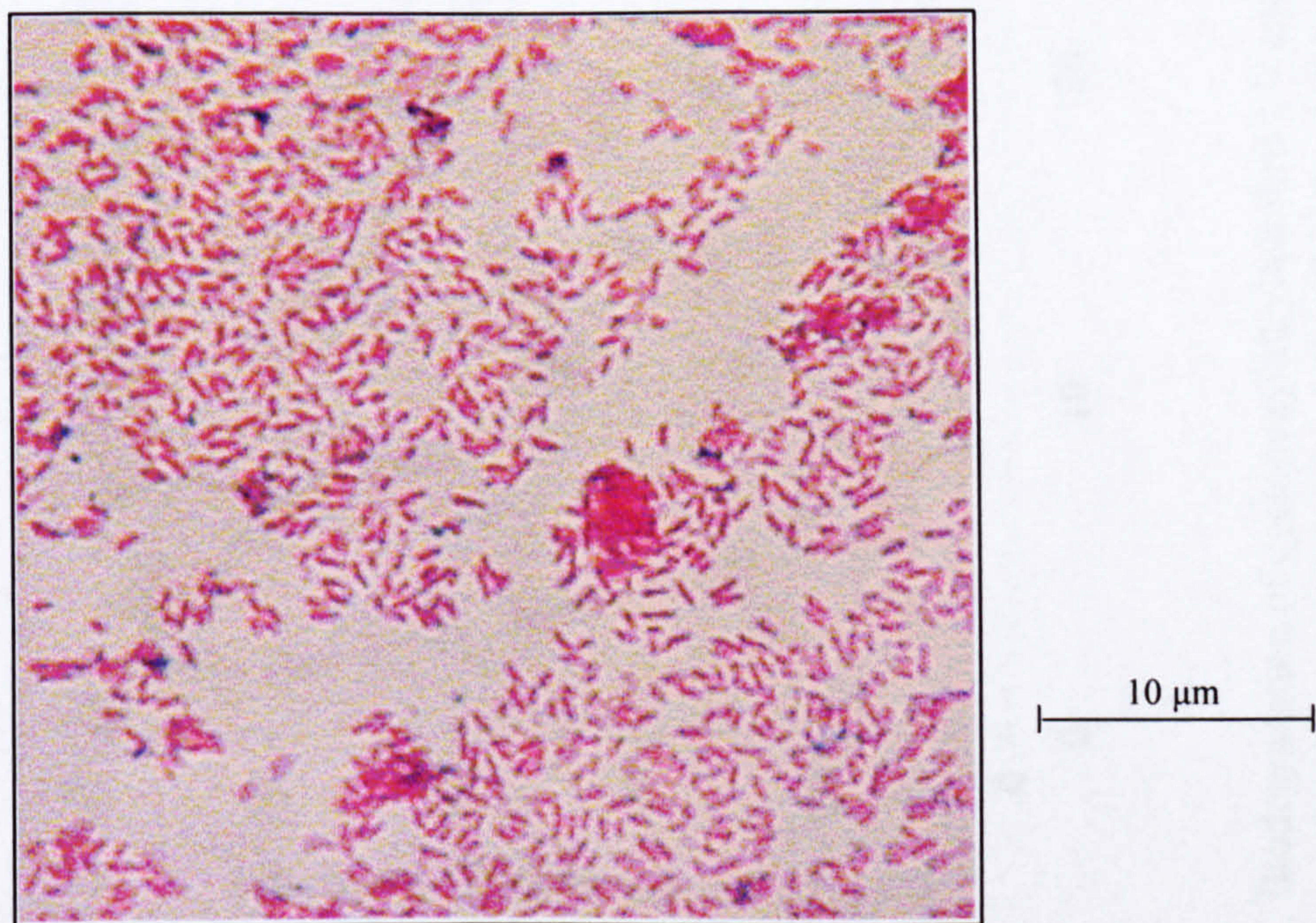


Figure 2.10. Digital photograph of healthy, uncontaminated, *P. fluorescens* colonies grown from the viability test sample taken on day 50. Bacteria transferred to a slide, and the photograph taken through a microscope.

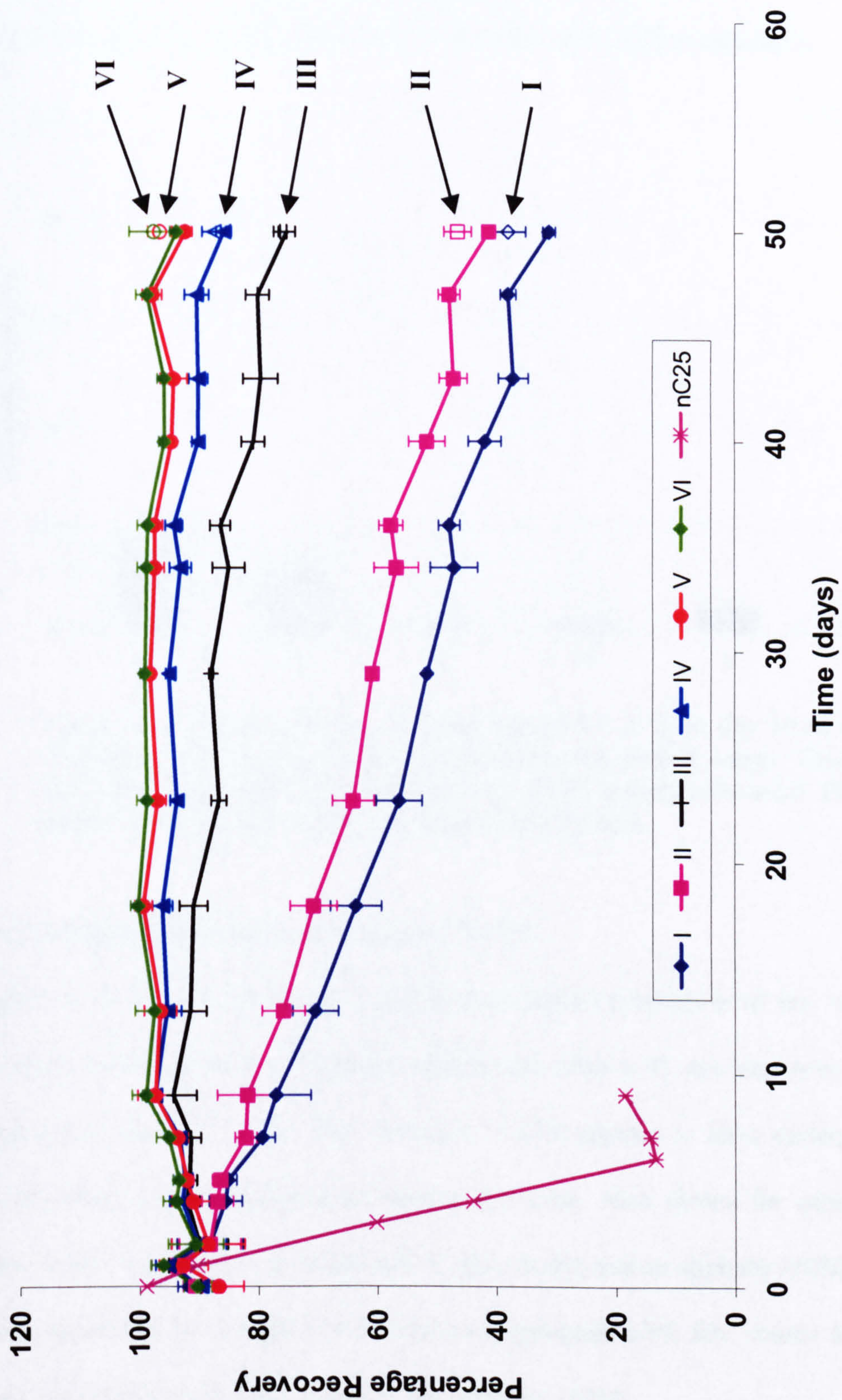


Figure 2.11. Biodegradation of cyclohexylalkyltetralins I-V and cyclohexylnaphthalene VI by *Pseudomonas fluorescens*. Results are presented as the percentage of each compound recovered with time. ♦ = I, ■ = II, + = III, ▲ = IV, ● = V, ◆ = VI, x = n-C₂₅ (n-pentacosane). The results from the n-pentacosane study have been included for comparison. Unfilled symbols correspond to the respective controls.

To determine the overall degree of biodegradation experienced by each compound, the data for day 50 were compared to those for the abiotic controls. Figure 2.12 shows the percentage loss of each of the hydrocarbons that can be attributed to biotic processes. These biotic losses are all <20% over 50 days for each of the compounds.

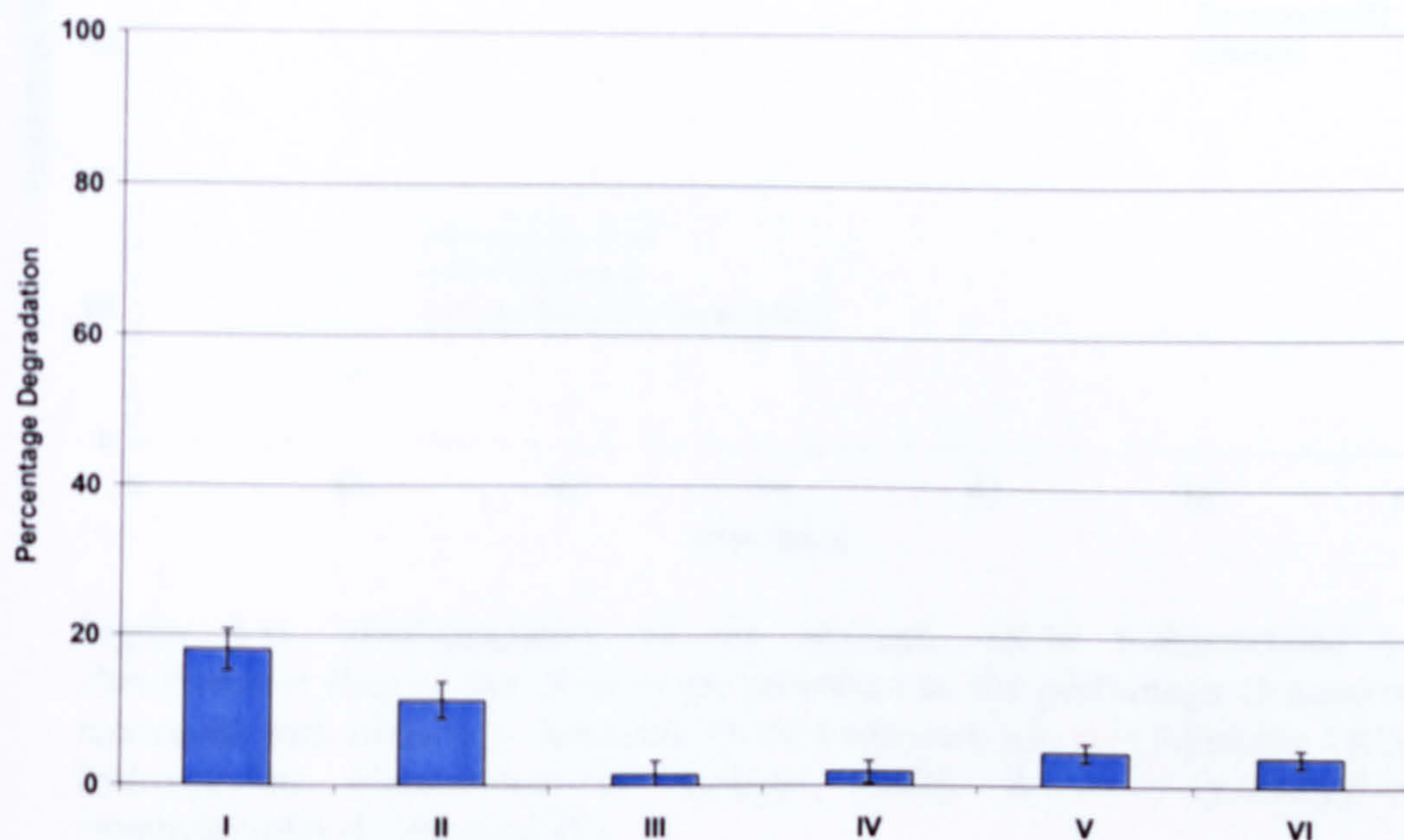


Figure 2.12. Biodegradation of compounds I-VI (n=3) at day 50 of the study. The values were calculated by comparison to the control values. This indicates the percentage difference between the final sample recoveries (biotic and abiotic loss) and the control recoveries (abiotic loss).

2.2.1.3 Biodegradation of an 'aromatic' UCM

Figure 2.13, shows the mean (\pm standard deviation) proportion of the 'aromatic' UCM (Section 5.4) recovered in triplicate experiments after a 41 day exposure period with *P. fluorescens* (Section 5.8.2). The 'aromatic' UCM appears to have undergone very little biodegradation (~5% degradation) during this time. Also shown for comparison are the data for the degradation of compound IV (this study) and an aliphatic UCM exposed to the same bacterium by Gough (1989). Unlike compounds I-VI, few abiotic losses appear to have occurred to the hydrocarbons in the aromatic UCM.

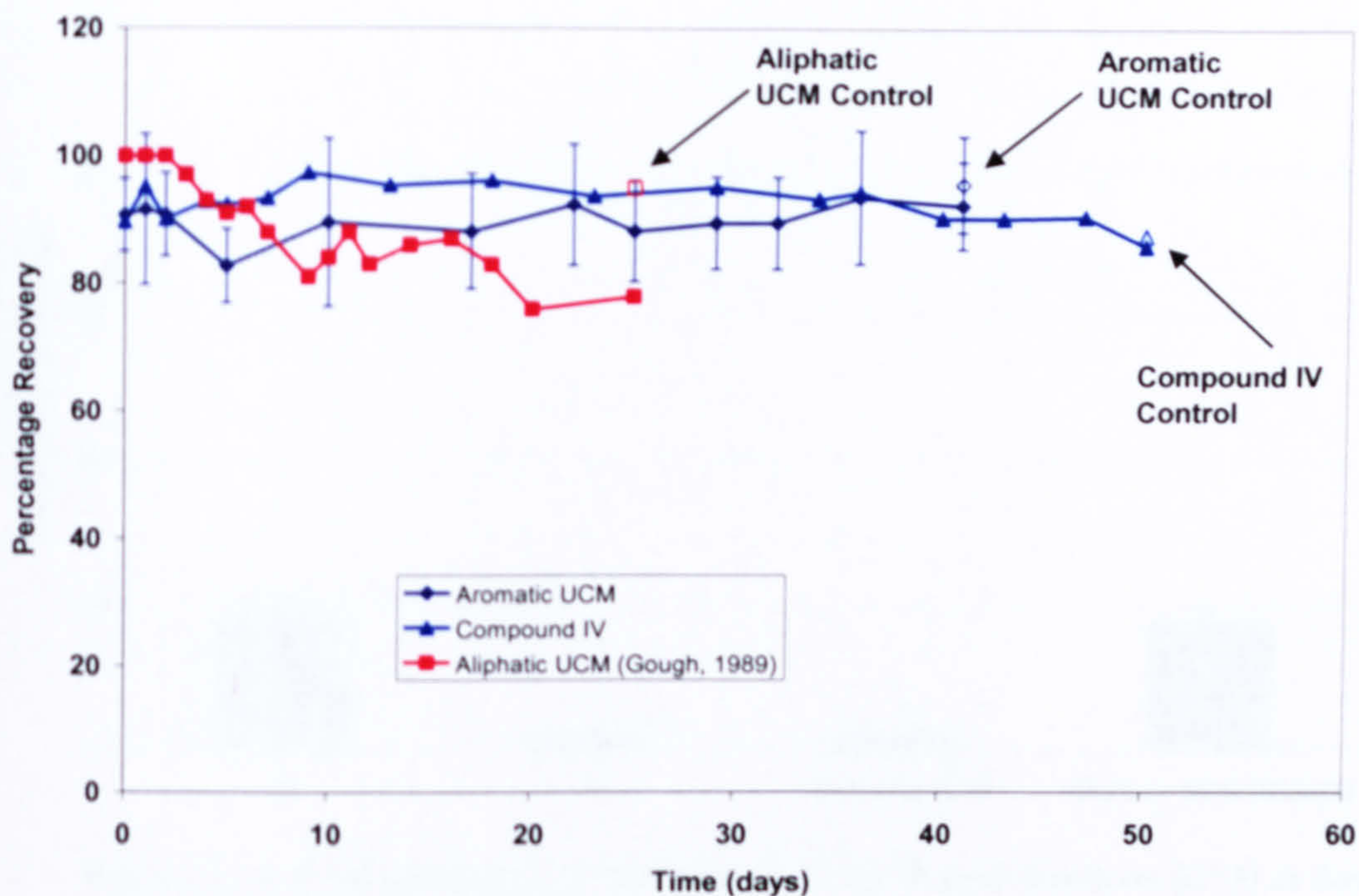


Figure 2.13. Biodegradation of the aromatic UCM hydrocarbons by *Pseudomonas fluorescens*. Results are presented as the percentage of material recovered with time. ♦ = Aromatic UCM hydrocarbons, ■ = Aliphatic UCM hydrocarbons (determined by Gough, 1989), ▲ = 7-cyclohexyl-1-isoamyltetralin (Compound IV).

The overall degree of biodegradation undergone by the ‘aromatic’ UCM hydrocarbons was determined by comparing the data for day 41 to that of the corresponding abiotic control. Figure 2.14 shows the percentage loss of ‘aromatic’ UCM hydrocarbons which can be attributed to biotic processes. Also included for comparison are the data for compound I and IV (Figure 2.7) and also for the aliphatic UCM studied by Gough (1989). These values represent the fraction of the total hydrocarbons that were lost as a result of biotic processes alone.

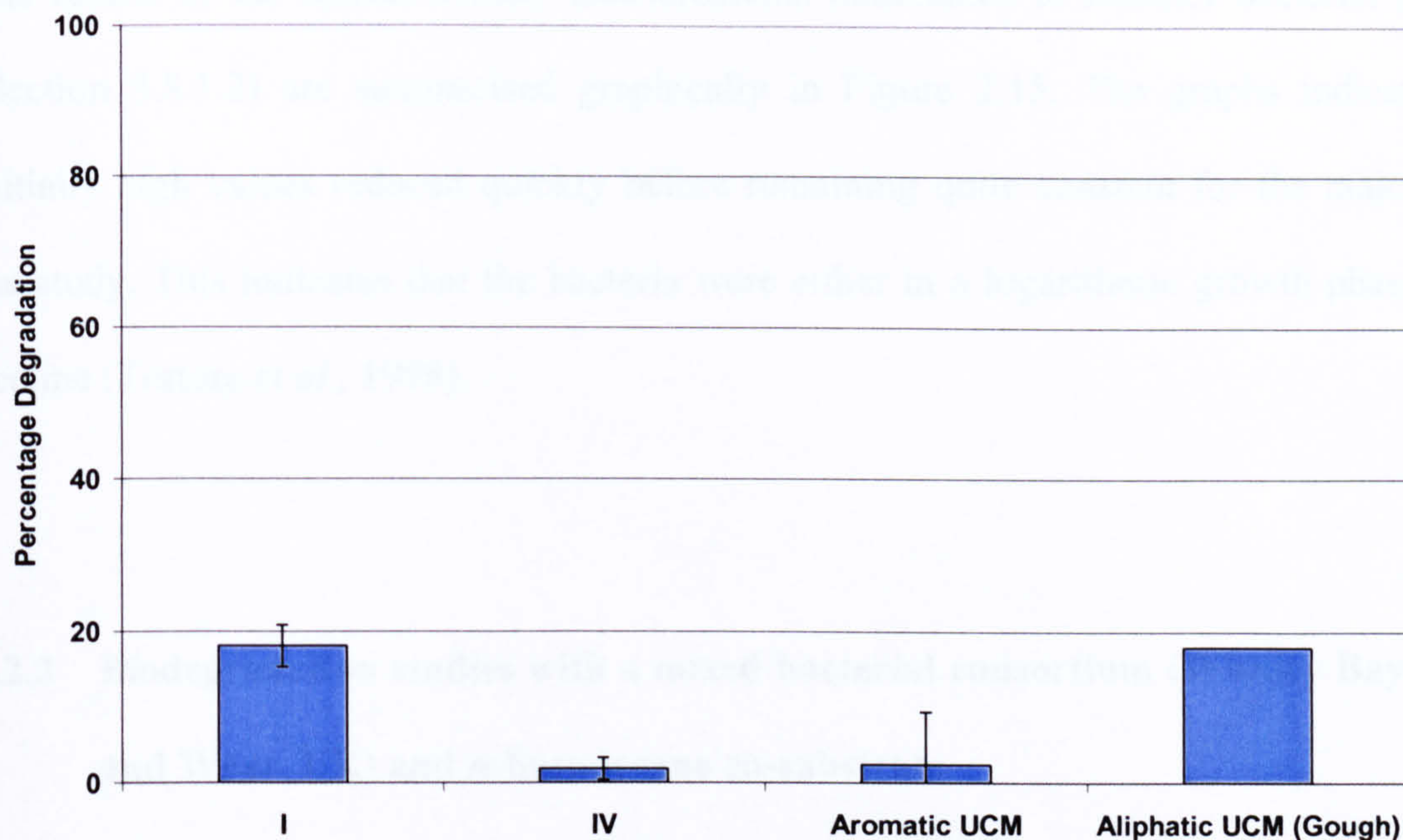


Figure 2.14. Biodegradation of the ‘aromatic’ UCM hydrocarbons (n=3) at day 41 of the study. The values were calculated by comparison to the control values. This indicates the percentage difference between the final sample recoveries (biotic and abiotic loss) and the control recoveries (abiotic loss). Also included for comparative purposes are the data for compounds I and IV, determined in this study and that of the aliphatic UCM hydrocarbons as determined by Gough (1989). Replicate data for the latter study were unavailable.

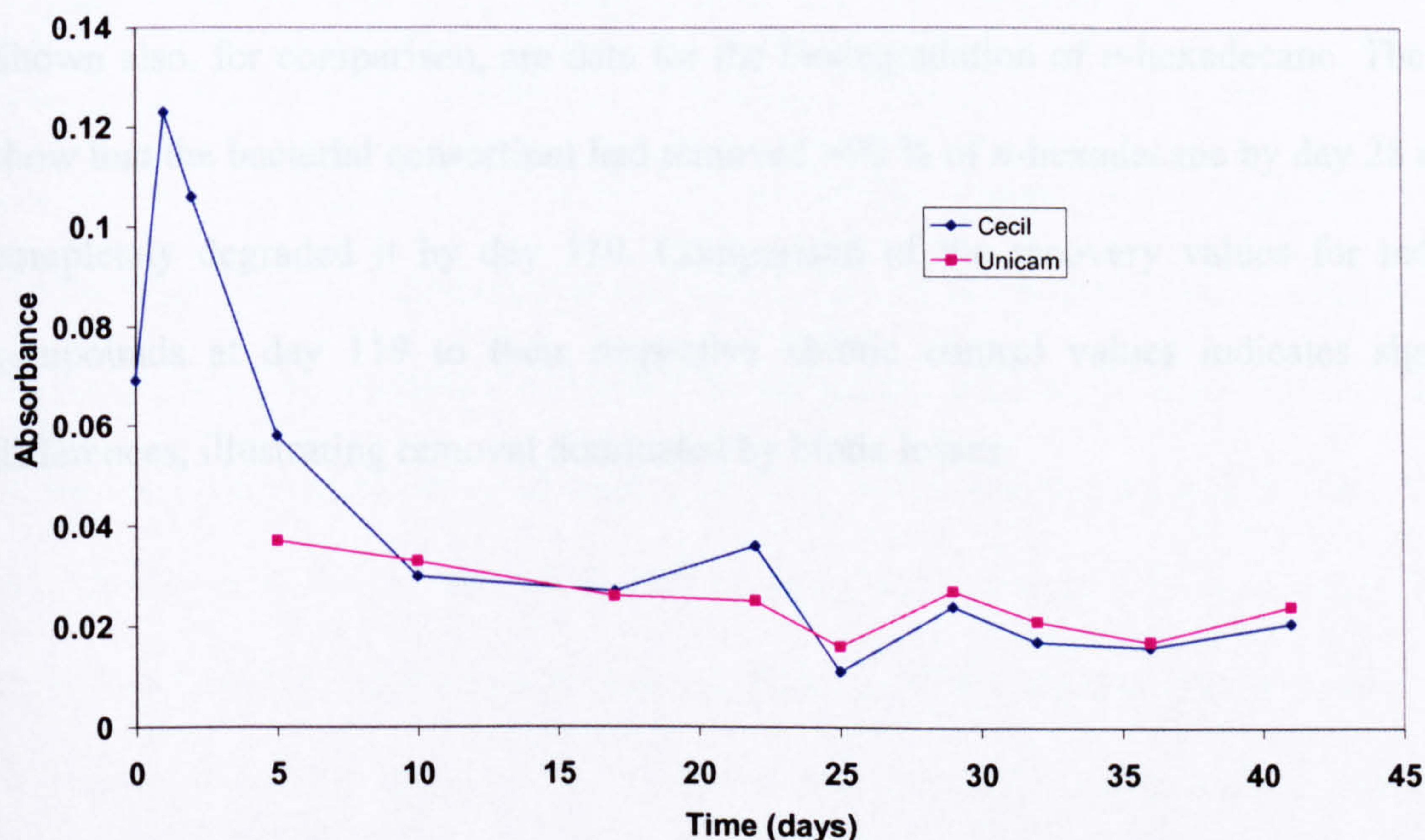


Figure 2.15. Optical density values for each sample, used as a measure of bacterial activity and growth (if occurring). Optical density was measured using two spectrophotometers, (a) Cecil CE 1010 (1000 series) and (b) Unicam Helios Epsilon.

The results of the optical density measurements undertaken to monitor bacterial growth (Section 5.8.1.2) are summarised graphically in Figure 2.15. The graphs indicate that initially high values reduced quickly before remaining quite constant for the majority of the study. This indicates that the bacteria were either in a logarithmic growth phase or in decline (Tortora *et al.*, 1998).

2.2.2 Biodegradation studies with a mixed bacterial consortium (Whitley Bay, Tyne and Wear, UK) and *n*-hexadecane co-substrate

The results of the biodegradation of synthetic compounds I (45 µg), II (34 µg), IV (59 µg) and VI (39 µg) at 20 °C in the presence of ~1000 x excess of *n*-C₁₆ are summarised in Figure 2.16, which shows how each of the compounds were affected by exposure to the Whitley Bay consortium of bacteria over a 119-day period. Compounds I and II appeared to suffer the greatest losses (both 100 % degraded by day 119), whereas compounds IV and VI were still present at day 119 (32% and 61% degraded with respect to starting 100%). Shown also, for comparison, are data for the biodegradation of *n*-hexadecane. The results show that the bacterial consortium had removed >90 % of *n*-hexadecane by day 28 and had completely degraded it by day 119. Comparison of the recovery values for individual compounds at day 119 to their respective abiotic control values indicates significant differences, illustrating removal dominated by biotic losses.

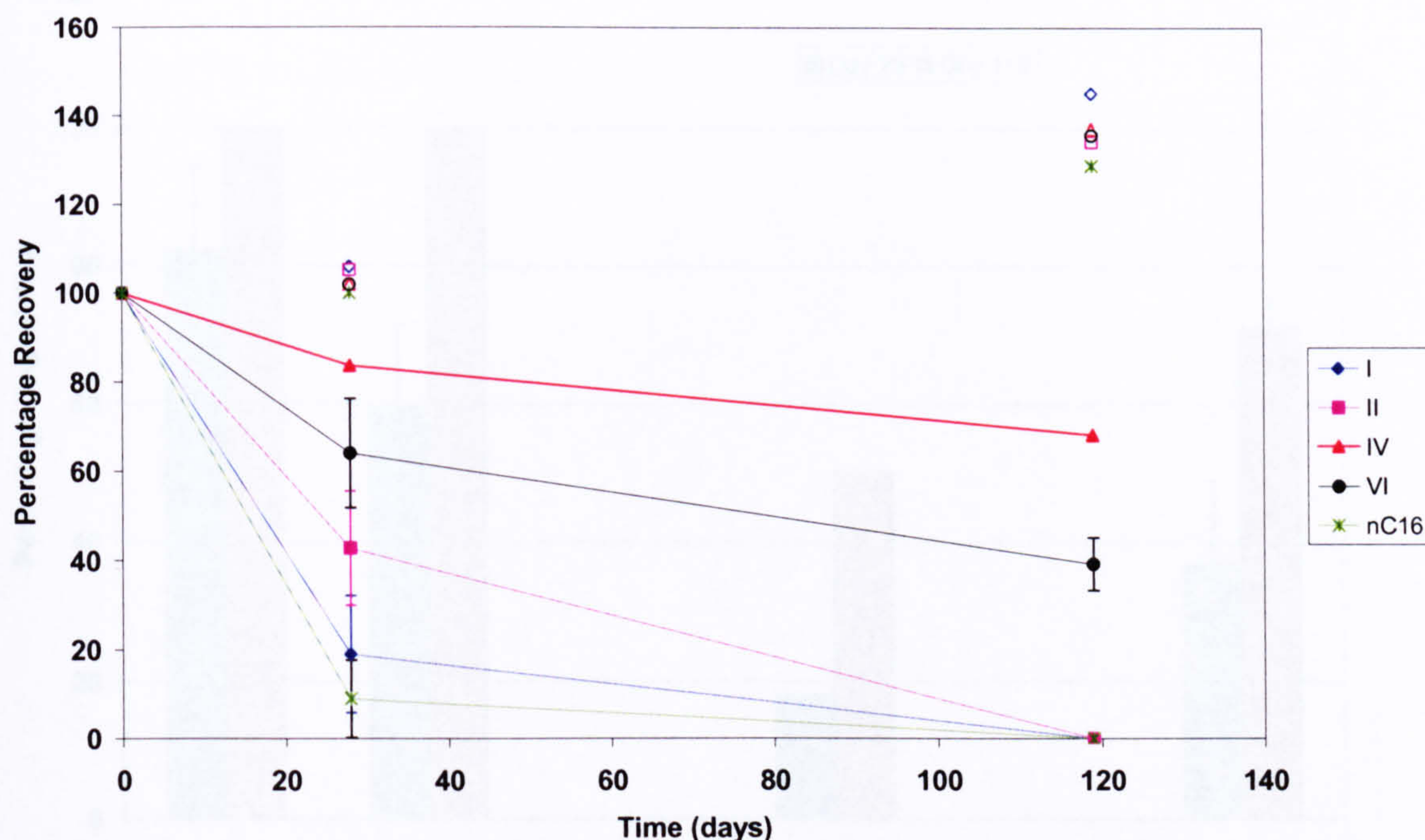


Figure 2.16. Graph showing the degradation of the alkyltetralins (I, II, IV) and alkylnaphthalene (VI) after exposure to the Whitley Bay bacterial consortium for 28 and 119 days. The degradation of the hexadecane ($n\text{-C}_{16}$) co-substrate is also shown. Open symbols correspond to control data points.

To determine the degree of biodegradation that the compounds I, II, IV and VI had undergone, the recovery data for day 28 and day 119 were compared to those of the abiotic control values. Figure 2.17 shows the percentage losses of each of the synthetic alkyltetralins and the alkylnaphthalene which can be attributed to biotic processes. The biotic losses vary considerably between the different compounds. After 28 days all of the compounds showed evidence of biodegradation, with compounds I and II being the most heavily degraded (82 % and 59 % respectively) and compound IV exhibiting the most resistance to biodegradation under these conditions (<20% degradation). A similar trend in susceptibility can be seen in the results at day 119, with compounds I and II being completely degraded and compounds IV and VI still present in significant quantities (IV = 50 %; VI = 29 % remaining).

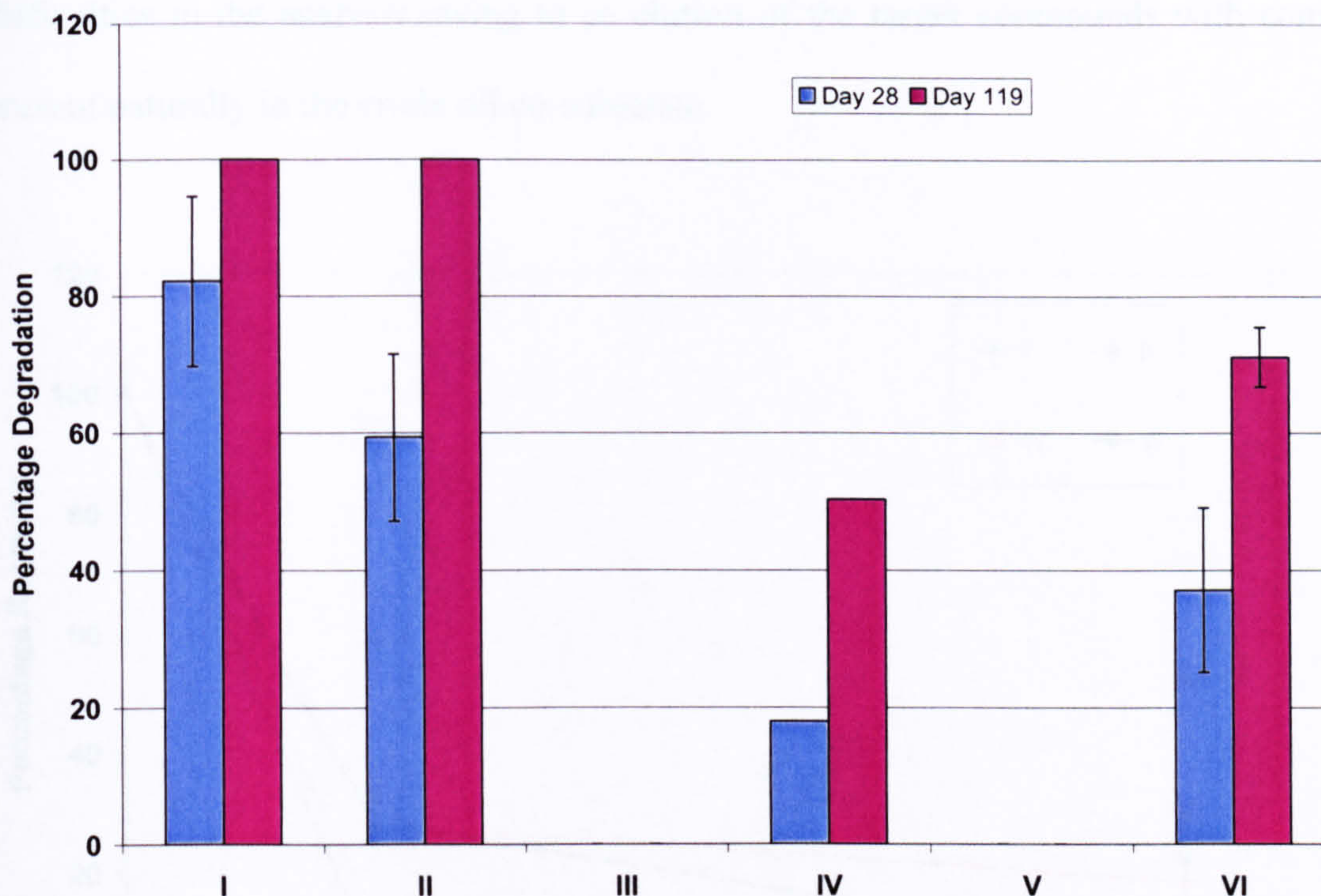


Figure 2.17. Summary of the biodegradation of compounds I, II, IV and VI (mean \pm range, $n=2$) in presence of 1000 x excess *n*-hexadecane, by a mixed consortium of bacteria at day 28 and day 119 compared to values for abiotic controls. Compounds III and V were not used in the present study.

2.2.3 Biodegradation studies with a mixed bacterial consortium (Whitley Bay, Tyne and Wear, UK) and North Sea crude oil co-substrate

The results of the biodegradation of synthetic compounds I (45 μg), II (34 μg), IV (59 μg) and VI (39 μg) at 20 $^{\circ}\text{C}$ in the presence of 45 mg of North Sea crude oil are summarised in Figure 2.18, which shows how each of the compounds were affected by exposure to the Whitley Bay consortium over a 119-day period. Compounds I and II again appeared to have suffered the greatest losses (both 100 % degradation by day 119), whereas compounds IV and VI are still present at day 119 (82% and 94% degradation respectively). Comparison of individual compounds at day 119 to their respective controls indicates significant differences in their recoveries, thus these results appear to be dominated by biotic losses. However, the recovery of the control samples is quite low, reflecting

difficulties in the analysis owing to co-elution of the target compounds with compounds present naturally in the crude oil co-substrate.

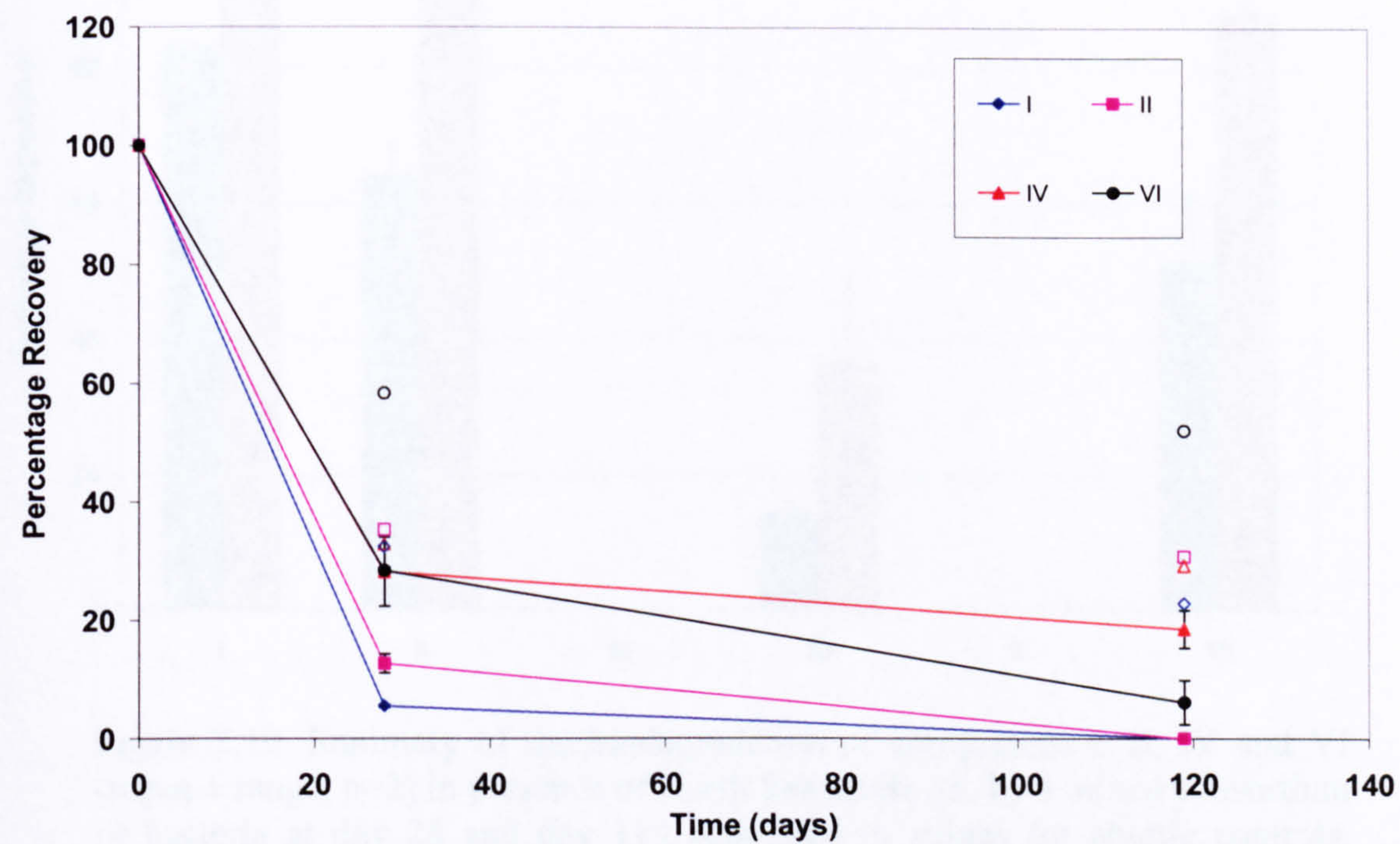


Figure 2.18. Graph showing the degradation of the alkyltetralins (I, II, IV) and alkylnaphthalene (VI) after exposure to the Whitley Bay bacterial consortium for 28 and 119 days in the presence of North Sea crude oil. Open symbols correspond to control data points.

It is therefore important to determine the degree of biodegradation that the compounds I, II, IV and VI had undergone compared to that of the abiotic controls so that an accurate measurement of the extent of biodegradation could be reported. The recovery data for both day 28 and day 119 were compared to those of the abiotic control values. Figure 2.19 shows the percentage losses of each of the alkyltetralins and the alkylnaphthalene, attributed to biotic processes.

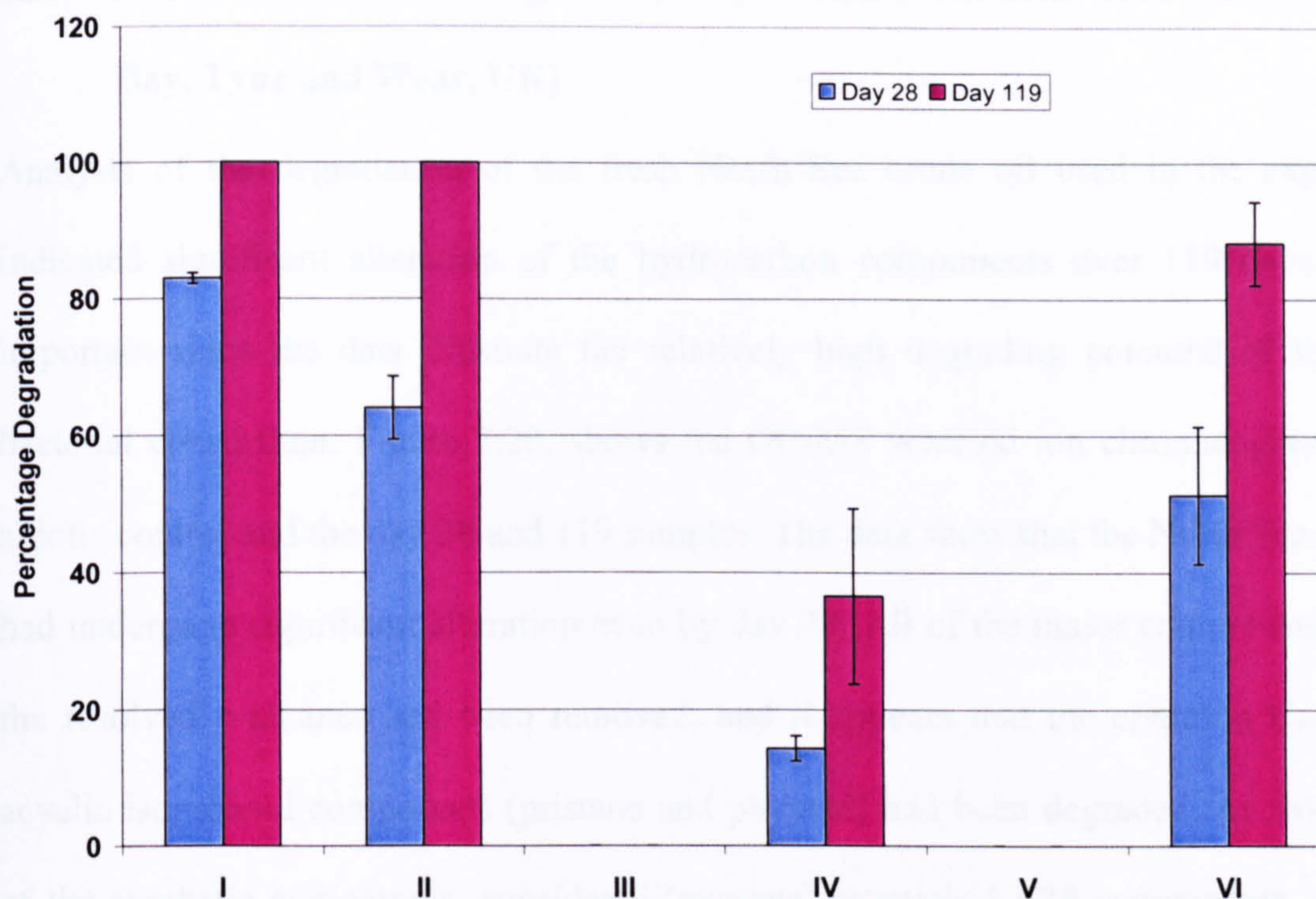


Figure 2.19. Summary of the biodegradation of compounds I, II, IV and VI (mean \pm range, $n=2$) in presence of North Sea crude oil, by a mixed consortium of bacteria at day 28 and day 119 compared to values for abiotic controls. Compounds III and V were not used in the present study.

The biotic losses appear to vary considerably between the different compounds as was also seen in the *n*-hexadecane study (e.g. Figure 2.16), with again compounds I and II undergoing the greatest losses and compounds IV and VI being the most resistant to biodegradation. Adjustment of the biodegradation values to the abiotic controls shows that less material has been biodegraded than initially appears from Figure 2.18. After 28 days all of the compounds showed some evidence of biodegradation, with compounds I and II most heavily degraded (82 % and 64 % respectively) and compound IV exhibiting the most resistance to biodegradation under these conditions (only 14 %). A similar trend in susceptibility was seen in the results for the day 119 experiment, with compounds I and II being completely degraded and compounds IV and VI still present in significant quantities (IV = 64 %; VI = 12 % remaining).

2.2.4 North Sea crude oil degradation by a mixed bacterial consortium (Whitley Bay, Tyne and Wear, UK)

Analysis of the degradation of the fresh North Sea crude oil used in the experiments indicated significant alteration of the hydrocarbon components over 119 days. This is important since the data illustrate the relatively high degrading potential of the mixed bacterial consortium. Figure 2.20, shows the GC-MS selected ion chromatograms of the abiotic control and the day 28 and 119 samples. The data show that the North Sea crude oil had undergone significant alteration even by day 28. All of the major components such as the resolved *n*-alkanes had been removed, and it appears that the common C₁₉ and C₂₀ acyclic isoprenoid compounds (pristane and phytane) had been degraded. At day 28 each of the synthetic compounds, considered 'average' aromatic UCM components, were still present. By day 119 degradation had proceeded to such an extent that an enrichment of the hopanes (pentacyclic terpanes) can be clearly observed. However, two of the 'average' compounds (IV and VI) had still not undergone complete mineralisation.

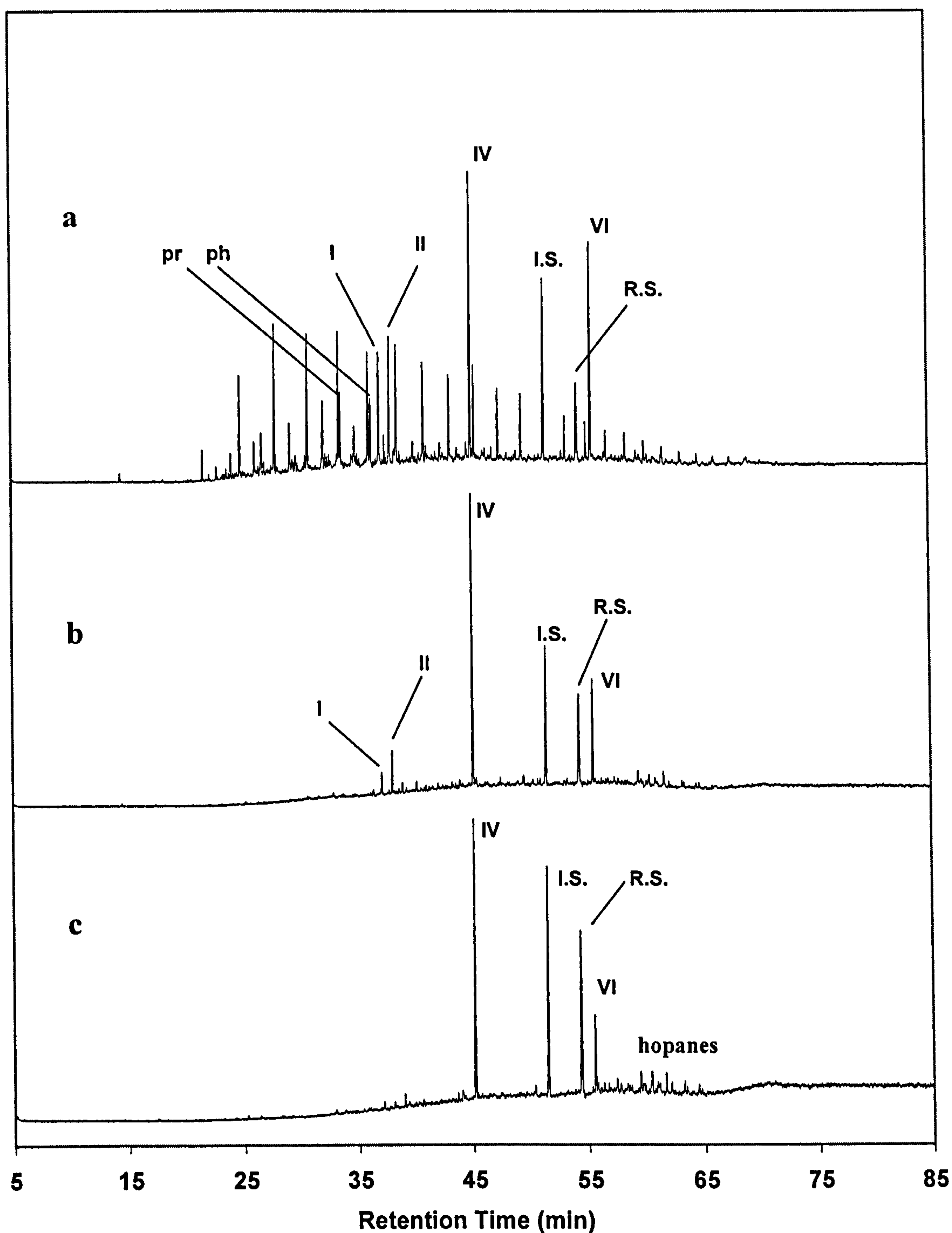


Figure 2.20. GC-MS m/z 85 selected ion chromatograms illustrating distributions of model compounds (I, II, IV and VI) and crude oil hydrocarbons in incubations with Whitley Bay consortium of aerobic bacteria: a. Sterilised day 28 control; b. Biotic day 28 incubation; c. Biotic day 119 incubation. I.S. = internal standard (n -pentacosane). R.S.= recovery standard (squalane). Hopanes = C_{29-33} hopanes (Watson *et al.*, 2002).

Examination of the sterane and hopane biomarkers over the course of the study showed that by day 28 the steranes (Figure 2.21), and the diasteranes (Figure 2.22) had appeared to begin to undergo biodegradation. The C₂₇ steranes have been highlighted in Figure 2.21, and for example, the $\alpha\beta\beta$ 20S isomer seems to be preferentially degraded to the $\alpha\beta\beta$ 20R isomer. By day 119 both isomers appeared to have undergone biodegradation compared to the C₂₈ and C₂₉ steranes. The C₂₇ diasteranes are highlighted in Figure 2.22, and it can be seen that the $\alpha\beta\beta$ 20S isomer seems to be degraded preferentially to the $\alpha\beta\beta$ 20R isomer, and by day 119 both isomers appeared to have undergone biodegradation compared to the other resolved compounds. Figure 2.23 shows that even the hopane biomarkers (C₃₀ – C₃₃), seem to have undergone some biodegradation by day 119. These compounds have been proposed as resistant biomarkers suitable for monitoring crude oil degradation (*e.g.* Wang *et al.*, 1994; Wang and Fingas, 1997; Wang *et al.*, 1998). From Figure 2.23 the $\alpha\beta$ 22R isomer appears to have been degraded preferentially to the $\alpha\beta$ 22S isomer, with the C₃₃ hopanes being degraded more rapidly than the C₃₁ and C₃₂ hopanes.

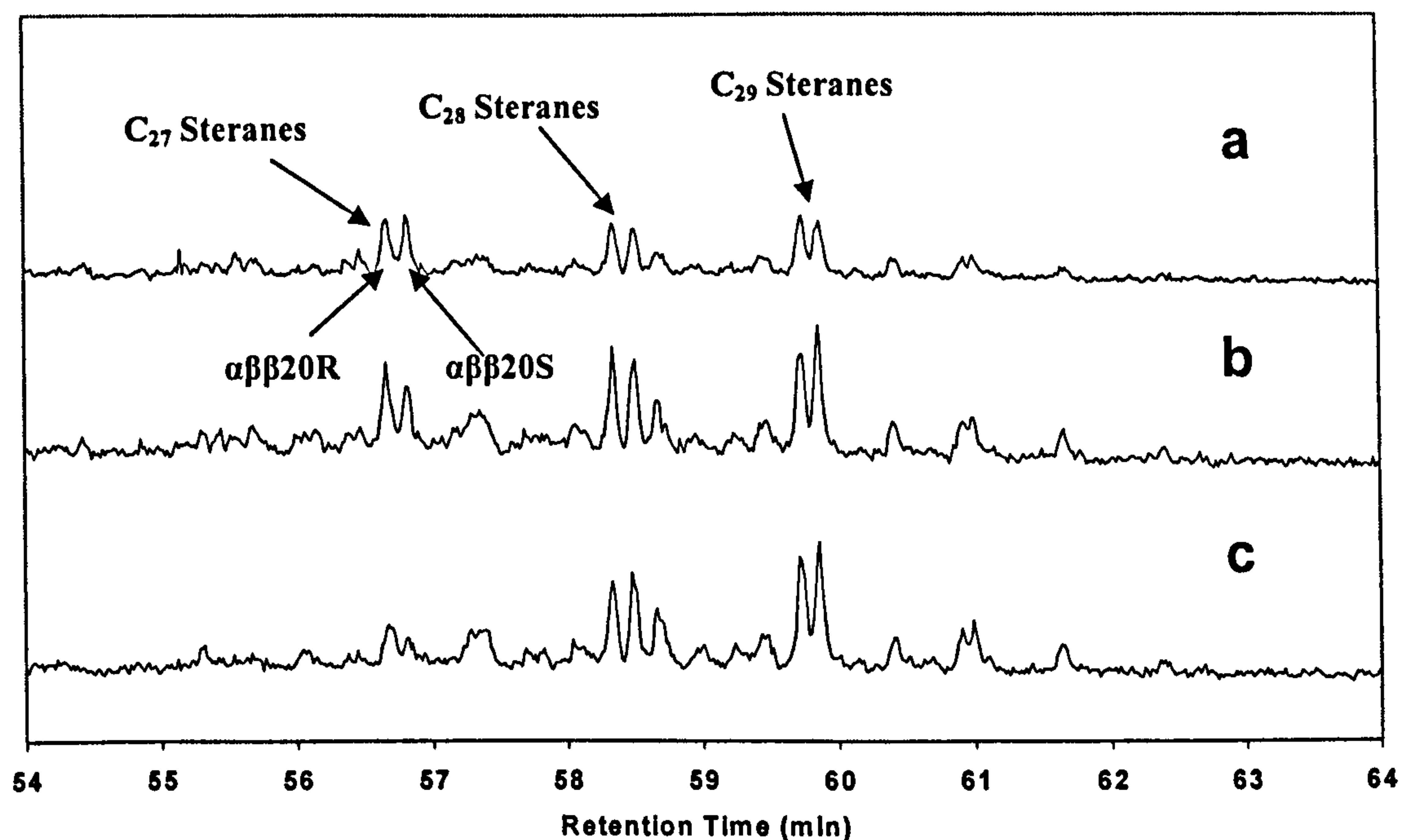


Figure 2.21. GC-MS m/z 218 selected ion fragmentograms illustrating distributions of sterane biomarkers with the Whitley Bay consortium of aerobic bacteria: a. Sterilised day 28 control; b. Biotic day 28 incubation; c. Biotic day 119 incubation. Degradation of the $\alpha\beta\beta$ 20S C₂₇ sterane isomer compared to the $\alpha\beta\beta$ 20R C₂₇ sterane isomer appears to have occurred.

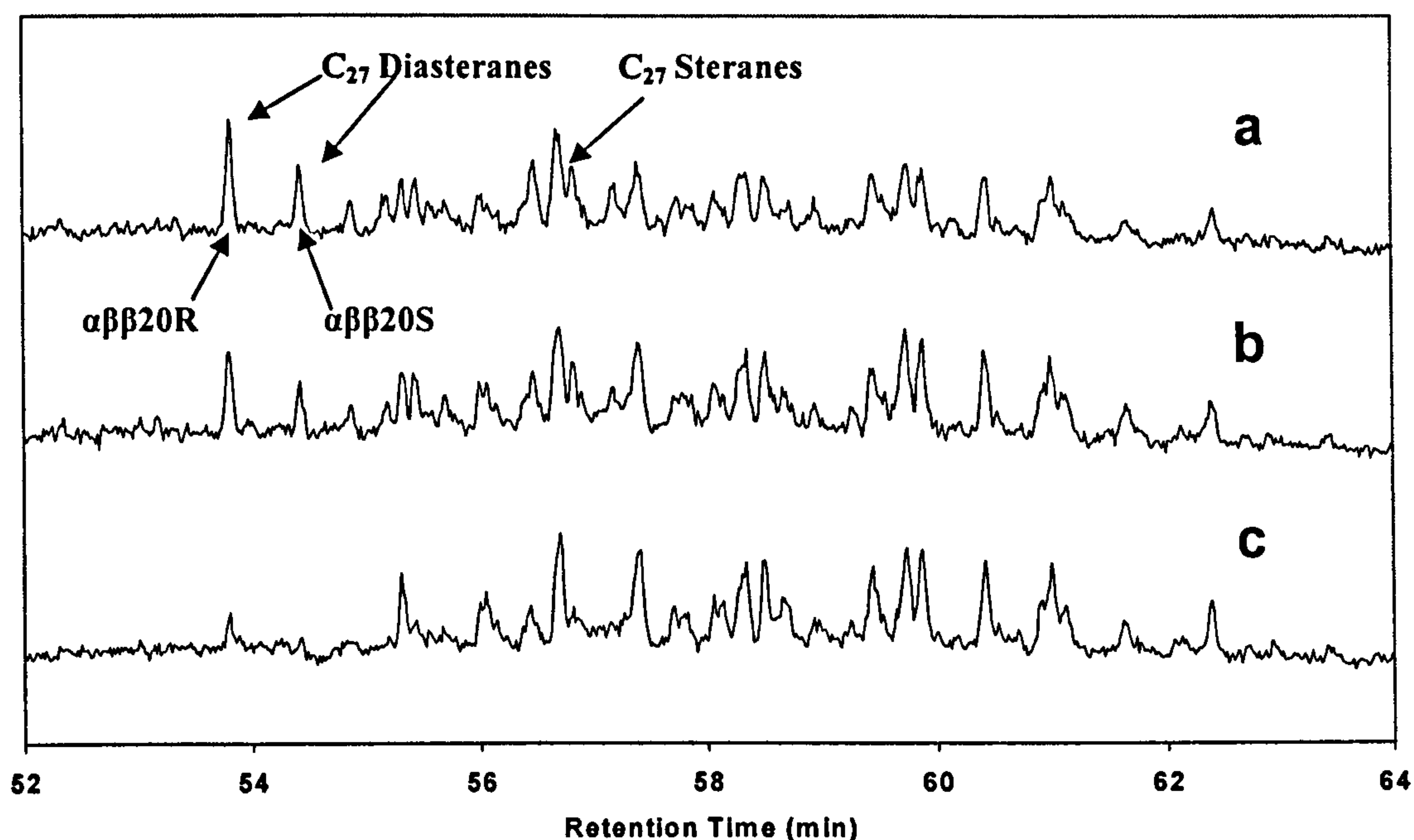


Figure 2.22. GC-MS m/z 217 selected ion fragmentograms illustrating distributions of diasterane biomarkers with the Whitley Bay consortium of aerobic bacteria: a. Sterilised day 28 control; b. Biotic day 28 incubation; c. Biotic day 119 incubation. Degradation of the $\alpha\beta 20S$ C_{27} diasterane isomer compared to the $\alpha\beta 20R$ C_{27} diasterane isomer appears to have occurred.

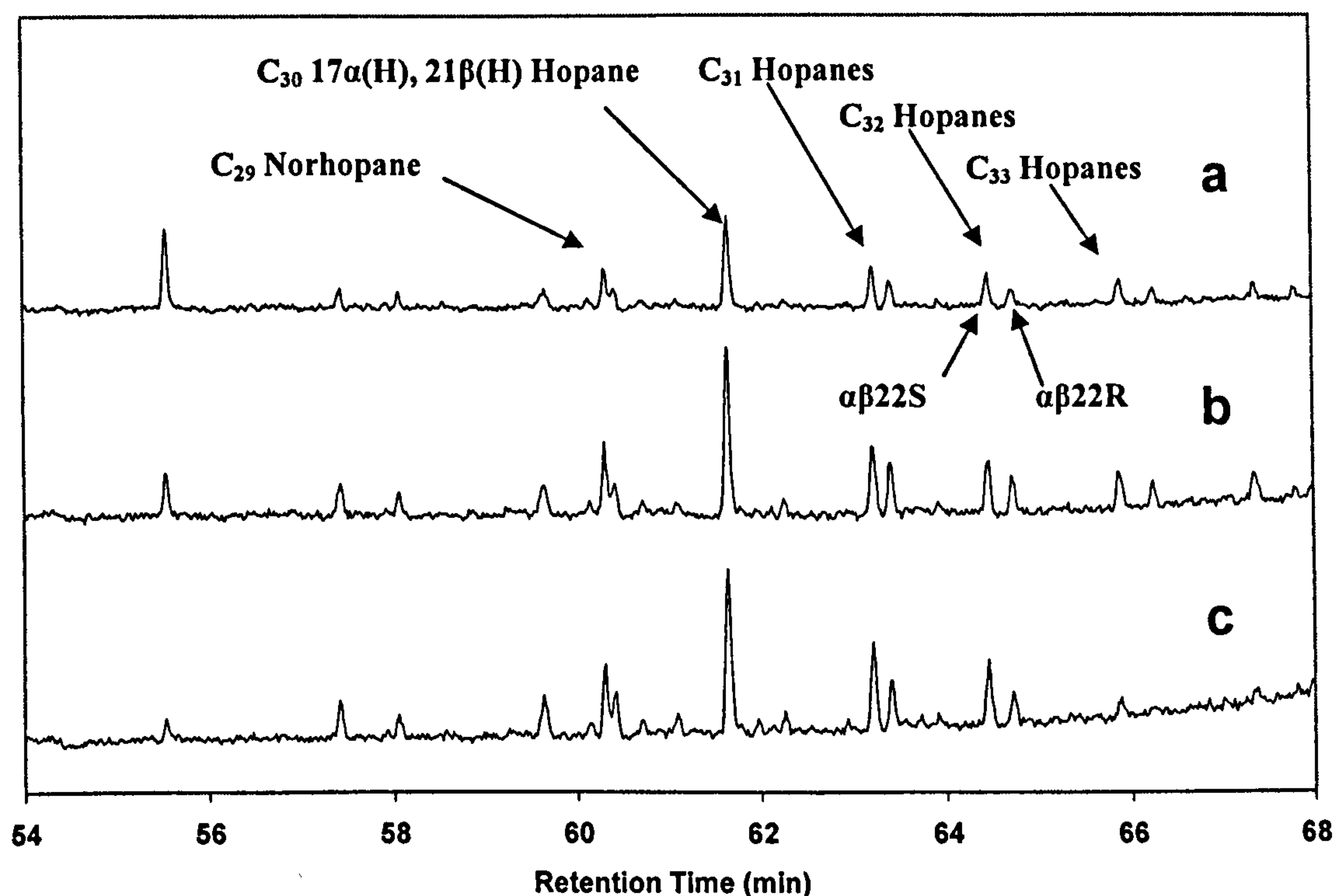


Figure 2.23. GC-MS m/z 191 selected ion fragmentograms illustrating distributions of hopane biomarkers with the Whitley Bay consortium of aerobic bacteria: a. Sterilised day 28 control; b. Biotic day 28 incubation; c. Biotic day 119 incubation. Preferential degradation of the $\alpha\beta 22R$ C_{32} hopane isomer compared to the $\alpha\beta 22S$ C_{32} hopane isomer appears to have occurred.

2.3 Discussion

2.3.1 Mono-culture studies

The rapid biodegradation of *n*-pentacosane (*n*-C₂₅) in the present study by *Pseudomonas fluorescens* is not only in agreement with the observations of Gough *et al.* (1992), but also with the general findings of most investigations into the biodegradation of different aliphatic hydrocarbons by this bacterium (*e.g.* Šepić *et al.*, 1996; Jensen *et al.*, 2000). For instance, Heath *et al.* (1997) showed that a *n*-C₂₀ to *n*-C₆₀ alkane mixture exposed to *P. fluorescens* was rapidly degraded by 80% after 14 days. The most resistant hydrocarbons were in the range *n*-C₄₀₋₆₀. Heath *et al.* (1997) reported that *n*-pentacosane used in the experiment decreased rapidly within 3 days (88% removal) and was completely degraded after 14 days of incubation. These results suggested that the conditions used in the present study were appropriate for a comparison of the relative degradation rates of different compound classes. Indeed, the data shown in Figure 2.9 are clearly in agreement with this; a rapid degradation of *n*-C₂₅ being recorded within 5 days.

In contrast, under the same conditions, the synthetic compounds I-VI were resistant to biodegradation by *P. fluorescens*. Compared with the abiotic controls, the maximum extent of degradation for any compound after 50 days was $18.1 \pm 3\%$ ($n=3$) for compound I (Figure 2.12). The statistical differences between the extent of degradation at day 50 for most of the pairs of compounds were significant (I/II, II/III, IV/V $P=0.01$) except for III/IV and V/VI where the extent of biodegradation was not statistically different from each other at any level (Table 2.2). Thus 7-cyclohexyl-1-*n*-propyltetralin (III) and 7-cyclohexyl-1-isoamyltetralin (IV) were both equally resistant to degradation, as were 7-cyclohexyl-1-*n*-nonyltetralin (V) and 7-cyclohexyl-1-*n*-nonylnaphthalene (VI).

Table 2.2. Summary of the t-test statistical analysis. ✓ = statistically different, x = not statistically different.

	I-II	II-III	III-IV	IV-V	V-VI
t=	5.49	9.88	0.53	4.21	1.01
P = 0.01 (critical value = 2.92)	✓	✓	x	✓	x
P = 0.1 (critical value = 1.75)	✓	✓	x	✓	x

Abiotic losses (volatilisation) were considerable however for the C₁₆ and C₁₇ alkyltetralins (I and II), as expected for relatively low molecular weight compounds. Such compounds are also probably susceptible to comparable rapid evaporation in the natural environment. Budzinski *et al.* (1998) observed significant abiotic losses of dimethylnaphthalenes (50-70%) compared to trimethylnaphthalenes, dibenzothiophenes and phenanthrenes (<30%) using a similar experimental method to that used in the present study. These losses were attributed to evaporation and oxidation. In the present study the C₁₉, C₂₁ and C₂₅ alkyltetralins (III-V) were much less volatile (mean recovery of abiotic controls ≥ 100%) and these were also more bioresistant than I and II (Figure 2.12). In all the biotic experiments the bacteria remained viable (though not rapidly growing) even after 50 days, as monitored by plating on agar and also by examination by spectrometry (Figure 2.10; *c.f.* Heath *et al.*, 1997). Under the same conditions *n*-C₂₅ was rapidly degraded (Figure 2.9) as was also observed previously under very similar conditions (Gough *et al.*, 1992). Under such conditions, C₂₅ methyl-branched alkanes were also readily degraded (95% in 25 d), whereas C₂₅ polymethyl branched and so-called highly branched acyclic isoprenoids were more slowly degraded (but up to 41% degradation in 25 d; Gough *et al.*, 1992). Under these conditions a C₂₅ 'T'-branched alkane and 'T'-branched alkylbenzene were also found to be resistant (*e.g.* alkylbenzene; 39% degradation in 25 d). Thus, to *P. fluorescens*, under the conditions specified, some of the alkyltetralins (in particular III, IV and V) appear to satisfy the criteria for classification as persistent monoaromatic pollutants, and thus as potential components of monoaromatic UCMs in the environment. The diaromatic 7-cyclohexyl-1-nonylnaphthalene also exhibited significant resistance to biodegradation by

P. fluorescens, and may be illustrative of some of the compounds found in the diaromatic fractions of oil UCMs.

An assessment of the biodegradability of the aromatic fraction of an unresolved complex mixture of hydrocarbons has not been made previously. In this study an aromatic fraction of the weathered crude oil Tia Juana Pesado (Venezuela) was isolated using open column chromatography and exposed to *P. fluorescens* under the same conditions as the synthetic compounds (I-VI). The main aims of this study were to determine whether an aromatic UCM from previously weathered crude oil was susceptible to further degradation, and to quantify the results observed for the synthetic compounds (I-VI). Figure 2.14 shows that there was little or no (further) degradation of the hydrocarbons in the aromatic UCM under these conditions. This observation is not unexpected, as UCMs are persistent in the environment following the biodegradation of simple GC resolvable compounds. Reddy *et al.* (2002) suggest that many of the compounds in the UCM may be too structurally complex to be biodegraded. Clearly, under the conditions used here, the synthetic compounds I-VI were nearly as resistant to biodegradation as the aromatic UCM hydrocarbons remaining in a fraction of TJP crude oil after it had been biodegraded naturally over geological time. In fact compound IV (7-cyclohexyl-1-isoamyltetralin) exhibited nearly identical resistance to biodegradation by *P. fluorescens* as the naturally occurring hydrocarbons in the aromatic UCM. The suggestion (Smith *et al.*, 2001) that some of the components occurring within the monoaromatic UCM may be highly branched alkyltetralins or structurally similar compounds is thus consistent by the above findings.

2.3.2 Mixed culture studies

Previous studies have shown that a consortium of bacteria isolated from seawater and sediments of Whitley Bay and Cullercoats, UK was capable of extensively and rapidly degrading the hydrocarbons of crude oil (Watson *et al.*, 2002). Indeed, incubations of artificially weathered Light Arabian crude oil with the bacteria for up to 80 days showed that even the polycyclic hopanoid alkanes, which are amongst the most resistant resolved alkanes of crude oils, are partially degraded to the corresponding hopanoic acids by this consortium (Watson *et al.*, 2002). Thus, incubation with an inoculum of these bacteria should provide an indication of susceptibility to biodegradation in a typically polluted environment.

In biodegradation studies using the mixed culture isolated from Whitley Bay and either *n*-C₁₆ or North Sea crude oil as co-substrates compounds I and II appeared to be significantly more biodegraded than compounds IV and VI (Figures 2.17 and 2.19). This trend is very similar to that observed in the biodegradation experiments conducted with *Pseudomonas fluorescens*. However, biodegradation in the monoculture study was less significant than following exposure to this more aggressive mixed consortium. Although compounds I and II were completely degraded by the end of the study (119 days), significant amounts of compounds IV and VI remained undegraded by the consortium after this lengthy period.

The data, however, should be treated with caution. Figures 2.16 and 2.18 show the percentage recovery of the compounds I, II, IV and VI after 28 and 119 days. Compounds I and II are both completely removed by day 119, but may have reached this zero point prior to sampling at day 119. This makes the assignment of accurate degradation rates to these compounds difficult. In the case of the crude oil co-substrate study (Figure 2.18), the recovery of the control samples is quite low, reflecting difficulties in the analysis owing to co-elution of the target compounds with compounds present naturally in the crude oil co-

substrate. However, the study using $n\text{-C}_{16}$ as a co-substrate exhibited very high ($\sim 140\%$) recovery values for the controls at day 119. It is unknown why this occurred as the control values for the samples taken at day 28 are approximately 100%, as expected. As the day 0, 28 and 119 samples and controls were all analysed at the same time instrumental error can be discounted as the reason for the unexpectedly high control values observed at day 119.

The removal of the n -alkanes and acyclic isoprenoids of the North Sea crude oil co-substrate by the above bacterial consortium appeared to be at least as rapid as the degradation of n -pentacosane by *Pseudomonas fluorescens*. The extent of oil degradation was assessed as 'heavy' on well-accepted environmental or in-reservoir scales of biodegradation reported by Volkman *et al.* (1984), Peters and Moldowan (1993) and Wang *et al.* (1994). This is reflected in the removal of the n -alkanes and acyclic isoprenoids and the alteration of some tetracyclic steranes, observed in the chromatograms. Quantification by selected ion monitoring GC-MS showed that there was no reliably measurable alteration of the total C_{29-33} hopanes over the time period of this study (Figure 2.23). However, preferential degradation of some isomers was observed (Figure 2.23) indicating significant degradation of the oil. Watson *et al.* (2002) observed 1-3.5% alteration of hopanes after 80 days with concomitant production of hopanoic acids. It is unlikely that the present method would allow reliable measurement of such small losses but the relative degradation of isomers is clear. These compounds have been used as 'conservative' hydrocarbon biomarkers (Wang *et al.*, 1994) but it is clear that even these polycyclics are degraded somewhat by this consortium. Figure 2.24 shows ratios of individual alkyltetralin/ C_{30} hopane, where a decrease in the ratio is a measure of the relative extent of biodegradation of the alkyltetralin (*e.g.* McMillen *et al.*, 1995). All of the compounds were shown to biodegrade, but compound IV was shown to undergo the least transformation relative to control values.

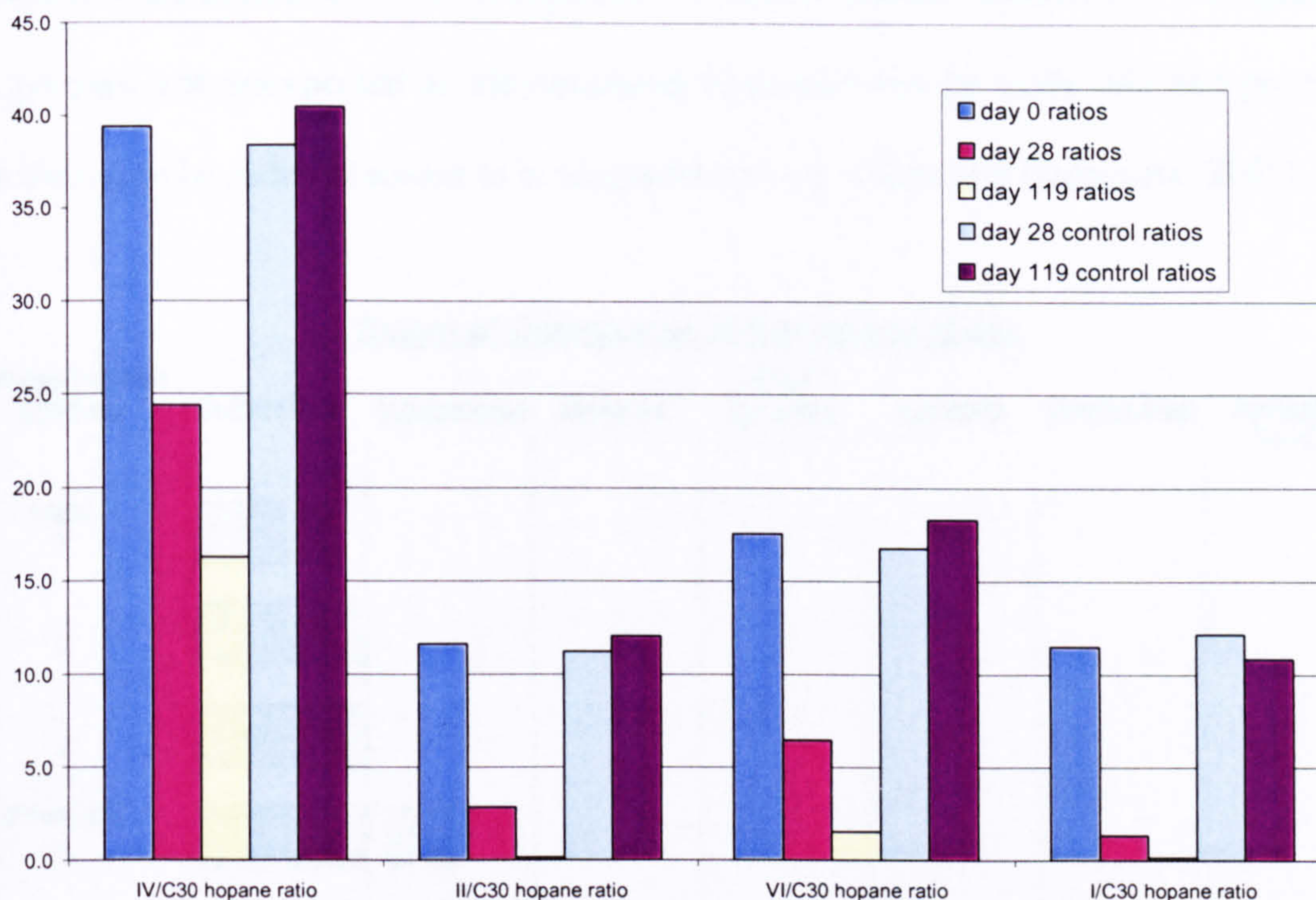


Figure 2.24. Comparison of the alkyltetralin/C30 hopane ratios with time. C30 hopane is considered resistant to biodegradation by the Whitley Bay bacterial consortium over the time period of this experiment (c.f. Wang et al., 1994; Watson et al., 2002).

Analysis of the biodegradation of different components and component classes of the North Sea crude oil allows the alkyltetralins to be positioned on the scale suggested by Peters and Moldowan (1993). An adjusted version of the biodegradation scale published by Peters and Moldowan (1993) is shown in Figure 2.25. The authors used the differential resistance to biodegradation of biomarker types to rank the extent of biodegradation of oils by comparing their relative amounts. From this they were able to suggest a scale on which crude oils could be ranked according to their degradation based on the relative abundances of the various hydrocarbons classes. Figure 2.25 shows the effects of various levels of biodegradation, ranked from 1-10. The authors suggest that a sharp division occurs between ranks 5 and 6, once the isoprenoids are removed, but prior to degradation of the steranes. The alkyltetralins were also ranked herein on the scale suggested by Volkman *et al.* (1984), and the adjusted scale can be seen in Table 2.3. Volkman *et al.* (1984) presented a revised nine point scale which ranked oils according to the extent which the hydrocarbon

distribution had been altered by biodegradation. This observed resistance to biodegradation was perhaps not unexpected as alkylaromatic hydrocarbons in crude oils had previously been shown to be rather resistant to biodegradation (*e.g.* Dutta and Harayama, 2001).

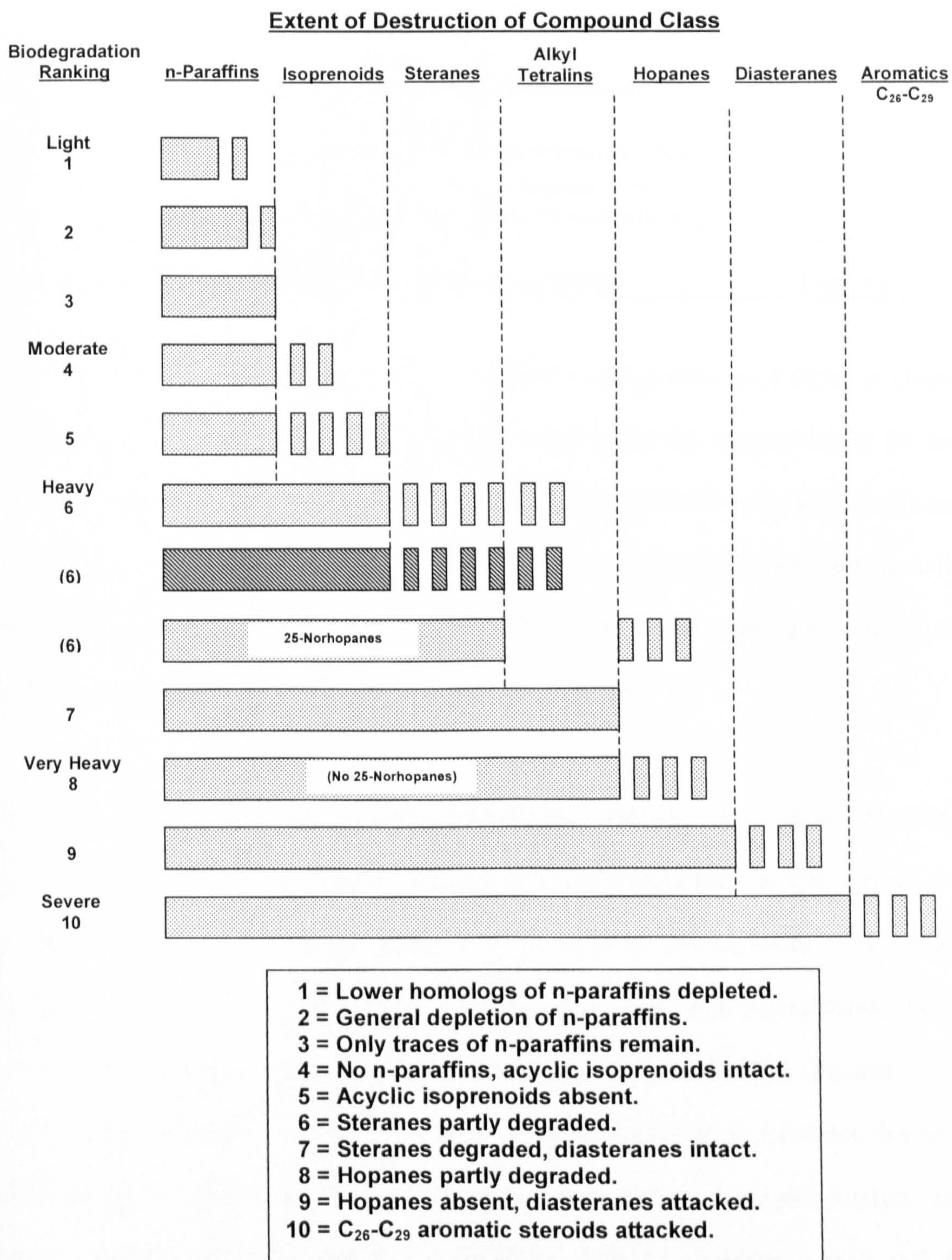


Figure 2.25. Biodegradation scale of Peters and Moldowan (1993) illustrating the relative position of the bioresistant alkyltetralins (*e.g.* compound IV) compared with other widely used biomarker classes.

Table 2.3. The effects of biodegradation on the composition of saturated and aromatic hydrocarbons in a typical mature paraffinic oil (Volkman *et al.*, 1984).

Level	Chemical composition	Extent of biodegradation
1	Abundant n-alkanes	Not degraded
2	Light-end n-alkanes removed	Minor
3	>90% n-alkanes removed	
4	Alkylcyclohexanes and alkylbenzenes removed; acyclic isoprenoid alkanes and naphthalene reduced	Moderate
5	Isoprenoid alkanes and methylnaphthalenes removed; selective removal of C ₂ -naphthalenes	
6	C ₁₄ -C ₁₆ bicyclic alkanes removed; alkyltetralins altered	Extensive
7	>50% (20R) - 5 α (H),14 α (H),17 α (H) steranes removed	Very extensive
8	Distribution of steranes and triaromatic steroids altered; demethylated hopanes abundant	Severe
9	No steranes; demethylated hopanes predominate	Extreme

Bost *et al.* (2001) suggested that the order or pattern of degradation of different classes of biomarkers can differ between oils, which may indicate that the composition of the carbon source exerts control on microbial consortia. This may explain why degradation of some of the steranes was observed with respect to other known bioresistant hydrocarbon markers. In another oil, with a different suite of hydrocarbons components and concentrations different trends in biodegradation may have been observed.

It is therefore suggested that the alkyltetralins studied here show a much higher resistance to biodegradation than the acyclic alkanes, and are comparable in this respect to the steranes. As with the previous experiments (*P. fluorescens*) abiotic losses were substantial for compounds I and II. Interestingly, the same order of average bioresistance (IV>II>I) was observed with both the consortium of bacteria and *P. fluorescens* (Figures 2.12 and 2.17). It is also interesting to note that there appeared to be slightly increased degradation of the synthetic compounds when they were present as part of a complex mixture, except for compound IV. This is shown by a comparison of the oil co-substrate study and the *n*-C₁₆ co-substrate study. Figure 2.26 shows the extent of degradation for the compounds studied (I, II, IV and VI) at days 28 and 119. At day 28 compounds I, II and VI are all more degraded in samples containing the North Sea crude oil than in the samples

containing excess *n*-hexadecane. This observation supports the occurrence of co-oxidation processes during the biodegradation of complex mixture of hydrocarbons such as crude oil (e.g. Volkman *et al.*, 1984; Cooney *et al.*, 1985; Bayona *et al.*, 1986). However, the opposite appears to be the case for compound IV.

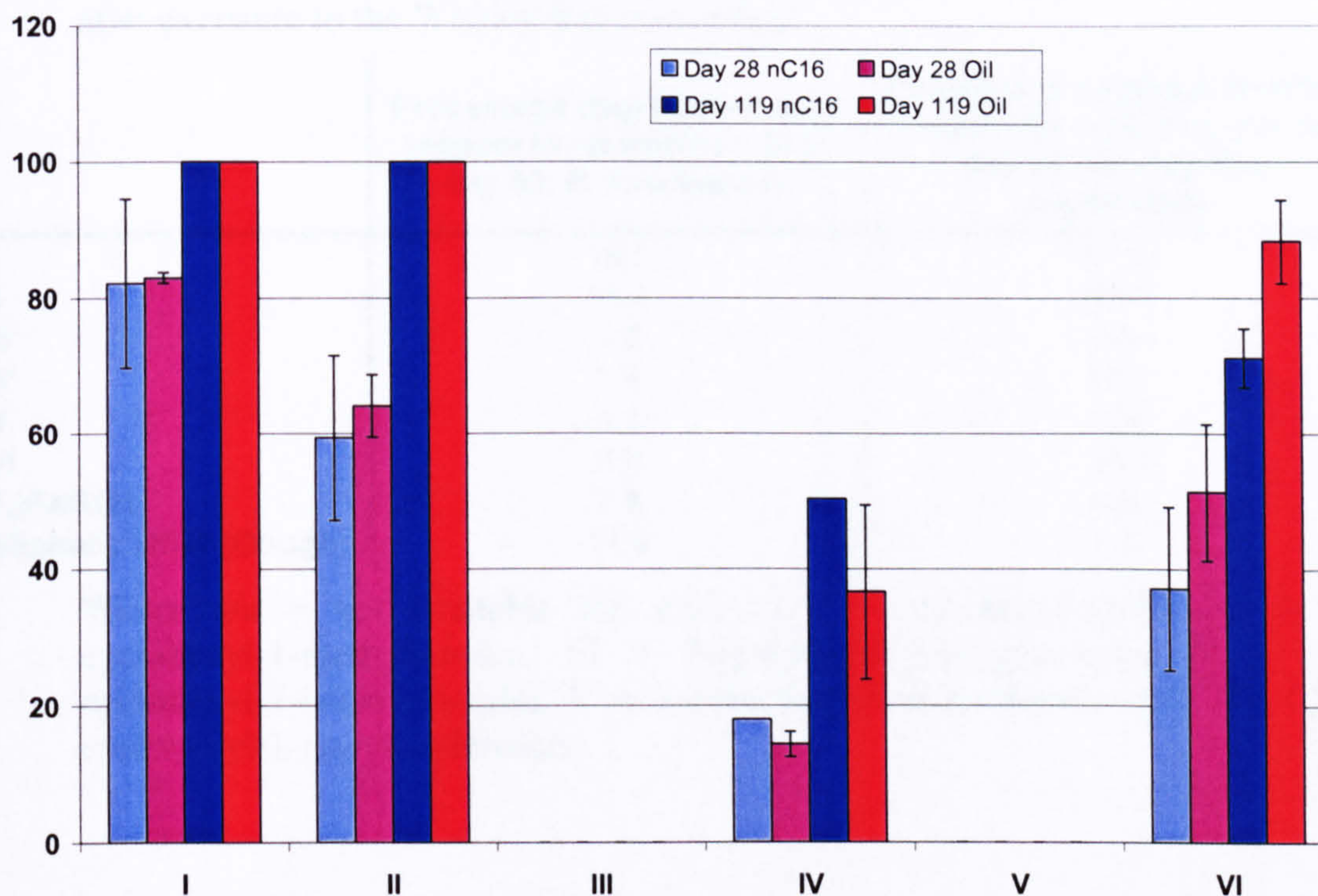


Figure 2.26. Comparison of the extent of biodegradation, relative to the abiotic controls, of the model compounds I, II, IV and VI when incubated with *n*-hexadecane and North Sea oil at days 28 and 119.

2.3.3 Comparison to legislative tests

When the data in this study are compared to the current legislation regarding the persistence of substances in the environment (Section 2.1.7) it is clear that after exposure to the *P. fluorescens* mono-culture, compounds I-VI and the aromatic and aliphatic UCMs are all less than 20% biodegraded after at least 28 days (Table 2.4). Thus, under the conditions employed in this study these substances are deemed resistant to ‘inherent primary biodegradation’ in the environment (OECD 301 series tests). When the half-life ($T_{1/2}$) of the compounds is calculated (Table 2.5) all of the compounds have half-lives

greater than 50 days which would qualify them as environmentally persistent according to the OSPAR Commission (2002).

Table 2.4. Summary of the percentage degradation of compounds I-VI and aromatic (TJPARO1) and aliphatic UCMs at day 50, after exposure to the bacterium *Psuedomonas fluorescens*, and compounds I, II, IV and VI at day 28, after exposure to the Whitley Bay consortium.

	Percentage degradation with respect to control (n = 3) at day 50, <i>P. fluorescens</i>	Percentage degradation with respect to control (n = 3) at day 28, Whitley Bay consortium
I	18.1	82.9
II	11.4	64.0
III	1.5	n/a
IV	1.9	14.3
V	4.5	n/a
VI	3.9	51.3
TJPARO1	2.4	n/a
Aliphatic UCM (Gough)	17.9	n/a

Where n/a = not available for study, I = 6-cyclohexyltetralin, II = 7-cyclohexyl-1-methyltetralin, III = 7-cyclohexyl-1-propyltetralin, IV = 7-cyclohexyl-1-isoamyltetralin, V = 7-cyclohexyl-1-nonyltetralin and, VI = 7-cyclohexyl-1-nonylnaphthalene.

Table 2.5. Summary of the calculated half-lives (days) of compounds I-VI and the aromatic and aliphatic UCMs when exposed to *Pseudomonas fluorescens*, and compounds I, II, IV and VI when exposed to the Whitley Bay consortium.

	Calculated biodegradability half-life (t _{1/2}), <i>P. fluorescens</i>	Calculated biodegradability half-life (t _{1/2}), Whitley Bay consortium
I	140	30
II	220	39
III	1720	n/a
IV	1350	175
V	560	n/a
VI	640	49
TJPARO1	1050	n/a
Aliphatic UCM (Gough)	140	n/a

Where n/a = not available for study, I = 6-cyclohexyltetralin, II = 7-cyclohexyl-1-methyltetralin, III = 7-cyclohexyl-1-propyltetralin, IV = 7-cyclohexyl-1-isoamyltetralin, V = 7-cyclohexyl-1-nonyltetralin and, VI = 7-cyclohexyl-1-nonylnaphthalene.

When compounds I, II, IV and VI were exposed to a natural consortium of bacteria (Whitley Bay) a greater extent of degradation was observed. Whilst compounds I, II, and VI are biodegraded by more than 20% after 28 days compound IV is still less than 20% biodegraded (Table 2.4), indicating that it would be classed as a persistent substance resistant to 'inherent primary biodegradation' by the OECD 301 series tests. Compounds II and VI are still less than 70% biodegraded, thus there is evidence for 'primary' but not 'ultimate' inherent biodegradability. Only compound I can be classed as being susceptible to ultimate inherent biodegradability during the experiments undertaken in this study. The calculated half-life of the compounds (Table 2.5) indicates that compound IV is again sufficiently resistant to biodegradation to be classed as environmentally persistent and compound VI is on the very limit of classification, having a half-life of 49 days. However it must be stressed that these data were not generated using one of the accepted standard tests, and the experimental procedures employed in this study are different from those tests, in particular the use of a mono-culture to measure biodegradation.

2.3.4 Modelling the biodegradation of the alkyltetralins and an alkylnaphthalene

A standardised test of chemical biodegradability has been devised by the Japanese Ministry of International Trade and Industry (MITI test; Tunkel *et al.*, 2000). This has been adopted by the Organisation for Economic Co-operation and Development (OECD) as a regulatory test for industrial chemicals. Increasingly, computer modelling of the test results is used to predict the biodegradability of chemical pollutants. The test involves incubation at 25 °C of test chemicals with a mixed consortium of aerobic bacteria cultured from a number of industrial and sewage effluent sludges, natural river, lake and seawaters and sediments. The composition of the consortium is not standardised. Compounds are tested at 100 µg mL⁻¹ concentrations for 14-28 days under conditions in which aniline is degraded by at least 65% in 28 days. The effects of the MITI test conditions on chemical biodegradation

have been shown to be predictively modelled with 81% accuracy for a validation set of 295 chemicals by a modelling program known as BIOWIN (v 4.00; Tunkel *et al.*, 2000). BIOWIN v 4.00 is one of the ten stand-alone programs that form the EPI (Estimation Programs Interface) Suite™ developed by the Environment Protection Agency's (EPA) Office of Pollution Prevention Toxics and Syracuse Research Corporation (SRC). This model has been used to predict the MITI biodegradability of the alkyltetralins I-V and the alkylnaphthalene (VI). Although the MITI conditions are rather different to those involved in the present experiments it is important to place the biodegradation of the alkyltetralins and alkylnaphthalene in the context of a regulatory test, particularly as regulation of hydrocarbon mixtures is increasing (*e.g.* Environment Agency, 2003). On a scale where a value of ≥ 0.5 indicates that a compound is 'readily degradable' and a value of < 0.5 indicates 'not readily degradable', compounds I-VI were all classified as "not readily degradable" in the non-linear BIOWIN model (I, 0.16; II, 0.16; III, 0.17; IV, 0.19; V, 0.08; VI, 0.11). For reference, *n*-C₂₅ had a calculated value of 0.9 and was classified as "readily degradable". The value of 7-cyclohexyl-1-pentyltetralin (unbranched version of compound IV) was determined as 0.18. This indicates that branching on an alkyl chain can significantly reduce the susceptibility of a hydrocarbon to biodegradation. Thus, the generally resistant nature of the model alkyltetralins and alkylnaphthalene and in particular the resistance of compound IV observed in the laboratory experiments, is also predicted by the BIOWIN (v 4.00) model.

BIOWIN v 4.00 is based upon original versions of the modelling software, adding two new predictive biodegradation models to the four already available in previous versions. Initially, the program used fragment constants developed using linear and non-linear regressions and evaluated biodegradation data (Howard *et al.*, 1992). Version 3 of the software added new expert survey data, and uses a slight revision of the previous fragments and molecular weight. This allows calculation of the probability of rapid

biodegradation from experimental data and an estimate of the primary and ultimate biodegradation times for complete degradation (days, weeks, months, longer) using evaluations of 200 chemicals by 17 biodegradation experts (Boethling *et al.*, 1994). The biodegradation of the alkyltetralins (I-V) and alkylnaphthalene (VI) was also predicted using these models and the results, along with those from the MITI test are summarised in Table 2.6. The results of the other predictive models indicate that all of the alkyltetralins and the alkylnaphthalene are quickly biodegraded, with ultimate biodegradation (complete mineralisation) predicted within weeks to months. These results somewhat contradict those of the MITI predictive tests, especially the standard linear and non-linear models which predict slow biodegradation of all the compounds. The ultimate and primary biodegradation models provide data that is more consistent with that determined by experimentation with the microbial consortium. Primary biodegradation is predicted to occur within 'days-weeks', and the experimental data indicate that all compounds studied had undergone some degradation after 28 days (Figure 2.26). Ultimate biodegradation is predicted to occur within the 'weeks-months' timeframe, which again is observed in most cases with the experimental data. Compounds I and II are completely degraded after 119 days (~3 months) and compound VI had undergone over 80% degradation in the same period (Figure 2.26). Only compound IV appears to differ significantly from the predicted data. The use of the BIOWIN v 4.00 highlights the advantages and limitations of using predictive software to determine the biodegradability of hydrocarbons in the environment. Whilst predictive software can be a useful tool in supporting and complementing 'real' data, alone it does not provide sufficiently reliable data to qualify as a substitute for experimentally generated results.

Table 2.6. Summary of the predicted biodegradability of the alkyltetralins (I-V) and alkylnaphthalene as calculated by the models available in the BIOWIN (v 4.00) software.

BIOWIN v 4.00 results						
Compound	Linear model prediction	Non-linear model prediction	Ultimate biodegradation timeframe	Primary biodegradation timeframe	MITI linear model prediction	MITI non-linear model prediction
I	Fast	Fast	Weeks-Months	Days-Weeks	Not readily degradable	Not readily degradable
II	Fast	Fast	Weeks-Months	Days-Weeks	Not readily degradable	Not readily degradable
III	Fast	Fast	Weeks-Months	Days-Weeks	Not readily degradable	Not readily degradable
IV	Fast	Fast	Weeks-Months	Weeks	Not readily degradable	Not readily degradable
V	Fast	Fast	Weeks-Months	Days-Weeks	Not readily degradable	Not readily degradable
VI	Fast	Fast	Weeks-Months	Days-Weeks	Not readily degradable	Not readily degradable

2.3.5 Previous studies of biodegradation of tetralin

A review of the literature available for alkyltetralin biodegradation suggests that only tetralin has been extensively studied previously. It is clear that even the unsubstituted compound is rather resistant to the action of aerobic bacteria. Schreiber and Winkler (1983) reported that none of the 41 strains investigated utilised tetralin as sole carbon source in pure culture although *Pseudomonas stutzeri* strain AS39 grown on salicylate was found to metabolise the hydrocarbon poorly, but only when present as a vapour. The authors concluded that the organism utilised an adaptation from the well-known naphthalene degradation pathway. If this is also the case for I-V, it is possible that the cyclohexyl substitution on the aromatic ring may hinder attack. The rather slow degradation of the cyclohexyl-substituted naphthalene (VI, Figure 2.26.) suggests this may indeed be the case. Sikkema and de Bont (1991) reported the isolation of a further eight bacteria which could grow on tetralin but even species known to degrade tetralin were unable to do so when the compound was added to the aqueous phase. Of 28 strains, only 4 could utilise tetralin as sole carbon source and, again, only when the substrate was added in the vapour phase or in a 2-phase system with fluorocompound 40. A further 4 species isolated from hydrocarbon-polluted areas were a mixture of Gram-negative and positive species but all eight grew very slowly on tetralin. More recently, Hernaez *et al.* (1999), isolated a new bacterial strain (tentatively assigned as *Sphingomonas macrogoltabidus*) from Rhine river mud by enrichment in a liquid, carbon-free minimal medium to which tetralin was again added *via* the vapour phase. Obviously vapour phase addition is not an environmentally realistic scenario for less volatile higher homologues.

Thus, the resistance of the present synthetic dialkyltetralins to a pure culture of *P. fluorescens* and a natural consortium of hydrocarbon degrading bacteria is consistent with previous findings for tetralin with single bacterial strains.

2.3.6 Biodegradation of the alkylcyclohexyltetralins (I-VI)

Using the mechanisms reported for the aerobic biodegradation of aliphatic and aromatic compounds in Section 2.1.4, it may be possible to postulate the method of biodegradation for the alkylcyclohexyltetralins (I-VI). Microbial attack could be towards the aromatic ring, creating a dihydrodiol, where two OH groups are introduced, usually on adjacent carbon atoms (Gibson and Subramanian, 1984; Iqbal and Mason, 1999; Wackett and Hershberger, 2001). However, it could also occur on either of the alicyclic rings (compounds I-V) forming hydroxy or keto derivatives, which would undergo further degradation. Finally, oxidation could begin on the alkyl chain (compounds II-VI). Terminal attack is the most likely mechanism, but compounds with larger alkyl groups (*e.g.* V and VI) could also under go sub-terminal oxidation (Swisher, 1987). The possible modes of microbial attack on the alkylcyclohexyl tetralins (I-V) are summarised in Figure 2.27.

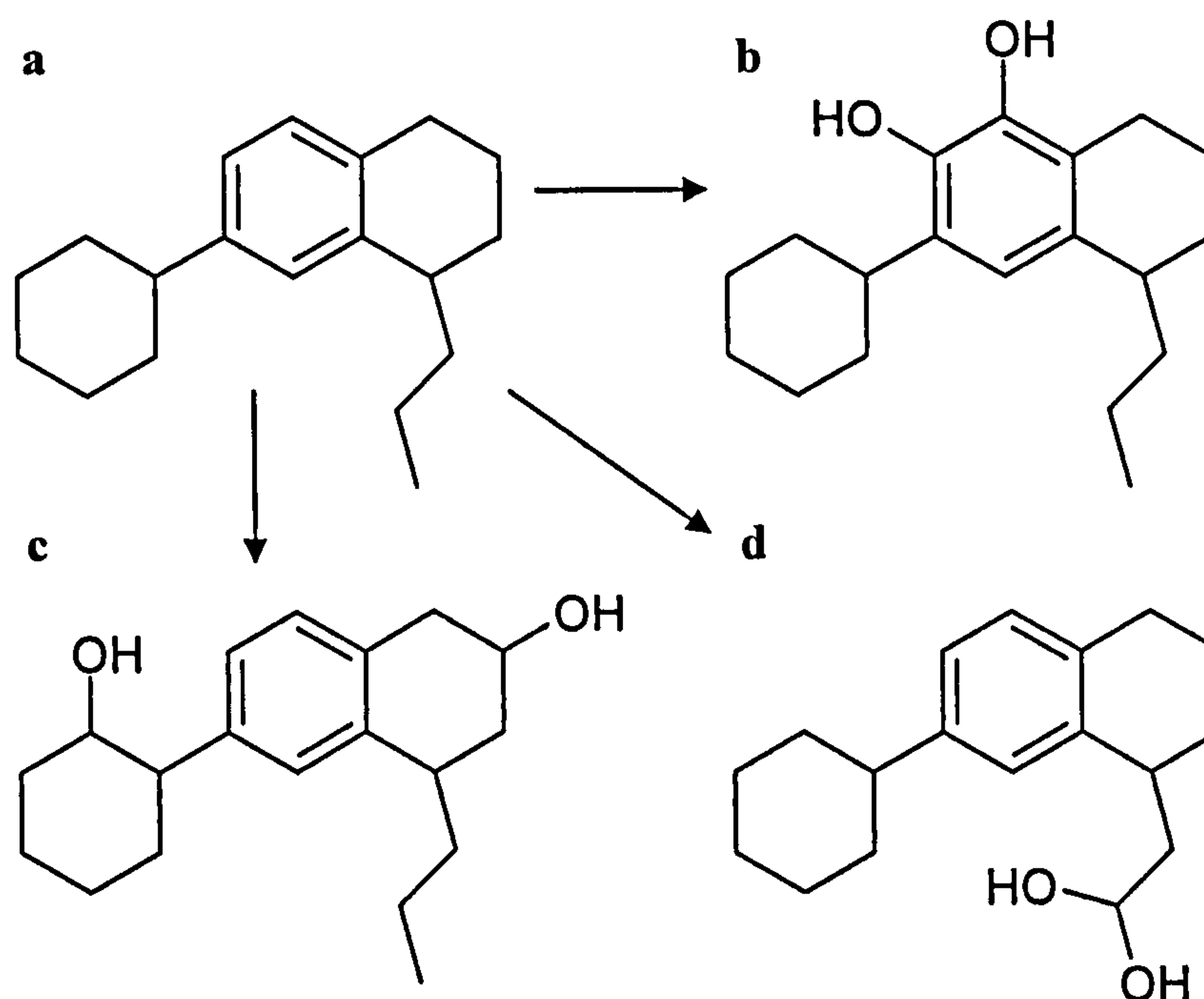


Figure 2.27. Three possible oxidation products of 7-cyclohexyl-1-propyltetralin. (a) Original compound, (b) Aromatic oxidation, (c) Oxidation of either of the cycloalkane constituents, (d) oxidation of the alkyl chain which can occur at the end (terminal) or before the end (subterminal) of the chain.

Biodegradation is thought not to occur *via* the aromatic ring as there is only one place where two adjacent carbon atoms are free for the oxidation to the dihydrodiol (Figure 2.27b). Furthermore, once the ring has been opened the presence of the cycloalkane and the hexa-cycle substituent would present a branching system, which may prevent degradation.

The core structure of compounds I-V is identical and yet there are significant differences in their degradation rates. As the alkyl chain is the only structural difference between the compounds it is suggested that this exerts the greatest influence on their degradation. However, it is unclear whether the alkyl chain itself is the target of oxidation or whether it hinders the degradation of either the aromatic or cycloalkane components. To help determine the effects of the alkyl chain, 3-dimensional models were created of the compounds studied (I-VI). Details of the modelling software and process have been reported in Section 5.3. Computer modelling indicates the alkyl chains on compounds II-VI do not appear to hinder attack of the aromatic or the cycloalkane groups, nor are they hindered to attack themselves. Figure 2.28 shows the 3-dimensional model of 7-cyclohexyl-1-isoamyltetralin. It is therefore suggested that the main mechanism of biodegradation of the alkylcyclohexyltetralins is *via* the alkyl chain.

6-cyclohexyltetralin is the most rapidly degraded of the compounds I-VI, and does not contain an alkyl chain. In this instance it is possible that degradation is occurring on the fused cycloalkane group of the tetralin-base structure. The presence of an alkyl chain (*e.g.* compounds II – V) may hinder attack of the cycloalkane group, or it may be easier to attack than the latter and degradation proceeds on the alkyl chain itself. The faster rate of degradation observed for 7-cyclohexyl-1-nonyltetralin compared to compounds with shorter alkyl chains may be due to the increased length of the alkyl chain being more

accessible to microbes in the mono-culture experiment. The longer chain may also promote sub-terminal or even multiple site oxidation.

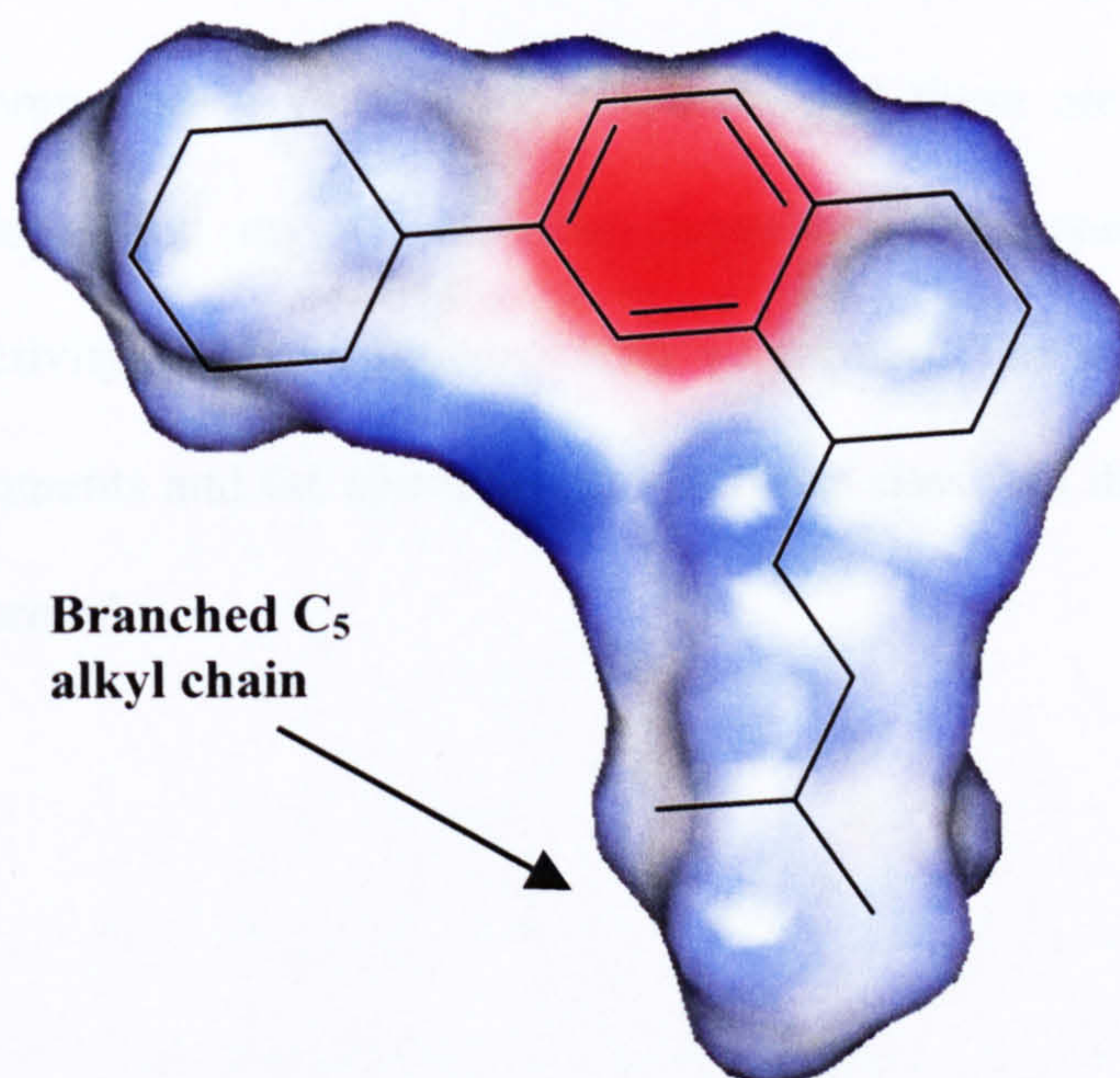


Figure 2.28. 3-dimensional model of 7-cyclohexyl-1-isoamyltetralin, created using ChemDraw 4.0, Chem3D 4.0 and WebLab Viewerlite software packages. The alkyl chain does not hinder biodegradation of other parts of the molecule, nor is it hindered itself. A skeleton structure of the compound drawn has also been placed over the 3D molecule.

Further evidence for microbial attack on the alkyl chain is provided by the branched compound, 7-cyclohexyl-1-isoamyltetralin (IV). The results indicate that this compound exhibits a greater resistance to biodegradation than those with linear substituents. It is possible that the branching in compound IV, particularly as it is at the end of the alkyl chain, hinders biodegradation, by preventing the onset of β -oxidation (*e.g.* Britton, 1984; Swisher, 1987; Alexander, 1999). These results are consistent with the observations of hydrocarbon structure reported in other studies (*e.g.* Budzinski *et al.*, 1998; Garret *et al.*, 1999; Holder *et al.*, 1999; Alexander, 1999; Leblond *et al.*, 2001).

In the case of the microbial consortium experiments, it is possible that there are sufficiently diverse species of bacteria to allow a combination of some or all of these methods of

microbial attack to occur. The microbial consortium may also contain species of bacteria more specialised or suited to the biodegradation of the alkylcyclohexyltetralins and alkylcyclohexylnaphthalene than *Pseudomonas fluorescens* used in the mono-culture studies. This would then result in the greater and possibly more comprehensive degradation of compounds I-VI. It must be stated that these are proposed degradation products, and, depending on which micro-organisms are present and their relative abundance and activity, different products may be formed. The microbial populations of individual environments and the abiotic factors at those sites will determine what products will actually be formed.

2.4 Conclusions

The low biodegradability of the tetralins in the present study by a common, known hydrocarbon degrader (*P. fluorescens*), and literature evidence for the comparative bioresistance of tetralin, supports suggestions from previous oxidative degradation and toxicological studies, that alkyltetralins are sufficiently bioresistant to be present in some monoaromatic UCMs of biodegraded crude oils. Experiments with a wider consortium of bacteria known to efficiently degrade GC detectable components of a weathered Light Arabian crude oil (Watson *et al.*, 2002) were conducted in order to further investigate the bioresistance of alkyltetralins. The alkyltetralins exhibited 15-80 % degradation after 28 days with 60% of the branched chain C₅ homologue remaining even after 119 days. Under these severe conditions a North Sea crude oil was extensively degraded and of compounds accepted as markers of oil degradation only the very bioresistant pentacyclic hopanes were largely undegraded. This places some of the alkyltetralins as resistant to all but heavy biodegradation on accepted scales of biodegradation (Peters and Moldowan, 1993), and further supports their candidacy as bioresistant pollutants and possibly representative of components of monoaromatic UCMs of hydrocarbons. In the same respect, the synthetic alkylcyclohexylnaphthalene also exhibits significant resistance to biodegradation and may be representative of the diaromatic compounds contributing to the aromatic hydrocarbon UCMs.

However, the data generated in the biodegradation experiments using the Whitley Bay consortium should be treated with some caution. In particular, the low control values in the crude oil co-substrate study caused by co-elution of the target compounds with crude oil constituents and the inexplicable high control values in the study using the *n*-C₁₆ co-substrate should be taken into consideration. Additionally, a more frequent sampling regime is necessary for accurate degradation rates to be assigned to the study compounds.

Chapter 3

Water solubility experiments

The water solubility of a compound is one of the most important physicochemical properties effecting its fate and behaviour in the aquatic environment. Aqueous solutions of 6-cyclohexyltetralin were prepared to calibrate a generator column technique at 25 °C in distilled water. The water solubility determined ($109 \pm 3 \mu\text{g L}^{-1}$) compared very well with a previous study. Aqueous solutions of an 'aromatic' and 'monoaromatic' unresolved complex mixture isolated from in-reservoir biodegraded Tia Juana Pesado (TJP) crude oil and, considered to be an 'average' aromatic UCM hydrocarbon structure, were prepared using the same method.

The data indicated that the generator column technique was not ideal for achieving reproducible solutions of complex hydrocarbon mixtures such as UCMs. However, the most 'soluble' components of the 'aromatic' and 'monoaromatic' UCMs entered the aqueous phase, forming secondary UCMs in the aqueous extracts. Characterisation of these solubilised UCM fractions may allow the most bioavailable and toxic UCM components to be identified and measured.

3.1 Introduction

The physical and chemical characteristics of crude oil change almost instantly when it is spilled in the marine environment. This is due to processes such as evaporation, dispersion, emulsification, dissolution, photooxidation, sedimentation, and biodegradation¹. The fate of a hydrocarbon in the environment is primarily controlled by the physicochemical properties of the substance and the prevailing environmental conditions (Mackay *et al.*, 1991; Tolls *et al.*, 2002). Immediately following a spill, evaporation of crude oil (particularly volatile compounds, b.p. <200 °C) is considered the most important weathering process, with up to 50% of the initial volume lost within the first few days (Jordan and Payne, 1980; National Research Council, 1989; Readman *et al.*, 1992; Nicodem *et al.*, 1997; ITOPF, 1999; Neff *et al.*, 2000). Heavier crude oils will lose a much smaller volume over the same period (Michel, 2001; National Research Council, 2003). Harsh weather conditions can increase the rate of evaporation and lead to emulsification. This is the formation of various states of water in oil, often called “chocolate mousse” or “mousse”. For example, high waves quickly formed a stable mousse containing 50-70% water from the oil spilled by the *Amoco Cadiz* (Gundlach *et al.*, 1983) and the *Exxon Valdez* (Galt *et al.*, 1991). These emulsions can significantly change the properties and characteristics of spilled oil in aqueous environments, including increases in the spill volume, density and viscosity (ITOPF, 1999; National Research Council, 2003).

Dissolution or solubilisation is also an important physical weathering process which affects crude oil and other organic compounds released into aqueous environments (*e.g.* Jordan and Payne, 1980; Shiu *et al.*, 1988; Readman *et al.*, 1996). Although the low water solubility of hydrocarbons has led to many of them being deemed virtually ‘insoluble’ in water, in reality all compounds are found to dissolve to some extent (Boylan and Tripp, 1971; Eganhouse *et al.*, 1996; Neff *et al.*, 2000). Solubility is the amount of an oil or petroleum product that will dissolve in the water column on a molecular basis (National

Research Council, 2003). When the molecules of a compound are partitioned so that its concentration reflects equilibrium between the pure material and aqueous solution, it is said to have reached saturation concentration. This saturation concentration in the aqueous phase is known as the water solubility of the compound (Tolls *et al.*, 2002; Miller *et al.*, 1984; Mackay *et al.*, 1980; Schwarzenbach *et al.*, 2003). The water solubility of a given compound is typically classed as the amount that can be dissolved in 1 litre (1000 mL) of pure water at a given temperature.

Partitioning of hydrocarbons into water is thought to only account for a small proportion of oil loss compared with evaporation (Shiu *et al.*, 1990; Riazi and Edalat, 1996; Hibbs *et al.*, 1999). Evaporation is considered to be 100 times faster than dissolution for aromatics and 10,000 times faster for alkanes, although the ratio will depend significantly upon environmental conditions and crude oil composition (Jordan and Payne, 1980; National Research Council, 1989; Cline *et al.*, 1991; ITOPF, 1999). However, it is important because the soluble components of crude oil, particularly small aromatic compounds which can comprise up to 20% of a crude oil, are thought to be the most toxic to aquatic organisms (*e.g.* Seymour and Geyer, 1992; Neff *et al.*, 2000; Rowland *et al.*, 2001; Smith, 2002; Tolls *et al.*, 2002). Solubility is also an important criterion for establishing other effects of oil partitioning. For instance, Banerjee (1984) and Tolls *et al.* (2002) suggest that solubility (and the somewhat related octanol-water partition coefficient) is required for the calculation of bioconcentration factors, sediment adsorption coefficients, toxicity, and biodegradation rates. Additionally, the water solubility of a chemical is an important characteristic for establishing potential for movement and distribution within the environment (Mackay *et al.*, 1980; Miller *et al.*, 1984; Page *et al.*, 2000; Tolls *et al.*, 2002).

¹ Biological processes affecting crude oil in the environment have been discussed in detail in Chapter 2.

Table 3.1 summarises the relative importance of dissolution on the different sources of petroleum hydrocarbon inputs into the environment. In particular, dissolution is most effective in the removal of gasolines, light distillates and low molecular weight fractions of crude oil spilled in the oceans (water-soluble fractions dominated by one- to three-ringed PAH). Dissolution from persistent oil slicks and stranded oil, however, can continue for years after an initial spill (National Research Council, 2003).

Table 3.1. Table summarising the relative importance of the process of dissolution of petroleum hydrocarbons. Adapted from the National Research Council (2003). L = Low, M = Medium, H = High.

Input type	Persistence	Dissolution
Seeps	years	M
Spills		
<i>Gasoline</i>	days	M
<i>Light distillates</i>	days	H
<i>Crudes</i>	months	M
<i>Heavy distillates</i>	years	L
Produced water	days	M
Vessel operation	months	M
Atmospheric	days	M
Land based	unknown	L

Table 3.1 is of course a very qualitative evaluation of the importance of solubilisation. Quantitative and detailed compositional data are more useful, for instance, for computer modelling oil spill fate/prediction. Although some progress has been made in understanding processes such as evaporation, (e.g. Jordan and Payne, 1980; Hibbs *et al.*, 1999) more knowledge is required about other processes, including dissolution, which have been the subject of limited study thus far.

3.1.1 Mechanism of dissolution of organic compounds in aqueous solutions

When a pure liquid or solid solute comes into contact with water at a defined temperature, the solution will approach a unique, constant saturation concentration, that is, the solubility. When the solute is a mixture, an equilibrium is achieved for each component, between the aqueous and organic phases (Shiu *et al.*, 1988). The process of solubilisation of an apolar hydrophobic organic compound in water is complex. Schwarzenbach *et al.* (2003) provide a detailed review of the mechanisms involved in the solubilisation of hydrophobic organic compounds in an aqueous medium. These are reviewed briefly below:

The water surrounding a nonpolar solute is thought to maintain, but not enhance, its hydrogen-bonding network. In doing this, water molecules are able to host an apolar solute of limited size without losing a significant number of H-bonds. Thus, if a small apolar organic solute is introduced to water it will primarily undergo van der Waals interactions. This should not result in the breaking of H-bonds among the water molecules, which would lead to a loss in enthalpy (Schwarzenbach *et al.*, 2003). Examples of such compounds include monoaromatics such as benzene, methylbenzene, dimethylbenzene and short chain alkanes such as *n*-pentane and *n*-hexane. Larger compounds such as PAHs exhibit greater positive excess enthalpies (Schwarzenbach *et al.*, 2003). Thus, most hydrocarbons only acquire a weak energy gain when dissolved in water, due to attractive dispersion forces. This amount of energy is far outweighed by the expenditure of energy required to accommodate apolar hydrocarbon molecules in the hydrogen-bonding network of the water molecules (Tolls *et al.*, 2002). With increasing apolar solute size, water is not able to maintain a maximum of hydrogen bonds among the water molecules involved (Tolls *et al.*, 2002; Schwarzenbach *et al.*, 2003). Thus, it can be summarised that the solvation of an organic solute in water is dependent not only upon the size of the molecule, but also the van der Waals and hydrogen-bonding interactions.

3.1.2 Toxicity dependence upon the water solubility of a hydrocarbon

When a hydrocarbon mixture, such as crude oil, is spilled in the marine environment, its partitioning or dissolution behaviour into the water column is of considerable interest and concern (*e.g.* Shiu *et al.*, 1990; Siron *et al.*, 1991; OSPAR Commission, 2000; Michel, 2001). Whilst the amount of oil that dissolves in the water is usually small (<1 ppm, ITOPF, 1999), this is the fraction to which many aquatic organisms are exposed (Shiu *et al.*, 1990; Siron *et al.*, 1991; Neff *et al.*, 2000). However, the level of exposure will depend upon individual organisms. Readman *et al.* (1992) suggest lower levels of contamination observed in fish from the Arabian Gulf compared to bivalve molluscs could be due to the fish being able to avoid oil spills and/or because they are capable of rapidly metabolising or depurating ingested oil. Chemicals dissolved in the ambient water are considered more bioavailable to organisms than chemicals in solid or adsorbed forms (National Research Council, 2003). Therefore, the extent to which aquatic organisms are exposed to toxicants such as petroleum hydrocarbons is largely controlled by the water solubility of the toxicant (May *et al.*, 1978b; Siron *et al.*, 1991; Michel, 2001; National Research Council, 2003).

The degree to which a substance can be absorbed into the tissues of organisms and so influence their physiology is termed 'bioavailability' (OSPAR Commission, 2000). As well as the properties of a substance (*e.g.* its solubility in water) it is suggested that bioavailability also depends on the habitats and feeding mechanisms of the organisms concerned (OSPAR Commission, 2000; National Research Council, 2003). When an aquatic animal is exposed to a nonpolar organic compound dissolved in the ambient water, the chemical partitions across permeable membranes into tissue lipids. This continues until an equilibrium, approximated by the octanol/water partition coefficient (K_{ow}) for the chemical is reached. At this equilibrium the rates of absorption into and desorption from the lipid phase of the organism are equal (National Research Council, 2003). Hydrocarbons

of crude oil origin (e.g. some PAH) and crude oil itself have been found to bioaccumulate in organisms *via* this process (e.g. Siron *et al.*, 1991; Franke *et al.*, 1994; Neff *et al.*, 2000; Ziolli and Jardim, 2002).

3.1.3 Factors affecting the solubilisation of hydrocarbons

3.1.3.1 Structural features of hydrocarbons

The structures of individual hydrocarbons can have a significant impact on their water solubility. Factors that have been found to affect the water solubility include size/surface area, aromaticity, polarity and degree of substitution. Three factors that are very closely linked to each other are molecular weight, molecular size and surface area. In general, as the molecular weight of a hydrocarbon increases, a corresponding decrease in the water solubility occurs (e.g. McAuliffe, 1966; Dunnivant and Elzerman, 1988; de Maagd *et al.*, 1998; Tolls *et al.*, 2002; Schwarzenbach *et al.*, 2003). Thus, lower molecular weight hydrocarbons such as alkylbenzenes and alkylxylenes are often found in elevated concentrations in contaminated water samples, compared with larger PAH compounds (e.g. Eganhouse *et al.*, 1993). Tolls *et al.* (2002) determined the water solubility of a series of C₁₀-C₁₉ saturated hydrocarbons, including linear, branched and cyclic alkanes. Water solubility was found to decrease with increasing carbon number. Furthermore, the relationship was found to be linear (Figure 3.1), indicating that the solubility of the *n*-alkanes was decreased by a constant increment for each additional CH₂ unit. Tolls *et al.* (2002) also compared solubility data for *n*-decane, cyclodecane and decahydronaphthalene, all C₁₀ hydrocarbons, and demonstrated that a small variation in molecular weight resulted in a significant change in the water solubility (Table 3.2).

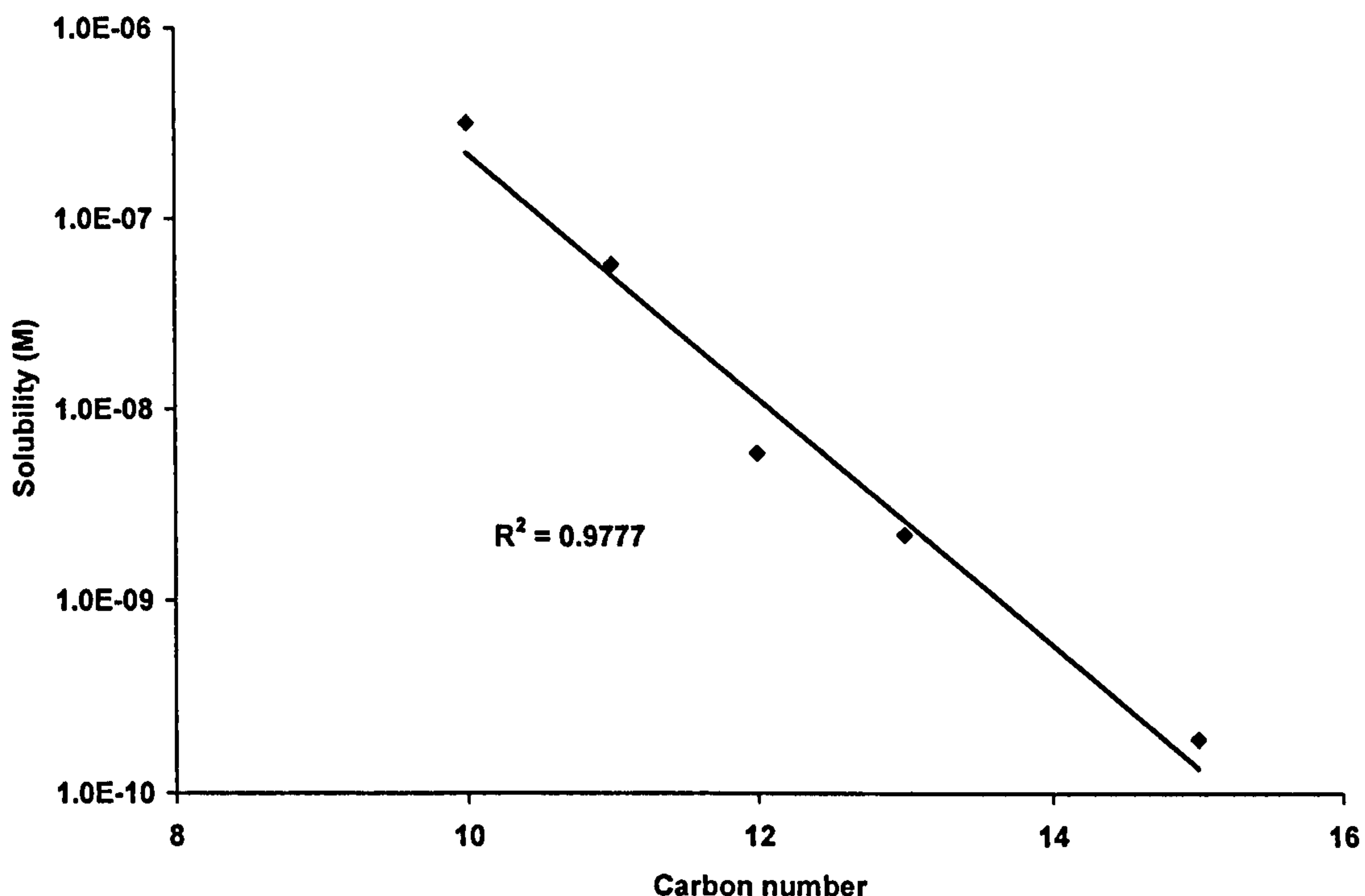

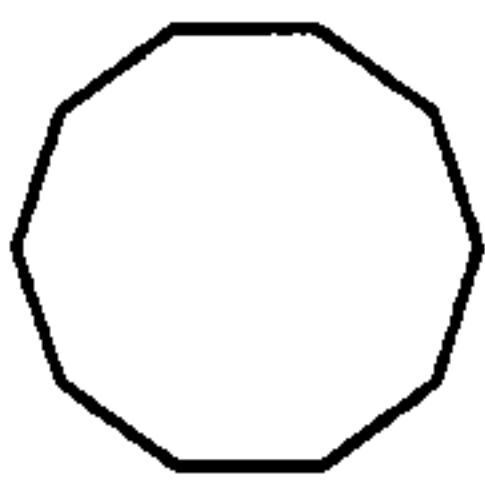
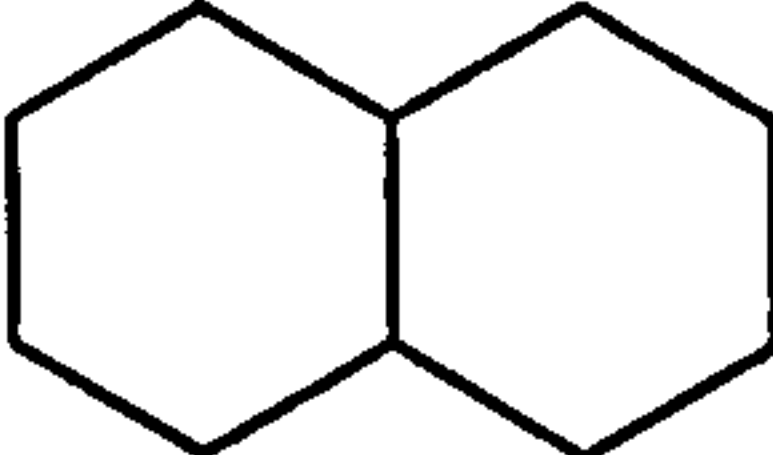


Figure 3.1. Plot of the logarithm of the water solubility (distilled water at 25 °C) of the *n*-alkanes decane, undecane, dodecane, tridecane and pentadecane versus molecular weight. Diagram reproduced from data reported by Tolls *et al.* (2002).

Table 3.2. Summary of the water solubility values (distilled water at 25 °C) determined for a set of C₁₀ hydrocarbons (decane, cyclodecane and decahydronaphthalene. Data reproduced from Tolls *et al.* (2002).

Compound name	Compound structure	Molecular weight	Water solubility (µg L ⁻¹)
<i>n</i> -decane		142	46
cyclodecane		140	330
decahydronaphthalene		136	850

Water solubility is driven by the solute-water interactions and the free energy penalty to build the cavity around the solute (*e.g.* Schwarzenbach *et al.*, 2003). The latter is energetically unfavourable due to entropy loss of water (the cavity can no longer be populated by water molecules) and a partial loss of favourable water:water interactions. In

general, this phenomenon will become more significant with increasing molecular size and surface area. Tolls *et al.* (2002) suggest that rather than molecular weight, the molecular size and/or surface area is a more accurate descriptor. When comparing different alkanes, molecular size is actually the primary determinant of water solubility. Increasing molecular size results in a decrease in water solubility, attributed to the increased free energy penalty for cavity formation in water. Indeed, molecular size and surface area measurements of hydrocarbons have routinely been analysed for their effect on water solubility (*e.g.* Sherblom *et al.*, 1992; Page *et al.*, 2000). For example, Silla *et al.* (1992) studied the effect of compound surface area or molecular area of a suite of 82 hydrocarbons, esters, ethers, alcohols and ketones including linear and branched homologues. The results showed that water solubility of a compound correlated well with its molecular surface area for groups of compounds.

A common way of expressing the bulk size of the molecules of a given compound is to use “molar volume” (Schwarzenbach *et al.*, 2003), derived from the molar mass and the liquid density at a given temperature. Schwarzenbach *et al.* (2003) suggest that within a homologous series of hydrocarbons, correlations between molar volume and water solubility are clear. However, even sets of quite closely related compounds such as *n*-alkanes and highly branched alkanes, or primary, secondary and tertiary aliphatic alcohols, exhibit different linear relationships (Schwarzenbach *et al.*, 2003). Thus, differences can arise from factors such as the shape of compounds (*e.g.* linear vs branched alkanes) and the number and type of substituents (*e.g.* aliphatic alcohols).

The effect of branching on a hydrocarbon can be viewed in two ways. First, for a given carbon number, the effect of branching is thought to reduce the surface area (Silla *et al.*, 1992; Figure 3.2). Second, branching on a fixed length hydrocarbon chain will increase the

carbon number and thus the branching acts as substituents on the parent hydrocarbon (Figure 3.3).

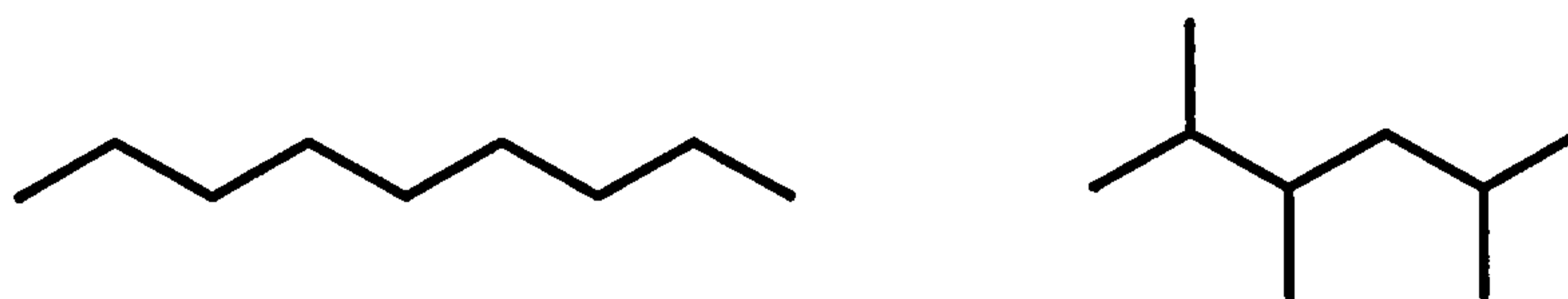


Figure 3.2. Comparison of an C_9 *n*-alkane and a C_9 branched alkane.



Figure 3.3. Comparison of a C_9 *n*-alkane and a trimethyl branched/substituted C_9 alkane (C_{12} in total).

Within a series of dodecane isomers (C_{12}) Tolls *et al.* (2002) observed a trend of increasing solubility with an increasing degree of branching. Silla *et al.* (1992) also studied the influence of branching on solubility. Linear compounds were less soluble in water than the corresponding branched compounds with the same carbon number. The authors attributed these observations to the linear compounds having a higher molecular surface area.

Structurally similar organic compounds, with differing degrees and types of substitution, are found to exhibit significantly different water solubility values (*e.g.* McAuliffe, 1966; Dunnivant and Elzerman, 1988; Smith, 2002; Schwarzenbach *et al.*, 2003). In particular, Schwarzenbach *et al.* (2003) suggest the presence of polar substituents (*e.g.* hydroxyl or nitrogen groups) results in hydrogen-bond interactions between the water molecules and the organic solute, leading to a decrease in the enthalpy and an increase in the entropy. The result can be a significant increase in water solubility of several orders of magnitude (*e.g.* Boylan and Tripp 1971; Siron *et al.*, 1991; Silla *et al.*, 1992). For example, Silla *et al.* (1992) observed that the presence of polar groups on organic compounds increased their water solubility compared with the saturated hydrocarbon homologues. Siron *et al.* (1991)

also found that water soluble fractions of crude oil were dominated by highly polar components.

In contrast, the presence of apolar hydrocarbon substituents (*e.g.* C₂, C₃, C₄ etc) may reduce the water solubility of a compound. Dunnivant and Elzerman (1988) observed a decrease in the solubility of polychlorinated biphenyl (PCB) congeners, with an increase in chlorine substitution. Lee *et al.* (1979a) and Sokol *et al.* (1992) also observed that aqueous solutions of PCB congeners were dominated by mono- and di-chlorinated biphenyls. This indicates that increasing substitution, in this case chlorination, can reduce the water solubility of a compound.

An increase in the size of an apolar substituent has also been found to reduce its water solubility (*e.g.* Sherblom *et al.*, 1992; Kuo, 1994; Page *et al.*, 2000; Smith, 2002). For example, Smith (2002) observed a decrease in the solubility of a suite of alkylcyclohexyltetralins with increasing chain length of the alkyl substituent. Sherblom *et al.* (1992) report the same phenomenon with a suite of long-chain linear alkylbenzenes (LABs). Kuo (1994) observed phenanthrene and dibenzothiophene to be depleted in preference to their methylated homologues, indicating that substitution of hydrocarbons results in a decrease in their water solubility. This decrease becomes more pronounced with an increase in the size of the substituent, indicating a close link to the resulting size of the compound (Page *et al.*, 2000).

Dunnivant and Elzerman (1988) observed that PCB congeners with no substitution at the *ortho*-position were significantly less soluble than those with the same number of chlorine substituents but with at least one in that position. Sherblom *et al.* (1992) investigated the position of the phenyl substitution on the alkyl chain in a series of linear alkyl benzenes. The authors found that the position of the phenyl group on a branched alkyl chain affected

the water solubility of the compound. Thus, the position of substituents on a compound (particularly aromatics) can also affect the level of solubility.

For a given carbon number, ring formation and increasing unsaturation of compounds is also found to increase the solubility of hydrocarbons in water (*e.g.* McAuliffe 1966; Eganhouse and Calder, 1976; Kuo, 1994). For example, McAuliffe (1966) observed that butene was more soluble than butane, and benzene was found to be more soluble than cyclohexane. Thus, the presence of one or more aromatic rings in a hydrocarbon structure can significantly affect the water solubility of the compound (*e.g.* Figure 3.4). Indeed, aromatic hydrocarbons are up to several orders of magnitude more soluble than saturated compounds with similar carbon numbers, due to the increased polarity of ring closure (*e.g.* McAuliffe 1966; Eganhouse and Calder, 1976; Kuo, 1994; de Maagd *et al.*, 1998).

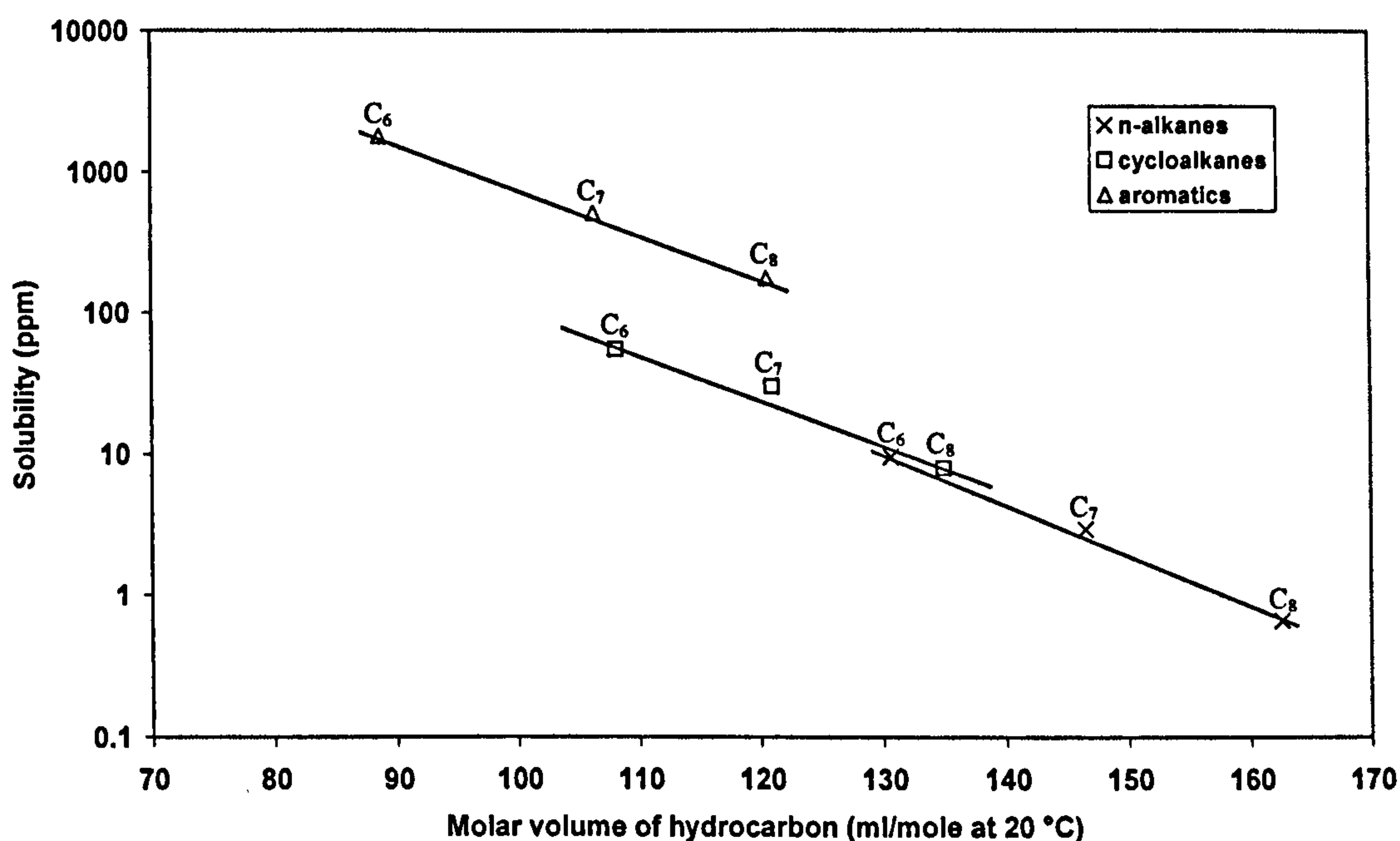


Figure 3.4 Comparison of the water solubility (room temperature) of a suite of C₆-C₈ *n*-alkanes, cycloalkanes and aromatics as a function of their molar volumes. Reproduced from McAuliffe (1966).

3.1.3.2 Environmental conditions

A combination of environmental factors such as high temperatures, intensive solar radiation (photooxidation) and a high rate of mixing of the local water column, are suggested to accelerate the weathering (*e.g.* dissolution) and degradation processes of oils (Badaway *et al.*, 1993). Wave action will break slicks up and disperse the oil as small droplets in the water column. The aqueous concentrations of the compounds will increase more rapidly due to the larger surface area of the droplets (Hibbs *et al.*, 1999; Hibbs and Gulliver 1999). Once in solution, crude oil is then mixed to low concentrations (Seymour and Geyer, 1992). Six months after the *Amoco Cadiz* spill oil concentrations in the water column were found in the ranges of 3-20 $\mu\text{g L}^{-1}$ offshore and 2-200 $\mu\text{g L}^{-1}$ nearshore (Gundlach *et al.*, 1983). Thus, crude oil or hydrocarbons released into high-energy systems will be dispersed rapidly. In contrast, oil trapped along a shore in protected waters can sometimes persist for long periods of time, as it is sheltered from such weathering processes (Gundlach *et al.*, 1983; Seymour and Geyer, 1992).

The dispersion and subsequent solubilisation of crude oil hydrocarbons is also dependent to some degree on other weathering processes, in particular, evaporation. If evaporation is slow, compounds will remain in the slick where they continue to dissolve, thus increasing the aqueous concentrations. The opposite occurs if evaporation is high, with smaller more volatile components (also generally the most soluble), being removed. In this case, only low aqueous concentrations are formed (Shiu *et al.*, 1988 and 1990; Hibbs and Gulliver, 1999). Thus, the removal of volatile hydrocarbons due to weathering processes can reduce the total solubility of the remaining crude oil mixture.

Table 3.3. Comparison of the water solubility values determined for a series of alkylcyclohexyltetralins in distilled water at 25 °C and 15 °C. Data reproduced from that published by Smith (2002).

Compound	Water solubility $\mu\text{g L}^{-1}$ (distilled water, 25 °C)	Water solubility $\mu\text{g L}^{-1}$ (distilled water, 15 °C)
6-cyclohexyltetralin	109 ± 6	95 ± 5
7-cyclohexyl-1-methyltetralin	45 ± 4	40 ± 2
7-cyclohexyl-1-propyltetralin	23 ± 3	17 ± 2

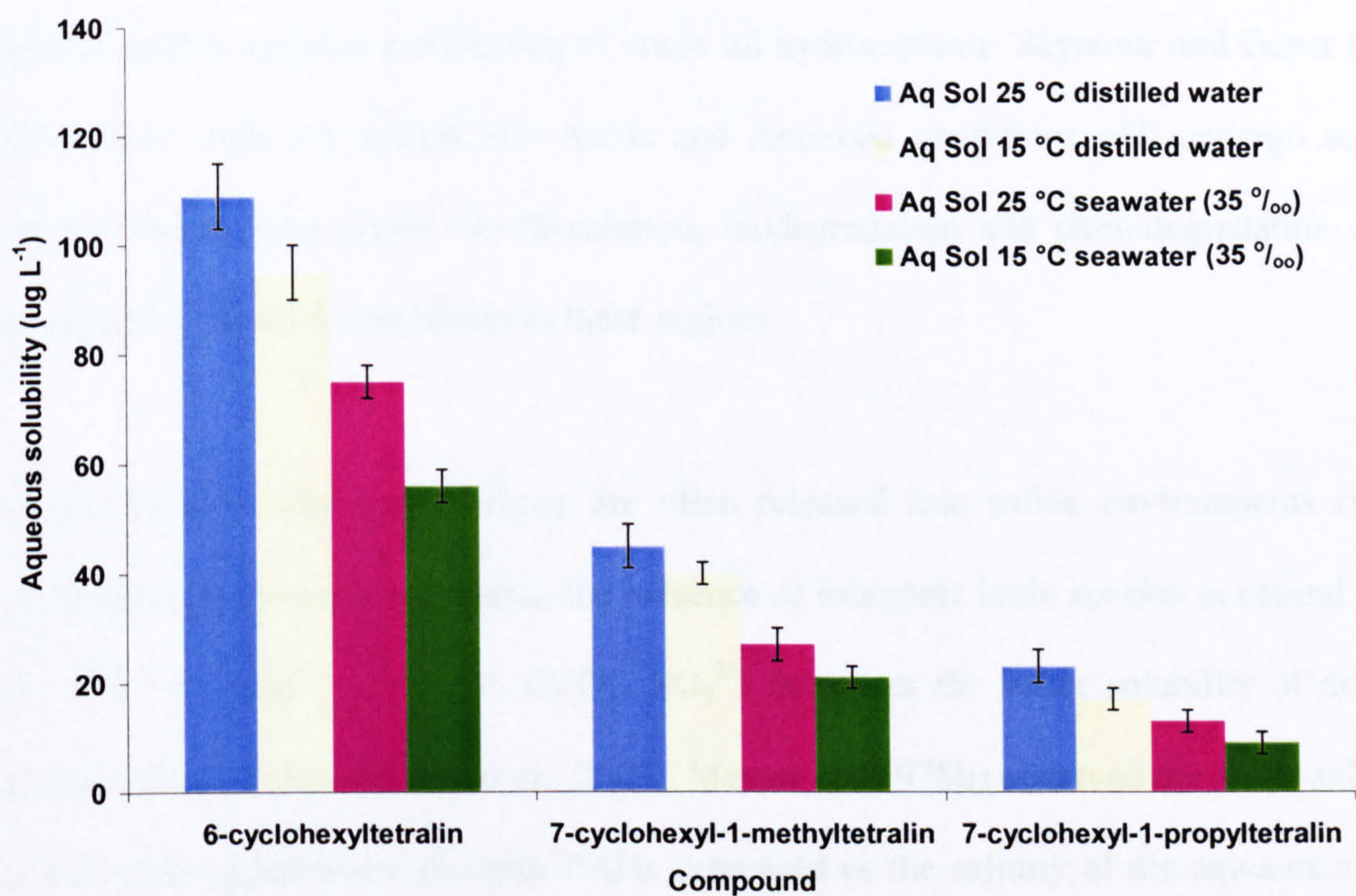


Figure 3.5. Summary of data obtained by Smith (2002) from solubility studies using a generator column technique. Water solubility increases with increasing temperature, but decreases with increasing salinity and molecular size.

The effect of temperature can also influence the amount of material that will dissolve into an aqueous medium. May *et al.* (1978a and b) observed that the water solubility of benzene, naphthalene and nine PAHs increased as the temperature of the aqueous medium increased. Other researchers *e.g.* Dickhut *et al.* (1986), Shiu *et al.* (1988 and 1990), Wolfe *et al.* (1998) and Smith (2002) have also reported this effect. However, Schwarzenbach *et al.* (2003) suggested that for the majority of liquid compounds, the change in solubility

with increasing temperature is relatively small, varying typically by less than a factor of 2 over a temperature range of 0-30 °C. This is evidenced by Smith (2002) who reported statistically significant but small differences in the water solubility of a series of alkylcyclohexyltetralins at different temperatures (Table 3.3 and Figure 3.5).

However, for large apolar compounds (*e.g.* PAHs and PCBs) the effect of temperature on water solubility can be significant (Schwarzenbach *et al.*, 2003). Wolfe *et al.* (1998) observed dramatically reduced concentrations of naphthalene in the WSF of Prudhoe Bay crude oil generated at 12 °C compared with those at 20 °C. Thus, lower temperatures can greatly inhibit aqueous partitioning of crude oil hydrocarbons. Seymour and Geyer (1992) report that crude oil spilled into Arctic and Antarctic conditions will undergo severely reduced weathering action *via* dissolution, biodegradation and photodegradation due in part to the reduced temperatures in these regions.

Crude oil and other hydrocarbons are often released into saline environments such as seawater and estuaries. In general, the presence of inorganic ionic species in natural waters (*i.e.* Na⁺, K⁺, Mg²⁺, Ca²⁺, Cl⁻, HCO₃⁻, SO₄²⁻) decreases the water solubility of nonpolar compounds (Schwarzenbach *et al.*, 2003). May *et al.* (1978b) observed the water solubility of benzene, naphthalene and nine PAHs decreased as the salinity of the aqueous medium increased. This “salting out” effect has also been reported in other studies (*e.g.* Sutton and Calder, 1974; Shiu *et al.*, 1988 and 1990; Wolfe *et al.*, 1998; Smith, 2002). For example, Smith (2002) reported significant differences in the water solubility of a series of alkylcyclohexyltetralins at different salinities (Table 3.4 and Figure 3.5).

Table 3.4. Comparison of the water solubility values determined for a series of alkylcyclohexyltetralins in distilled water at 25 °C and seawater (35‰) at 25 °C. Data reproduced from that published by Smith (2002).

Compound	Water solubility $\mu\text{g L}^{-1}$ in distilled water at 25 °C	Water solubility $\mu\text{g L}^{-1}$ in seawater at 25 °C
6-cyclohexyltetralin	109 ± 6	75 ± 3
7-cyclohexyl-1-methyltetralin	45 ± 4	27 ± 3
7-cyclohexyl-1-propyltetralin	23 ± 3	13 ± 2

Thus, dissolved ions compete successfully with organic compounds for the water molecules. The water solubility of larger organic compounds is affected to a greater extent than smaller and/or polar compounds (Schwarzenbach *et al.*, 2003). Sutton and Calder (1974) suggested that this process may be important in estuarine regions where fresh water containing dissolved organic compounds mixes with seawater. The rise in salinity of the fresh water may result in the “salting out” of the hydrocarbons in the river water. The solubility of organic compounds in pure and saline waters (at constant temperature) can be related by the Setschenow equation (*e.g.* May *et al.*, 1978b; Rossi and Thomas, 1981; Rawling, 1998).

$$\log\left(\frac{S_{sw}}{S_{pw}}\right) = KC$$

Where S_{pw} is the water solubility of the compound in pure water, S_{sw} is the water solubility of a compound in water of salinity C (mol L^{-1}). K corresponds to the Setschenow solubility constant.

3.1.4 Review of methods available for determining water solubility of hydrocarbons in water

3.1.4.1 Long-term dissolution

In experimental studies, the long-term dissolution method is frequently used to generate water-soluble fractions (WSFs) of hydrocarbons (*e.g.* Boylan and Tripp, 1971; Lee *et al.*, 1979a; Sherblom *et al.*, 1992; Eganhouse *et al.*, 1996; Luthy *et al.*, 1997). This method involves the careful placement of the neat target material in water and its slow dissolution. Importantly, this method allows the formation of WSFs that do not contain micro-emulsions, which can lead to erroneous results. Whilst some studies have used equilibration times of a few days (Sherblom *et al.*, 1992) several months are typically required (Lee *et al.*, 1979a; Ghosh *et al.*, 1998) making this method time consuming. Other limitations to this technique include the need for large quantities of water due to the low solubility of organic compounds. Additionally, substrate losses from the aqueous phase during transfer, concentration and adsorption to container walls is also likely (Stolzenburg and Andren, 1983).

3.1.4.2 Shaking / stirring methods

A common method employed for the generation of water-soluble fractions of organic compounds and mixtures is the ‘shake flask method’. This involves equilibrating an excess amount of the organic material with water by gentle shaking or stirring (*e.g.* Banerjee, 1984; Bennett *et al.*, 1990; Siron *et al.*, 1991; Wolfe *et al.*, 1998; Page *et al.*, 2000; Ziolli and Jardim, 2003). Whilst this method is much faster than the long-term dissolution technique, it can still take days to reach equilibrium, and is subject to the same limitations (Stolzenburg and Andren, 1983). Additional experimental difficulties include: incomplete equilibration of the hydrocarbons with the aqueous medium; the formation of micro-

emulsions; dispersion rather than true dissolution; and microcrystal suspensions (May *et al.*, 1978a; Billington *et al.*, 1988; Bennett *et al.*, 1990; Sokol *et al.*, 1992;).

3.1.4.3 Water miscible co-solvents

Water miscible co-solvents (*e.g.* methanol or acetone) are sometimes used in the generation of aqueous solutions of hydrocarbons (*e.g.* Gschend and Wu, 1985; Pinal *et al.*, 1990; Rao *et al.*, 1990; Potter and Pawliszyn, 1994; Nzengung *et al.*, 1996). Hydrocarbons are dissolved in the co-solvent prior to addition to the aqueous medium. This method is useful for enhancing the movement of sparingly soluble organic compounds (Nzengung *et al.*, 1996). However, the solvent has a significant impact on the solubility of organic compounds (Pinal *et al.*, 1990), and can alter the physical/chemical/biological properties of the matrix studied (Ghosh *et al.*, 1998; Schwarzenbach *et al.*, 2003). As a result, systems generating water soluble fractions of hydrophobic organic compounds may not be environmentally realistic. This can lead to higher than expected amounts of the solute being present in the aqueous phase.

3.1.4.4 Generator column technique

The 'generator column technique' is finding increased usage for the determination of accurate water solubility values. This is based on the coupled column liquid chromatographic method first proposed by May *et al.* (1978a). Water is pumped through a column containing glass beads coated with the target substrate, allowing the generation of known or measurable concentrations of sparingly soluble organic compounds. May *et al.* (1978a) determined that the precision of replicate measurements was better than $\pm 3\%$. The technique is becoming more popular due to its increased repeatability (*e.g.* Stolzenburg and Andren, 1983; Miller *et al.*, 1984; Dickhut *et al.*, 1986; Shiu *et al.*, 1988;

Mackay *et al.*, 1991; Sokol *et al.*, 1992; Coyle *et al.*, 1997; Ghosh *et al.*, 1998; Sijm *et al.*, 1999). See also Section 5.9 and Figure 5.7.

After an initial purge volume of 100-500 mL equilibrium is established, the hydrocarbon concentration is independent of flow rate between 0.1 and 5 mL min⁻¹ (e.g. May *et al.*, 1978a; Dunnivant and Elzerman, 1988). Adsorption losses of hydrocarbons to containers and transfer tools are minimised because the volume between the generator column and the extraction vessel is only a few µL. Furthermore, the walls of the transfer lines are pre-saturated with the substrate being studied during the column conditioning process. This reduces the possibility of adsorptive losses of the substrate. Other advantages include the elimination of emulsification, microcrystals and evaporation problems (Billington *et al.*, 1988; Sokol *et al.*, 1992). However, the method still carries certain limitations, with solubility data in the submicromolar range still remaining scattered (Dickhut *et al.* 1986). Furthermore, Mackay *et al.* (1991) suggest depletion of the more soluble components, especially of complex mixtures, may occur at the water inlet end of the column. Therefore, the mixture in the column is unlikely to remain as homogenous as that initially coated onto the glass beads.

3.1.5 Mixtures of organic compounds

3.1.5.1 Simple mixtures of organic compounds

Due to the complex nature of environmental samples, recent studies have begun to investigate the water solubility of hydrocarbons and other organic compounds when they are present as simple mixtures (e.g. Dickhut *et al.*, 1986; Luthy *et al.*, 1997; de Hemptinne *et al.*, 1998; Ghosh *et al.*, 1998). Many of these studies have focused on known organic

pollutants, in particular polychlorinated biphenyl (PCB) congeners (*e.g.* Ghosh *et al.*, 1998; Luthy *et al.*, 1997; Sokol *et al.*, 1992; Lee *et al.*, 1979).

The ability to observe the change in distribution of components with time, is an advantage of analysing mixtures of compounds, rather than single substrates (Ghosh *et al.*, 1998). Compound distribution in an aqueous solution obtained from a hydrocarbon mixture is found to be different from the distribution in the mixture itself. Often, different compounds become more predominant, with others becoming only minor components of the generated solution (*e.g.* Sokol *et al.*, 1992; Luthy *et al.*, 1997; Ghosh *et al.*, 1998). Thus, lower molecular weight compounds would be expected to dissolve more rapidly and to a greater extent than higher molecular weight compounds. For example, Ghosh *et al.* (1998) observed that over time PCB congener mixtures eluting from a generator column initially became depleted in the lower molecular weight material. Higher molecular weight material dissolved out in increasing amounts after this occurred.

Luthy *et al.* (1997) also observed that the WSF was dominated by a higher concentration of the less substituted compounds (most soluble), compared with the pure PCB mixture. Lower water solubility values are often reported for a compound diluted in a mixture (*e.g.* hydraulic oil) than those determined for the pure compound (*e.g.* Lee *et al.*, 1979a; Sokol *et al.*, 1992; Luthy *et al.*, 1997). This significant reduction in compound aqueous dissolution, with decreasing mole fraction in a mixture, is consistent with a 'Raoult's law' relationship (see below).

3.1.5.2 Complex mixtures of organic compounds

There are a number of studies concerning the water solubility of complex mixtures of hydrocarbons, such as crude oils, in the environment (*e.g.* Bennett *et al.*, 1990; Mackay *et*

al., 1991; Badaway *et al.*, 1993; Eganhouse *et al.*, 1993; Ali, 1994; Eganhouse *et al.*, 1996; Luthy *et al.*, 1997; Page *et al.*, 2000). These studies indicate that the interaction of components in such mixtures can cause significant changes in the solubilities of their constituents. The degree of the effect depends upon chemical types, phase, and composition of the mixture involved (Coyle *et al.*, 1997).

Banerjee (1984) and Sherblom *et al.* (1992) observed that mixtures of structurally related liquids (*e.g.* chlorobenzenes) were found to display near ideal behaviour. Deviations from ideality were found to occur with mixtures containing a diverse range of compounds, *e.g.* crude oil. Thus, the behaviour of a compound in a mixture may not correspond to that predicted from pure component data. The solubility of individual components can also be affected by interaction in the aqueous phase with other dissolved components.

Some of the trends observed in the dissolution of complex mixtures can be explained. Eganhouse *et al.* (1993) observed water soluble fractions of crude oil in groundwater dominated by a complex mixture of monoaromatic hydrocarbons. This indicates that dissolution of a complex mixture can give rise to another complex mixture of organic compounds in the WSF. However, alteration in the relative proportions of the compounds in the aqueous phase, compared with the initial organic mixture, often occurs (*e.g.* Shiu *et al.*, 1988 and 1990; Siron *et al.*, 1991; Eganhouse *et al.*, 1996; Smith, 2002). A strong enrichment of lower molecular weight hydrocarbons with higher solubility values (*e.g.* monoaromatics) is typically observed. For example, Smith (2002) reported that lower molecular weight compounds dominated the WSF of a monoaromatic UCM. The solubility of crude oil decreases, and the parent mixture eventually undergoes a significant compositional change as the more soluble components become depleted (Shiu *et al.*, 1988; Eganhouse *et al.*, 1996; Schwarzenbach *et al.*, 2003).

The water solubility of complex mixture components is generally different from the solubility of the compounds when measured individually (Coyle *et al.*, 1997). In some cases, water solubility of individual compounds increases when they are present in a mixture. For example, Sherblom *et al.* (1992) observed that the water solubility of two *n*-alkylbenzenes significantly increased when they were present in a mixture of other linear alkylbenzenes. This enhanced water solubility is indicative of cosolute or other multicomponent influences occurring in a mixture of structurally similar compounds.

In contrast, when compounds are present as part of a complex mixture containing many structurally different components (*e.g.* crude oil) the water solubility can also be reduced. Page *et al.* (2000) report that the solubility of naphthalene and its substituted homologues, when present as part of a crude oil, was much less than that of the pure compounds. This phenomenon can be explained by 'Raoult's law', which states that 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. Thus, as the mole fraction of a component in a mixture decreases, so does the water solubility of that component (Luthy *et al.*, 1997). In the case of crude oil, where a compound (*e.g.* naphthalene) is just one of thousands, the mole fraction is very small, hence the apparent reduction in water solubility compared with the pure compound. The solubility of weathered crude oils is much lower and falls more slowly than fresh crude oils. This can result in a relative enrichment of less volatile and less soluble hydrocarbons such as PAHs. Under these conditions, Hibbs *et al.* (1999) suggest that such compounds continue to dissolve into water, resulting in high concentrations. This too is consistent with 'Raoult's law', where the mole fraction of the less soluble material increases as the more soluble material dissolves or evaporates. As a result, there is a potential for high aqueous concentrations of low solubility, low volatility compounds.

3.1.6 Solubility data reported in publications

A number of authors have expressed concern with the reliability of much of the published data regarding the dissolution of hydrocarbons and hydrocarbon mixtures (*e.g.* Pontolillo and Eganhouse 2001; Eganhouse and Pontolillo 2002; Tolls *et al.*, 2002; Schwarzenbach *et al.*, 2003). For example, Pontolillo and Eganhouse (2001) and Eganhouse and Pontolillo (2002) have published a comprehensive review of available water solubility and octanol-water partition coefficient (K_{ow}) data for hydrophobic organic compounds. The review highlighted significant problems: errors in reporting of data and references, and poor data quality and/or inadequately documented procedures. Data compilations were found to be dominated by non-original data and also liable to omissions. Pontolillo and Eganhouse (2001) suggest that the cumulative effect of these errors is to obscure the extent and reliability of the original database, therefore, making deduction of reliable solubility or K_{ow} values difficult.

These problems are compounded by the fact that water solubilities determined by different methods and/or different laboratories may vary by as much as an order of magnitude (Pontolillo and Eganhouse 2001; Schwarzenbach *et al.*, 2003). Discrepancies are more evident with decreasing solubility of a compound. These can reflect the increased difficulty in experimentally determining solubility for very hydrophobic compounds (Tolls *et al.*, 2002; Schwarzenbach *et al.*, 2003). Furthermore, small amounts of hydrocarbons present in the water as microemulsions can exceed the fraction of dissolved hydrocarbon and lead to erroneous results (Tolls *et al.*, 2002). It is suggested that the true K_{ow} and solubility values for many compounds are still unknown. Worryingly, solubility and K_{ow} values are increasingly estimated using computational software rather than experimental determination. The data used to develop such software is based on that reviewed by Pontolillo and Eganhouse (2001) who found it to be flawed and unreliable.

3.1.7 Aims of the present study

From the foregoing discussion it is clear that determination of the solubility of crude oil-derived hydrocarbon mixtures is a difficult but important goal, particularly as the solubilised fraction is toxic to many marine organisms (*e.g.* Neff *et al.*, 2000). The solubility of the major volatile hydrocarbons of fresh crude oil, *e.g.* BTEX chemicals, has been quite well studied (*e.g.* Page *et al.*, 2002; Erben *et al.*, 2003; Huang *et al.*, 2003). However, the loss of such compounds by evaporation may mean that under some circumstances higher molecular weight complex mixtures, particularly containing aromatic hydrocarbons dominate the WSF. Very few studies of the latter appear to have been made. Smith (2002) investigated the water solubility of a series of alkylcyclohexyltetralins (I-III, Figure 3.6), thought to be representative of some crude oil UCM components, using a generator column technique at different temperatures and salinities. A cursory examination of a UCM was also made (Smith, 2002).

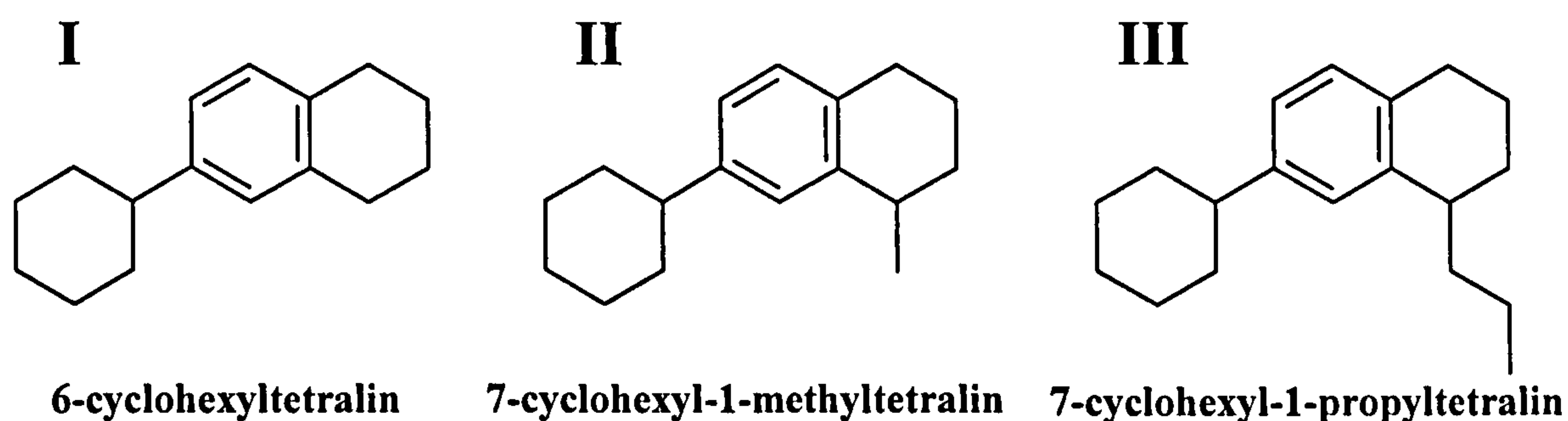


Figure 3.6. Alkylcyclohexyltetralins I-III proposed as monoaromatic UCM components. The water solubility of these compounds were determined using a generator column by Smith (2002).

In the present study, the solubilisation of an isolated aromatic UCM (Section 5.4) and an isolated monoaromatic UCM (Section 5.5) was examined using the generator column method (Section 5.9). For reference, and calibration with the methods of Smith *et al.* (2001), the water solubility of 6-cyclohexyltetralin previously synthesised by Wraige (1997) was also determined (Sections 5.6 and 5.9).

3.2 Results

3.2.1 Water solubility of 6-cyclohexyltetralin

The aim of this initial experiment was to investigate the reproducibility of the generator column method for the determination of the water solubility of an individual hydrocarbon between operators. The water solubility of 6-cyclohexyltetralin (Section 5.9.1), determined using a generator column in distilled water at 25 °C, is presented in Table 3.5. Glass beads coated with 6-cyclohexyltetralin were packed into the generator column and distilled water was eluted through the column at 1 mL min⁻¹ (Sections 5.9.2 and 5.9.3). An internal standard (7-cyclohexyl-1-methyltetralin) was added to the aqueous solutions prior to extraction (Section 5.9.4). Analysis was by GC and quantification was achieved using calibration graphs. Also shown are data from Smith (2002), who conducted the same experiment with 6-cyclohexyltetralin (and also with 7-cyclohexyl-1-methyltetralin and 7-cyclohexyl-1-propyltetralin). Values are reported as the mean ± standard deviation (n = 10).

Table 3.5. Water solubilities of 6-cyclohexyltetralin, 7-cyclohexyl-1-methyltetralin and 7-cyclohexyl-1-propyltetralin as determined by the generator column method in distilled water at 25 °C.

Compound	Water solubility µg L ⁻¹ mean ± st. dev. (n=10)
6-cyclohexyltetralin (this study)	109 ± 3
6-cyclohexyltetralin ^b	109 ± 6
7-cyclohexyl-1-methyltetralin ^b	45 ± 4
7-cyclohexyl-1-propyltetralin ^b	23 ± 3

^b denotes data reported by Smith (2002) in similar experiments.

The mean water solubility of 6-cyclohexyltetralin determined in the present study was found to be not statistically different (Students t-test) from that determined by Smith

(2002). Figure 3.7 shows the concentration ($\mu\text{g L}^{-1}$) of the 6-cyclohexyltetralin dissolved into each of the successive 50 mL aliquots collected from the generator column. The first 500 mL of effluent was discarded, as this was the period used to reach equilibrium in the system. The results indicate that the concentration of 6-cyclohexyltetralin remained constant once equilibrium had been achieved.

The volume of effluent from 1000 mL to 1500 mL was collected as 50 mL aliquots, and the results are presented in Table 3.6. Values are reported as $\mu\text{g L}^{-1}$.

Table 3.6. Water solubility of 6-cyclohexyltetralin in successive 50 mL aliquots of effluent from the generator column.

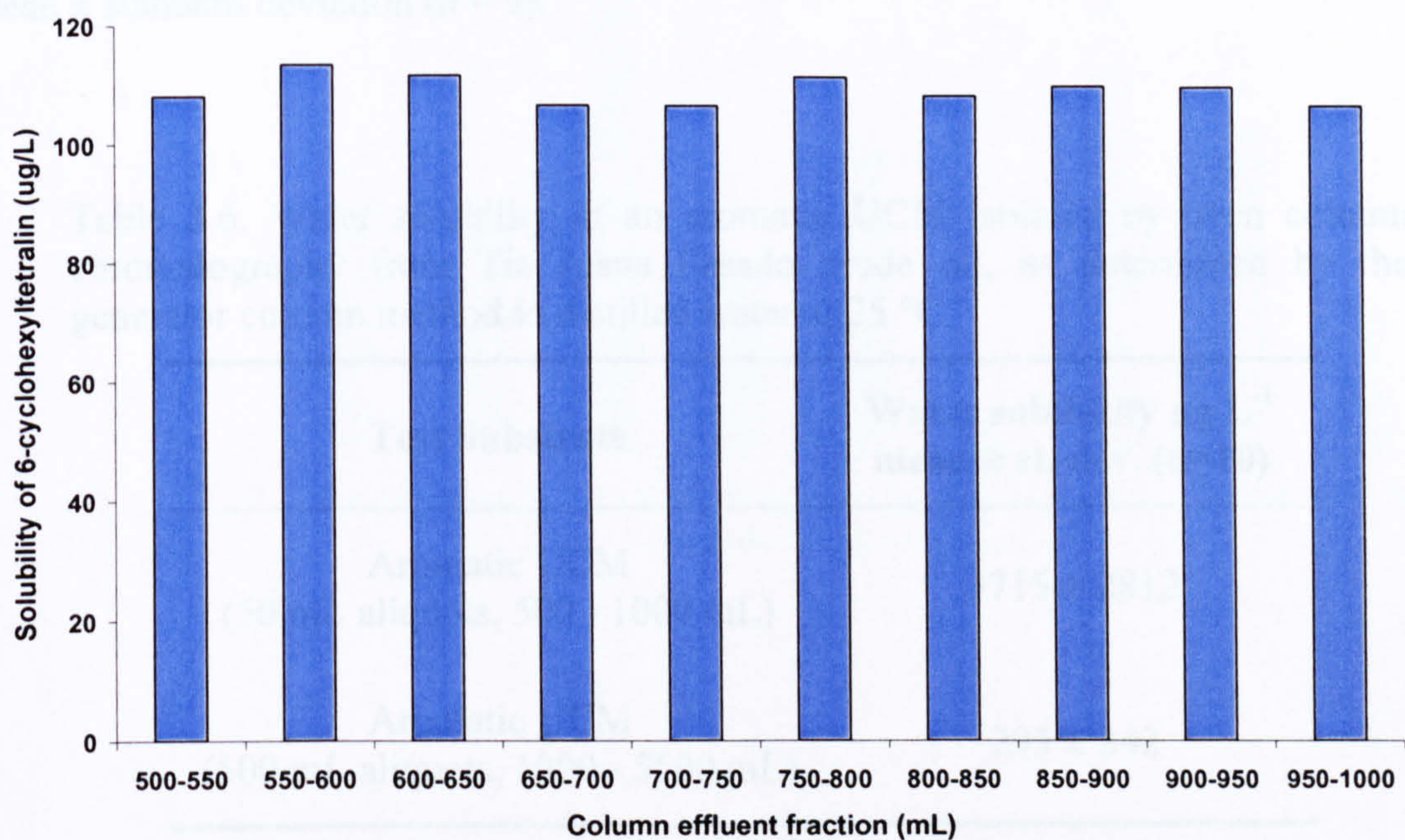


Figure 3.7. Comparison of the concentration of 6-cyclohexyltetralin in each of the successive 50 mL water soluble fractions produced from the generator column.

3.2.2 Water solubility of the aromatic UCM

The water solubility of an aromatic UCM (isolated from Tia Juana Pesado crude oil, Section 5.4) was determined using a generator column in distilled water at 25 °C, and is presented in Table 3.6. Glass beads coated with the aromatic UCM were packed into the generator column and distilled water was eluted through the column at 1 mL min⁻¹ (Sections 5.9.2 and 5.9.3). The first 500 mL were discarded as this is the accepted volume used to reach equilibrium in the system (*e.g.* May *et al.*, 1978a; Ghosh *et al.*, 1998). The next 500 mL (500-1000 ml) of water effluent was collected as 50 mL aliquots. An internal

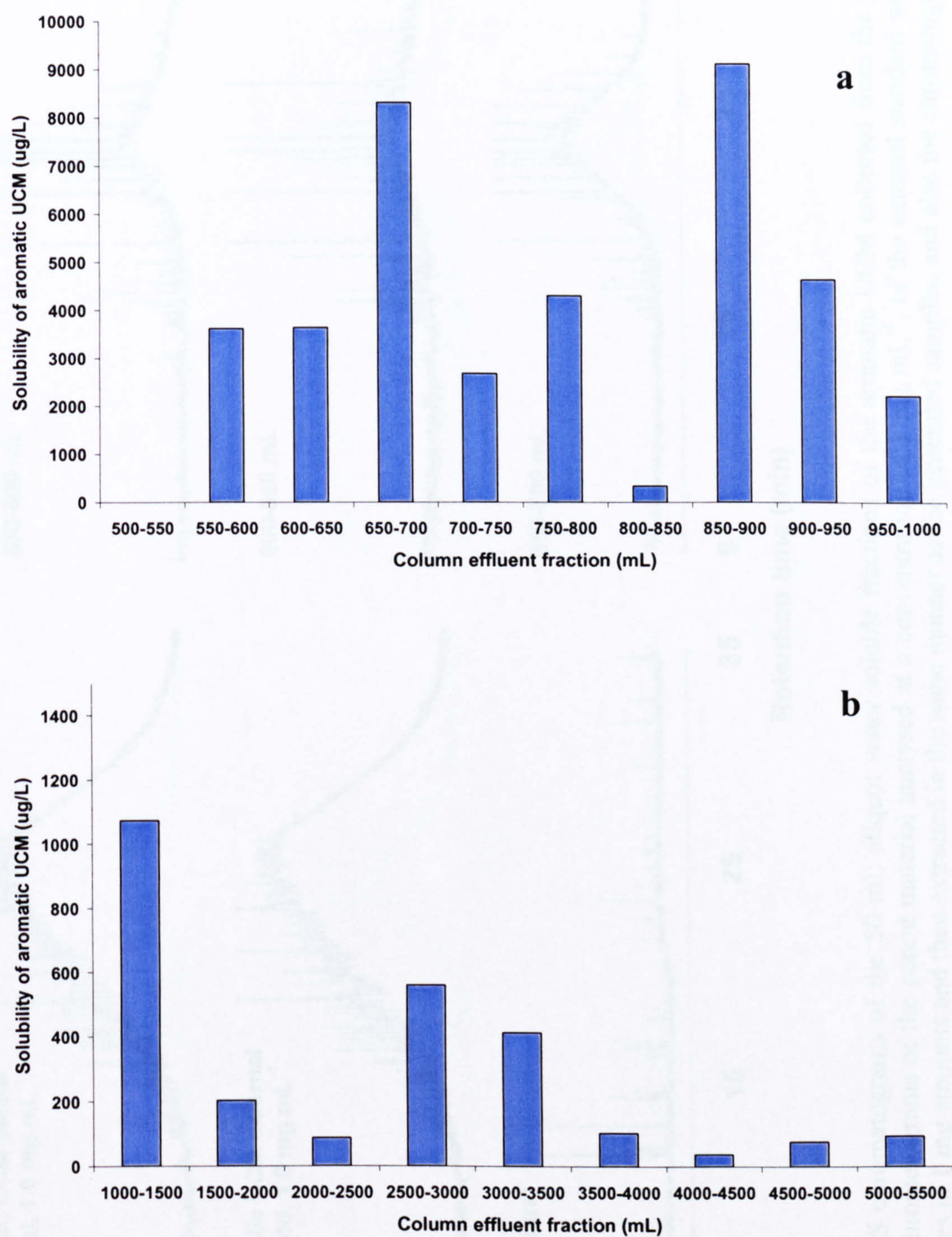
standard could not be used owing to coelution with the aromatic UCM, therefore external calibration was used to quantify the concentration of the aromatic UCM in the aqueous solutions. 1 mg of aromatic UCM was added to 500 mL of pure distilled water and extracted in the same manner as the aqueous solutions collected from the generator column (Sections 5.9.5). Analysis was by GC and concentrations were determined using calibration graphs. The volume of effluent from 1000 mL to 5500 mL was collected as 500 mL aliquots, and the results are also summarised in Table 3.6. Values are reported as the mean \pm standard deviation (n = 9).

Table 3.6. Water solubility of an aromatic UCM isolated by open column chromatography from Tia Juana Pesado crude oil, as determined by the generator column method in distilled water at 25 °C.

Test Substrate	Water solubility $\mu\text{g L}^{-1}$ mean \pm st. dev. (n=10)
Aromatic UCM (50 mL aliquots, 500 - 1000 mL)	3715 \pm 2812
Aromatic UCM (500 mL aliquots, 1000 - 5500 mL)	293 \pm 342

Clearly the ‘dissolution’ of the complex mixture of aromatic hydrocarbons in the TJP UCM has not been reproducibly achieved, despite the use of a calibrated generator column method. The variability in both the initial 50 mL and the later 500 mL aliquots was large. The water solubility of the 500 mL fractions appears to be an order of magnitude lower than those measured in the 50 mL fractions initially collected from the generator column. Although a ‘compositional equilibrium’ in the effluent is unexpected given the large number of different hydrocarbons in the UCM, a total hydrocarbon ‘concentration equilibrium’ could be expected. The results of this study suggest that such a ‘concentration equilibrium’ had not been achieved when sample collection began.

Figures 3.8a and 3.8b show the concentration ($\mu\text{g L}^{-1}$) of the isolated aromatic UCM dissolved into each of the successive 50 mL aliquots (Figure 3.8a) and 500 mL aliquots (Figure 3.8b) collected from the generator column.



Figures 3.8a and 3.8b. Comparison of the aqueous concentration of aromatic UCM with increasing water volume through the generator column. Figure **a** is the 50 mL extracts, and Figure **b** is the 500 mL extracts.

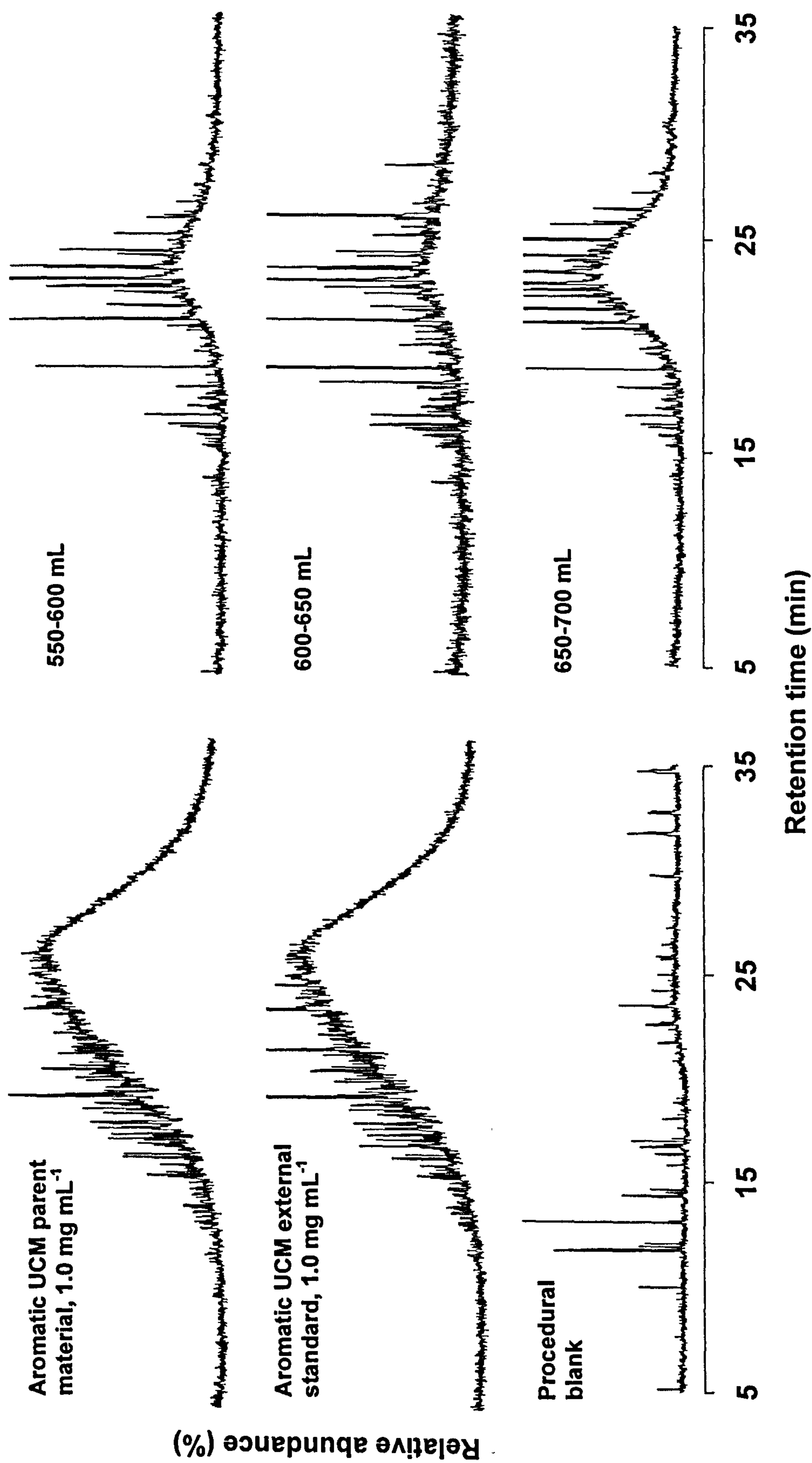


Figure 3.9. GC-MS chromatograms of the 50 mL aliquot water soluble fractions of the aromatic UCM collected from the generator column. Included are the chromatograms of the parent material analysed at a concentration of 1 mg mL⁻¹, of the external standard which was added to blank water samples in 1 mg amounts and then extracted in the same manner as the generated samples, and also the chromatogram obtained from analysis of a procedural blank. Figure 3.9 is continued on the next page.

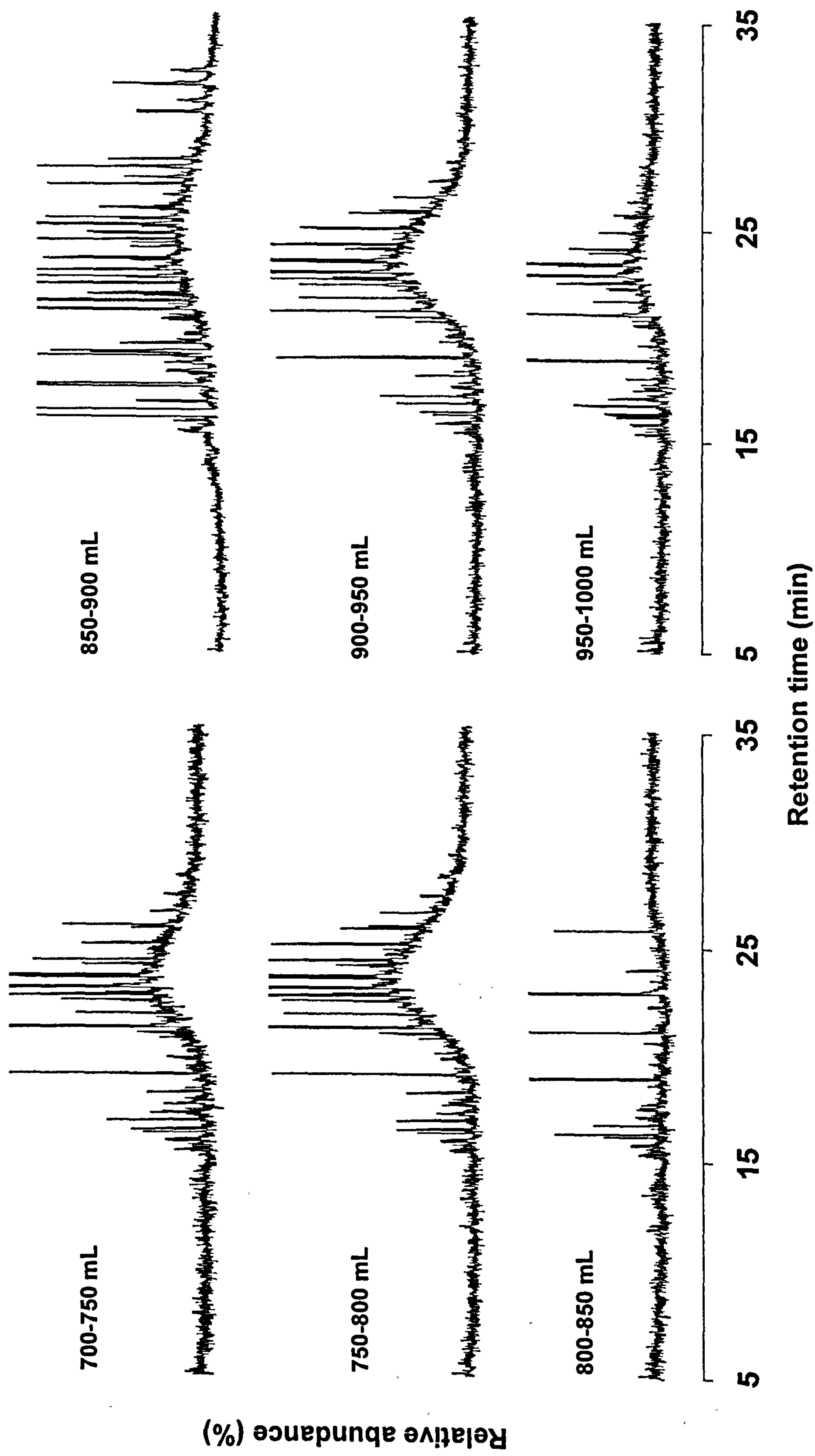


Figure 3.9. Continued from the previous page.

Figure 3.9 shows the GC-MS chromatograms of the generated aqueous fractions (50 mL) of the aromatic UCM. Chromatograms of the original material and the procedural blank are also shown. There is a significant chromatographic difference between the aromatic UCM isolated from Tia Juana Pesado crude oil and that present in the WSF. The chromatograms indicate that a significantly reduced number of compounds are present above the instrumental limits of detection in the water soluble fraction. When compared with the original material, the UCM is greatly reduced in size and component number. Figure 3.10 is a comparison of the water-soluble fraction (750-800 mL) and the original aromatic UCM. The 750-800 mL fraction was chosen as it is the middle aliquot in the series collected. The diagram clearly shows that only a small proportion of the hydrocarbons in the original aromatic UCM were water soluble above the limits of analytical detection. Interestingly, there also seem to be significantly more resolved peaks in the water soluble fraction, compared with the original aromatic UCM material.

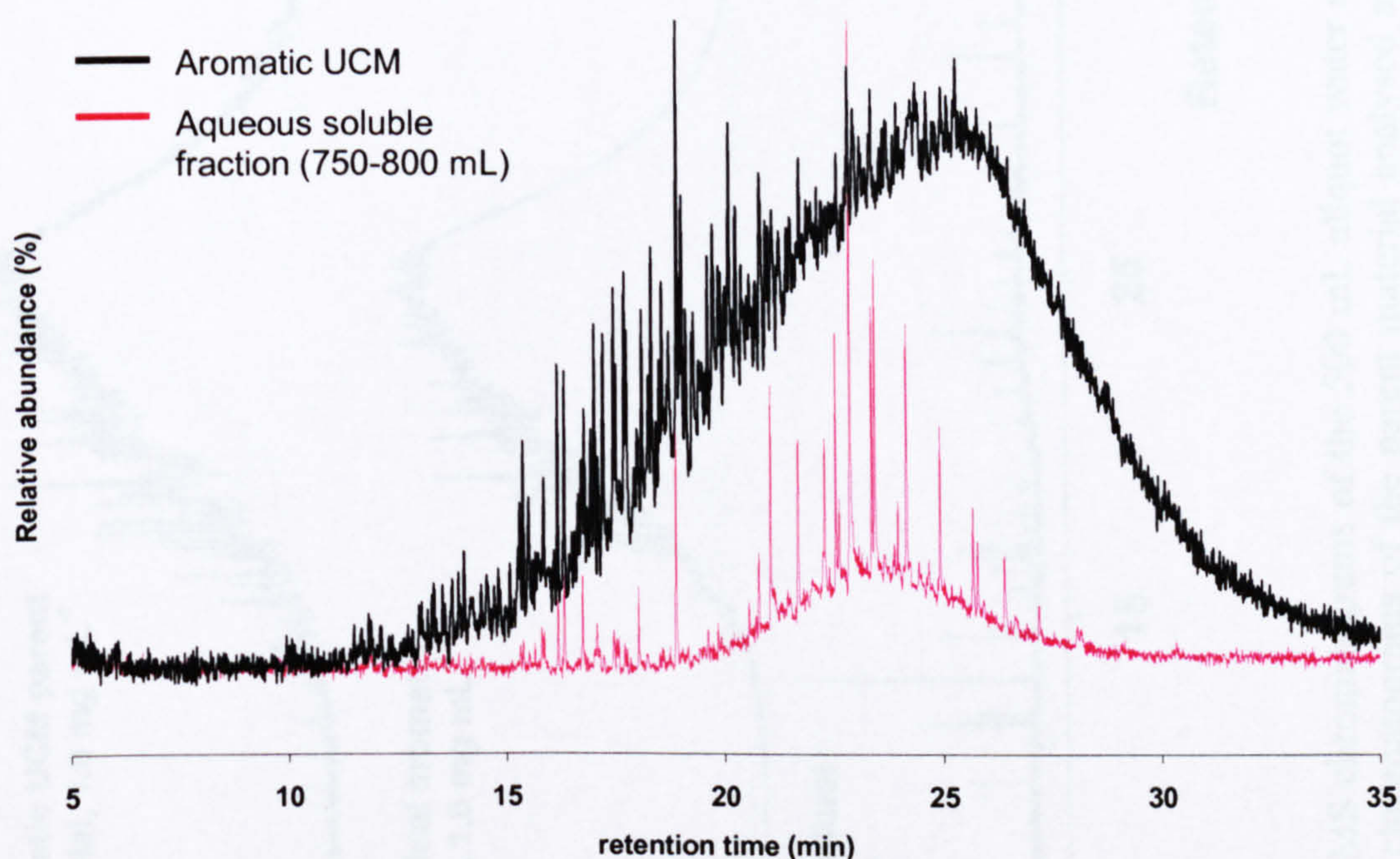


Figure 3.10. Comparison of the chromatograms of the original aromatic UCM material and the generated water soluble aromatic UCM fraction (750-800 mL).

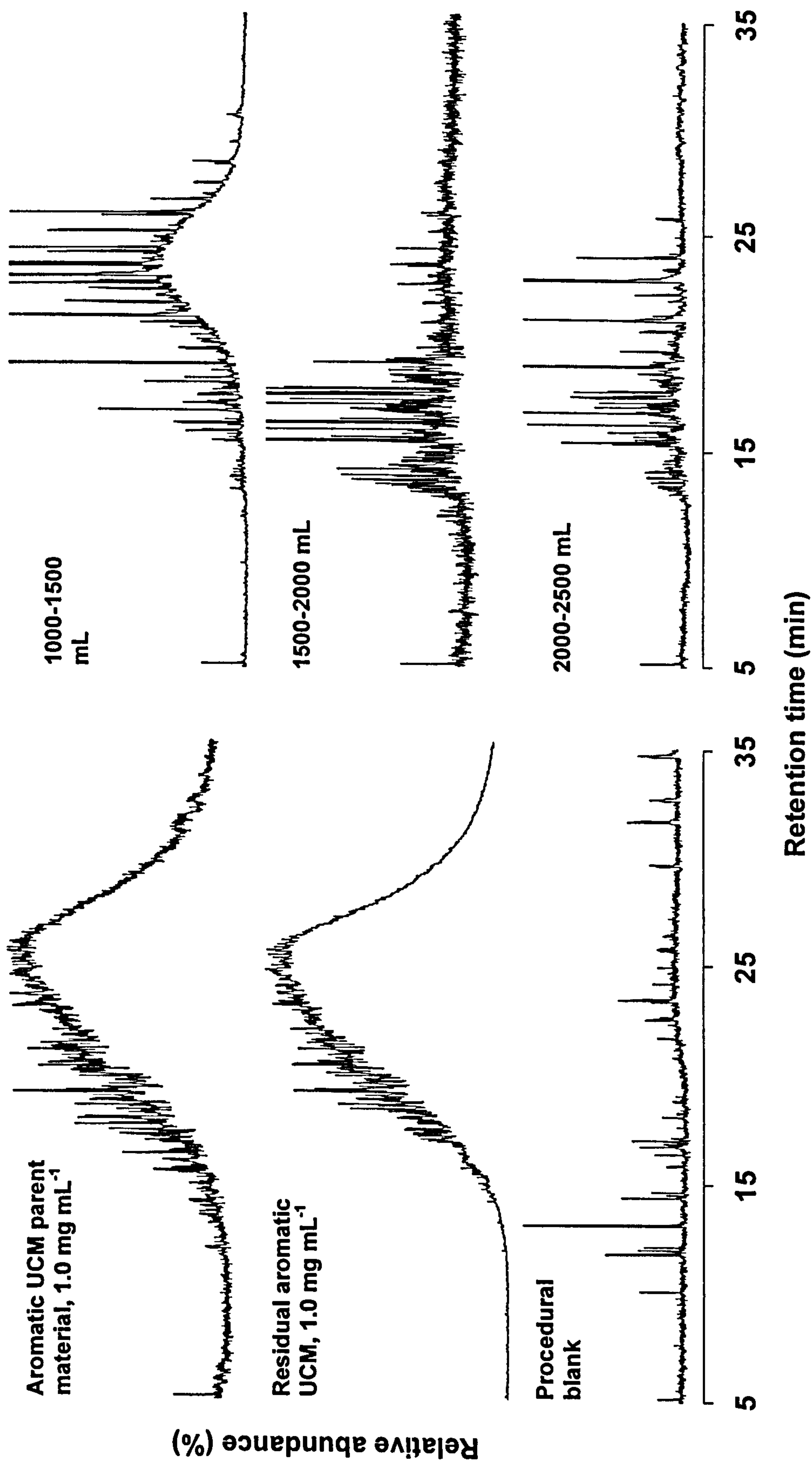


Figure 3.11. GC-MS chromatograms of the 500 mL aliquot water soluble fractions of the aromatic UCM collected from the generator column. Included are the chromatograms of the parent material analysed at a concentration of 1 mg mL⁻¹, the chromatogram of the residual material which was removed from the column and analysed at 1 mg mL⁻¹, and also the chromatogram of the procedural blank sample. Figure 3.11 is continued on the next page.

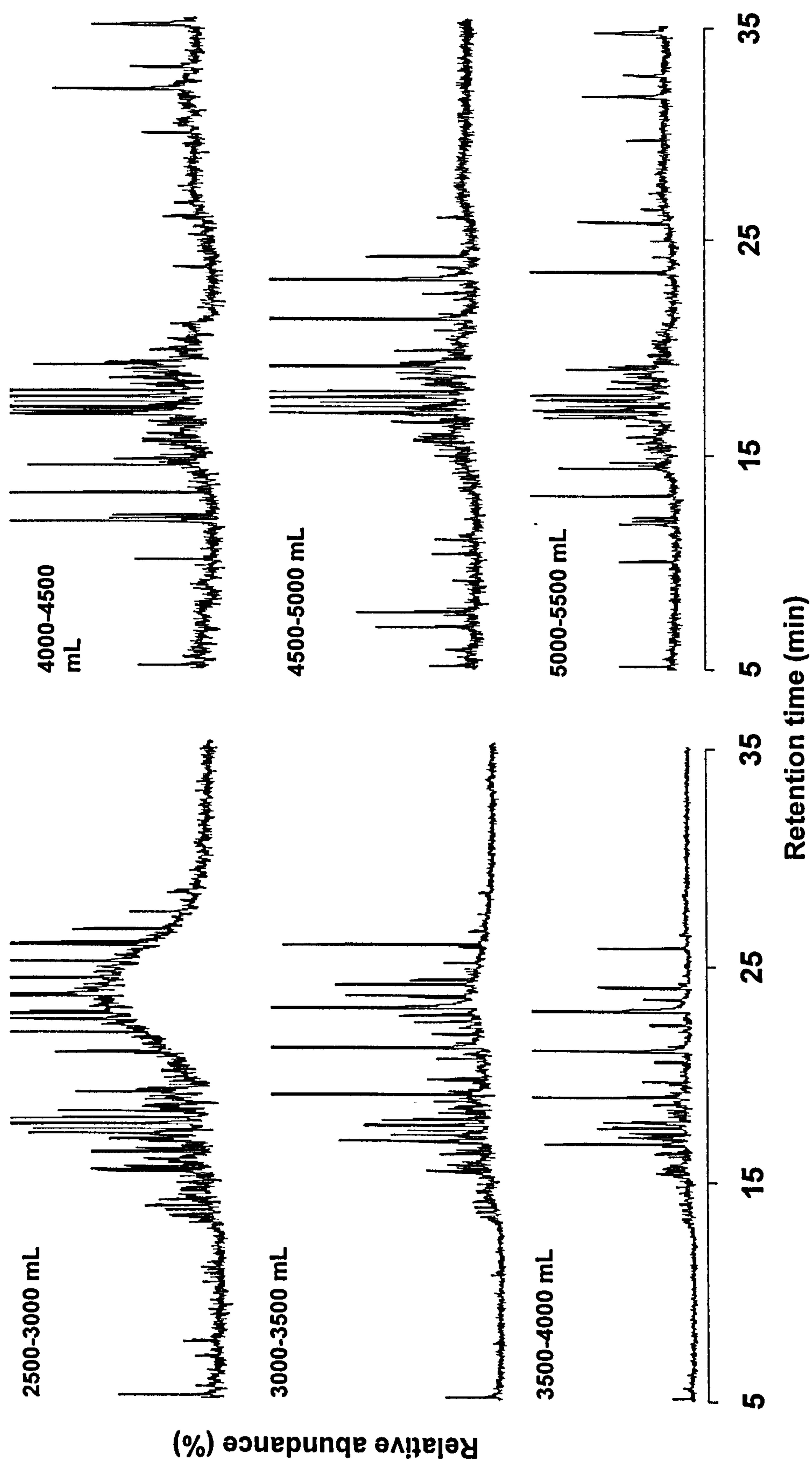


Figure 3.11. Continued from the previous page.

Figure 3.11 shows the GC-MS total ion current chromatograms of the generated 500 mL aqueous fractions (1000 – 5500 mL) of the ‘solubilised’ aromatic UCM. Chromatograms of the original material, the procedural blank and the residual material remaining on the column are also shown. The chromatograms indicate that there is a significant difference between the material in the original aromatic UCM and that present in the WSFs. Fewer compounds appear to be present in the water soluble fraction above the limits of analytical detection, indicating fewer components occur in the soluble UCM. Figure 3.12 shows a comparison of the water-soluble fraction (3000-3500 mL) and the original UCM. The 3000-3500 mL fraction was chosen as it is the middle aliquot in the series collected. It is evident that only a small proportion of the hydrocarbons in the original aromatic UCM were water soluble over the limits of analytical detection. Furthermore, an increased number of resolved compounds can be seen in the chromatogram of the WSF, compared with the original UCM.

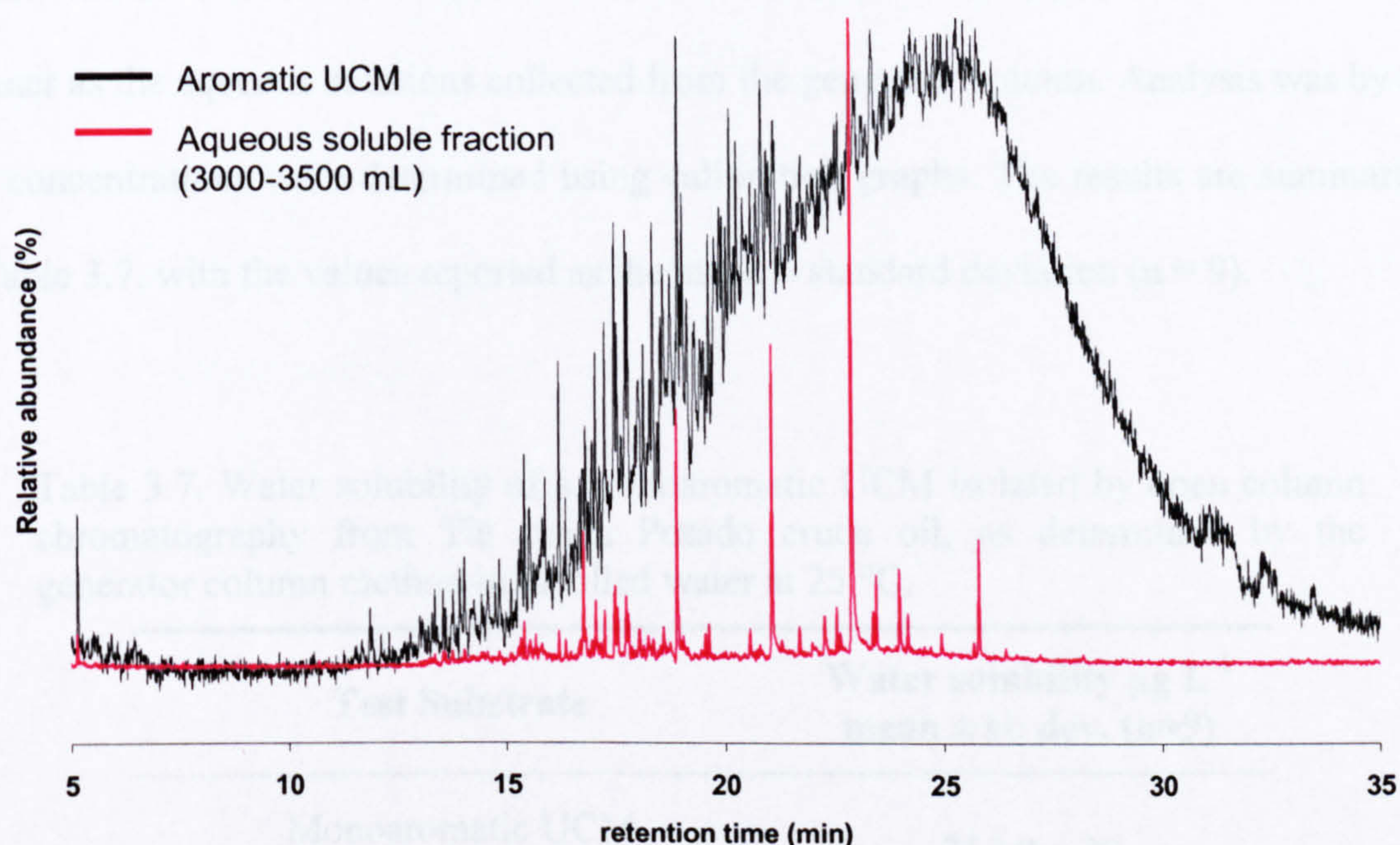


Figure 3.12. Comparison of the chromatograms of the parent aromatic UCM material and the generated water soluble aromatic UCM fraction (2500-3000 mL).

3.2.3 Water solubility of the monoaromatic UCM (50 ml and 500 ml aliquots)

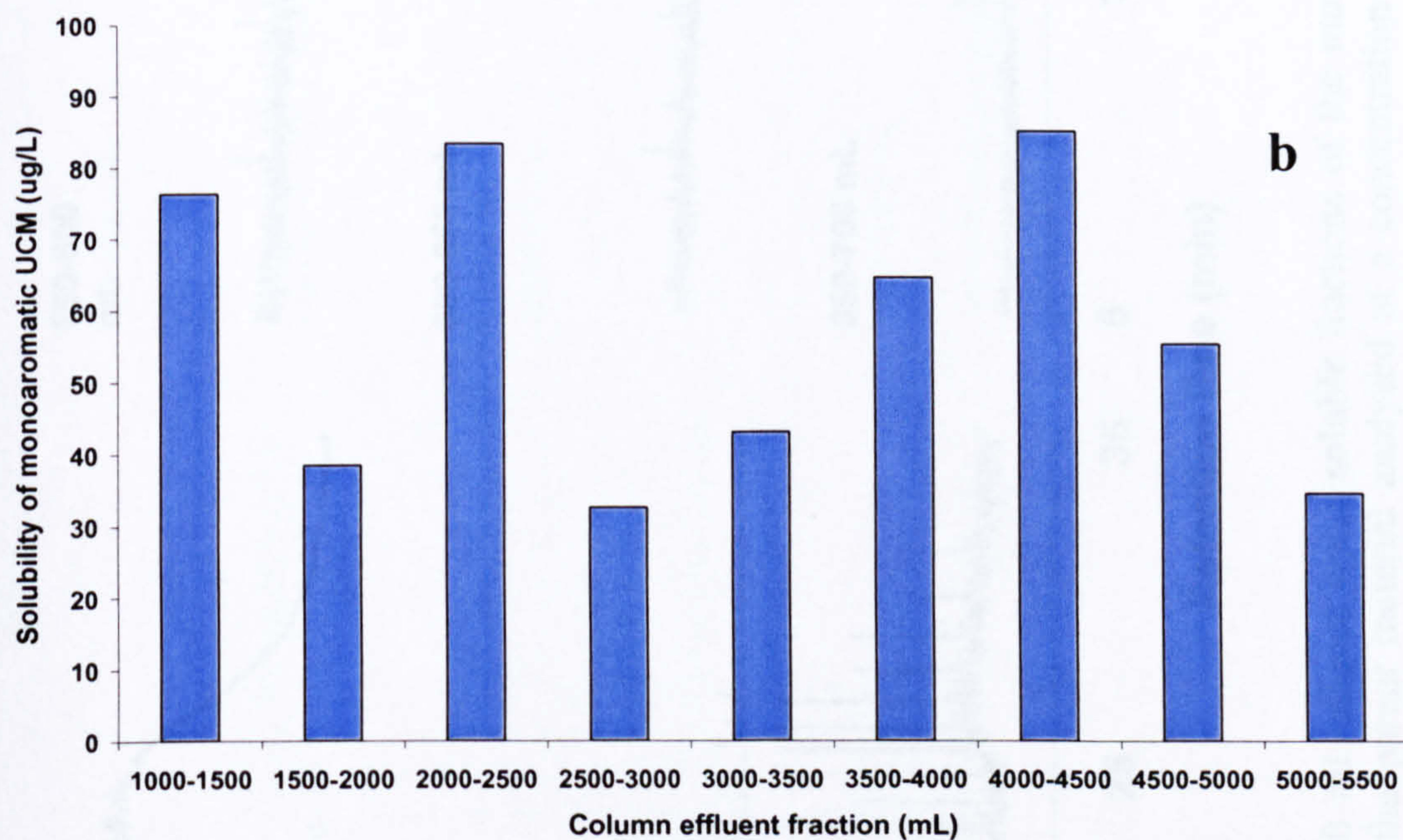
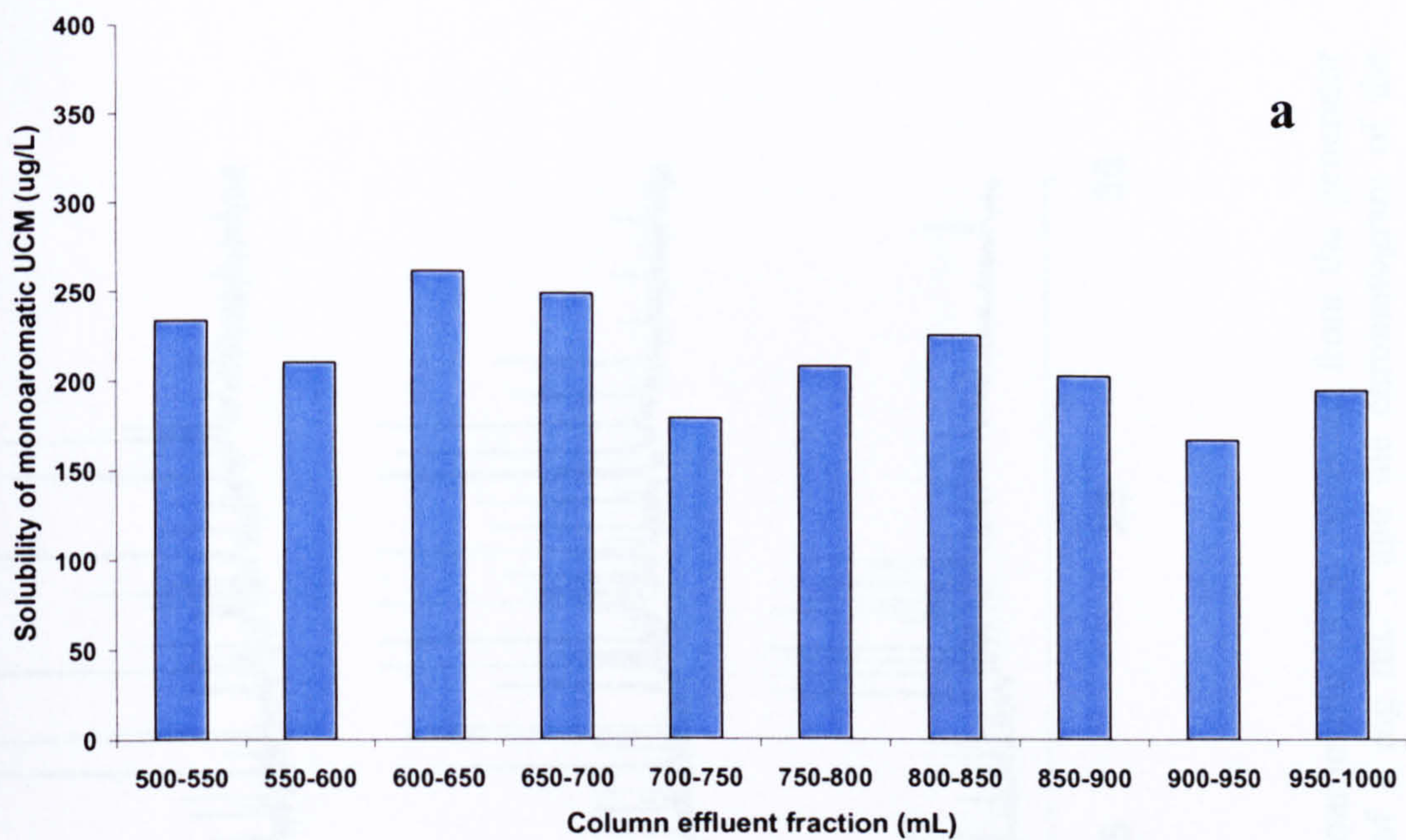
Since the generator column method appears unsuccessful in supplying reproducible solutions of the total aromatic UCM, it was decided to investigate the dissolution of a somewhat less complex fraction. The water solubility of a monoaromatic UCM, isolated from the aromatic fraction of Tia Juana Pesado crude oil using HPLC (Section 5.5), was therefore determined using a generator column in distilled water (25 °C). The monoaromatic UCM was coated to glass beads and packed into the generator column. Distilled water was eluted through the column at 1 mL min⁻¹. The first 500 mL was discarded whilst the system reached equilibrium (*e.g.* May *et al.*, 1978a; Ghosh *et al.*, 1998). The next 500 mL (500-1000 ml) of effluent was collected as 50 mL aliquots, and the volume of effluent from 1000 mL to 5500 mL was collected as 500 mL aliquots. An internal standard could not be used owing to coelution with monoaromatic UCM components, therefore external calibration was used for quantification. 1 mg of monoaromatic UCM was added to 500 mL of distilled water and extracted in the same manner as the aqueous solutions collected from the generator column. Analysis was by GC and concentrations were determined using calibration graphs. The results are summarised in Table 3.7, with the values reported as the mean ± standard deviation (n = 9).

Table 3.7. Water solubility of a monoaromatic UCM isolated by open column chromatography from Tia Juana Pesado crude oil, as determined by the generator column method in distilled water at 25 °C.

Test Substrate	Water solubility µg L ⁻¹ mean ± st. dev. (n=9)
Monoaromatic UCM (50 mL aliquots, 500-1000 mL)	213.0 ± 30
Monoaromatic UCM (500 mL aliquots, 1000-5500mL)	57 ± 21

The dissolution of the monoaromatic hydrocarbons in the TJP UCM appears less variable for both the 50 mL and the 500 mL aliquots than that determined for the total aromatic UCM hydrocarbons. However, the standard deviation values are still too large for the calibrated generator column method to be deemed reproducible in the production of aqueous solutions of a monoaromatic UCM. The water solubility of the 500 mL fractions appears to be an order of magnitude lower than those measured in the 50 mL fractions initially collected from the generator column. Although a 'compositional equilibrium' in the effluent is unexpected given the large number of different hydrocarbons in the UCM, a total hydrocarbon 'concentration equilibrium' could be expected. The results of this study suggest again that a 'concentration equilibrium' had not been achieved when sample collection began.

Figures 3.13a and 3.13b show the concentration ($\mu\text{g L}^{-1}$) of the monoaromatic UCM present in the 50 mL aliquots (Figure 3.13a) and 500 mL aliquots (Figure 3.13b) collected from the generator column. Figures 3.13a and 3.13b show in more detail the variation in the monoaromatic UCM in the generated water soluble fractions. The material in both the 50 mL and 500 mL fractions appears to decrease in concentration with increasing volume of water through the column.



Figures 3.13a and 3.13b. Comparison of the aqueous concentration of monoaromatic UCM with increasing water volume through the generator column. Figure **a** is the 50 mL extracts, and Figure **b** is the 500 mL extracts.

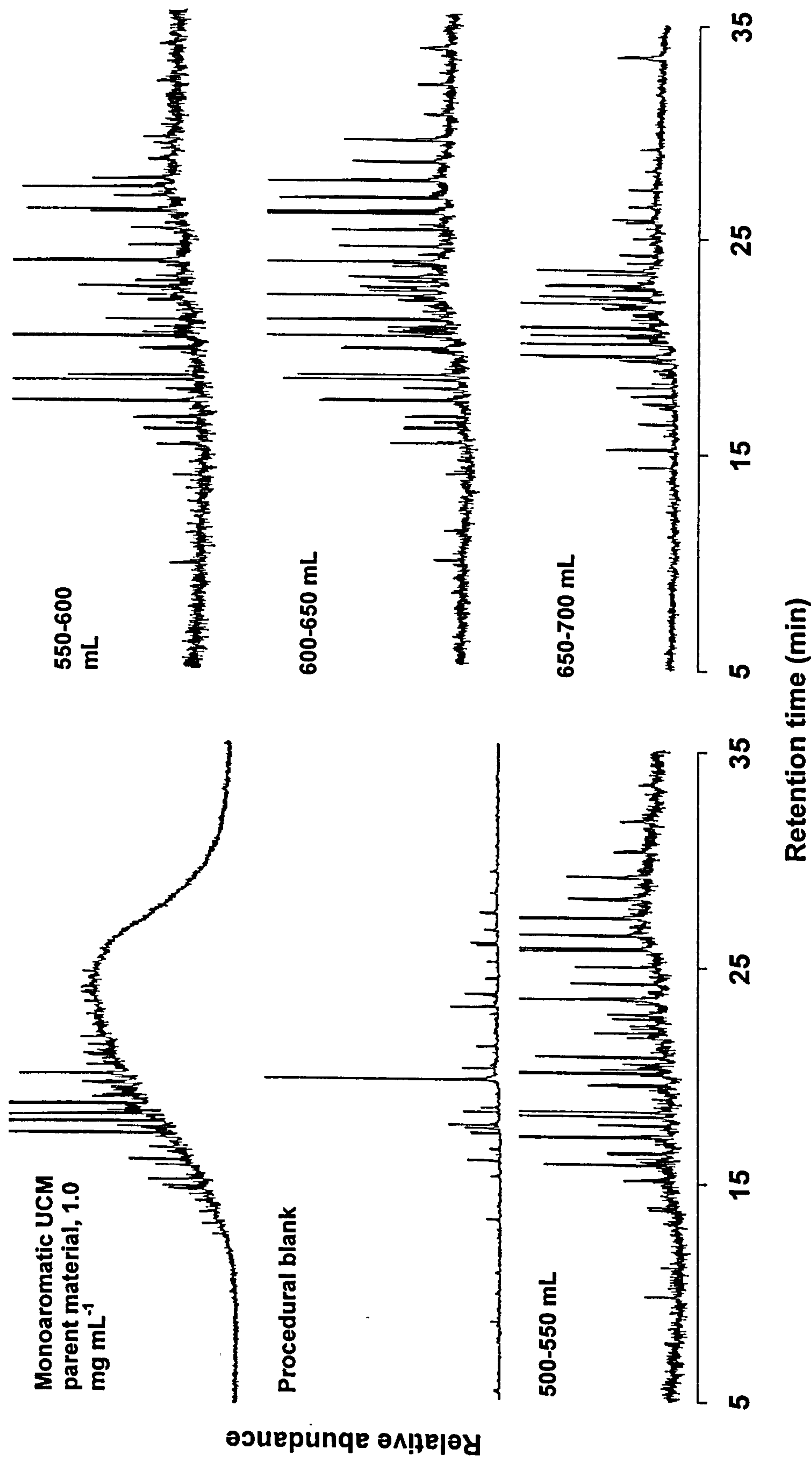


Figure 3.14. GC-MS chromatograms of the 50 mL aliquot water soluble fractions of the monoaromatic UCM collected from the generator column. Included are the chromatograms of the parent material analysed at a concentration of 1 mg mL⁻¹, and the chromatogram of the procedural blank sample. Figure 3.14 is continued on the next page.

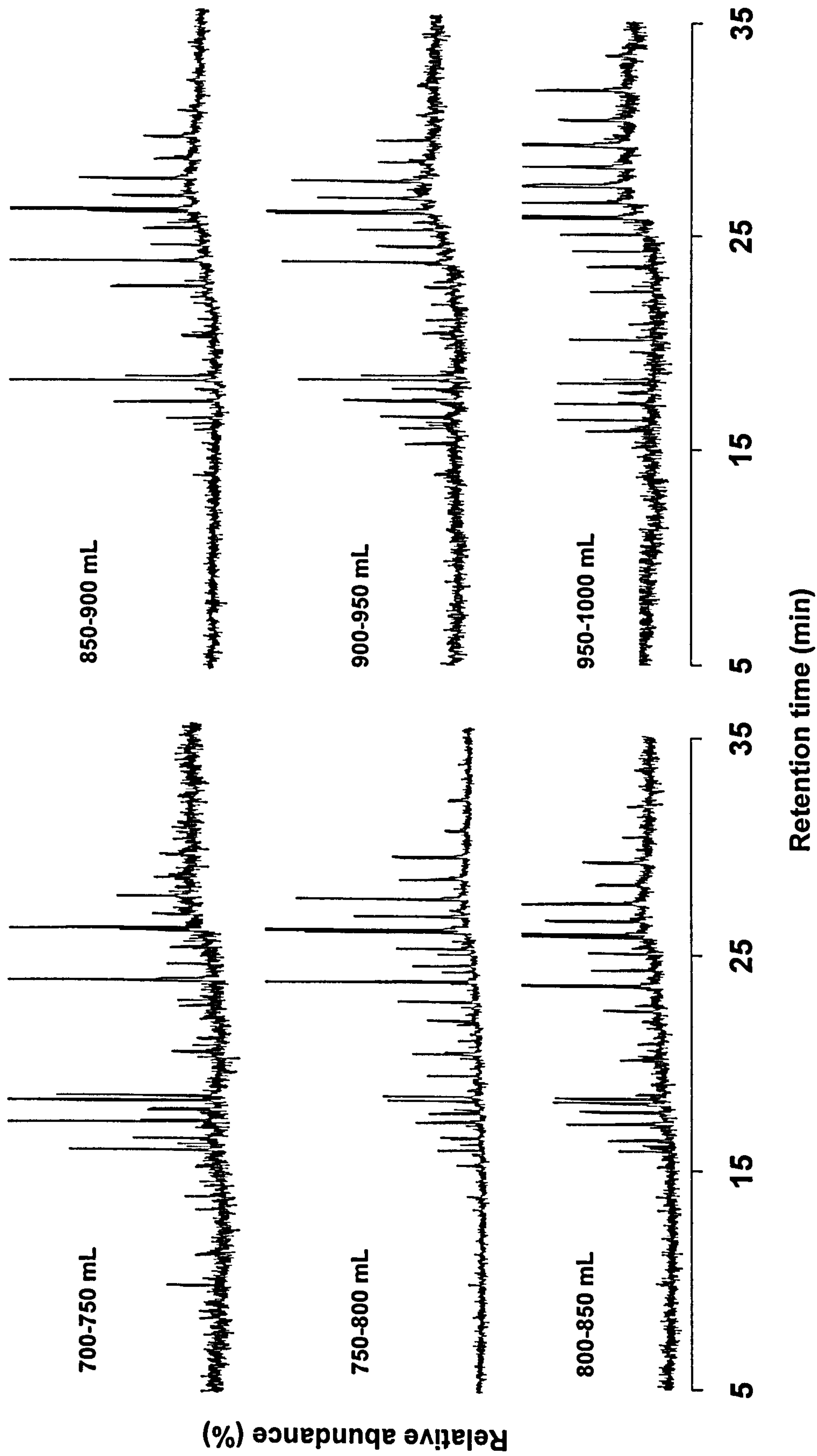


Figure 3.14. Continued from the previous page.

Figure 3.14 shows the GC-MS chromatograms of the extracted 50 mL WSFs (500-1000 mL) of the monoaromatic UCM. Chromatograms of the original material and the procedural blank are also shown. The chromatograms represent the water soluble material present in the 50 mL fractions when dissolved in 100 μ L of dichloromethane (DCM). Analysis of 1 μ L of this solution using GC-MS produced chromatograms bordering on the limits of instrumental detection, indicating that very little material was present in these fractions. There was still a significant chromatographic difference between the original monoaromatic UCM and the material present in the WSF. The chromatograms indicate that only a few compounds are present in the WSFs above the instrumental limits of detection. Therefore, it is unclear whether a UCM is present in the WSFs but below the limits of analytical detection, or whether it is absent. As the analytical response of the 50 mL water soluble fractions of the monoaromatic UCM is so low, it is difficult to compare the chromatograms of the WSFs to that of the original monoaromatic material. To determine the influence of impurities, Figure 3.15 shows a comparison of the water-soluble fraction (750-800 mL) and the procedural blank chromatograms. The 750-800 mL fraction was chosen as it is the middle aliquot in series collected. The two chromatograms show significant differences, which indicates that some material from the monoaromatic UCM did dissolve. However, the compounds present appear to be in the form of resolved peaks rather than an unresolved complex mixture.

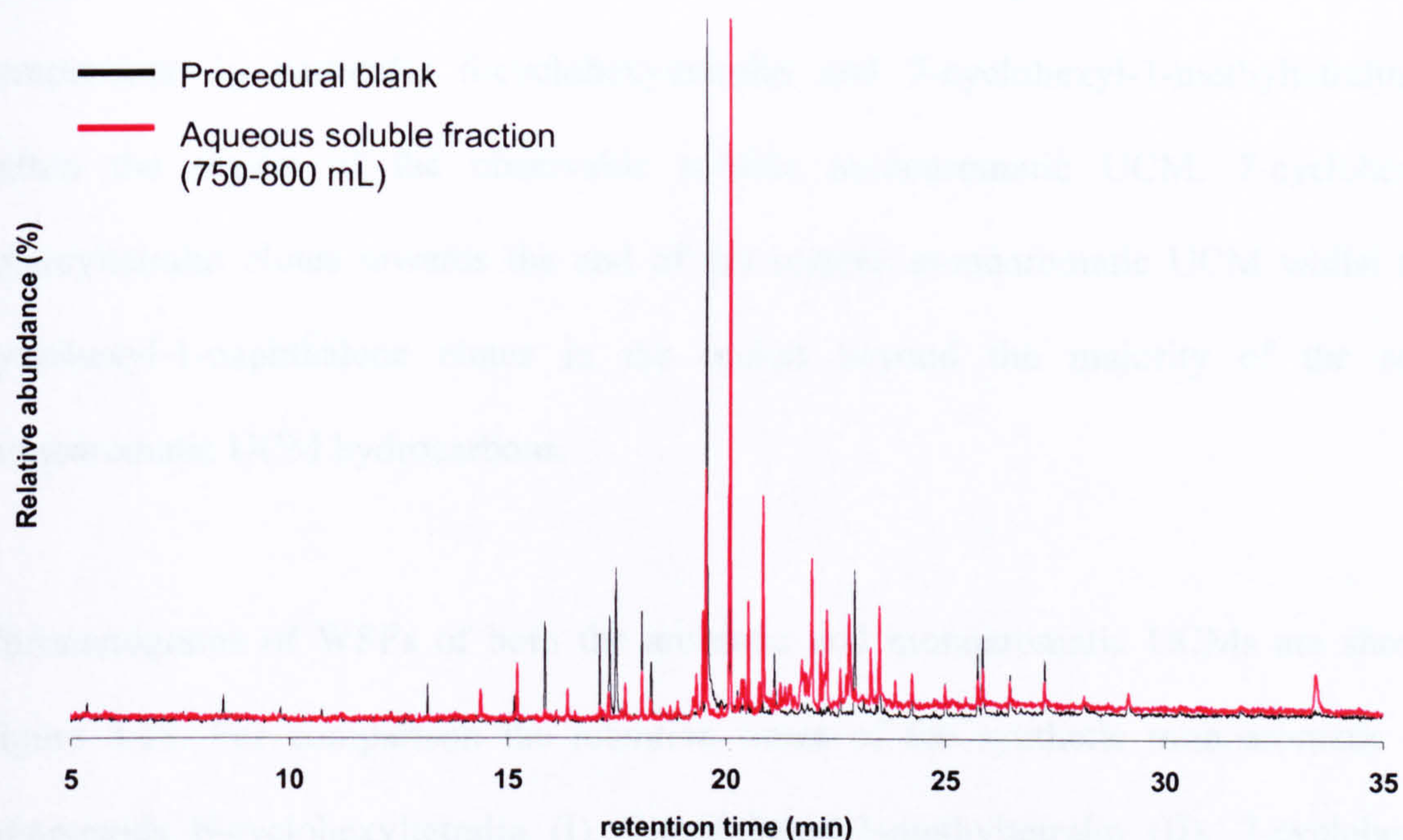


Figure 3.15. Comparison of the chromatograms of the procedural blank and the generated water soluble monoaromatic UCM fraction (750-800 mL).

Figure 3.16 shows the GC-MS chromatograms of the extracted 500 mL water soluble fractions (1000-5500 mL) of the monoaromatic UCM. Chromatograms of the original material and the procedural blank are also shown. The chromatograms provide clear evidence for a substantial difference between the original monoaromatic UCM and the UCM present in the WSFs. In the WSFs, the UCM is notably reduced in size, indicating a reduced number of compounds present compared with the original material. Figure 3.17 shows a comparison of the water-soluble fraction (2000-2500 mL) and the original monoaromatic UCM. The 2000-2500 mL fraction was chosen for the clear UCM exhibited in the chromatogram. Also shown for comparison are the retention times of the synthetic monoaromatic UCM hydrocarbons 6-cyclohexyltetralin (I), 7-cyclohexyl-1-methyltetralin (II), 7-cyclohexyl-1-isoamyltetralin (IV), and 7-cyclohexyl-1-nonylnaphthalene (VI). The diagram reveals that only a limited number of hydrocarbons in the original monoaromatic UCM are water soluble above the limits of analytical detection. Furthermore, some of these compounds become increasingly resolved in the chromatogram of the WSF

compared with the original UCM. The retention times of the synthetic monoaromatic UCM components, in particular 6-cyclohexyltetralin and 7-cyclohexyl-1-methyltetralin, fall within the region of the observable soluble monoaromatic UCM. 7-cyclohexyl-1-isoamyltetralin elutes towards the end of the soluble monoaromatic UCM whilst the 7-cyclohexyl-1-naphthalene elutes in the region beyond the majority of the soluble monoaromatic UCM hydrocarbons.

Chromatograms of WSFs of both the aromatic and monoaromatic UCMs are shown in Figure 3.18. For comparison the retention times of the synthetic monoaromatic UCM compounds 6-cyclohexyltetralin (I), 7-cyclohexyl-1-methyltetralin (II), 7-cyclohexyl-1-isoamyltetralin (IV) and 7-cyclohexyl-1-nonylnaphthalene (VI), are shown. Study of these chromatograms highlights a distinct difference in the most soluble components in each of the two source UCMs. The aromatic water soluble fraction appears to be dominated by higher molecular weight material, whereas the monoaromatic water soluble fraction is dominated by lower molecular weight material. There is very little overlap between the two UCMs. Whilst the diaromatic model compound 7-cyclohexyl-1-nonylnaphthalene eluted away from the monoaromatic UCM region, its retention time falls in the middle of the water soluble aromatic UCM. Due to its diaromatic nature, a retention time later than the soluble monoaromatic UCM is unsurprising.

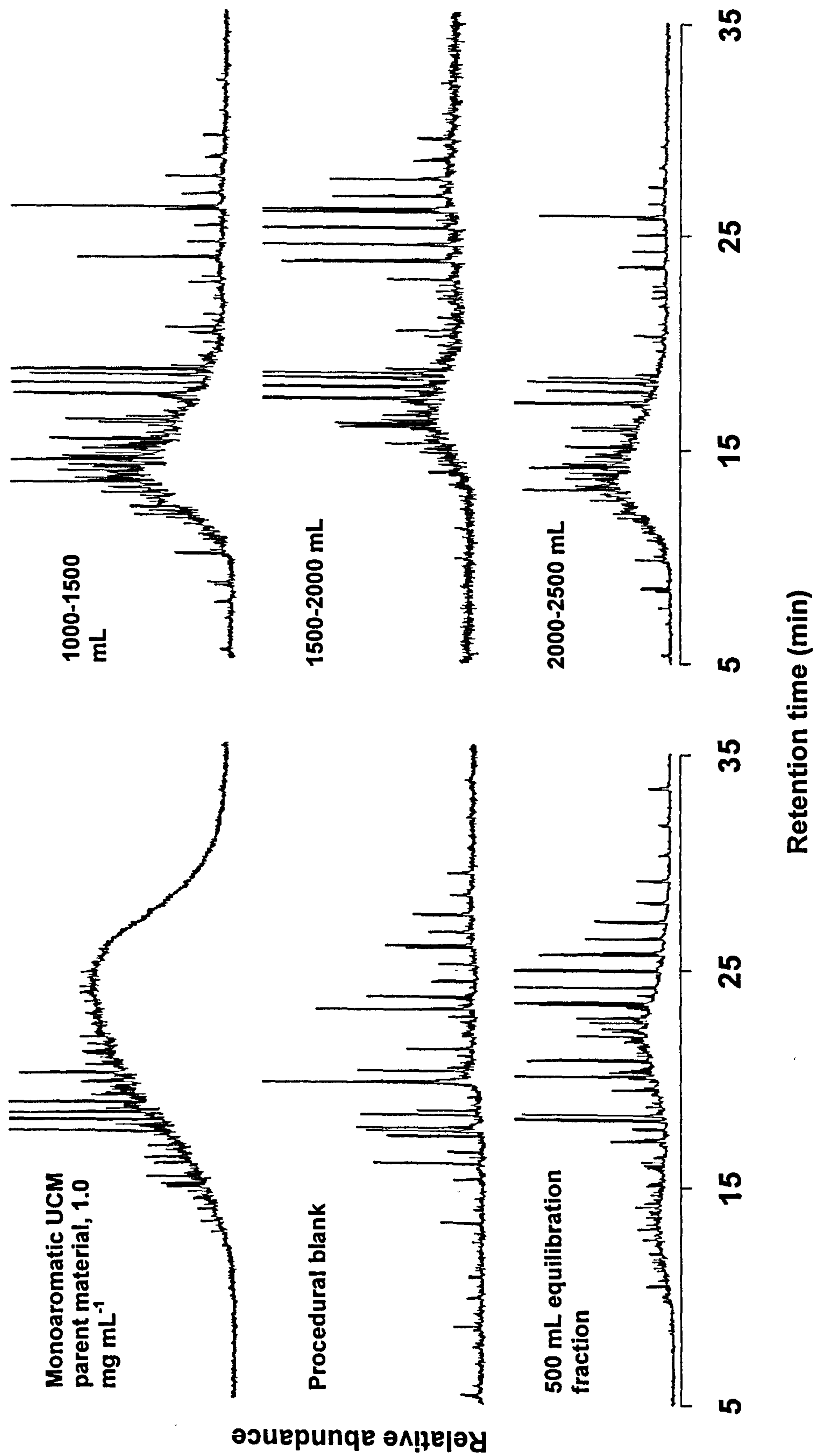


Figure 3.16. GC-MS chromatograms of the 500 mL aliquot water soluble fractions of the monoaromatic UCM collected from the generator column. Included are the chromatograms of the parent material analysed at a concentration of 1 mg mL⁻¹, and the chromatogram of the procedural blank sample. Figure 3.16 is continued on the next page.

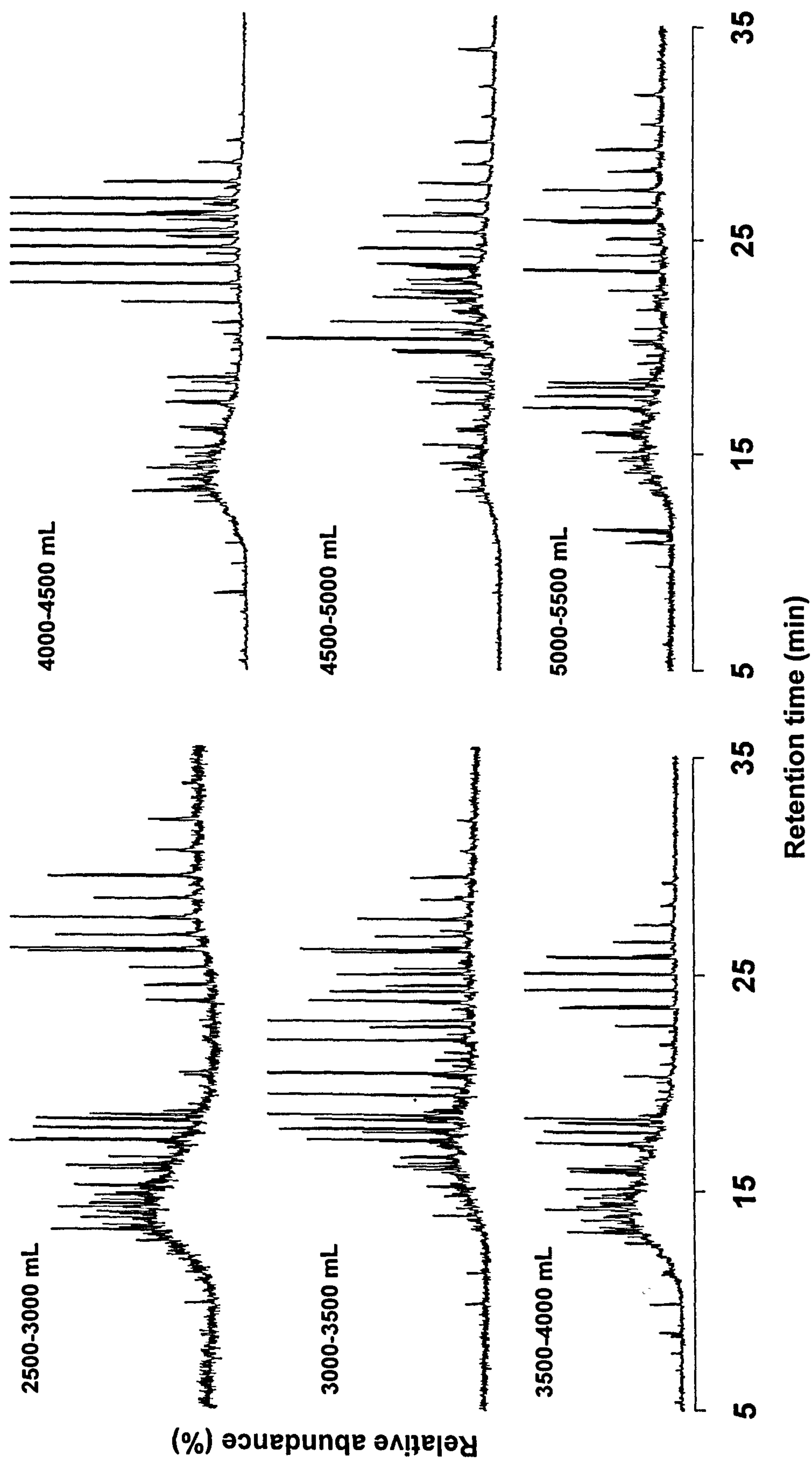


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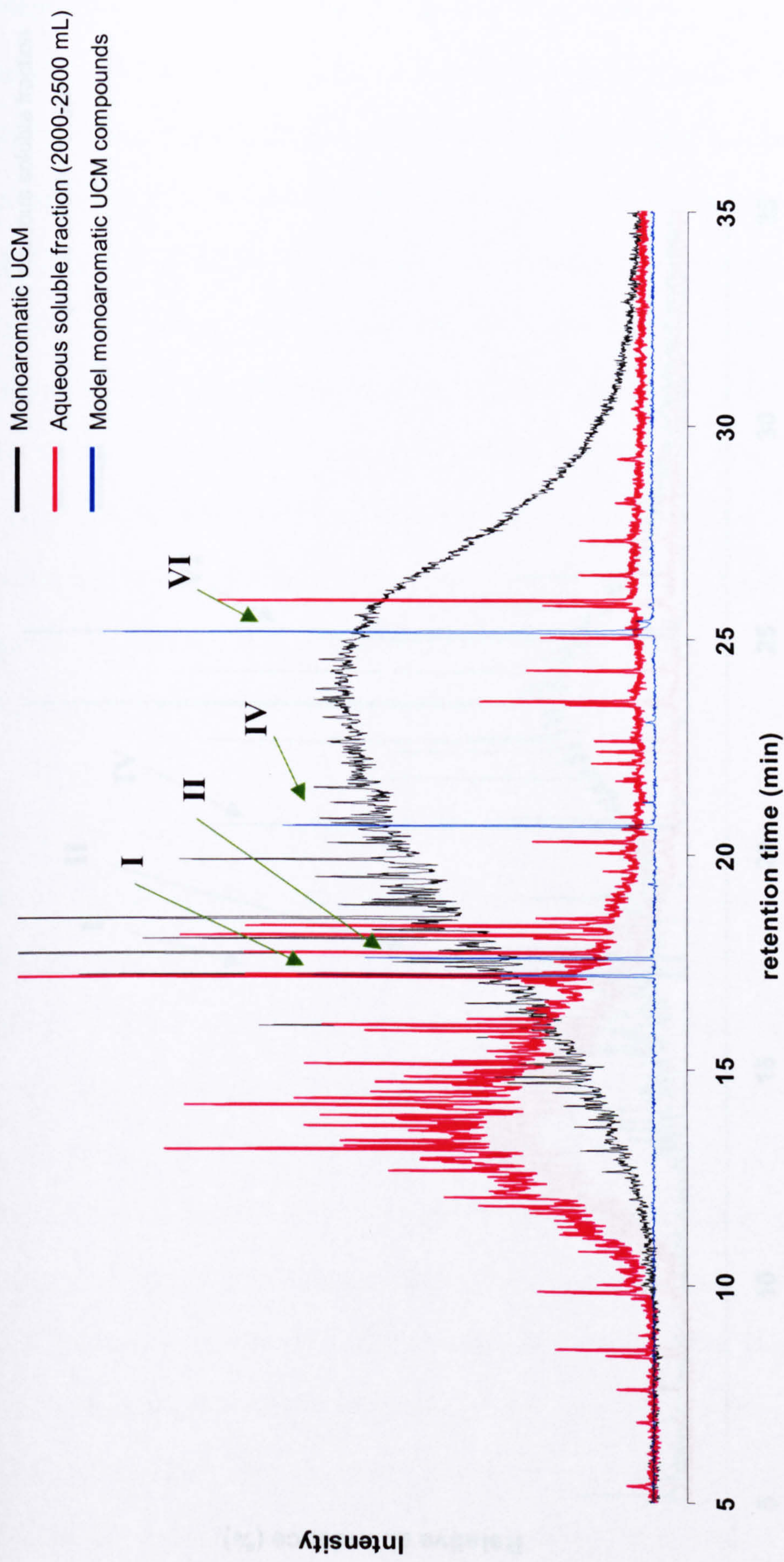


Figure 3.17. Comparison of the chromatograms of the parent monoaromatic UCM material and the generated water soluble monoaromatic UCM fraction (2000-2500 mL). Also shown for comparison are the retention times of the model monoaromatic UCM hydrocarbons 6-cyclohexyltetralin (I), 7-cyclohexyl-1-methyltetralin (II), 7-cyclohexyl-1-isoamyltetralin (IV) and 7-cyclohexyl-1-nonylnaphthalene (VI).

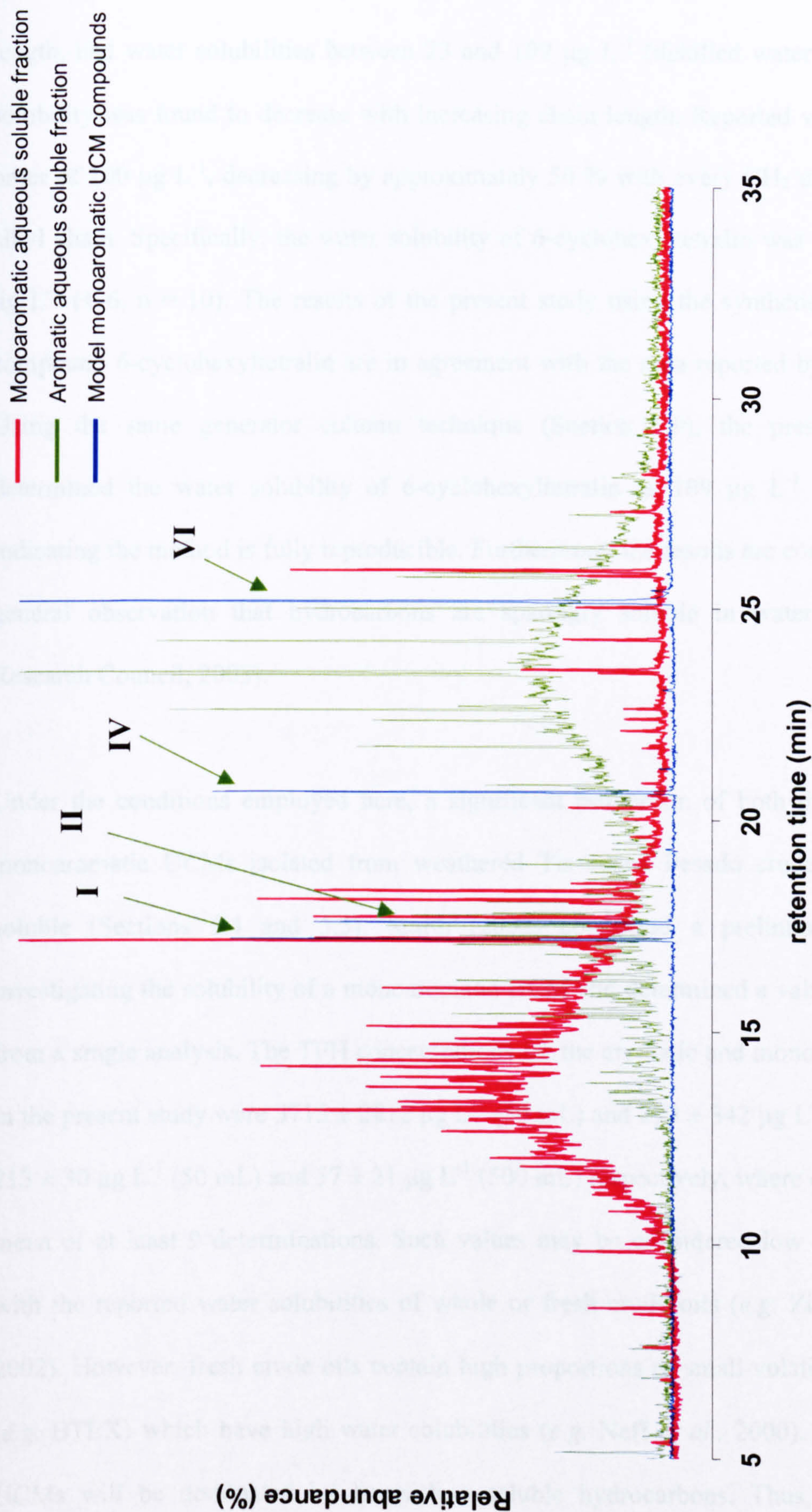


Figure 3.18. Chromatographic comparison of a water soluble aromatic UCM fraction (2500-3000 mL) and a water soluble monoaromatic UCM fraction (2000-2500 mL). Also shown for comparison are the retention times of the model monoaromatic UCM hydrocarbons 6-cyclohexyltetralin (I), 7-cyclohexyl-1-methyltetralin (II), 7-cyclohexyl-1-isoamyltetralin (IV) and 7-cyclohexyl-1-nonylnaphthalene (VI).

3.3 Discussion

Smith (2002) showed that a series of alkylcyclohexyltetralins, which varied only in chain length, had water solubilities between 23 and 109 $\mu\text{g L}^{-1}$ (distilled water at 25 °C). The solubility was found to decrease with increasing chain length. Reported values are of the order of 100 $\mu\text{g L}^{-1}$, decreasing by approximately 50 % with every CH_2 unit added to the alkyl chain. Specifically, the water solubility of 6-cyclohexyltetralin was reported as 109 $\mu\text{g L}^{-1}$ (± 6 , $n = 10$). The results of the present study using the synthetic monoaromatic compound 6-cyclohexyltetralin are in agreement with the data reported by Smith (2002). Using the same generator column technique (Section 5.9), the present study also determined the water solubility of 6-cyclohexyltetralin as 109 $\mu\text{g L}^{-1}$ (± 3 , $n = 10$), indicating the method is fully reproducible. Furthermore, the results are consistent with the general observation that hydrocarbons are sparingly soluble in water (e.g. National Research Council, 2003).

Under the conditions employed here, a significant proportion of both the aromatic and monoaromatic UCMs isolated from weathered Tia Juana Pesado crude oil was water soluble (Sections 5.4 and 5.5). Smith (2002) conducted a preliminary experiment investigating the solubility of a monoaromatic UCM and determined a value of 560 $\mu\text{g L}^{-1}$ from a single analysis. The TPH concentrations for the aromatic and monoaromatic UCMs in the present study were $3715 \pm 2812 \mu\text{g L}^{-1}$ (50 mL) and $293 \pm 342 \mu\text{g L}^{-1}$ (500 mL), and $213 \pm 30 \mu\text{g L}^{-1}$ (50 mL) and $57 \pm 21 \mu\text{g L}^{-1}$ (500 mL) respectively, where each value is the mean of at least 9 determinations. Such values may be considered low when compared with the reported water solubilities of whole or fresh crude oils (e.g. Ziolli and Jardim, 2002). However, fresh crude oils contain high proportions of small volatile hydrocarbons (e.g. BTEX) which have high water solubilities (e.g. Neff *et al.*, 2000). By their nature, UCMs will be dominated by larger less soluble hydrocarbons. Thus, UCMs can be expected to be less soluble than fresh crude oils.

Ziulli and Jardim (2002) studied the dissolution of different Brazilian crude oils and found that the concentrations varied by over an order of magnitude from 4.9 - 47 mg C L⁻¹. This indicates that the solubility of a crude oil or hydrocarbon mixture is highly dependent upon its chemical composition. Previous studies (*e.g.* Tolls *et al.*, 2002) have suggested that determination of the water solubility of hydrocarbons can often result in problems with regard to the limits of analytical detection. This is exemplified by the results from the 50 mL fractions prepared from the monoaromatic UCM. The amount of material present in the fractions is too little for suitable analytical quantification. The effect of using crude oil fractions, rather than a whole oil must also be taken into consideration. In a whole oil there are a large number of polar compounds, which are more water soluble than their hydrocarbon homologues. These compounds may be expected to dissolve to higher concentrations in the aqueous phase leading to high TPH values for whole oils (*e.g.* Boylan and Tripp 1971; Siron *et al.*, 1991; Silla *et al.*, 1992).

Polar compounds may also act as co-solvents or co-solutes, thus increasing the water solubility of the non-polar hydrocarbons (*e.g.* Mackay *et al.*, 1991; Schwarzenbach *et al.*, 2003). In the fractions studied here, these polar components are removed during the open column chromatography procedure. It is therefore expected that the TPH concentration in the WSFs will be reduced compared with the whole oil. Furthermore, the co-solvency effect may be greatly reduced or removed completely (*e.g.* Mackay *et al.*, 1991; Schwarzenbach *et al.*, 2003). Shiu *et al.* (1988) report that crude oil solubility decreases, as the more soluble components are depleted (*e.g.* BTEX) during weathering processes. As a result, the solubility of weathered crude oils was found to be significantly lower than fresh crude oils (*e.g.* Neff *et al.*, 2000). As the fractions used in the present study are isolated from a weathered oil, the water solubility may therefore be expected to be lower than that of a fresh crude oil.

The presence of a predominant UCM in the WSFs of crude oil and crude oils fractions have been previously reported (e.g. Barron *et al.*, 1999a; Page *et al.*, 2000; Smith, 2002; Ziolli and Jardim 2003). Barron *et al.* (1999a) observed WSFs prepared from weathered oils exhibited substantially larger UCMs and fewer resolved peaks than WSFs generated from fresh oils. Barron *et al.* (1999a) also noted UCM elution times indicative of a C₁₀-C₃₀ carbon range. In the present study, the approximate carbon range for the original aromatic and monoaromatic UCMs, and the respective WSFs has been calculated using both the Kovat's and the Lee retention indices (Figure 3.19). Graphs of the determined Kovat's retention index (Figure B1) and Lee retention index (Figure B2) are presented in Appendix B. The Lee index (Lee *et al.*, 1979b) is based upon unsubstituted aromatic compounds (naphthalene, phenanthrene, chrysene) and Kovat's index is based upon the saturated alkanes (C₁₀-C₃₀).

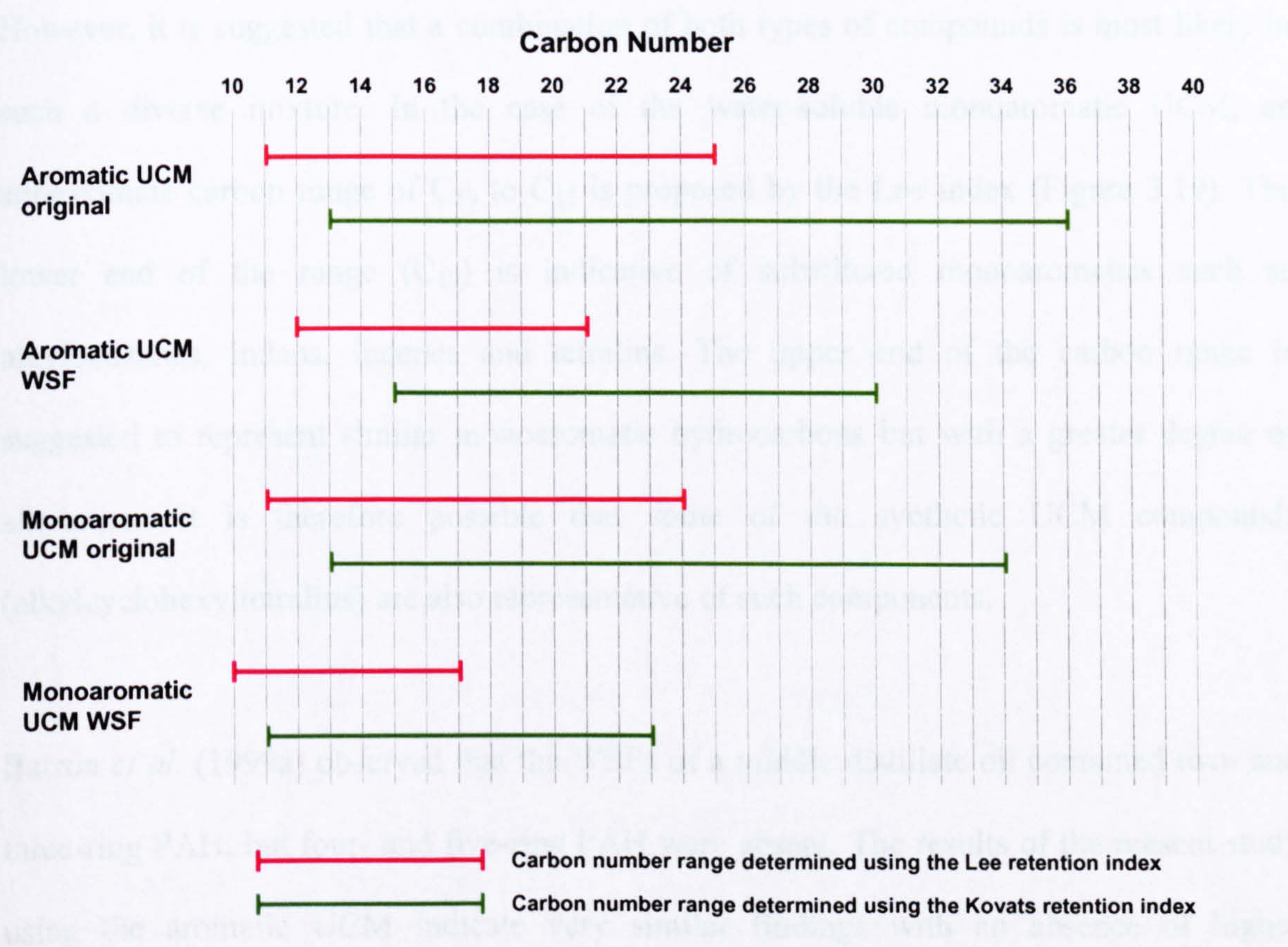


Figure 3.19. Comparison of the carbon ranges of the UCMs for both the aromatic and monoaromatic WSFs and the original aromatic and monoaromatic mixtures. Values have been calculated using both the Lee retention index and the Kovats retention index.

As the water-soluble UCMs are thought to be comprised predominantly of substituted aromatic hydrocarbons, neither of the indices is an 'ideal' reference for the determination of their carbon ranges. Compounds in the water-soluble UCMs are expected to exhibit retention times influenced by both aromatic and aliphatic components. Under these conditions the Lee retention index appears to be a more accurate reference than Kovat's retention index, therefore only the former will be discussed in detail herein.

Figure 3.19 shows that the water-soluble aromatic UCM has an approximate carbon range of C₁₂-C₂₁. The higher molecular weight compounds (C₁₉-C₂₁) are indicative of component hydrocarbons that contain at most four aromatic rings, with some degree of alkylation. The lower end of the range may represent highly substituted monoaromatic compounds (*e.g.* hexylbenzene, C₁₂) or alkylated naphthalenes (*e.g.* dimethyl- or ethylnaphthalene, C₁₂). However, it is suggested that a combination of both types of compounds is most likely in such a diverse mixture. In the case of the water-soluble monoaromatic UCM, an approximate carbon range of C₁₀ to C₁₇ is proposed by the Lee index (Figure 3.19). The lower end of the range (C₁₀) is indicative of substituted monoaromatics such as alkylbenzenes, indans, indenenes and tetralins. The upper end of the carbon range is suggested to represent similar monoaromatic hydrocarbons but with a greater degree of alkylation. It is therefore possible that some of the synthetic UCM compounds (alkylcyclohexyltetralins) are also representative of such components.

Barron *et al.* (1999a) observed that the WSFs of a middle distillate oil contained two- and three-ring PAH, but four- and five-ring PAH were absent. The results of the present study using the aromatic UCM indicate very similar findings with an absence of higher molecular weight PAH compounds. C₁-C₄ alkyl naphthalenes were the predominant PAHs in the WSFs analysed by Barron *et al.* (1999a). The presence of alkylbenzenes with up to a C₆ alkyl chain was also reported as dominant components. The alkylbenzenes and the

alkylnaphthalenes identified have carbon number ranges of C₆-C₁₂ and C₁₀-C₁₄ respectively. The results of this study indicate similar compounds may be typical components of the water-soluble fractions generated.

Figure 3.20 shows that there is a significant difference in the total petroleum hydrocarbon (TPH) concentration between the WSFs of the aromatic and monoaromatic UCM. Owing to the longer generation times, it is suggested that the 500 mL WSFs represent the more accurate picture of the continued dissolution of a crude oil into an aqueous phase. The reduced solubility of the monoaromatic UCM (57 µg L⁻¹) compared with the aromatic UCM (293 µg L⁻¹) may be due in part to the compound types present in the two mixtures (*i.e.* composition). Whilst the monoaromatic UCM will contain predominantly monoaromatic hydrocarbons, the weathered nature of the oil means it will be depleted in volatile compounds.

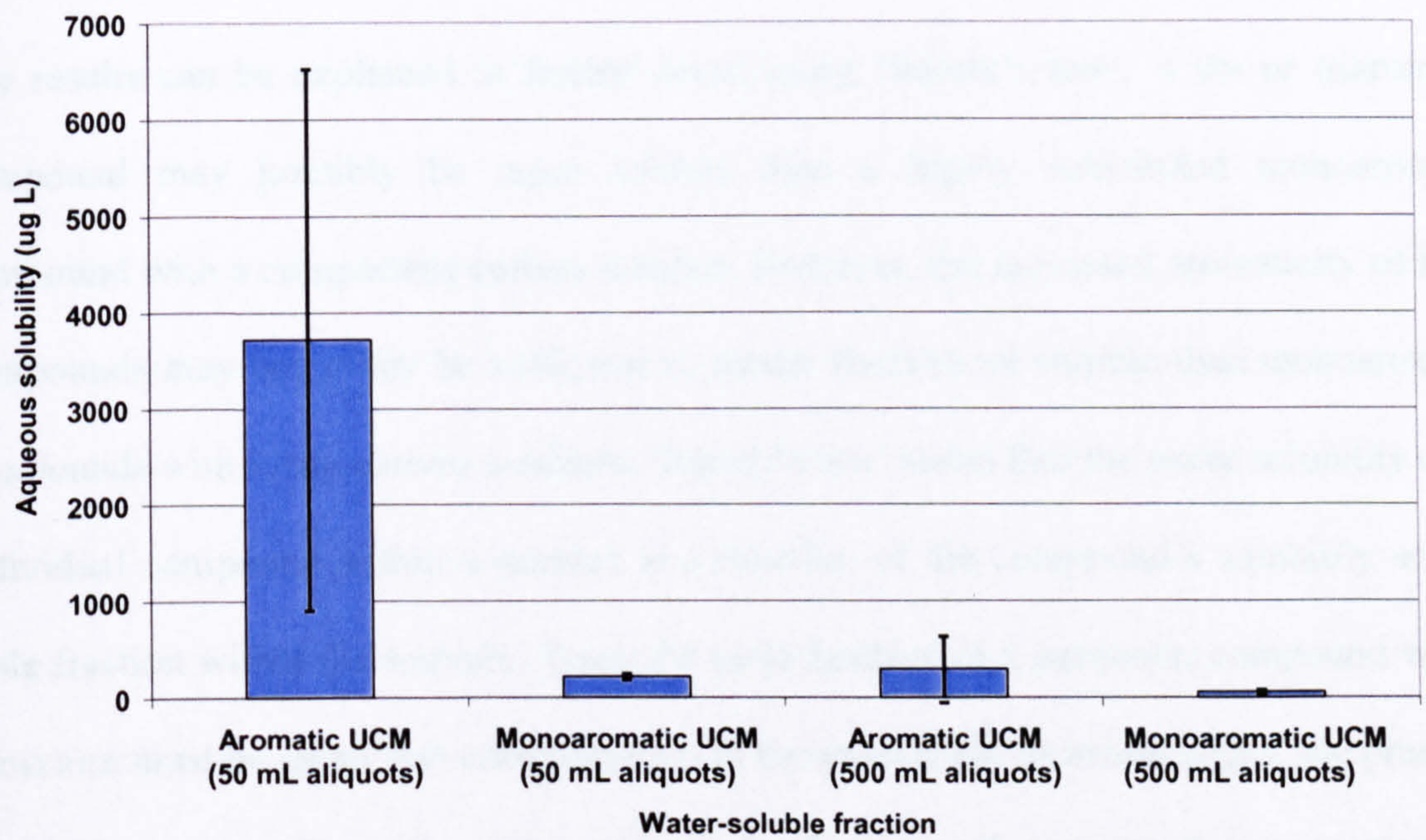


Figure 3.20. Comparison of the ‘water solubility’ of the aromatic and monoaromatic UCMs (n=9). Values were determined at 25 °C in distilled water.

The compounds in the original monoaromatic UCM which do not enter the aqueous phase in amounts amenable to analytical detection are suggested as those with significant degrees

of substitution. These compounds, though mono- or di-aromatic, may be characterised by larger molecular weights and surface areas due to the large size or number of substituents. This would result in lower volatility, and lead to a marked decrease in the water solubility of such compounds. In particular, saturated hydrocarbons (*e.g.* alkanes) are known to have very low solubilities compared with aromatic compounds of a comparable carbon number (*e.g.* McAuliffe 1966; Eganhouse and Calder, 1976; Kuo, 1994; de Maagd *et al.*, 1998). Therefore, substitution of aromatic hydrocarbons with large saturated groups would be expected to significantly reduce the water solubility of the resultant compound. The aromatic UCM WSF will probably still contain the monoaromatic compounds but also many PAH and their substituted homologues. It is possible that many of these compounds are characterised by higher water solubilities due to a greater degree of aromaticity and limited alkylation. Highly substituted PAHs, however, may be too insoluble to contribute to the aromatic UCM.

The results can be explained in further detail using 'Raoult's law'. A di- or triaromatic compound may possibly be more soluble than a highly substituted monoaromatic compound with a comparable carbon number. However, the increased aromaticity of PAH compounds may not solely be sufficient to render them more soluble than monoaromatic compounds with lower carbon numbers. 'Raoult's law' states that the water solubility of an individual compound within a mixture is a function of the compound's solubility and its mole fraction within the mixture. Thus, the mole fraction of a particular compound within a mixture must be taken into consideration. In the case of the aromatic UCM, the presence of PAH compounds will reduce the mole fraction of the smaller monoaromatic compounds. Therefore, compared to the monoaromatic UCM, their water solubility will be reduced. The concentration of individual compounds within the mixture will exacerbate this effect. Those components present in higher concentration will have a larger mole fraction within the mixture. A high mole fraction may increase a compound's solubility

within the mixture sufficiently to raise it above other hydrocarbons that exhibit a higher solubility as a pure compound. Thus, according to 'Raoult's law', it is possible for a hydrocarbon with a lower individual water solubility than another hydrocarbon to be more soluble when they are both present in a complex mixture such as petroleum.

Shiu *et al.* (1988) suggest increased weathering of crude oil results in a decrease in the solubility of the mixture and, therefore, TPH concentration. The weathering process removes the smaller, more volatile components (*e.g.* BTEX), which are generally the most soluble (Shiu *et al.*, 1988). This results in an increase in the mole fraction of the more environmentally persistent compounds in the remaining weathered mixture. However, these compounds generally have much lower individual water solubility. Therefore, it is not unexpected that the concentration of the TPH in the WSF is lower than that derived from a fresh crude oil containing more volatile compounds with higher individual water solubility. The dissolution of petroleum products can be significantly affected by the degree of weathering a crude oil has undergone (*e.g.* Ziolli and Jardim, 2003).

Operation of 'Raoult's law' can be seen more readily upon comparison of the WSFs of the aromatic and monoaromatic UCMs. The range of the two UCM retention times shown in Figure 3.18 indicates that each WSF is dominated by different solubilised material. In particular, the hydrocarbons dominating the soluble aromatic UCM are characterised by compounds with substantially higher average molecular weight, than those dominating the soluble monoaromatic UCM. It should be noted that the hydrocarbons present in the original monoaromatic UCM fraction are also present in the original aromatic UCM, as the former is an isolate of the latter. Increasing the number of aromatic rings on a compound will increase its polarity, and thus its solubility. This effect however, may be far outweighed by the reduction in solubility due to the effect of increasing molecular size. If 'Raoult's law' is considered, it is possible to explain why the soluble aromatic UCM is

dominated by higher molecular weight, though less soluble material, rather than the low molecular weight hydrocarbons present in the soluble monoaromatic UCM. It is suggested that the compounds dominating the WSFs of the monoaromatic UCM have a much higher mole fraction when present here than when in the aromatic UCM. This is because of the reduced number of compounds in the monoaromatic mixture. In the original aromatic UCM many of the larger PAH compounds may be present at a concentration greater than the monoaromatic components. Their higher mole fraction within the mixture may be sufficient to raise the relative solubility of these compounds above that of the monoaromatic components. Thus, compounds that are the most dominant in the WSF of the monoaromatic UCM may not be the same as those in the aromatic UCM.

The physicochemical characteristics of the compounds may also significantly influence the solubility of a compound in a mixture, as they determine the pure water solubility. This effect becomes more evident when the soluble monoaromatic UCM is compared with that of the original material. A dominance of lower molecular weight compounds in the WSF is apparent (Figure 3.17). The compounds in the original mixture must differ primarily by the degree and type of substitution, as they are predominantly monoaromatic. The results indicate that compounds with lower molecular weight, *i.e.* those with the least substitution, are the most soluble. This trend of reduced solubility with increasing size or number of alkyl substituents has been previously reported (Sherblom *et al.*, 1992; Kuo, 1994; Page *et al.*, 2000; Smith, 2002; Schwarzenbach *et al.*, 2003). In the case of the monoaromatic UCM, the physicochemical properties of the compounds appear to exert the greatest influence on solubility. This may indicate that the mole fraction of most components are similar.

The TPH concentrations in the water-soluble fractions of both the aromatic and monoaromatic UCMs exhibit large standard deviations, indicating this method was subject

to significant problems when applied to highly complex mixtures such as UCMs. Interestingly, the distribution of the soluble material remains similar in all of the aliquots collected from an individual column, but the total UCM hydrocarbon concentration varies greatly. Whilst the low concentrations of organic material in the WSF extracts can make analytical measurement problematic, other factors may be also contributing. For example, de Maagd *et al.* (1998) suggest that the relative standard deviation of a measured water solubility increases, as the solubility decreases. The authors report a 26 % standard deviation for the water solubility of pure chrysene. Alkyl substituted aromatic hydrocarbons are suggested to be components of the aromatic and monoaromatic WSFs in the present study. The presence of alkyl chains will serve to lower the water solubility of a compound compared with the unsubstituted parent hydrocarbon. Furthermore, the carbon number range for the soluble UCMs, estimated by the Lee index, indicate that some of the component hydrocarbons are also quite large (particularly in the aromatic UCM). Thus, in a mixture as complex as crude oil, where most of the compounds are sparingly soluble, large standard deviations in the data would be expected. The dynamic nature of the solubilisation of complex mixtures using a generator column, together with other complex processes affecting the solubilisation of hydrocarbons, will also contribute to the large standard deviation seen in the measured values. Another possible reason is that the system could be acting in the same way as a chromatographic column in a HPLC system, whereby the material on the column could be eluting as a series of 'peaks'. This could give rise to the significant differences in concentration between the collected aliquots, as some could contain more hydrocarbons than others. It is also possible that the system has led to the culturing of a microbial population within the column. Bacteria present in the system would be provided with suitable conditions for growth, including a carbon source and a constant temperature of 25 °C. This could also have contributed to the observed erratic nature of the results in a number of ways. Bacteria adhering to the UCM material could prevent normal dissolution of the hydrocarbons into the passing water. Secondly, it is also

possible that any UCM hydrocarbons which dissolve in the aqueous phase become more bioavailable to the microbes and could undergo some degree of degradation. Finally, the integration of UCMs present in the aqueous extracts at low concentration and the use of a UCM for calibration will also confound the problem, as such chromatographic features can be difficult to measure accurately and consistently.

A comparison of the retention times of the synthetic compounds 6-cyclohexyltetralin (I), 7-cyclohexyl-1-methyltetralin (II), 7-cyclohexyl-1-isoamyltetralin (IV) and 7-cyclohexyl-1-nonylnaphthalene (VI) to those of the water-soluble aromatic and monoaromatic UCMs is shown in Figure 3.18. The retention times of 6-cyclohexyltetralin (I) and 7-cyclohexyl-1-methyltetralin (II) coincide with those of the water soluble monoaromatic UCM, indicating that these compounds may be typical of those in the monoaromatic WSF. Compounds I and II contain 16 and 17 carbon atoms respectively, which is concurrent with the carbon number range determined for the UCM. 7-cyclohexyl-1-isoamyltetralin (IV), having a longer C₅ branched alkyl chain, has a retention time corresponding to the very end of the water-soluble monoaromatic UCM chromatographic range (Figure 3.18). Compounds such as this may represent those typical of a “solubility cut-off” for the monoaromatic material, where the physicochemical characteristics of the compound render it too insoluble for analytical detection. 7-cyclohexyl-1-isoamyltetralin is C₂₁, which is outside of the range determined by the Lee index for the aqueous monoaromatic UCM (C₁₀-C₁₇). However, the close proximity of its chromatographic elution to that of the monoaromatic UCM, indicates that the carbon range may actually be greater than estimated by the Lee index. Therefore, the Lee index, although a useful guide, does not accurately account for substituted aromatic compounds as it is based upon the unsubstituted parents. The diaromatic compound 7-cyclohexyl-1-nonylnaphthalene has a considerably longer retention time than the range of the monoaromatic UCM. Not unexpectedly, the retention time of this compound coincides with the middle of the water soluble aromatic UCM (Figure 3.18),

which is known to contain diaromatic compounds. Despite a detailed GC-MS study of both the aromatic and monoaromatic UCM water soluble fractions, none of the synthetic alkylcyclohexyltetralins (I-V) were found to be present. However, if the mole fraction of such compounds in the UCMs is very small, they may be present in concentrations below the analytical limits of detection. Thus, the existence of the synthetic compounds cannot be ruled out entirely.

Zioli and Jardim (2003) prepared WSFs of two Brazilian crude oils, and found in both cases the GC-MS chromatograms exhibited a large UCM. The WSFs were found to contain many low molecular weight hydrocarbons. These included alkylbenzenes and naphthalenes that the authors suggested would be highly susceptible to bacterial attack, and thus not persistent in the environment. However, the water soluble UCMs generated in the present study are prepared from isolated fractions of weathered Tia Juana Pesado crude oil, previously shown to be resistant to further biodegradation (Chapter 2). The water soluble UCMs in the present study may be expected to be persistent in the environment. This has implications with regard to the ecotoxicological impact of such material once present in the environment. Neff *et al.* (2000) have suggested that monoaromatic hydrocarbons are the main contributors to the acute toxicity of WSFs of fresh crude oils observed in aquatic organisms. The authors also reported that the toxicity of WSFs of crude oils could not be accounted for entirely by the toxicity contributed by the resolved components. They suggested that constituents of the UCM might be responsible for this additional toxicity. Whilst many of the volatile monoaromatic hydrocarbons are lost during weathering processes, a sufficient amount remains and contributes to monoaromatic UCMs observed in crude oil residues. It is therefore suggested that some of these compounds may be responsible for detrimental effects in aquatic organisms. Carls *et al.* (1999) and Heintz *et al.* (1999) report that chronic exposure of Pacific herring and pink salmon embryos to WSF of a weathered crude oil at concentrations as little as $1 \mu\text{g L}^{-1}$, were sufficient to cause

significant sublethal effects. Even allowing for the irreproducibility of the methods, the present study indicates that monoaromatic UCMs are capable of dissolving into the water column to concentrations far exceeding this value (57-213 $\mu\text{g L}^{-1}$).

Rowland *et al.* (2001) and Donkin *et al.* (2003) have previously reported that monoaromatic UCMs are indeed toxic to marine organisms. The present study indicates that perhaps only a proportion of the material in the monoaromatic UCM is sufficiently water soluble to reach the site of toxic action. Therefore, whilst the monoaromatic UCM has been shown to be toxic to marine organisms, perhaps only a percentage of the hydrocarbons can be responsible for the observed toxicological impacts. Banerjee (1984) suggests that the behaviour of a toxic component within a complex mixture will be different from the pure toxicant. The toxicity of a WSF derived from a mixture of compounds, will be less than that of a corresponding solution of the most toxic component. However, as the present study shows, the aqueous phase generally contains only a selection of the components present in the parent oil. The ratio of constituents in the aqueous phase will not therefore be the same as in the parent oil product (*e.g.* Bennett *et al.*, 1990). From a toxicological viewpoint this is important, as some toxic compounds may be present in higher concentration in the aqueous phase, than in the parent material. Therefore, the toxicity of a complex mixture of hydrocarbons dissolved in the water column may be difficult to predict, as it is potentially different from that of the parent material.

To determine if any changes occurred over time in the composition of the generated WSFs of the aromatic UCM, certain compounds were identified from the GC-MS chromatograms. Their presence was monitored in the fractions collected. Compounds were identified from their characteristic base ion fragments generated in the mass spectral detection step of the analysis (Killops and Killops, 1993). In the aromatic WSFs a range of

unsubstituted and alkylated PAHs were observed (Figure 3.21). In the first fraction collected (500-1000 mL) a wide range of compounds are identifiable, including phenanthrene, fluorene, dibenzothiophene and their alkylated homologues, as well as a series of alkylnaphthalenes. In the later fractions, the absence of the lower substituted homologues and lower molecular weight compounds was noted (*e.g.* alkylnaphthalenes, phenanthrene and fluorene). Instead, higher molecular weight compounds dominated the later WSFs. Whilst some compounds were not present in the later eluting fractions, other higher homologues not previously seen, became evident. For example, the trimethylphenanthrenes were absent in the early eluting fractions, but were clearly present in those eluting later. Again, these observations can be explained if 'Raoult's law' is considered. In the early eluting fractions the lower molecular weight and most soluble compounds are present in the aqueous phase. These compounds could be expected to deplete over time through dissolution, reducing their mole fraction in the UCM. Conversely, the mole fraction of other less soluble compounds (*e.g.* trimethylphenanthrenes) would increase, raising their relative solubility. Thus, it is not unexpected for them to become observable in the later eluting water-soluble fractions.

It is evident from these observations that the dissolution of hydrocarbons from a complex mixture such as crude oil is a dynamic process. It is suggested, therefore, that given a sufficient period of time, a significant number and range of UCM components may be expected to enter the aqueous phase. Despite these findings, the dissolution of hydrocarbons with very low pure compound solubility may still be constrained by their physicochemical properties. Such compounds may not enter the aqueous phase in concentrations high enough for routine analytical detection.

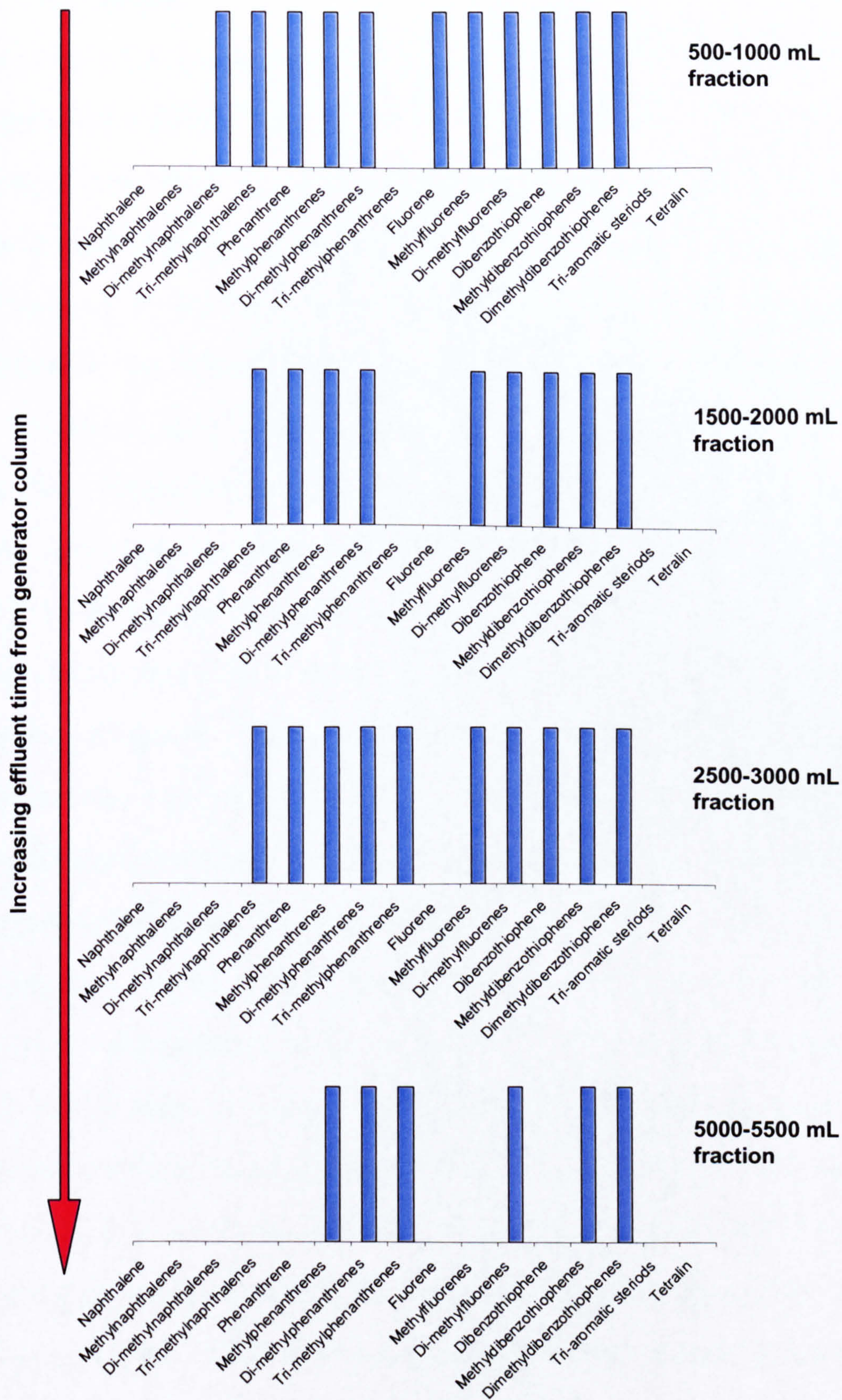


Figure 3.21. Comparison of the major compounds occurring in the water-soluble fractions of the aromatic UCM over time.

3.4 Conclusions

The results of the present study confirm that a large number of components of both an aromatic and a monoaromatic UCM are 'water soluble'. UCMs with chromatographic profiles different from those of the UCM substrates tested were observed in the aqueous phase extracts. Weathered crude oil residues, typically characterised by dominant UCMs, are known to be resistant to degradation, and therefore persistent in the environment. Fractions of these UCMs, in particular the monoaromatic hydrocarbons, have been shown to be toxic to marine organisms (Rowland *et al.*, 2001; Donkin *et al.*, 2003). The dissolution of these persistent, toxic monoaromatic UCM hydrocarbons in the present study confirms their potential bioavailability to aquatic organisms. The technique used in the study and the data collated indicate that hydrocarbons may continue to dissolve in the water column, long after an initial crude oil spill. Over time such hydrocarbons may dissolve into aqueous systems to high concentrations compounding the effect on marine organisms (*e.g.* Crowe *et al.*, 2004). A second UCM generated in the WSFs comprised a large number of compounds at similar concentrations. This implies such components have comparable solubilities and mole fractions within the mixture. The monoaromatic hydrocarbons in the WSF fraction, therefore, are thought to exhibit similar physicochemical properties, and are suggested to be C₄-C₁₁ substituted monoaromatics (*i.e.* total carbon range of C₁₀-C₁₇). The synthetic alkyltetralins, already shown to be bioresistant (Chapter 2) and toxic (Rowland *et al.*, 2001) have retention times that compare well to either the soluble monoaromatic (I, II and IV) or aromatic (IV and VI) UCMs. The present study and that made by Smith (2002) have shown that these compounds are water soluble when pure. This provides further evidence for the candidacy of alkyltetralins as representative components of some monoaromatic UCMs.

The dissolution of hydrocarbons from a complex mixture is a dynamic process, with the most soluble compounds being evident in the earliest fractions. The less soluble compounds are found to become more predominant in the later fractions. The most soluble components become exhausted from a mixture through dissolution into the aqueous phase and their mole fraction also decreases. The concentration/mole fraction of other components in the mixture then increases, raising their solubility. Over a sufficient period of time, a significant number of UCM components may be expected to enter the aqueous phase. However, dissolution of hydrocarbons with very low solubilities may still remain below the limits of analytical detection.

This study has highlighted the need for care when investigating the water solubility of hydrocarbon mixtures, in particular the influence mole fraction can exert on solubility. The fractionation of a mixture alters the mole fraction of the components, generally enhancing the dissolution of compounds compared with the original mixture. This can lead to abnormally high solubility values when fractions rather than whole mixtures are analysed. The chemical composition of a mixture will affect its dissolution (*e.g.* Barron *et al.*, 1999a), and this must be taken into consideration when estimating or predicting the dissolution characteristics of a complex mixture. The distribution or concentration of mixture components can have a significant bearing on their water solubility within a mixture.

Finally, the study has highlighted serious deficiencies in the generator column technique for the production of aqueous solutions of highly complex mixtures. Whilst this method has been shown to produce very repeatable data for individual test compounds, the results of the present study using aromatic and monoaromatic UCMs indicate this is not the case for complex mixtures of compounds. It is suggested that a much gentler transition from the analysis of single compounds to complex mixtures is required in future studies.

Chapter 4

Characterisation of the water soluble fraction of a monoaromatic UCM

The water ‘soluble’ monoaromatic UCM generated in Chapter 3 provided an opportunity to study the composition of what is predicted to be some of the most bioavailable and toxic UCM components. Resolution and characterisation of a water ‘soluble’ monoaromatic UCM was achieved using comprehensive gas chromatography x gas chromatography – time-of-flight – mass spectrometry (GCxGC-ToF-MS) analysis. Over 1200 compounds were separated by the chromatography, of which about 500 had distinct mass spectra. From a detailed characterisation of some of these monoaromatic UCM hydrocarbons *via* comparison to mass spectra registered in the NIST library the identities of over 100 monoaromatic UCM components were inferred. The monoaromatic UCM hydrocarbons identified included highly alkylated homologues of benzene, indene, indan, tetralin, biphenyl, diphenylmethane and tetrahydrophenanthrene. Importantly, the study indicated that UCMs comprise the geochemically minor isomers and analogues of known major crude oil constituents. Thus, UCMs may contribute significantly to the observed additive narcotic toxicity of resolved crude oil hydrocarbons.

4.1 Introduction

Until recently, the limitations of available analytical techniques have prevented all but a partial resolution and identification of crude oil components. In particular, mainly those compounds present in high concentrations lend themselves to chromatographic resolution. Such compounds have been successfully characterised using conventional chromatographic and detection techniques such as gas chromatography - mass spectrometry (GC-MS).

As a result, the large proportions of both fresh and weathered crude oils which are unresolvable (UCMs) have remained unidentified using conventional analytical techniques. This chromatographic feature or 'hump' is produced when standard analytical techniques (*e.g.* GC) are incapable of separating the many thousands of minor organic compounds present in a crude oil. The sheer number of compounds, together with their similar physical and chemical properties result in overlapping peaks, which produce the characteristic UCM feature.

A review of the previous techniques employed to resolve and identify the component hydrocarbons of the UCM was presented in Chapter 1. None of these studies were capable of resolving fully or of fully characterising the UCM hydrocarbons. A comparatively recent, but significant development, is the transfer of portions of the eluent partially separated on one chromatographic column, to a second chromatographic system. If the second system has a different method of separation (*i.e.* a column with a different stationary phase), compounds coeluting on the first column may be separated on the second. This technique became known as 'heart-cutting', hyphenated GC-GC (Schoenmakers *et al.*, 2000; Phillips and Xu, 1995), or multi-dimensional gas chromatography (MDGC). The technique has proved useful, but is limited as only portions of first-dimension chromatogram eluent can be transferred to the second dimension (Liu

and Phillips, 1991; Venkatramani and Phillips, 1993; Schoenmakers *et al.*, 2000; de Boer and Law, 2003; Johnson *et al.*, 2003; Marriott *et al.*, 2003). Thus, heart-cutting can only improve the resolution of a selected group of sample components from a single 'cut' due to the slow chromatography of the second dimension (Schoenmakers *et al.*, 2000; Adahchour *et al.*, 2003). If the entire sample were transferred, the final chromatogram would be equivalent to a single mixed-phase column and not two separate chromatographic steps. Sequential heart-cutting techniques can be used to analyse an entire sample. However, a loss of first dimension resolution occurs, and a very long analysis time is required (Liu and Phillips, 1991). It is therefore only possible to perform a small number of discrete heart-cut transfers in each analysis, and the gain in total peak capacity is limited (Marriott *et al.*, 2003). Whilst heart-cutting is classed as multidimensional gas chromatography, it cannot be termed 'comprehensive' due to its inability to provide second-dimension chromatography to the entire sample (Johnson *et al.*, 2003; Marriott *et al.*, 2003).

4.1.1 Gas chromatography x gas chromatography (GCxGC): A two-dimensional analytical approach

It was not long before the first fully comprehensive multi-dimensional gas chromatographic system was developed. This was first pioneered by Professor John Phillips (Liu and Phillips, 1991) in the early 1990s. In this development the entire sample injected into the first column also passes through the second column and into the detector. The sample is subject to independent chromatography in two dimensions, therefore the technique is termed 'comprehensive' (Gaines *et al.*, 1999; Dallüge, 2003; Hamilton and Lewis, 2003). This overcomes the problems associated with GC-GC heart cutting (Johnson *et al.*, 2003), and can be classed as a state-of-the-art analytical technique. Indeed, the First International Symposium on Comprehensive Multidimensional Gas Chromatography was held as recently as 6-7 March 2003 in Volendam, The Netherlands.

Liu and Phillips (1991) first used a thermal desorption modulator to generate high-speed chromatograms sampled from a continuously flowing stream. In this case, the flowing stream was the eluent from a slower chromatographic column (1st dimension). A second column is placed orthogonal to the inlet GC's retention time axis to form a two-dimensional data space (Venkatramani *et al.*, 1996; Gaines *et al.*, 1999; Schoenmakers *et al.*, 2000; Dallüge, 2003). Effluent from the first column is transferred to the second column *via* the thermal modulator. The modulator effectively 'digitises' the first dimension chromatogram and then focuses the sample material in a series of sharp, equidistant concentration pulses (Schoenmakers *et al.*, 2000). The eluent flow from the first column enters the modulator, where it is divided into a series of individual 'heart-cuts' or concentration pulses corresponding to approximately one peak width of time (*i.e.* just a few seconds).

These concentration pulses are then sequentially placed onto the second column, which has a different stationary phase, subjecting the material to a second chromatography step. The second column/system is made fast enough to generate at least one complete chromatogram during the time required for a peak to elute from the primary instrument column (Liu and Phillips 1991; Phillips and Beens, 1999; Marriott *et al.*, 2003). Each time a single datum would normally be recorded in the primary chromatogram, a complete high-speed secondary chromatogram is recorded (Venkatramani and Phillips, 1993). During this time the modulator prevents continuous elution from the first dimension, and accumulates material for the next concentration pulse by cooling the eluent (Liu and Phillips, 1991; Venkatramani and Phillips, 1993; Reddy *et al.*, 2002; Marriott *et al.*, 2003). Upon completion of a secondary chromatogram, the modulator is heated to 100°C above the GC oven temperature, volatilising the accumulated sample from the first column. The carrier gas then transfers the material to the second column; thus the modulator effectively acts as

an injector for the second chromatography step. As a result, the primary retention axis is still calibrated in minutes (standard operating speed) but the secondary axis is calibrated in seconds (Phillips and Xu, 1995). Frysinger and Gaines (2002) discuss the concept of modulation in further detail.

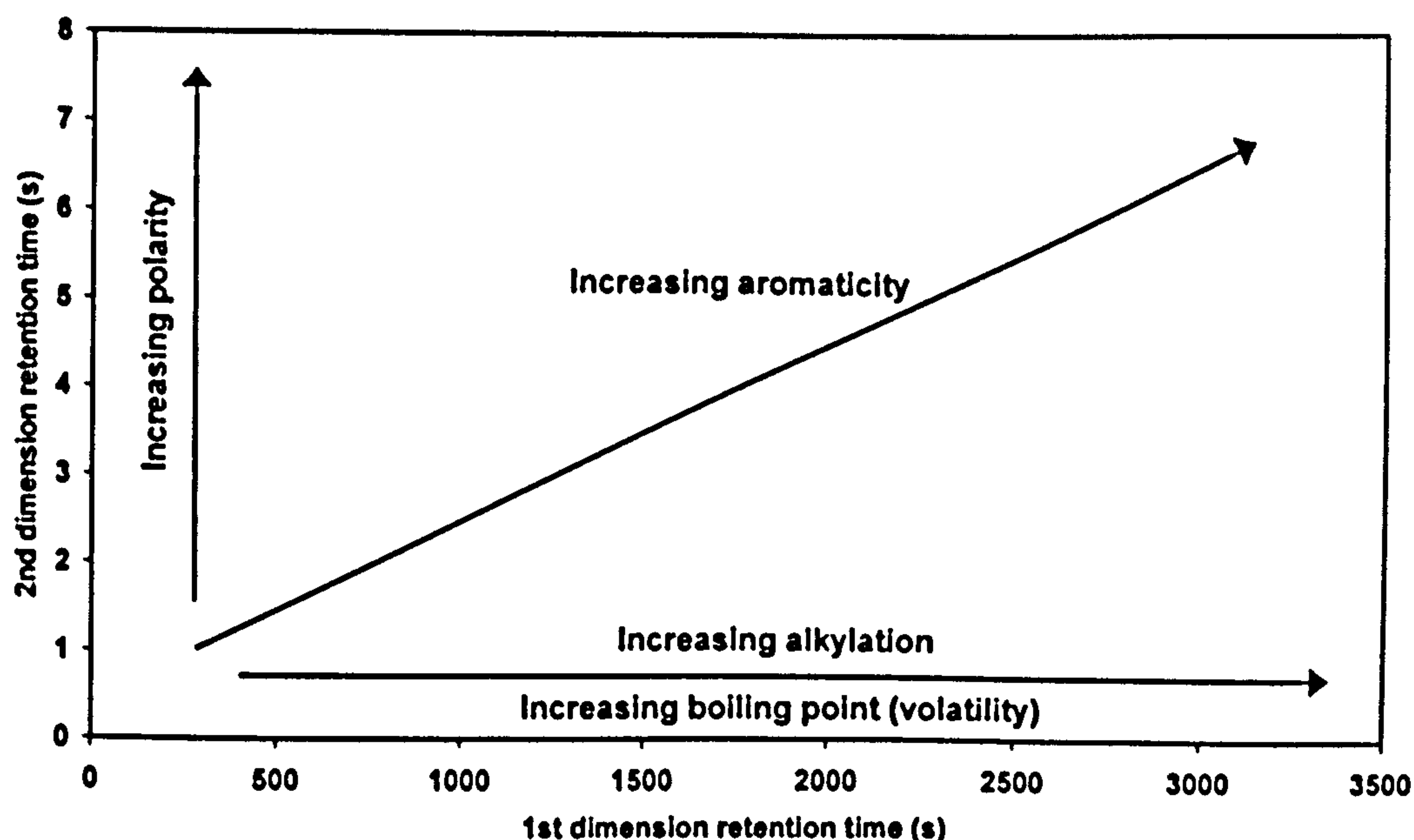


Figure 4.1. Diagram showing how a GCxGC system fitted with apolar (1st dimension) and polar (2nd dimension) columns separates hydrocarbons in a mixture based on their volatility and polarity.

The key to the separation of compounds is the use of the product resolving power of two stationary phases (Liu *et al.*, 1994). These must differ in their retention characteristics so that compounds not separated on the primary column are likely to be separated on the secondary. Typically, an apolar first-dimension and a polar second-dimension are employed (Figure 4.1). This gives a volatility-based separation, producing a boiling point elution order across the x-axis. A polarity-based separation results in class-type separation along the y-axis (*e.g.* Frysinger *et al.*, 2003). This column combination has resulted in the resolution of up to 10 peaks that coelute under normal one-dimensional conditions (Gaines *et al.*, 1999). Thus, the system has potential for the separation of complex mixtures of hydrocarbons which exhibit coelution on one-dimensional chromatography (*e.g.* UCMs).

Figure 4.2 shows a schematic of the comprehensive two-dimensional gas chromatograph using a thermal desorption modulator. In order to achieve high-speed chromatography the second column is much shorter than the first. However, to increase the resolution of the chromatography on the fast second dimension the column is generally narrower (Liu *et al.*, 1994; Shellie *et al.*, 2001; Adahchour *et al.*, 2003).

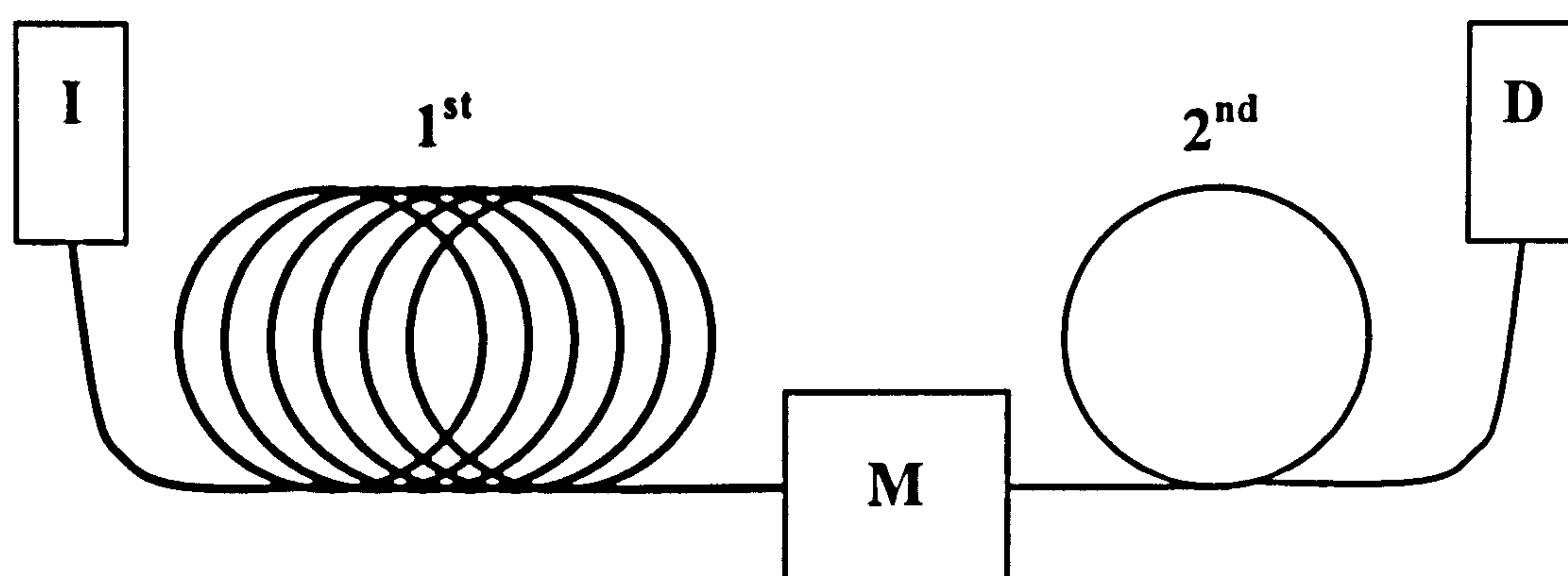


Figure 4.2. Schematic of a GCxGC system. I, injector; M, modulator; D, detector; 1st, first-dimension column; 2nd, second-dimension column.

The three important factors that make GCxGC possible are:

- (1) The modulator continuously ‘chopping up’ and accumulating the eluent from the first dimension, and effectively ‘injecting’ it in sharp pulses into the second dimension.
- (2) High-speed chromatography in the second dimension, allowing generation of a complete chromatogram in the time taken for a peak to elute from the first dimension.
- (3) Chromatography on two different stationary phases, allowing comprehensive two-dimensional separation of compounds about a plane.

The GCxGC technique produces a two-dimensional contour plot chromatogram (*e.g.* Figure 4.3). Each substance forms a peak on the plane defined by two retention times (one from each column). The computer generates a two-dimensional chromatogram by plotting each successive secondary chromatogram horizontally at the primary column retention time corresponding to the modulation pulse (*e.g.* Liu and Phillips, 1991; Gaines *et al.*,

1999; Xu *et al.*, 2001; Frysinger *et al.*, 2003). The two-dimensional nature allows substantially increased chromatographic peak capacity compared with a one-dimensional chromatogram generated on either column individually (Liu and Phillips, 1991; Liu *et al.*, 1994; Phillips and Beens, 1999; Gaines *et al.*, 1999; Dallüge, 2003). Under high-resolution conditions, Phillips and Xu (1995) have produced chromatograms of petroleum containing 4000 peaks.

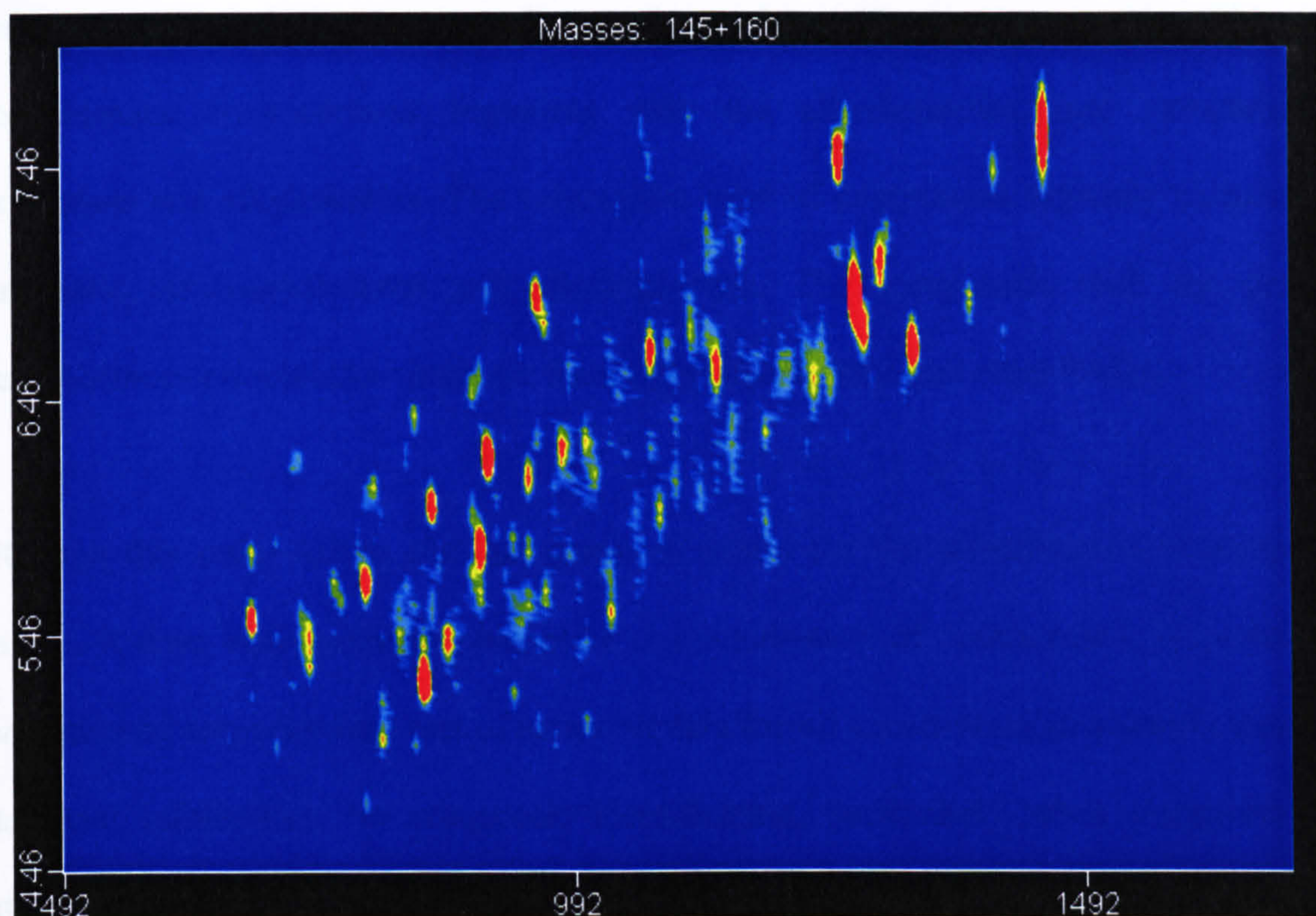


Figure 4.3. Portion of a two-dimensional (volatility vs polarity) contour plot for those compounds with fragment ions of m/z 145 and 160 (>90% abundance) present in the water soluble monoaromatic UCM sample analysed in the current study using GCxGC-ToF-MS. The retention times on both axes have been selected to provide the most detail for the central portion of the chromatogram. The background is blue. Low abundance peaks are white and high abundance peaks are red.

These two-dimensional chromatograms have also proven to be diagnostic in the identification of mixture components. Venkatramani and Phillips (1993) were possibly the first to recognise the ordered nature of hydrocarbons in GCxGC chromatograms. The authors observed that the aliphatics, monoaromatics and naphthalenes form distinct bands of peaks on the chromatographic plane resulting from their differing polarities. It is this

order to the chromatograms that aids in the identification of compounds (Phillips and Beens, 1999; Frysinger and Gaines, 1999; Gaines *et al.*, 1999; Dallüge, 2003).

The identification and ordering of chemical classes in petroleum products has subsequently been determined in more detail (*e.g.* Venkatramani and Phillips, 1993; Gaines *et al.*, 1999; Schoenmakers *et al.*, 2000; Dallüge, 2003; Frysinger *et al.*, 2003). The *n*-alkanes and branched alkanes are least polar, and have the shortest second-dimension retention times. These are followed by the cycloalkanes, beginning with the monocyclics. Then the monoaromatic hydrocarbons beginning with the alkylbenzenes, and including the alkylindans and alkyltetralins. The subsequent band of compounds typically includes the diaromatic alkyl-naphthalenes and alkylbiphenyls. The compounds with the greatest second-dimension retention times are the alkylphenanthrenes and other PAHs.

Although this order to the chromatograms is useful for the identification of compound classes, it does not enable the complete characterisation of components. However, the generation of two complete sets of retention data for all constituents in a sample provides additional information for compound identification, particularly when compared with authentic reference compounds (Liu *et al.*, 1994; Dallüge, 2003; Frysinger *et al.*, 2003). A retention time match in two dimensions should be considered reliable for peak identification because coelution in two-dimensions is less likely than in one-dimensional gas chromatography (Gaines *et al.*, 1999; Phillips and Beens, 1999; Reddy *et al.*, 2002; Frysinger *et al.*, 2003). Furthermore, coelution in two-dimensions generally occurs from the overlap of related isomers.

Further developments in the technique have allowed increased separation. For example, initial studies housed both chromatographic columns in the same GC oven. Because the second dimension separation is very fast (just a few seconds) the chromatography therefore

takes place under essentially isothermal conditions (Schoenmakers *et al.*, 2000). The most up to date instruments now commonly house the second column in a separate insulated secondary oven. This can be temperature programmed independently of the main GC oven controlling the primary column (Reddy *et al.*, 2002; Frysinger *et al.*, 2003; Marriott *et al.*, 2003). In some cases a third oven housing the modulator unit is also installed inside the main GC oven, where it can be controlled independently (*e.g.* Reddy *et al.*, 2002; Frysinger *et al.*, 2003).

4.1.1.1 Applications of GCxGC

Despite its recent development, GCxGC has already been used extensively, especially in the analysis of complex mixtures. As a result, GCxGC is increasingly used in the analysis of crude oils and other petroleum derived complex hydrocarbon mixtures (Blomberg *et al.*, 1997; Frysinger and Gaines, 1999). For example, Venkatramani and Phillips (1993) used GCxGC to analyse a kerosene sample isolated from crude oil. Phillips and Xu (1995) also used GCxGC to analyse kerosene samples. In both cases, the technique allowed a detailed separation of the component hydrocarbons of kerosene to be achieved. Frysinger *et al.* (1999) and Hamilton and Lewis (2003) have used GCxGC to study the composition of gasolines, providing useful data about the sample composition. This included the identification of alkylbenzenes, toluenes and xylenes and other aromatic compounds. Blomberg (2002) used GCxGC to study both heavy catalytically cracked cycle oil, and a light gasoil. Liu and Phillips (1991) used GCxGC to study the composition of a coal liquids sample. Gaines *et al.* (1999) have even used GCxGC to aid in the identification of oil spill sources. The technique was used to analyse a spill sample and two potential source samples, and allowed association of the spill to the correct source. Following this, Frysinger and Gaines (2002) used GCxGC in the forensic analysis of ignitable liquids in fire debris, with the aim of fingerprinting source materials.

GCxGC has also found other applications apart from petroleum analysis. Xu *et al.* (2001) and Johnson *et al.* (2003) used GCxGC to study the alkenone composition of Black Sea sediments. Analysis provided complete separation of coeluting isomers and other sample compounds. Dallüge (2003) used GCxGC to determine the pesticide content of vegetables, and also to study the composition of cigarette smoke. Hamilton and Lewis (2003) used GCxGC to study the organic content of aerosols collected from an urban area. Liu *et al.* (1994) achieved separation of pesticides present in human serum. Marriott *et al.* (2003) provide a comprehensive review of the application of GCxGC to the analysis of selected environmental toxins including dioxin, polychlorinated biphenyls and PAHs.

4.1.1.2 Limitations in the identification of compounds resolved using GCxGC

It is undeniable that GCxGC provides the opportunity for substantially greater separation and more information about sample composition than has previously been possible using conventional one-dimensional GC techniques. However, analysis is limited to group-type profiling using a fast flame ionisation detector (FID) to generate ordered chromatograms. Identification of individual components is generally only possible using authentic reference compounds (*e.g.* Gaines *et al.*, 1999; Reddy *et al.*, 2002; Frysinger *et al.*, 2003; Hamilton and Lewis *et al.*, 2003). Thus, for extremely complex mixtures such as petroleum, GCxGC only allows accurate identification of groups of compounds (*e.g.* benzenes, toluenes and xylenes, naphthalenes). Essentially, the technique provides a detailed chromatogram, but no method for the characterisation of components. Indeed, Shellie *et al.* (2001) suggest that the use of GCxGC can only truly be justified if separated peaks can be positively identified.

4.1.2 Gas chromatography x gas chromatography–time of flight–mass spectrometry (GCxGC-ToF-MS): A three-dimensional analytical approach

The need for mass spectrometric (MS) detection to identify the resolved peaks generated by GCxGC analysis has been proposed (Schoenmakers *et al.*, 2000; Frysinger *et al.*, 2003; Marriott *et al.*, 2003). The addition of an MS detector is considered to act as a third-dimension capable of generating the mass spectra for the separated analytes (*e.g.* Frysinger and Gaines, 1999). Mass spectrometry using a relatively slow-scanning quadrupole instrument has been reported for GCxGC (Frysinger and Gaines, 1999; Frysinger *et al.*, 2002). However, the analysis had to be slowed excessively (~7 hrs) to get just one reasonable scan across the peaks. Whilst the benefits of mass spectral identification of components were evident, conventional quadrupole mass spectrometers are too slow to act as detectors for the high-speed chromatograms eluting from the GCxGC second dimension (Shellie *et al.*, 2001; Hamilton and Lewis, 2003; Sinha *et al.*, 2003). A data acquisition rate of 50 Hz or more is required to properly record the very narrow peaks (60-6000 ms) eluting from the second column (Adahchour *et al.*, 2003).

Time-of-flight mass spectrometry (ToF-MS) offers a mass spectral detector with a high data acquisition rate (*e.g.* Phillips and Beens, 1999; Schoenmakers *et al.*, 2000; Shellie *et al.*, 2001). ToF-MS was developed over 50 years ago, and a detailed review of the technique is provided by Cotter (1997). However, its coupling to a GCxGC system has only recently been achieved (van Deursen *et al.*, 2000; Shellie *et al.*, 2001). ToF-MS is capable of recording spectra at a rate of $\geq 50 \text{ s}^{-1}$, which is equivalent to ~10 mass spectra per peak (Shellie *et al.*, 2001; Dalluge *et al.* 2002a; Sinha *et al.*, 2003). Additionally, ToF-MS uses the complete spectrum for identification, rather than the two to four qualifier ions used in conventional MS detection (Dalluge *et al.*, 2002a; Focant *et al.*, 2003). Thus, ToF-MS provides identical mass spectral patterns over a complete chromatographic peak (spectral continuity) for the same component (Focant *et al.*, 2003; Welthagen *et al.*, 2003).

This results in better peak purity and promotes the use of deconvolution algorithms (Hamilton and Lewis, 2003).

The ToF-MS data processing software is capable of performing mass spectral deconvolution of two coeluting compounds. The deconvolution algorithm allows the mass spectra to be mathematically separated to obtain 'clean' spectra (Dalluge *et al.*, 2002b; Focant *et al.*, 2003; Zrostlikova *et al.*, 2003). However, there are certain requirements for this to be achieved. First, the coeluting peaks must only be partially overlapping, with their apices separated by at least three scans (Adahchour *et al.*, 2003; Dalluge, 2003). Second, the mass spectra of each of the coeluting analytes must display characteristic m/z values (Dalluge, 2003). The deconvolution software used in the mass spectrometric acquisition can therefore be viewed as an additional tool to aid separation of mixture components (Adahchour *et al.*, 2003). This process can permit a 3- to 10-fold increase in the number of identifiable peaks compared with GCxGC-FID alone. Further detail regarding this process is presented by Dalluge *et al.* (2002a).

GCxGC-ToF-MS offers an analytical technique that exhibits superior separation and more accurate peak assignments than previously possible (Shellie *et al.*, 2001). The coupling of ToF-MS supplies mass spectral data for all compounds separated by GCxGC. These deconvoluted mass spectra can provide supplementary information in the preliminary classification of compounds into chemical classes (Welthagen *et al.*, 2003). Additionally, GCxGC-ToF-MS has much lower detection limits (5-50x) making it significantly more sensitive than one-dimensional GC or conventional MS techniques (*e.g.* Focant *et al.*, 2003; Sinha *et al.*, 2003; Zrostlikova *et al.*, 2003).

The GCxGC-ToF-MS technique has already seen use in the analysis of complex mixtures. Dalluge *et al.* (2002a) and Zrostlikova *et al.* (2003) monitored pesticides in vegetable and

fruit samples. Adahchour *et al.* (2003) analysed trace-level flavour compounds in food extracts, whilst Shellie *et al.* (2001) separated and identified previously coeluting components in lavender essential oil. GCxGC-ToF-MS has also been used to analyse the composition of cigarette smoke (Dalluge *et al.*, 2002b), and the organic material present in aerosols (Welthagen *et al.*, 2003). Sinha *et al.* (2003) and Focant *et al.* (2003) have used complex samples containing fuel components, pesticides, halogenated compounds and natural products to test the effectiveness of the GCxGC-ToF-MS technique.

4.1.3 GCxGC and GCxGC-ToF-MS analysis of UCMs

Despite its recent development, comprehensive GCxGC has already seen application in the resolution of many complex mixtures. This has included two studies of petroleum derived UCMs (Reddy *et al.*, 2002; Frysinger *et al.*, 2003). However, GCxGC-ToF-MS has yet to be employed in the analysis and characterisation of UCM hydrocarbons, although Welthagen *et al.* (2003) suggest it would be perfectly suited to this task.

Reddy *et al.* (2002) first used GCxGC to study the composition of a weathered crude oil UCM. The UCM was analysed without any prior fractionation into hydrocarbon classes, and so represents a whole oil analysis. The study indicated the presence of aliphatic compounds such as one and two ring cycloalkanes, monoaromatic hydrocarbons including alkylbenzenes, alkylindans and alkyltetralins, and the diaromatic alkyl-naphthalenes and alkylbiphenyls. Larger alkylated PAHs were also observed including fluorenes and phenanthrenes. Thus GCxGC separation was able to resolve hundreds of compounds, providing information on the group type composition of the UCM.

Following this preliminary study, research focused specifically on using GCxGC to fully resolve a UCM present in petroleum contaminated sediment (Frysinger *et al.*, 2003). Prior

to analysis the UCM was fractionated into saturates, monoaromatics, diaromatics and triaromatics using silica gel column chromatography. The use of authentic standards allowed identification of a few UCM components and inference of others. The ordered nature of the chromatogram also aided in class type identification of compounds.

In the triaromatic fraction, Frysinger *et al.* (2003) identified specific methyl and dimethyl phenanthrene isomers based on the standards, and inferred the occurrence of other C₁- and C₂-phenanthrene isomers. In the diaromatic fraction, identification of specific alkylnaphthalenes allowed bands of alkylnaphthalene isomers with C₃-C₆ substituents to be proposed. The diaromatic fraction also contained a large number of unidentified peaks, tentatively attributed as alkylbiphenyls and alkylbenzothiophenes. In the monoaromatic fraction, C₈-C₁₄ substituted alkylbenzenes were identified and the presence of alkylindans and alkyltetralins inferred. A very large number of peaks were evident in the alkylbenzene region, indicating the long alkyl chains give rise to many different isomers. In many cases these peaks appeared to be coeluting or partially co-eluting with each other.

Whilst polar columns generally provided good separation of compound classes, Frysinger *et al.* (2003) observed certain compound classes were coeluting due to similar polarities (*e.g.* naphthalenes and biphenyls). The chromatograms also contain coeluting compounds along the first dimension, probably due to large numbers of isomers exhibiting very similar volatility characteristics. Thus, it appears that fractionation of the UCM prior to analysis still resulted in mixtures too complex for complete resolution to be achieved. The use of authentic standards only permitted accurate identification of a few components, but was effective in mapping the location of compound classes. Whilst the GCxGC technique has provided the best separation of UCM hydrocarbons to date, full characterisation of components was impossible. Frysinger *et al.* (2003) suggest complete characterisation of resolved UCM components can only be achieved with mass spectral analysis.

4.1.4 Aims of the present study

Rowland *et al.* (2001) and Smith (2002) have previously shown that the monoaromatic hydrocarbons of a crude oil UCM appeared the most toxic to the blue mussel (*Mytilus edulis*). It is these monoaromatic UCM hydrocarbons that hold the greatest interest from an environmental perspective. Water soluble UCM hydrocarbons are considered to be those which are bioavailable to aquatic organisms, and therefore responsible for the observed toxicological impacts reported by Rowland *et al.* (2001). A study of the water solubility characteristics of the monoaromatic UCM isolated from Tia Juana Pesado (TJP) crude oil is reported in Chapter 3. Using a 'generator column' technique, water saturated with the water soluble components of the monoaromatic UCM was produced (Section 5.9).

The present investigation employed GCxGC-ToF-MS analysis to study the composition of the water soluble fraction of the monoaromatic UCM (Section 5.10). The study had three main aims. First, that GCxGC would provide a quick method for resolving the sample components. Second, that ToF-MS would provide clear mass spectra of each resolved component, aiding identification. Third, that identification of some unknown compounds could be achieved by comparison of mass spectra to those in a library (NIST library). It was expected that this would lead to an indication of those compounds responsible for the observed toxicity of the TJP monoaromatic UCM.

4.2 Results

The two-dimensional chromatogram generated by GCxGC-ToF-MS analysis of the water soluble monoaromatic UCM sample (Section 5.9) is presented in Figures 4.4 and 4.5. Individual peaks are spread about the chromatographic plane depending upon the component volatility and polarity, and abundance is represented by the peak height. A one-dimensional chromatogram of the first dimension (apolar column) is also shown in the background for comparison. The column bleed peaks, which elute below the monoaromatic hydrocarbons, have been removed from the chromatogram for clarity, however, the saturated impurities remain. Clearly, the monoaromatic UCM hydrocarbons are better resolved using the two-dimensional chromatography rather than conventional one-dimensional GC.

Figures 4.6a and 4.6b focus on the area of the GCxGC-ToF-MS chromatogram corresponding to the UCM hydrocarbons. The one-dimensional chromatogram is shown in the background, confirming the unresolved nature of the sample when analysed by one-dimensional gas chromatography. In all chromatograms the background is blue, with low intensity peaks coloured white and high intensity peaks coloured red. The chromatograms indicate that large numbers of individual hydrocarbons contribute to the UCM. From their close proximity to one another it can be inferred that these compounds have very similar volatility and polarity properties. There is also a range in compound abundance, with a few components clearly present in higher concentration (red peaks) in the sample than others (white peaks).

For comparison the water soluble monoaromatic UCM sample was also analysed by conventional GC-MS. The 1-D chromatogram of the sample is shown in Figure 4.7. This shows the unresolvable nature of the complex mixture of hydrocarbons present in the sample when analysed by this technique.

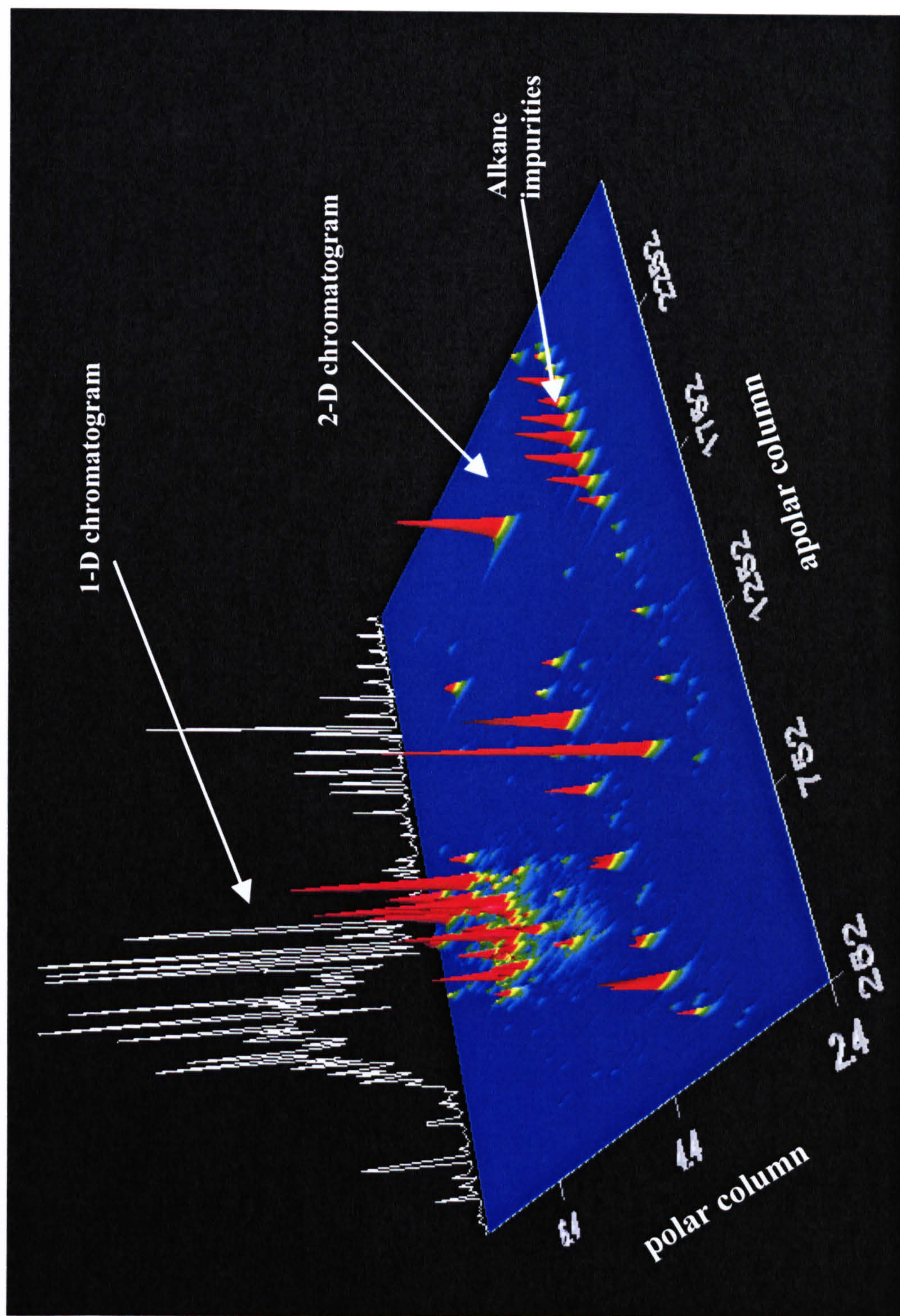


Figure 4.4. GCxGC-ToF-MS 2-D chromatogram of the water soluble monoaromatic UCM. The 1-D chromatogram of the first dimension (apolar) can be seen at the back of the 2-D chromatogram. The background is blue, with low intensity peaks coloured white and high intensity peaks coloured red. The peaks corresponding to the column bleed compounds have been removed for clarity.

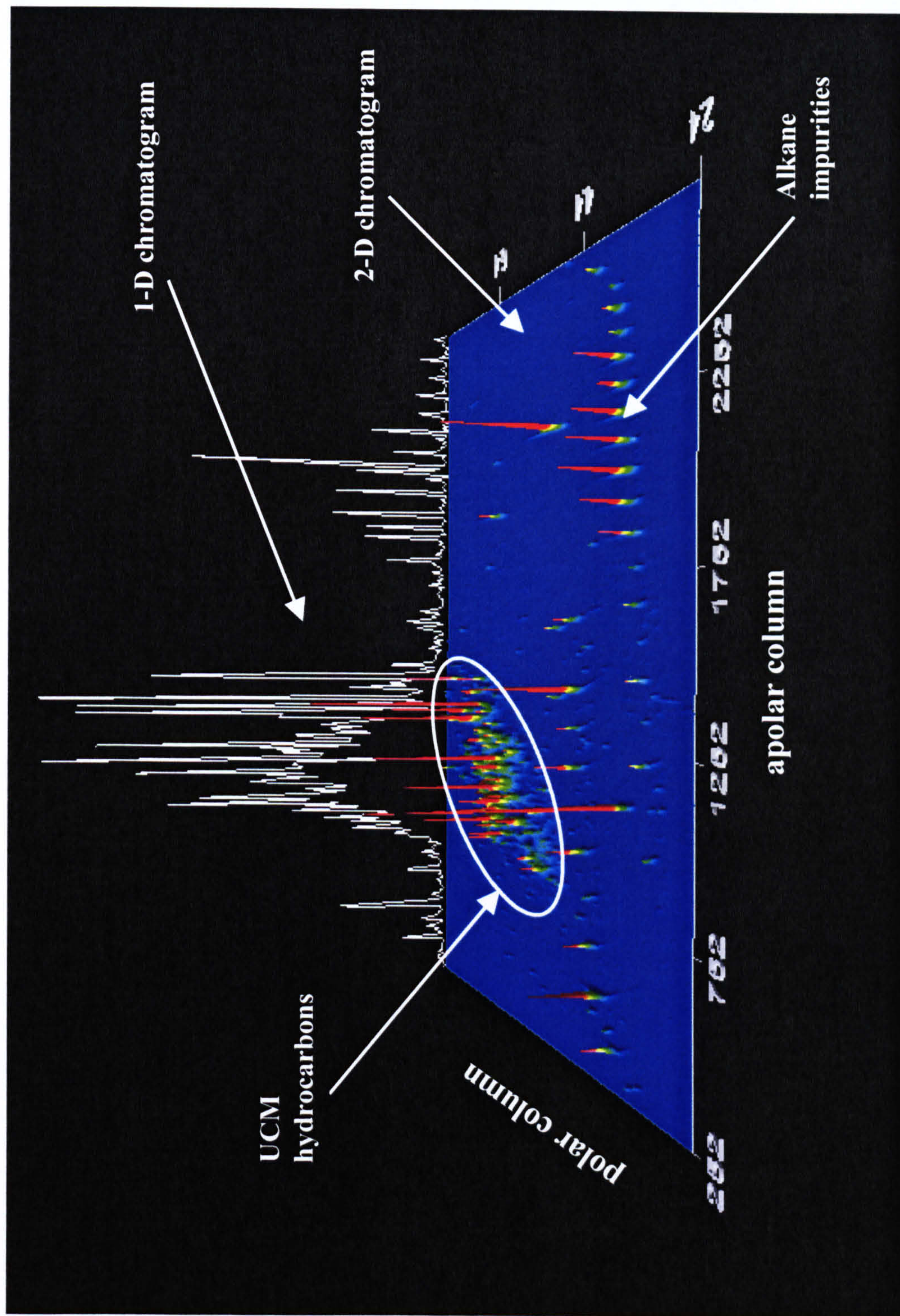
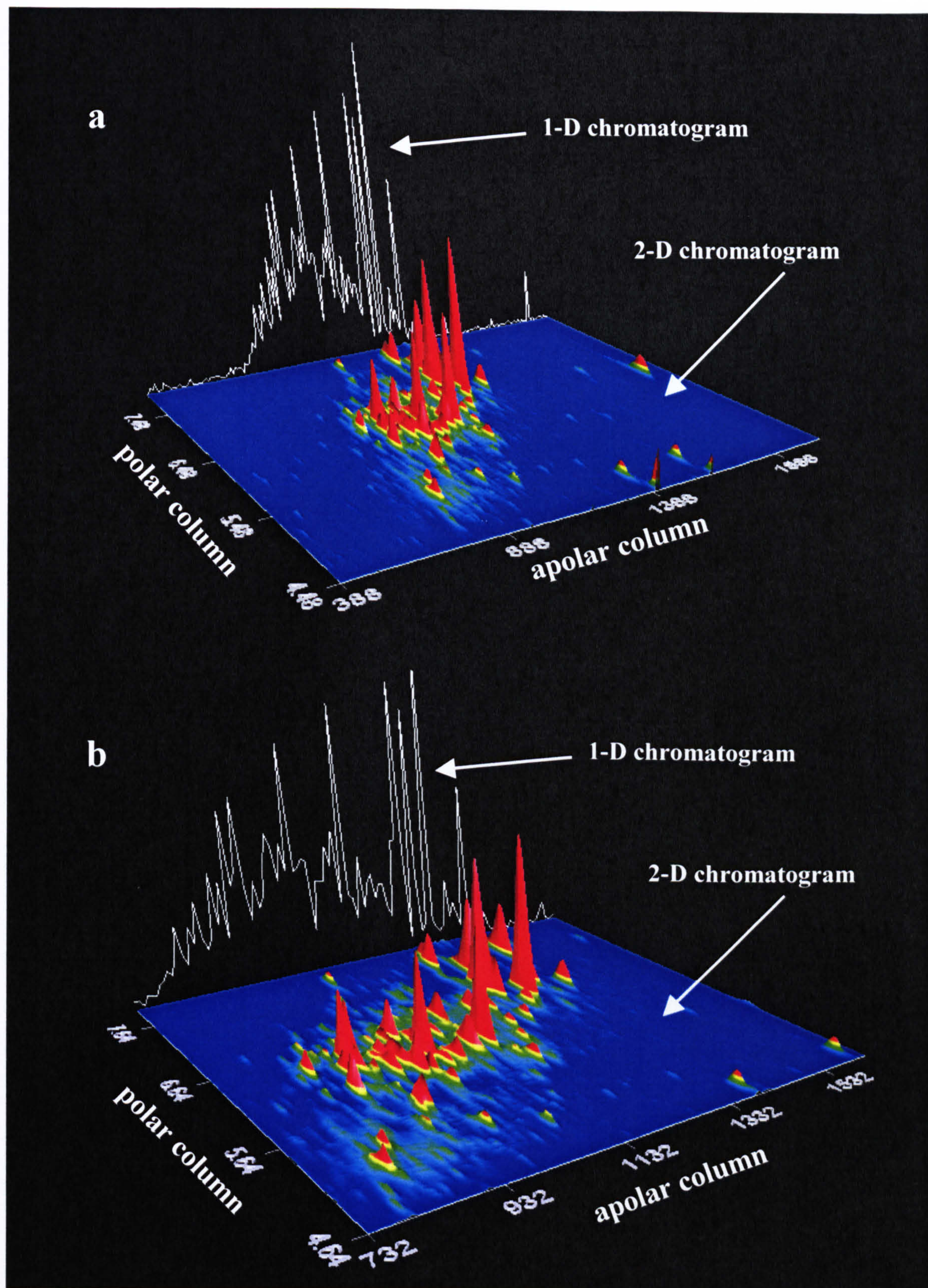


Figure 4.5. GCxGC-ToF-MS 2-D chromatogram of the water soluble monoaromatic UCM. The 1-D chromatogram of the first dimension (apolar) can be seen at the back of the 2-D chromatogram. This allows identification of the compounds responsible for the UCM observed in the 1-D chromatogram. The peaks corresponding to the column bleed compounds have been removed for clarity.



Figures 4.6a and 4.6b. GCxGC-ToF-MS 2-D chromatograms of the water soluble monoaromatic UCM. Figure 4.6a focuses in on the compounds responsible for the UCM, which can be seen in 1-D at the back of the chromatogram. Figure 4.6b shows the water soluble monoaromatic UCM in more detail, with the 1-D chromatogram shown in the background.

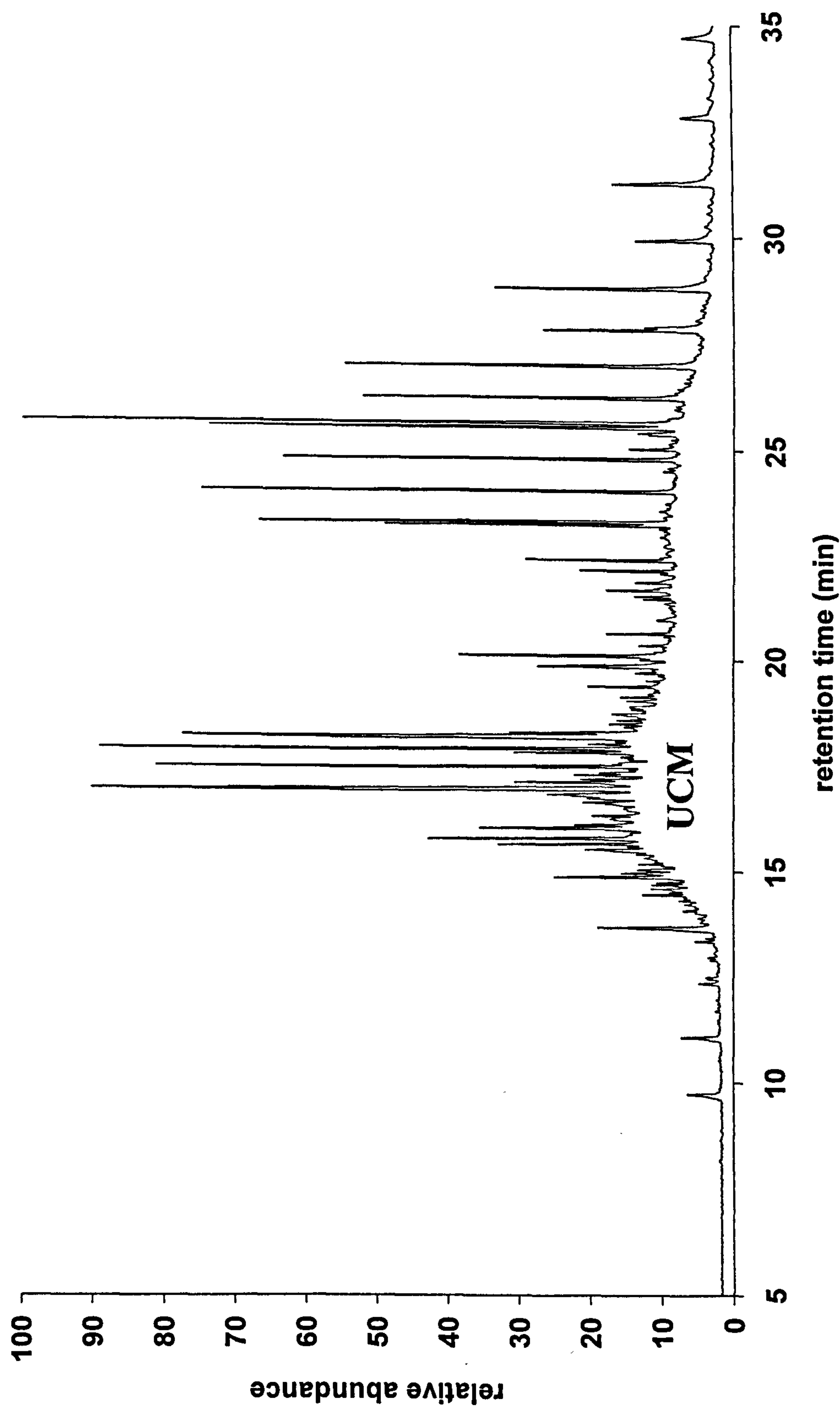


Figure 4.7. One-dimensional GC-MS chromatogram of the water soluble monoaromatic UCM. Analysis was performed on a Hewlett Packard 5890 gas chromatograph fitted with a HP5970 mass selective detector.

The ChromaToF™ v.2.01 data processing software (Leco Corporation, USA) is able to deconvolute regions within the chromatogram that have unique mass spectra (Section 5.10). These regions are then assigned an individual ‘peak marker’ denoting the compound causing the observed mass spectrum. The software can be adjusted to plot up to 5000 individual peaks, based on those components which are most abundant. In the present study the instrument was adjusted to plot the 2500 most intense peaks. A plot of the ‘peak markers’ assigned for the water soluble monoaromatic UCM hydrocarbons is presented in Figure 4.8. From the current sample, the instrument was able to determine 1860 regions with unique mass spectra above the limits of analytical detection. Of the 1860 peaks identified, a proportion corresponded to the stationary phase material ‘bleeding’ from the column. Other peak markers were identified as a series of alkanes; impurities introduced when the soluble monoaromatic UCM was generated. Those peaks corresponding to the column bleed material and the saturated impurities are highlighted in Figure 4.8.

The mass spectra of the column bleed and saturated compounds are typically characterised by diagnostic base ions, which can be used for their identification. These base ions have been summarised in Table C.1. (Appendix C). Peak markers corresponding to the column bleed (538 peaks; Figure C.1, Appendix C) and the saturate impurities (70 peaks; Figure C.2, Appendix C) were identified and removed. These are presented in appendix C as individual plots. Some of the diagnostic base ions for the column bleed compounds are also characteristic of other hydrocarbons. Care was taken to ensure only those compounds eluting in the column bleed region were removed and that any peaks which were possible UCM components were retained. Removal of the column bleed and saturated impurities left 1252 peaks considered as water soluble monoaromatic UCM components.

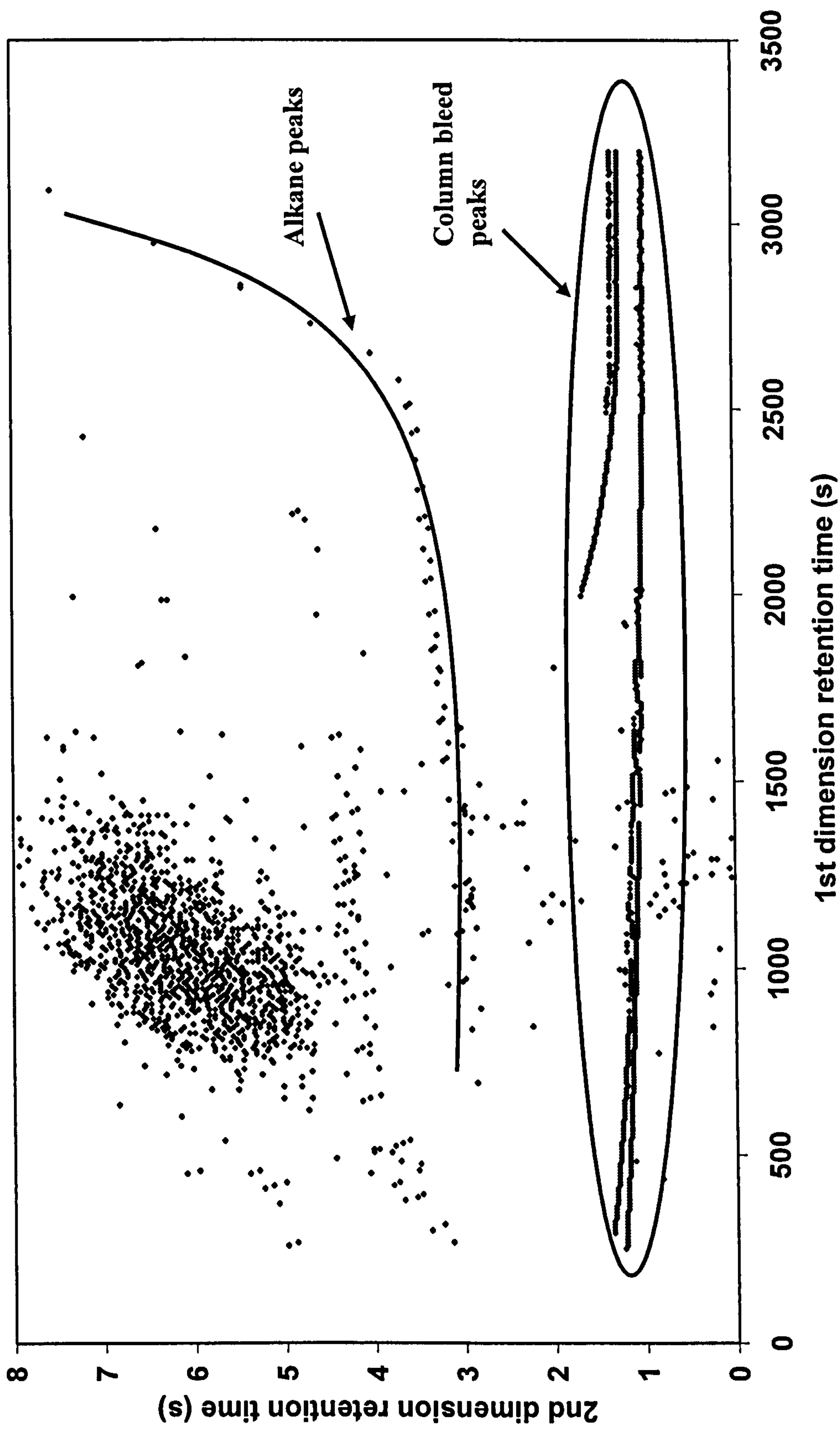


Figure 4.8. Plot of the 1860 peak markers assigned by the software to the water soluble monoaromatic UCM. Each mark represents a separate deconvoluted mass spectrum. Those peak markers corresponding to the column bleed peaks and the saturate hydrocarbons are indicated.

The mass spectra associated with these 1252 peak markers were compiled as a unique NIST library. The MS Search function in v.2.0. of the NIST software allowed the data to be viewed as single library, permitting analysis of the individual mass spectra. Some mass spectra exhibited a poor degree of clarity, providing inadequate diagnostic data for compound identification. However, a good proportion of the spectra appeared sufficiently 'clean' for potential identification of the compound to be achieved. The 'library editor' facility of the NIST software permits individual mass spectra to be added or removed from a library. Thus, it was possible to edit the 1252 component UCM library, removing unclear/poor spectra. Only those spectra that appeared most likely to enable compound identification were retained in the final UCM library (total 490). A peak marker plot of these remaining 490 compounds is presented in Figure 4.9. All compounds elute before 1800 seconds on the first dimension, thus, the x-axis has been reduced for enhanced clarity.

A number of compounds appear to have very early retention times on the second (polar) dimension, but elute late on the first dimension. These have been highlighted on Figure 4.9. Such compounds are thought to have second-dimension retention times longer than the 8-second modulation time and actually elute very early on the next chromatogram. This 'wrapping over' onto the next elution cycle has been observed previously (*e.g.* Phillips and Xu, 1995; Shellie *et al.*, 2001) and is discussed in more detail in Section 4.4.3.

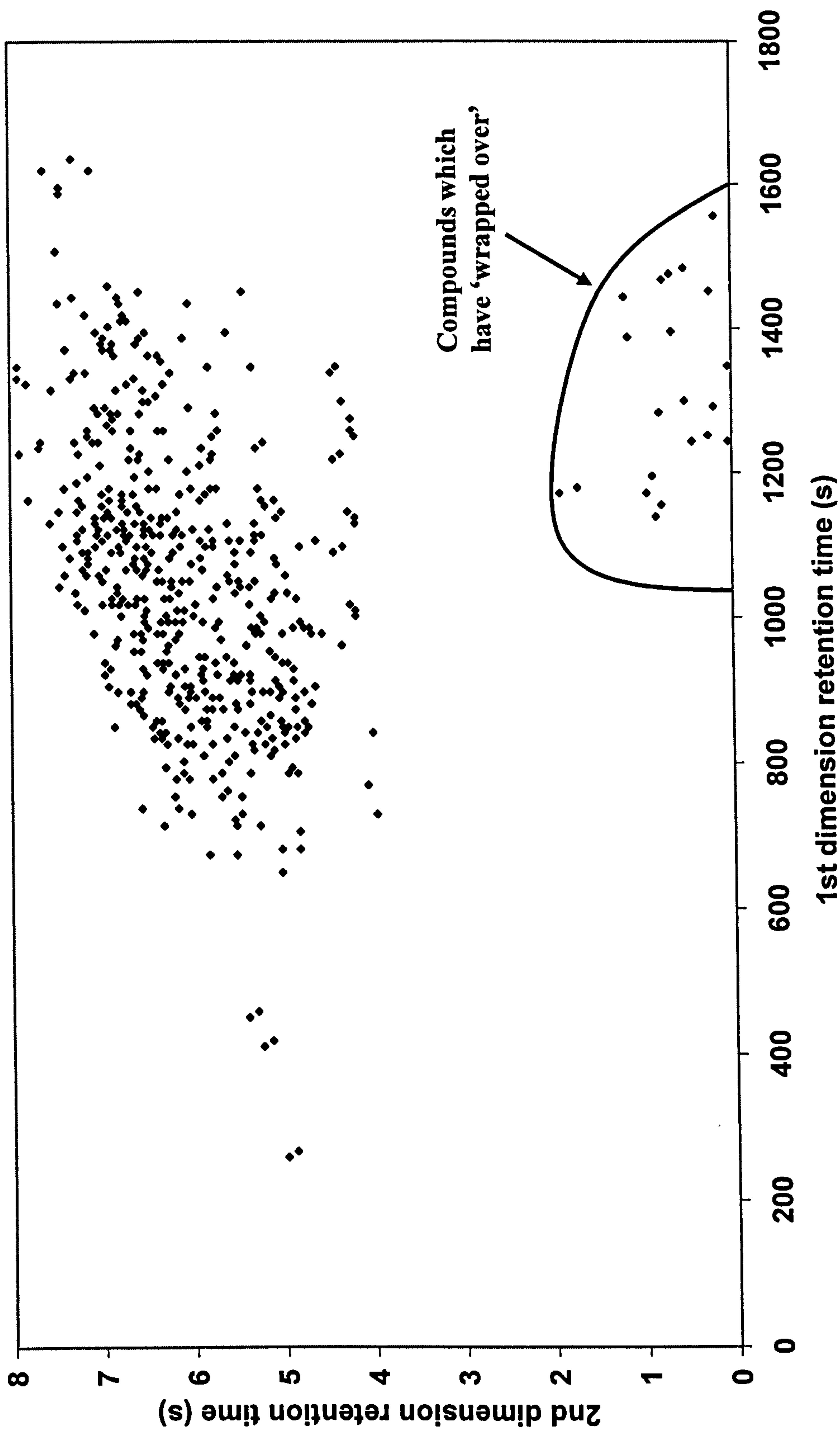


Figure 4.9. Plot of the 490 peak markers that have mass spectra suitable for possible identification of the parent hydrocarbon. The retention time of these peak markers are all before 1800 s on the first dimension, thus the scale has been reduced to enhance the resolution of the plot. Compounds that are thought to 'wrap over' due to retention times longer than 8.0 seconds on the 2nd dimension are indicated.

The mass spectrum for each of the UCM components contains a characteristic base ion. This is the most common ion fragment produced during mass spectrometry of a particular compound. As groups of related compounds typically have the same base ion they can be particularly diagnostic in compound identification. The number of mass spectra exhibiting each particular base ion has been calculated, and Figure 4.10 shows their distribution within the 490 unknown compounds. Each column represents at least one compound class, indicating the UCM contains a broad range of hydrocarbon structures. However, the distribution is most concentrated between those compounds with base ions of m/z 157 and m/z 210, indicating these groups of compounds are the most abundant in the UCM.

Table 4.1 lists the 15 most abundant base ions, and the number of compounds exhibiting each base ion. This is also presented as a percentage of the 490 'clean' mass spectra. The most abundant base ion is 169, accounting for nearly 7% of all of the compounds present. Those compounds with base ions of 169, 183, 195, 163, and 209 make up the five most abundant hydrocarbons in the UCM, accounting for 28.3% of the 490 compounds.

Figure 4.11 summarises the molecular weight ranges of the ten most abundant base ions as determined from their mass spectra. Those compounds with a base ion of 163 exhibit the largest diversity of molecular weights, ranging from 178 to 290 mass units. Compounds exhibiting base ions of 209 and 161 mass units had the most limited molecular weight ranges. The molecular weight range across all ten base ions was determined, with the lowest being 172 and the largest 290 mass units.

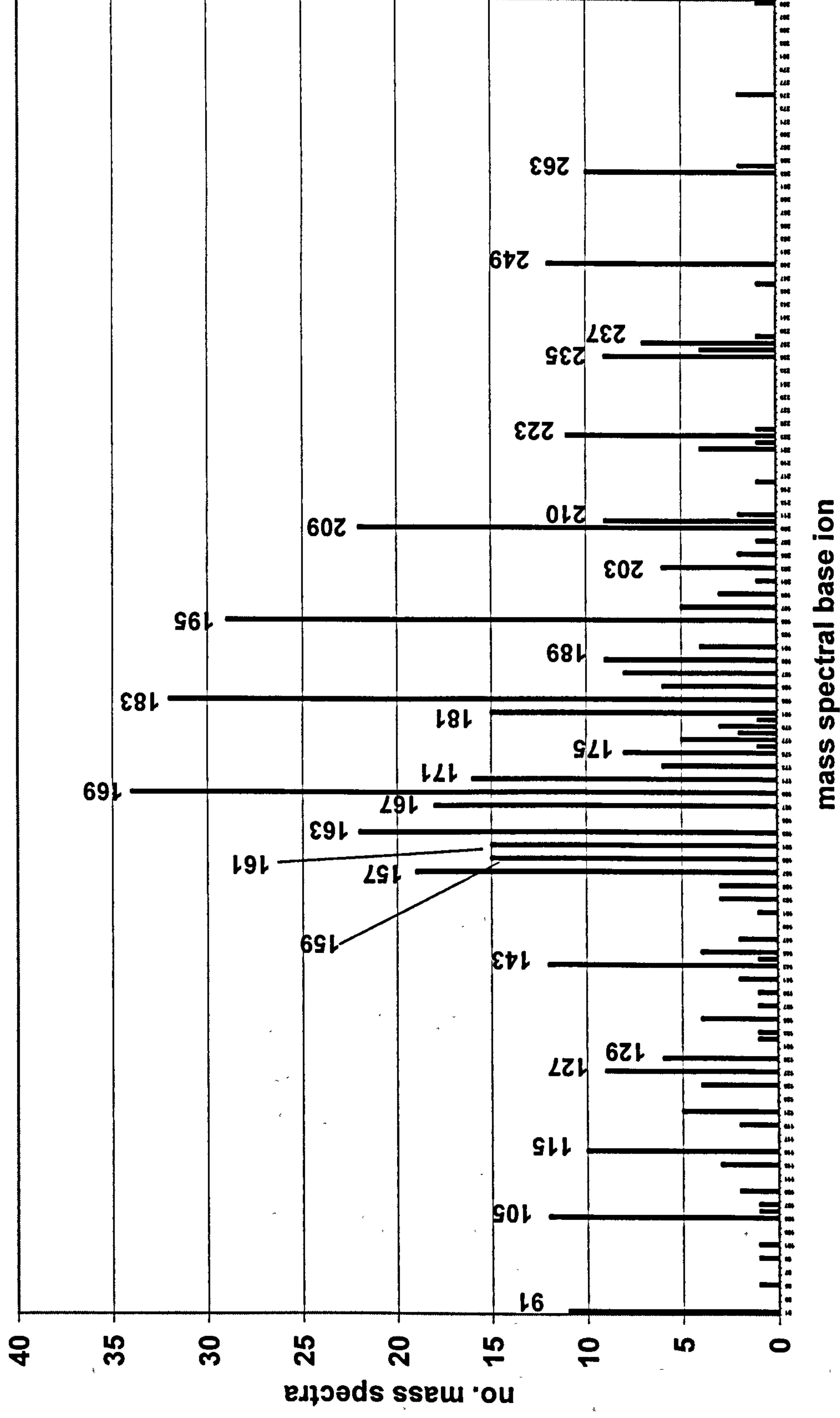


Figure 4.10. Distribution of hydrocarbon base ions (m/z) within the 490 mass spectra selected from the water soluble monoaromatic UCM. The most abundant base ions are labelled individually.

Table 4.1. Summary of the 15 most abundant base ions in the mass spectra generated by GCxGC-ToF-MS analysis of the water soluble monoaromatic UCM.

Base ion (m/z)	No. of compounds with base ion	Percentage of total (490) compounds
169	34	6.9
183	32	6.5
195	29	5.9
163	22	4.5
209	22	4.5
157	19	3.9
167	18	3.7
171	16	3.3
159	15	3.1
161	15	3.1
181	15	3.1
105	12	2.4
143	12	2.4
249	12	2.4
91	11	2.2

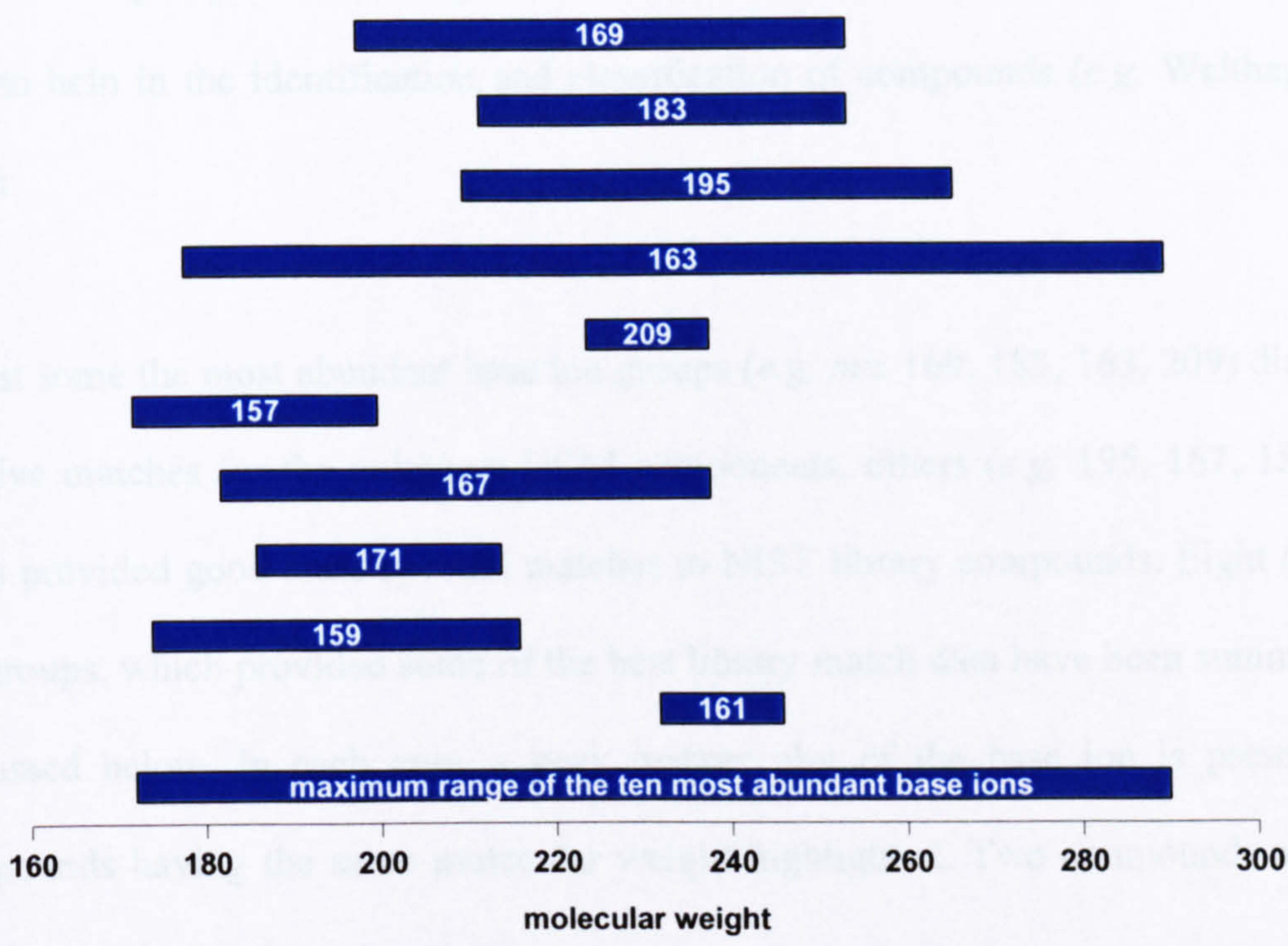


Figure 4.11. Summary of the molecular weight ranges for the ten most abundant base ions. The molecular weight range of the compounds is shown by the blue bars, with the base ion characteristic to those compounds listed on the individual bars. A maximum range for the 10 base ions is also included.

4.3 Characterisation of water soluble monoaromatic UCM hydrocarbons: Results and Discussion

The 490 mass spectra in the 'UCM library' were divided into sub-libraries, each containing those compounds sharing the same base ion (m/z). Comparison of these mass spectra with those listed in the NIST 'main library' aimed to bring about the positive identification of some UCM hydrocarbons. For each individual mass spectrum in the 'base ion libraries', the NIST software was able to search the 'main library' for the 100 best matches. From these, the most accurately matching spectra could be selected. In many cases, sufficiently accurate mass spectral matches were not observed, and for some of the base ions, the NIST software suggested no suitable compounds at all (*e.g.* mono- or diaromatic hydrocarbons). However, some good matches between unknown compounds and NIST library spectra were found. In fact, sufficient matches were found to provide two compound identifications for some base ion groups, allowing inference of other homologues within the base ion group. A knowledge of the fragmentation patterns in spectral data was also used to help in the identification and classification of compounds (*e.g.* Welthagen *et al.*, 2003).

Whilst some the most abundant base ion groups (*e.g.* m/z 169, 183, 163, 209) did not yield positive matches for the unknown UCM components, others (*e.g.* 195, 167, 159, 181) at times provided good mass spectral matches to NIST library compounds. Eight of the base ion groups, which provided some of the best library match data have been summarised and discussed below. In each case, a peak marker plot of the base ion is presented, with compounds having the same molecular weight highlighted. Two compounds are selected for characterisation in each case.

Frequently, more than one NIST library spectrum provided a good match for an unknown. In these cases, knowledge of the sample origin helped to distinguish between candidate compounds. The oil was fractionated into saturates, aromatics and polars using column chromatography, and the aromatic fraction used for further study. Therefore, those matches to compounds which are saturates or that contain hetero atoms (*e.g.* oxygen and nitrogen) could be ruled out. The constraints on the NIST library search could be set to look only for compounds containing carbon and hydrogen (hydrocarbons).

4.3.1 Compounds with a base ion of m/z 91

A peak marker plot of the compounds exhibiting a m/z 91 base ion is presented in Figure 4.12. Figure 4.13a shows the mass spectrum of A (retention time 1020,4.24), whilst Figure 4.13b shows the mass spectrum of 1-propyloctyl-benzene. This is proposed as the best match from the NIST library for A. Figure 4.14a shows the mass spectrum of B (retention time 1276,4.22), with Figure 4.14b showing the mass spectrum of 1-propyldecyl-benzene. This is suggested as the best match from the NIST library for B.

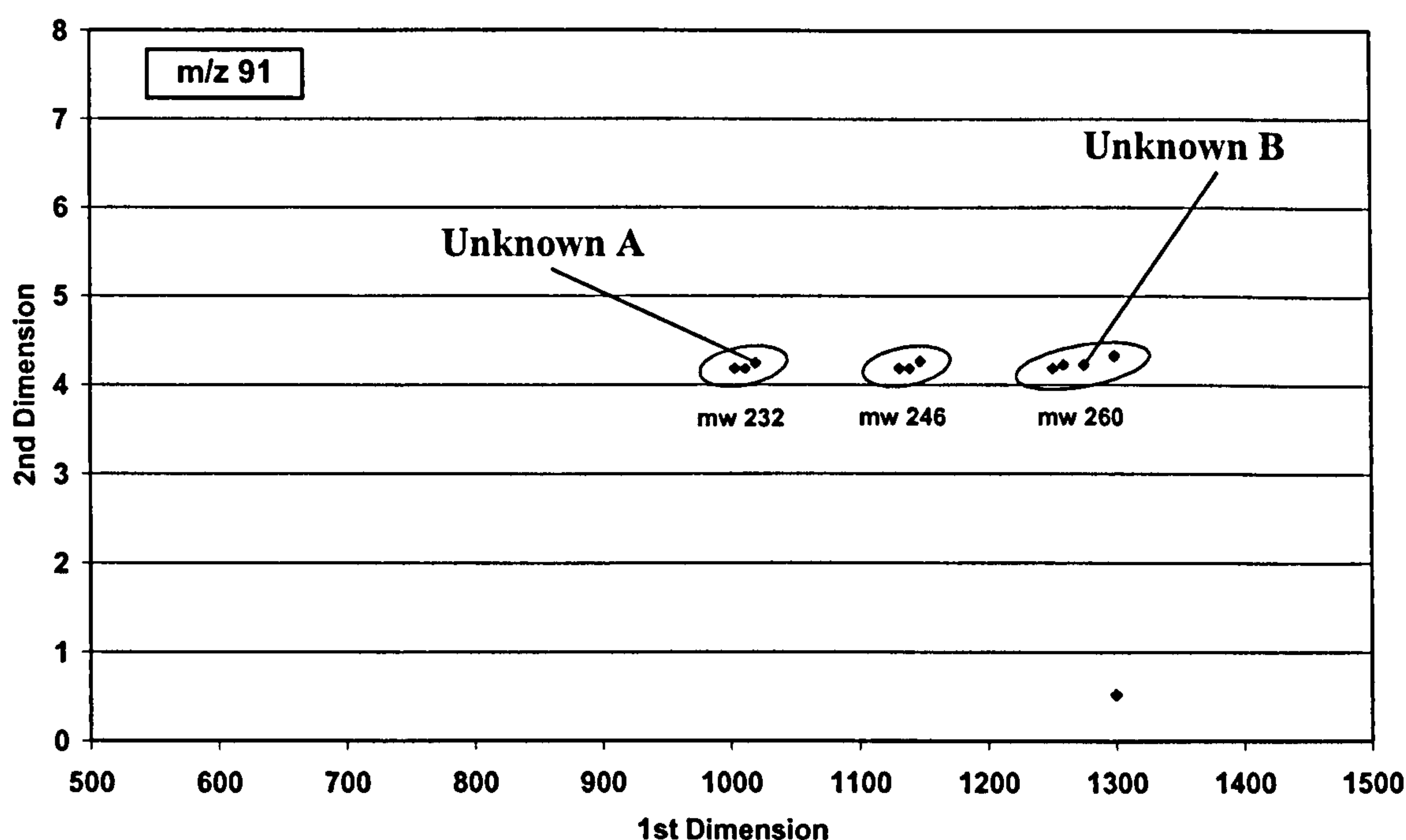


Figure 4.12. Peak marker plot of the compounds present in the water soluble monoaromatic UCM exhibiting a base ion of m/z 91. Those compounds displaying the same molecular weight are grouped together.

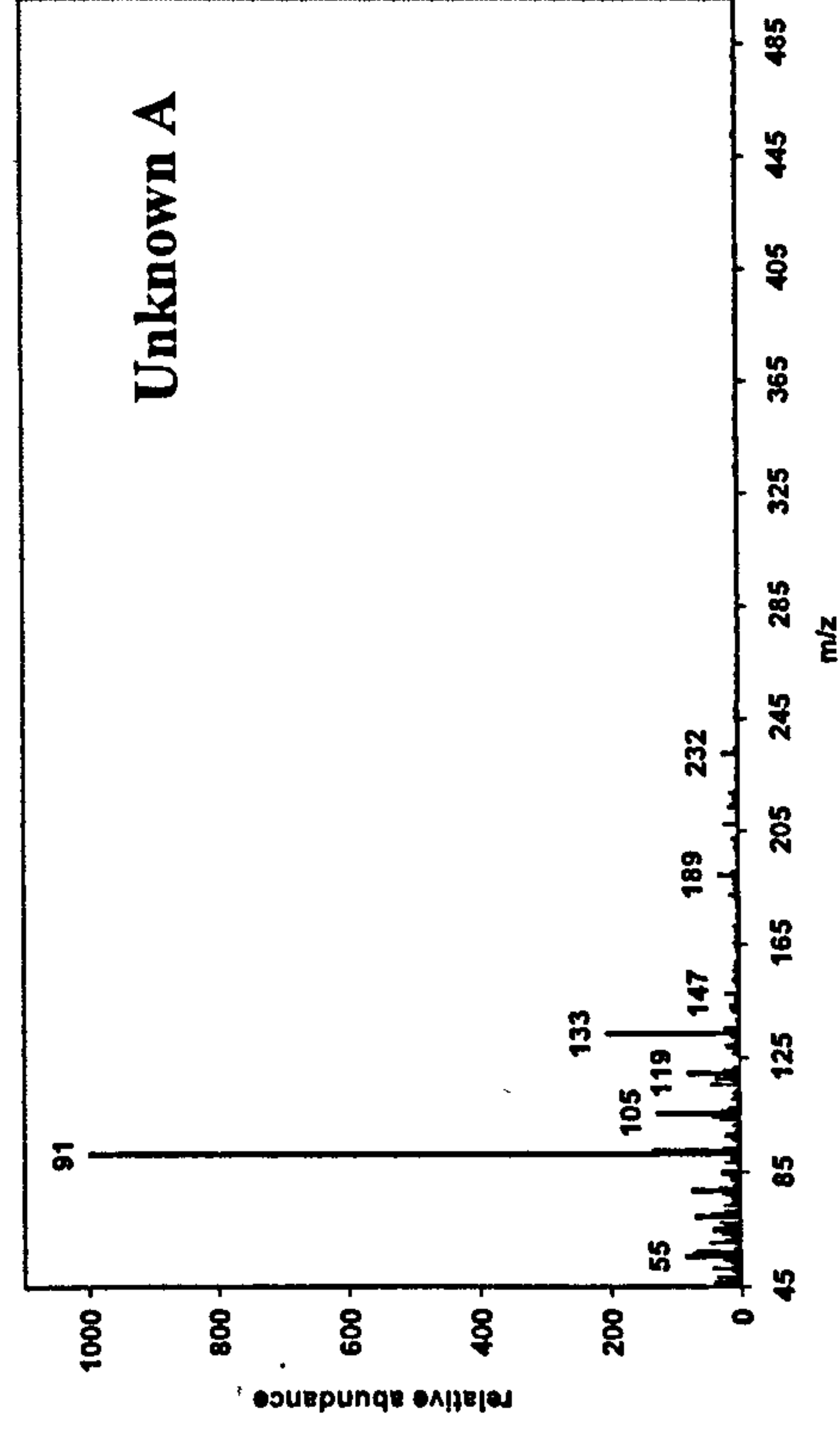


Figure 4.13a. Mass spectrum of the unknown compound A, which has a retention time of 1020,4.24.

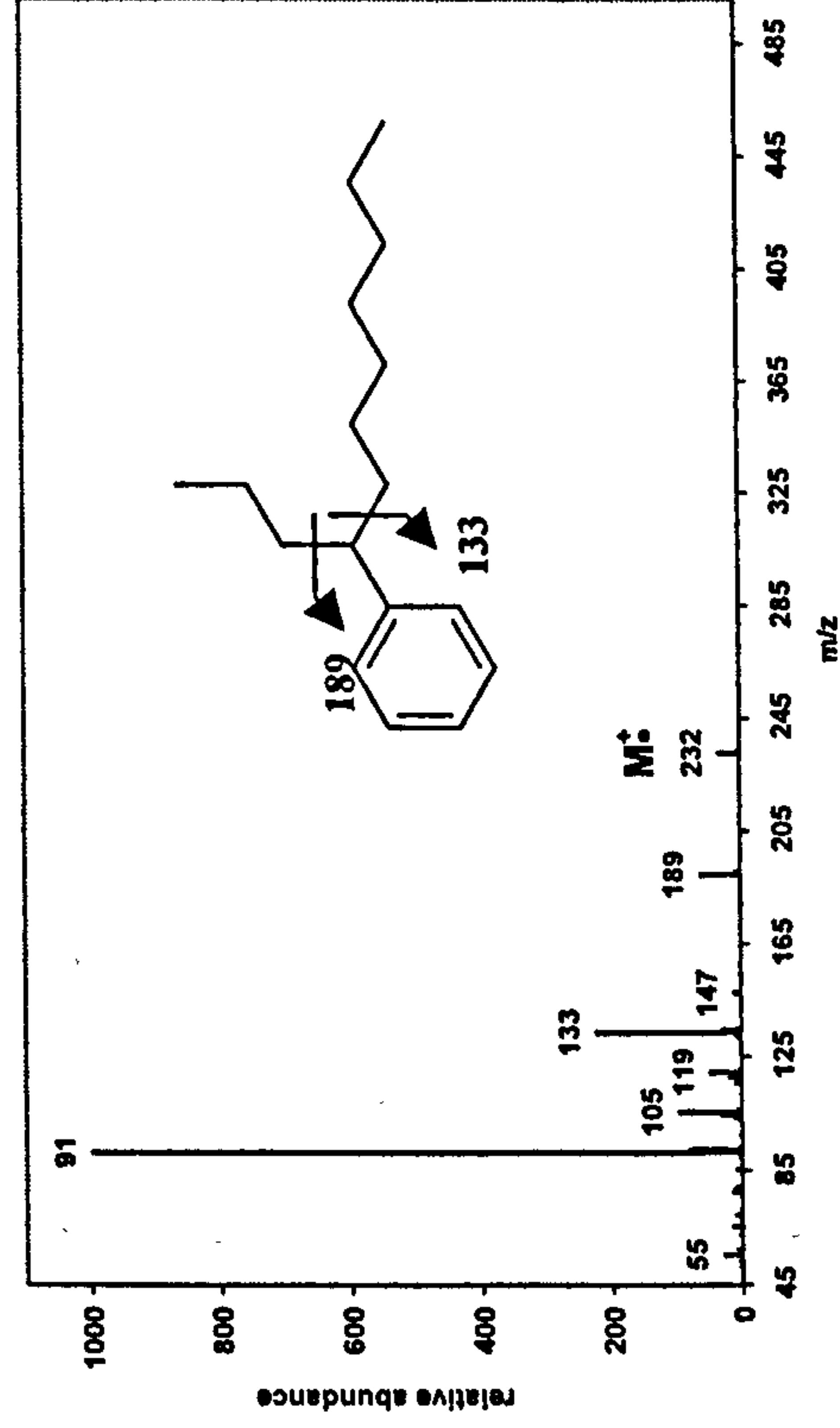


Figure 4.13b. Mass spectrum of the candidate NIST library compound, 1-propyloctyl-benzene.

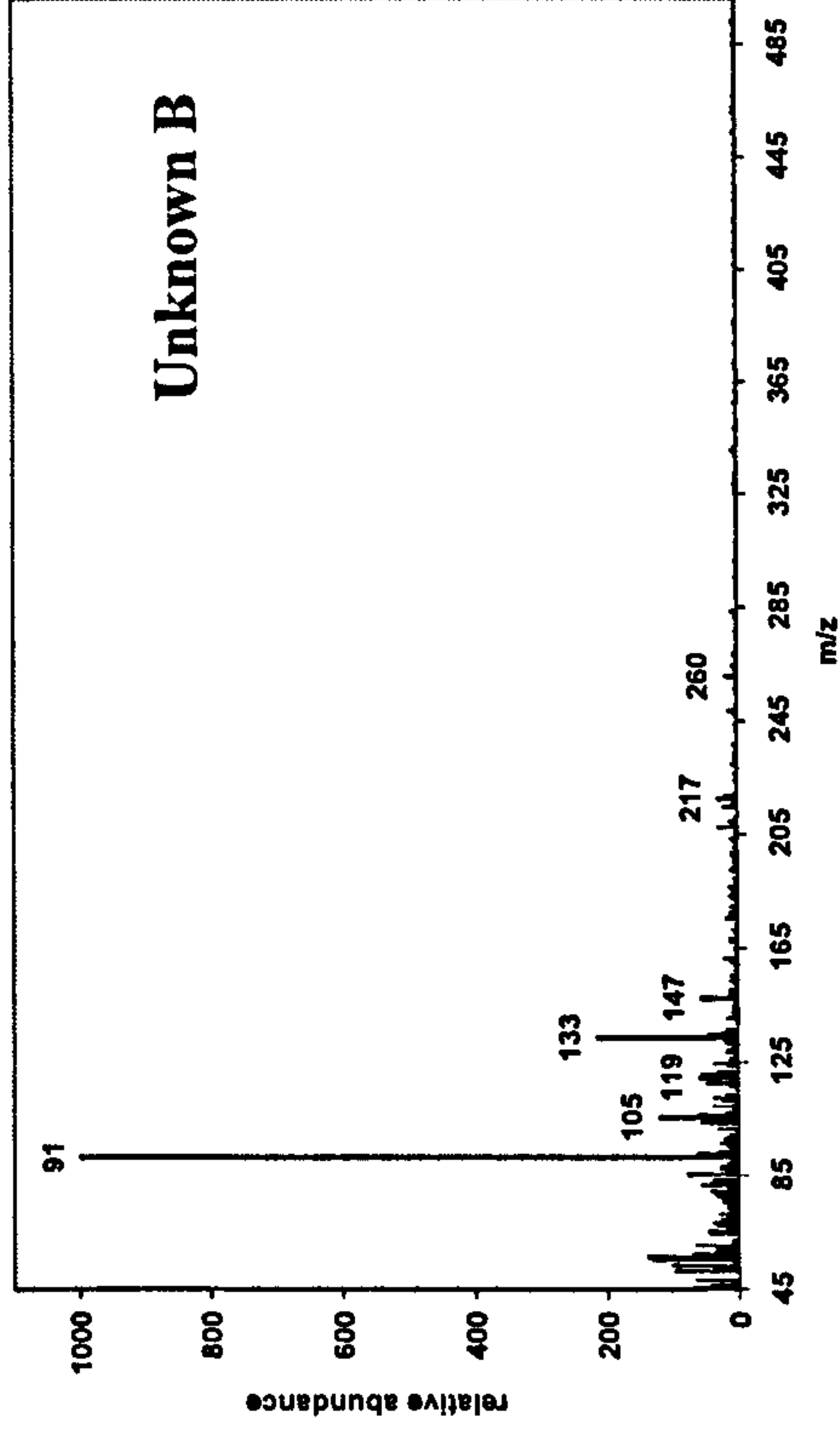


Figure 4.14a. Mass spectrum of the unknown compound B, which has a retention time of 1276,4.22.

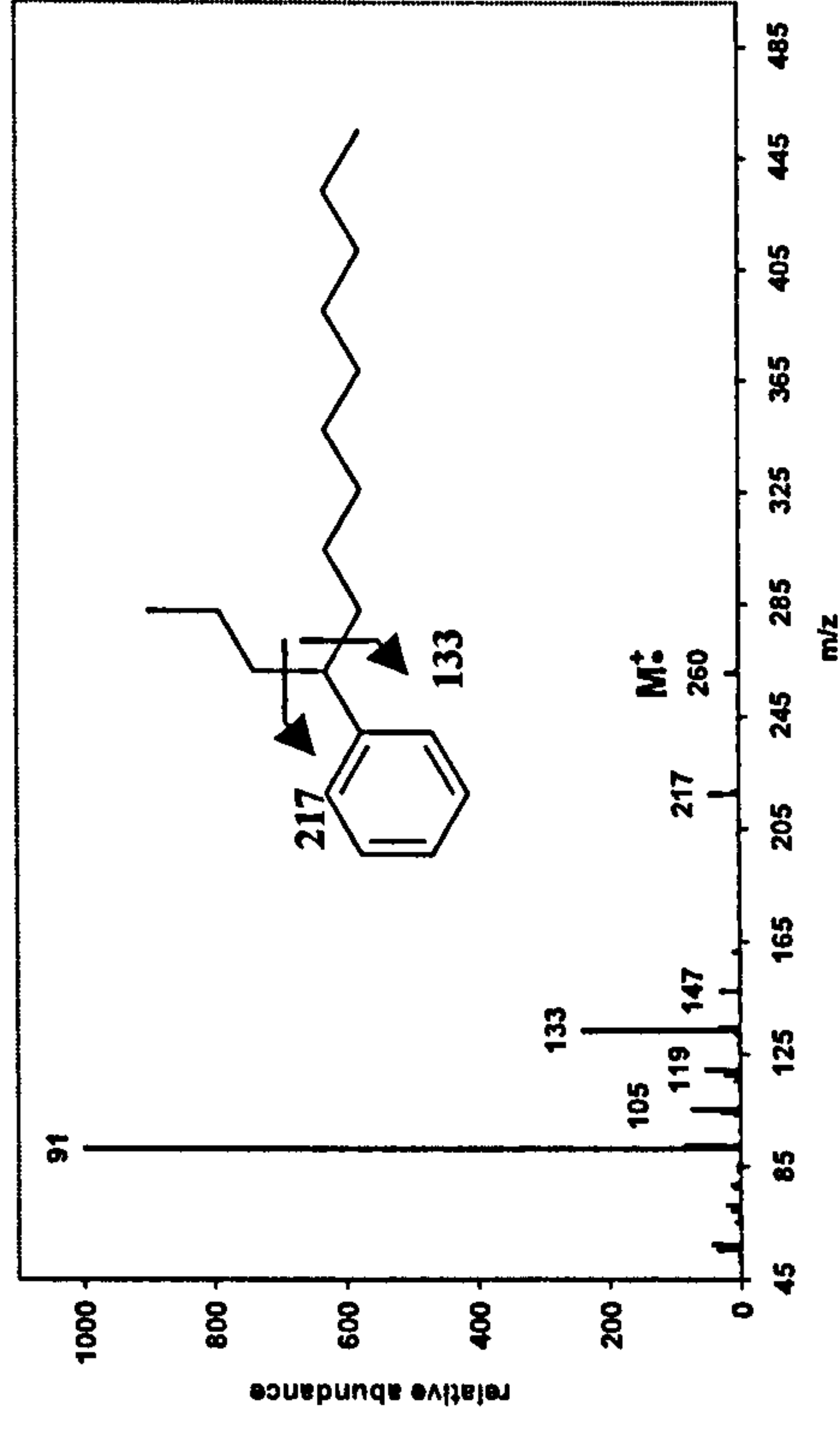


Figure 4.14b. Mass spectrum of the candidate NIST library compound, 1-propyldecyl-benzene.

In both cases, the mass spectra of the NIST library compounds exhibit an excellent match to the mass spectra of the unknown compounds. This indicates that linear alkylbenzenes (LABs) may indeed be components of the monoaromatic UCM. Their persistence in the environment has led to extensive study, particularly in terms of toxicity, as they are the parent material in linear alkylbenzene sulphonate (LABS) surfactant manufacture (*e.g.* Takada and Ishiwatari, 1990; Raymundo and Preston, 1992; Preston and Raymundo, 1993; Hellou *et al.*, 1994; Ellis *et al.*, 1996; Fernandez *et al.*, 2002).

Alkylbenzenes are also constituents of crude oil, with those compounds containing a lesser degree of alkylation being most frequently reported (*e.g.* Šimeková *et al.*, 1970; Matisova *et al.*, 1991; Dixit and Ram, 1996; Pal *et al.*, 1998). However, highly alkylated benzenes have also been observed (*e.g.* Radke *et al.*, 1984; Matisova *et al.*, 1991; Ellis *et al.*, 1996; Golovko *et al.*, 2000; Frysinger *et al.*, 2003). Frysinger *et al.* (2003) identified a series of alkylbenzenes (C₈-C₁₄ substituted) in a monoaromatic UCM using GCxGC analysis and authentic standards. A large number of peaks for each alkyl chain length were apparent, indicating many isomers, some possibly containing branched chains.

The *n*-alkylbenzenes of a comparable molecular weight to A and B exhibit a base ion of *m/z* 92 (*e.g.* Ellis *et al.*, 1992) rather than *m/z* 91. Furthermore, they would be readily susceptible to biodegradation (Dutta and Harayama, 2001) making them unlikely constituents of a UCM. LABs such as 1-propyloctyl-benzene and 1-propyldecyl-benzene still exhibit long straight alkyl chains, which could be susceptible to biodegradation (Takada and Ishiwatari, 1990; Dutta and Harayama, 2001). However, internal isomers where the phenyl group is attached near the middle of the chain are more resistant to biodegradation than those substituted at the end (Takada and Ishiwatari, 1990; Raymundo and Preston, 1992). Internal isomers are thought to be more sterically hindered and less accessible to bacteria than external isomers (Swisher, 1987; Alexander, 1999).

In the case of 1-propyloctyl-benzene (mw 232), the phenyl group is attached to a C₁₁ chain. Assuming the external isomers at positions 4, 5 and 6 (*) are susceptible to biodegradation, the internal isomers at positions 1, 2 and 3 (red) would be the most resistant to biodegradation (Figure 4.15). These internal isomers are most likely to represent compound **A** and the other peaks with molecular weight of 232. This indeed appears to be the case, as there are three peaks in the peak marker plot with molecular weight of 232 (Figure 4.12), and three possible internal isomers. Analysis of the mass spectra of the other two peaks indicates good matches for 1-butylheptyl-benzene and 1-pentylhexyl-benzene isomers.



Figure 4.15. Diagram indicating the internal (red) and external (*) isomers of a C₁₁ alkylbenzene.

The phenyl group in 1-propyldecyl-benzene (mw 260) is attached to a C₁₃ chain. Here, the bioresistant internal isomers are at positions 1, 2, 3 and 4 (Figure 4.16). Compound **B** and the other peaks with molecular weight of 260 most likely correspond to these internal isomers. With four peaks in the peak marker plot having molecular weight of 260 (Figure 4.12), and four possible internal isomers, this appears to be the case. The mass spectra of the other three peaks provided good matches for 1-butylnonyl-benzene and 1-pentylloctyl-benzene, and a reasonable match for 1-hexylheptyl-benzene.



Figure 4.16. Diagram indicating the internal (red) and external (*) isomers of a C₁₃ alkylbenzene.

Significant branching along the chain would provide high resistance to biodegradation (Alexander, 1999). Indeed, Gough (1989) observed significant resistance to biodegradation by the branched alkylbenzene 9-(2-phenylethyl)heptadecane. Compounds A and B are from a highly weathered crude oil UCM, in which only bioresistant compounds would be present. Thus, compounds A and B may be internal LABs or exhibit some degree of branching on the alkyl chains which would further increase their resistance to biodegradation. Unfortunately no examples of highly branched alkylbenzenes (BABs) exist in the NIST library for mass spectral comparison.

BABs have, however, been reported in crude oils and other fossil fuels (*e.g.* Vella and Holzer, 1992; Golovko *et al.*, 2000), including those exhibiting an isoprenoidal side chain (Figure 4.17; Ostroukhov *et al.*, 1982; Schwark and Püttmann, 1990; Gorchs *et al.*, 2003). Vorob'eva and Petrov (1998) observed phytanylnaphthalenes with an isoprenoidal chain in crude oil. In all cases mass spectra were not reported. The branching, particularly terminal branching (*e.g.* Figure 4.17) increases the resistance to biodegradation (Britton, 1984; Swisher, 1987).

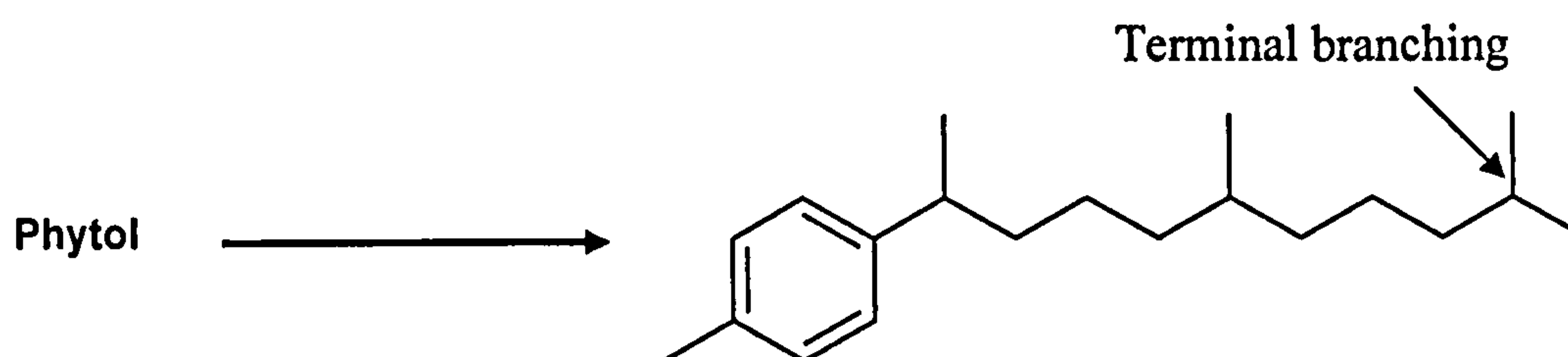


Figure 4.17. 1-Methyl-4-(1,5,9-trimethyl-decyl)-benzene, possibly formed by ring closure and aromatisation of phytol (Golokov *et al.*, 2000). The molecular weight is 274, similar to that of the compounds in the present study.

The water solubility of C₁₀ and C₁₈ substituted LABs is 40.4 and 9.6 µg L⁻¹ respectively (Fernandez *et al.*, 2002). This former value corresponds well to the water solubility of the monoaromatic UCM, determined as 57 ± 21 µg L⁻¹ (Chapter 3). Fernandez *et al.* (2002) also observed that LABs are toxic, inducing a narcotic affect in the aquatic crustacean

Daphnia magna. This toxic effect was found to be additive, the same mode of action as proposed for the monoaromatic UCM by Rowland *et al.* (2001).

Hellou *et al.* (1994) observed alkylbenzenes (m/z 91 and 105) in the mussel tissue of winter flounder (*Pseudopleuronectes americanus*) exposed to Hibernia crude oil. Analysis revealed these compounds had molecular weights of 232, 246 and 260, the same as those present in the water soluble monoaromatic UCM analysed herein (Figure 4.12). The aromatic hydrocarbon TIC chromatograms of the fish extract also exhibited a dominant UCM. Thus, such compounds appear, to be soluble, bioavailable and toxic to aquatic organisms, typical of the properties of some monoaromatic UCMs (Rowland *et al.*, 2001; Donkin *et al.*, 2003).

The evidence suggests that compounds A and B, together with the other components exhibiting a m/z 91 base ion, are alkylbenzenes. NIST library mass spectra matches indicate they are LABs, and the internal isomers of these compounds may be sufficiently bioresistant to be present in the monoaromatic UCM. However, it is likely that compounds A and B contain an even greater degree of branching than internal LABs. Comparison with authentic reference compounds is required to confirm their exact structures.

4.3.2 Compounds with a base ion of m/z 105

A peak marker plot of the compounds exhibiting a m/z 105 base ion is presented in Figure 4.18. Figure 4.19a shows the mass spectrum of **C** (retention time 1220,4.42), whilst Figure 4.19b shows the mass spectrum of 1-methylundecyl-benzene. This is proposed as the best match from the NIST library for **C**. Figure 4.20a shows the mass spectrum of **D** (retention time 1340,4.44), with Figure 4.20b showing the mass spectrum of 1-methyldodecyl-benzene. This is suggested as the best match from the NIST library for **D**.

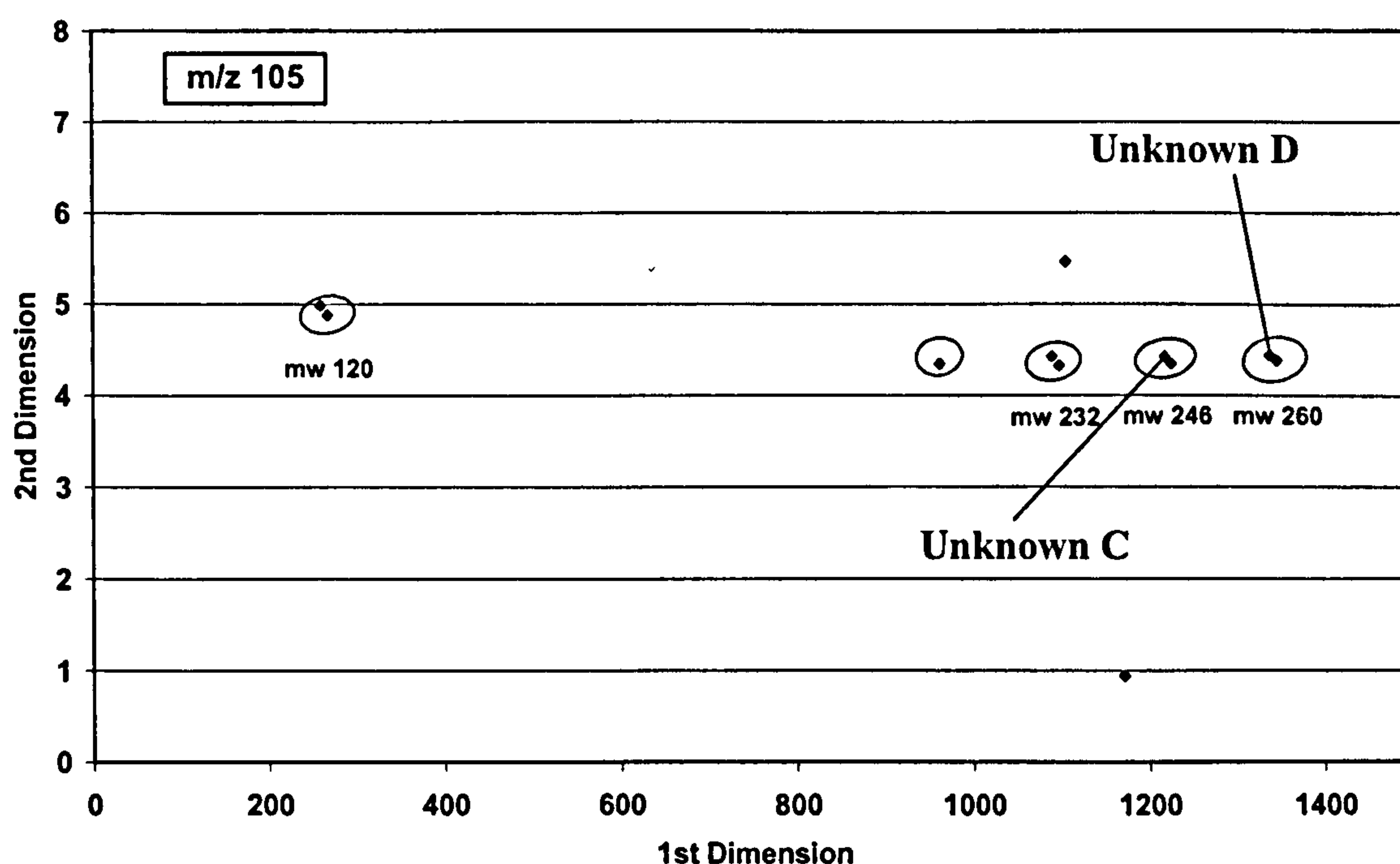


Figure 4.18. Peak marker plot of the compounds present in the water soluble monoaromatic UCM exhibiting a base ion of m/z 105. Those compounds displaying the same molecular weight are grouped together.

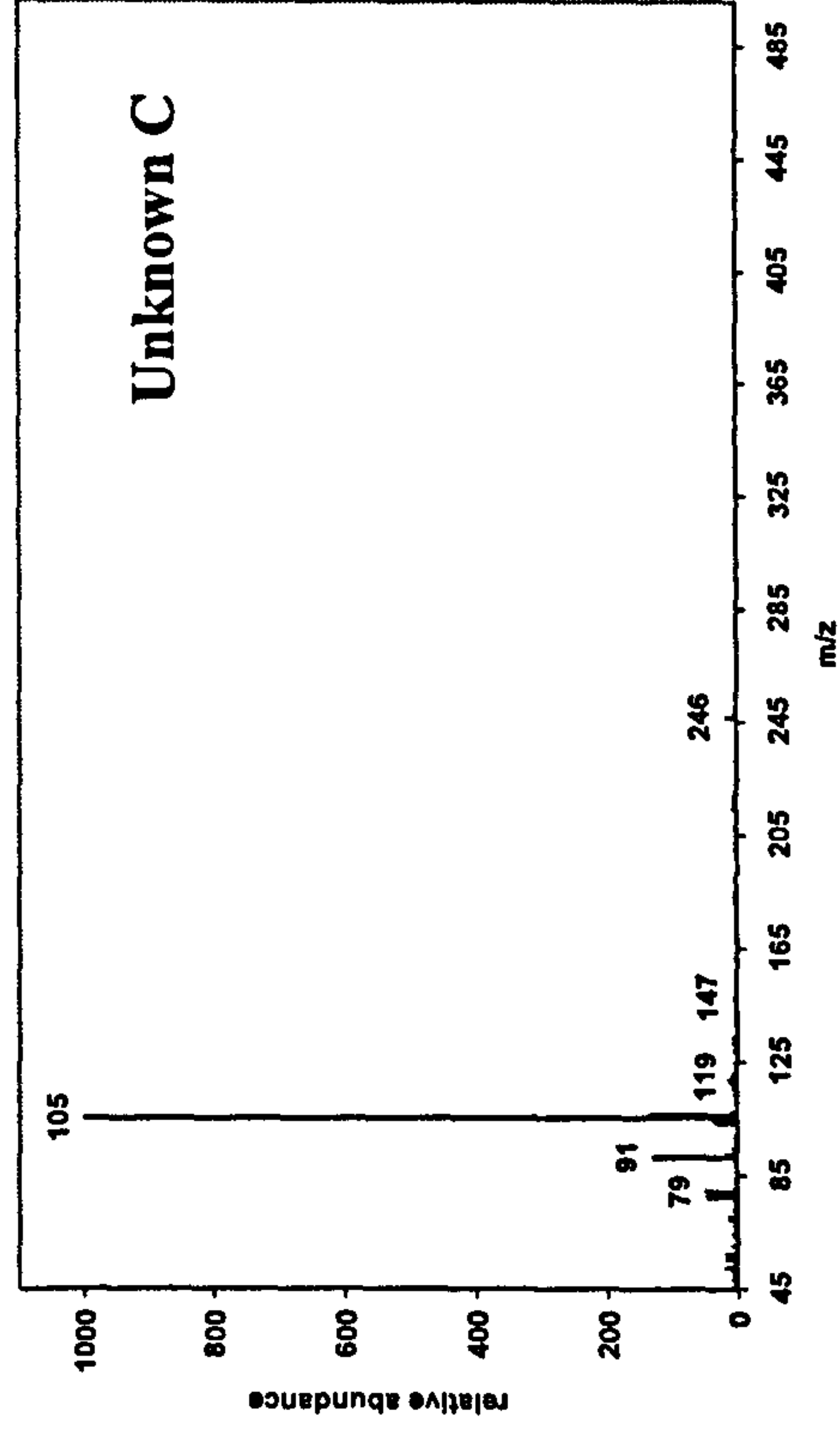


Figure 4.19a. Mass spectrum of the unknown compound C, which has a retention time of 1220,4.42.

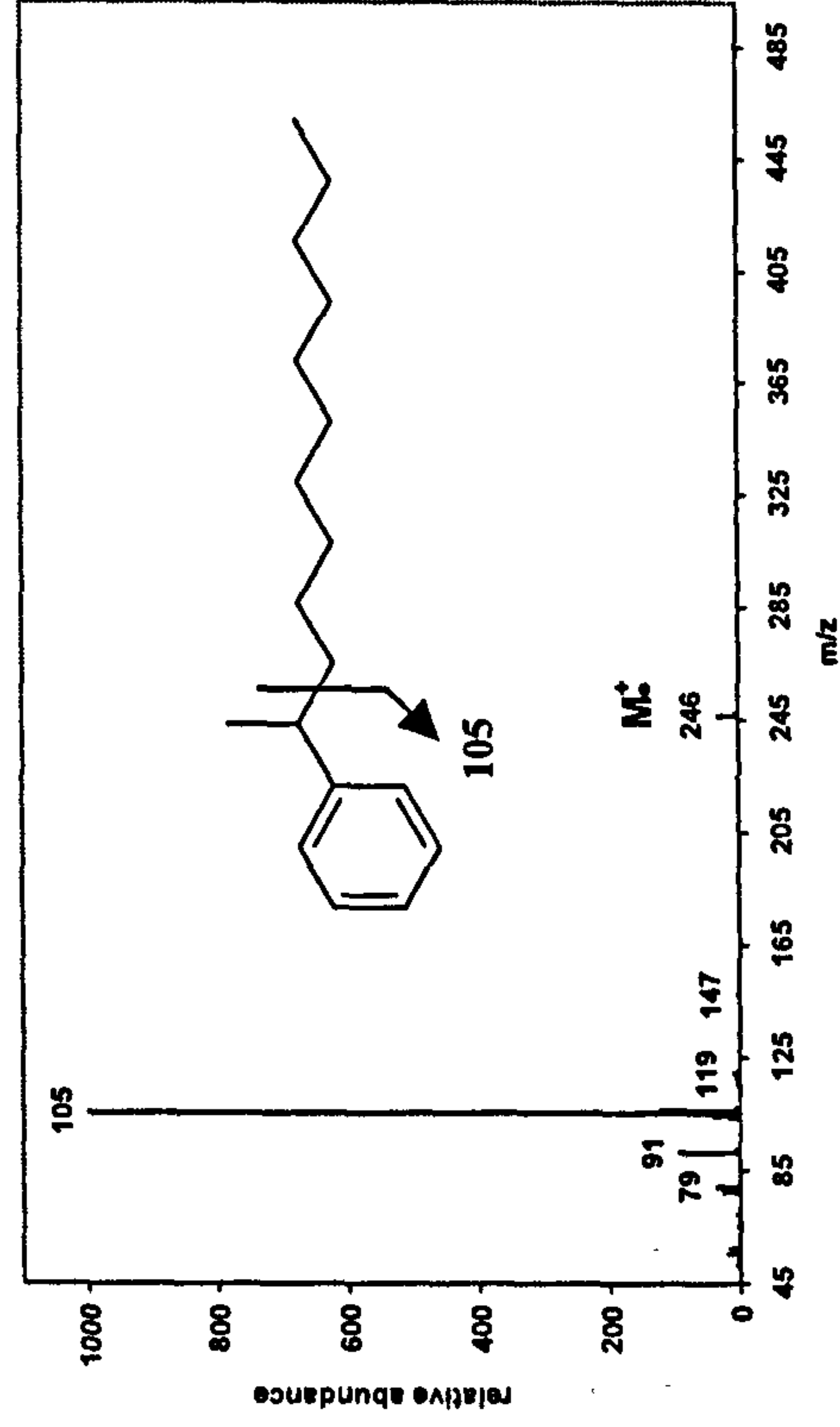


Figure 4.19b. Mass spectrum of the candidate NIST library compound, 1-methylundecyl-benzene.

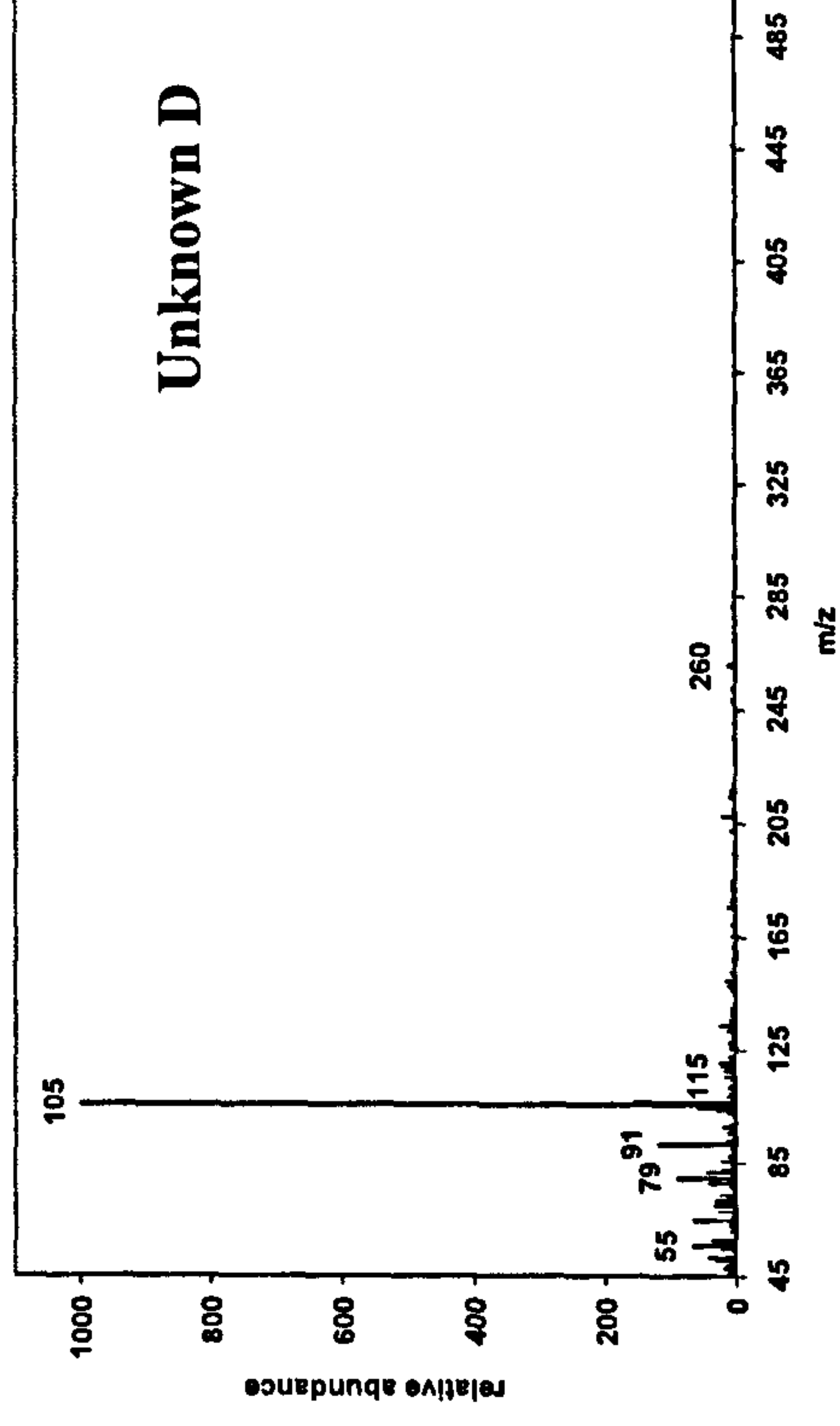


Figure 4.20a. Mass spectrum of the unknown compound D, which has a retention time of 1340,4.44.

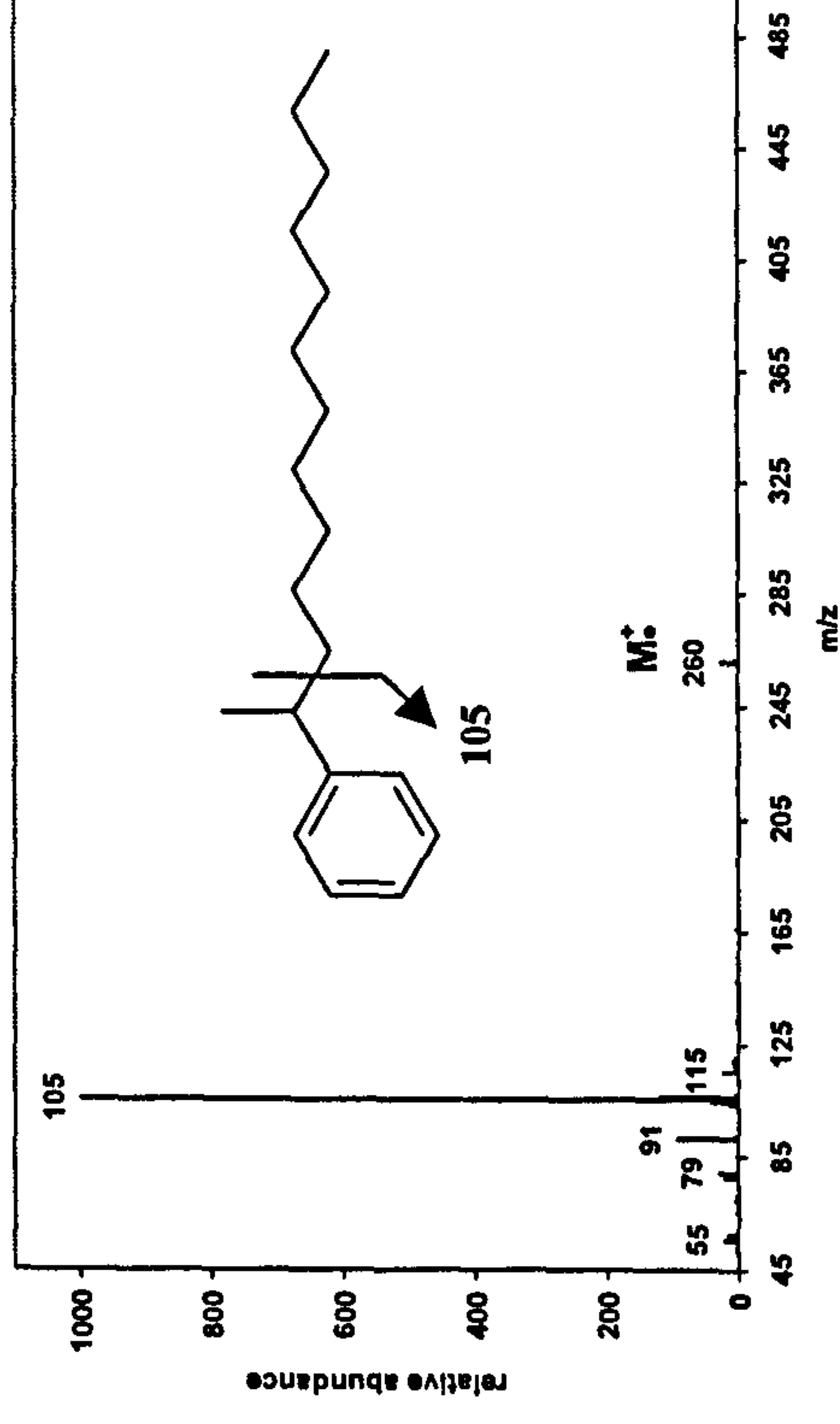


Figure 4.20b. Mass spectrum of the candidate NIST library compound, 1-methyldodecyl-benzene.

As these compounds are isomeric to the alkylbenzenes with a m/z 91 base ion discussed in Section 4.3.1, only information specific to these two compounds will be discussed herein. The phenyl group in 1-methylundecyl-benzene (mw 246) and 1-methyldodecyl-benzene (mw 260) is attached to the second carbon atom of the alkyl chain. These are external isomer positions, which are typically more susceptible to biodegradation than internal isomers (Swisher, 1987; Takada and Ishiwatari, 1990; Raymundo and Preston, 1992; Alexander, 1999).

Internal isomers of these compounds correspond to the LABs discussed in Section 4.3.1, which exhibit a base ion of m/z 91 rather than m/z 105. Study of the NIST library mass spectra shows that LABs with the phenyl group at position one (*n*-alkylbenzenes) have a base ion of m/z 92. Those at position two have a base ion of m/z 105, and those in more central positions exhibit m/z 91 base ions. Thus, if compounds C and D are alkylbenzenes they must be substituted at the two-position, making them external isomers.

If compounds C and D are external LAB isomers, such structures must be sufficiently bioresistant to preserve them in the monoaromatic UCM. This is possible given that that LABs used in the manufacture of LAS surfactants are still sufficiently persistent in the environment to cause concern (*e.g.* Takada and Ishiwatari, 1990; Preston and Raymundo, 1993; Hellou *et al.*, 1994; Ellis *et al.*, 1996; Fernandez *et al.*, 2002).

Although the mass spectra of LABs exhibit the best NIST library matches for compounds exhibiting m/z 91 and m/z 105 base ions, an absence of spectra for branched alkylbenzenes (BABs) in the library prevented comparison with such compounds. Since significant branching of the alkyl chain would increase the bioresistance of these compounds (Alexander, 1999), such structures should be considered. Figure 4.21 shows an isoprenoidal branched compound isomeric to 1-methyldodecyl-benzene. Fragmentation at

the same point is likely, although other minor fragment ions may be observed due to the branching.

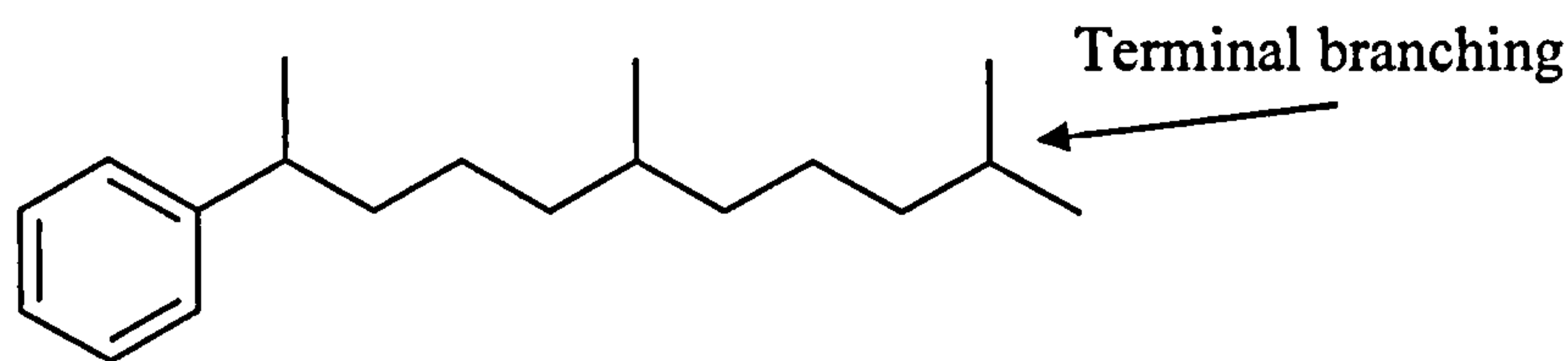


Figure 4.21. Rearrangement of the alkyl carbons in 1-methyldodecyl-benzene (mw 260) gives the isoprenoidal branched (1,5,9-Trimethyl-decyl)-benzene (mw 260) above.

If compounds **C** and **D** are part of a homologous series of methylalkylbenzenes, only one isomer giving a m/z 105 base ion is possible for each molecular weight (assuming no branching on the chain occurs). The peak marker plot in Figure 4.18 indicates that there are two compounds at each molecular weight exhibiting a base ion of m/z 105. This means there is either a branched isomer present, or a homologous series of a different compound with very similar volatility and polarity characteristics. Given the number of possible branched isomers, it seems unlikely that only one would be present at each molecular weight. Furthermore, the mass spectra of the other compounds in the peak marker plot exhibit a different fragmentation pattern to the LABs, indicating another homologous series of compounds.

Two other types of compound also exhibit a m/z 105 base ion, alkyltoluenes and 1,2-di-*n*-alkylbenzenes. Alkyltoluenes (Figure 4.22) have previously been reported in crude oil (*e.g.* Ellis *et al.*, 1992; Ellis *et al.*, 1995; Dutta and Harayama 2001). Unfortunately, the NIST library only has examples with C₆ alkyl substituents so mass spectral comparison with the compounds in the peak marker plot (Figure 4.18) was not possible.

Branched and linear alkyl chains are possible, but the absence of large numbers of isomers suggests the compounds are not branched alkyltoluenes. Even so, there are three possible

linear alkyltoluene isomers (Figure 4.22). Ellis *et al.*, (1995) observed that the abundance of the different alkyltoluene isomers in crude oils was not dependent upon oil maturity, but appeared more dependent upon the type of source rock. It is therefore unclear which alkyltoluene isomer is most likely to be observed in the monoaromatic UCM sample.

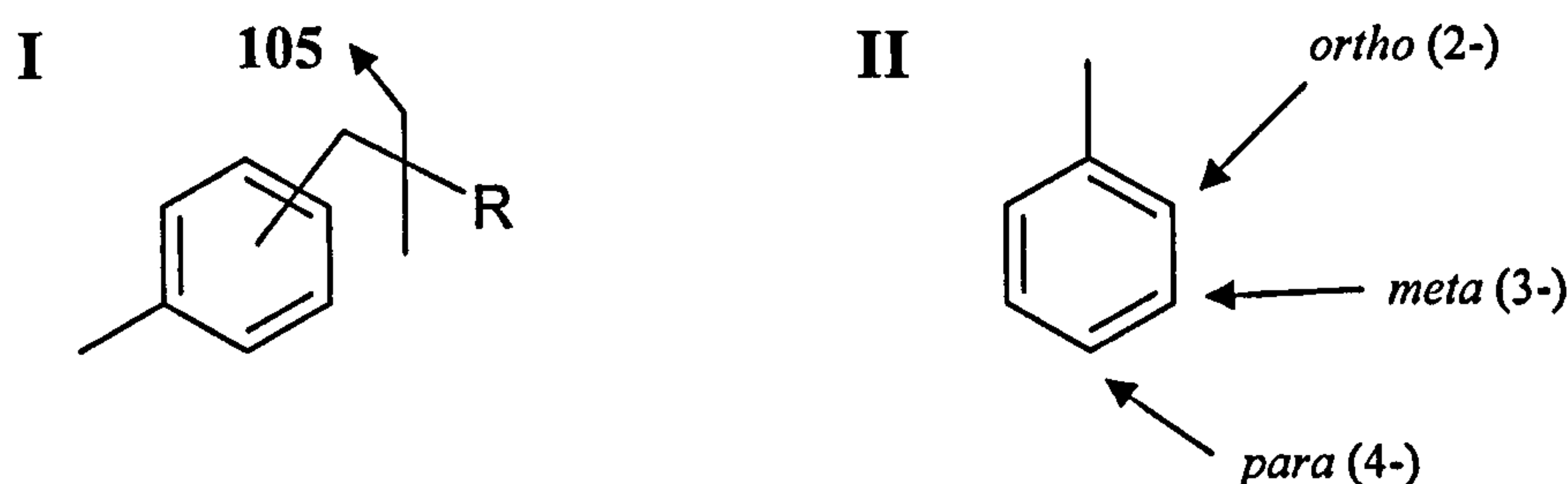


Figure 4.22. (I) Structure of the alkyltoluenes, which give a characteristic base ion (m/z) 105. R represents an alkyl chain of any length. (II) Three possible linear alkyltoluene isomers.

1,2-di-*n*-alkylbenzenes identified in Amposta crude oil (Sinninghe Damste *et al.*, 1991) have two linear alkyl chains attached to the benzene ring. The reported mass spectra exhibit close similarities to those compounds in the peak marker plot. However, the number of possible isomers for each molecular weight was much greater than the number seen in the peak marker plot. It is therefore suggested that the compounds in the peak marker plot correspond to a homologous series of LABs substituted at the two-position and another homologous series of hydrocarbons, possibly a linear alkyltoluene isomer. In both cases compounds with a branched chain are possible, but the data seem to indicate such compounds are not present due to the small number of isomers in the peak marker plot.

4.3.3 Compounds with a base ion of m/z 143

A peak marker plot of the compounds exhibiting a m/z 143 base ion is presented in Figure 4.23. Figure 4.24a shows the mass spectrum of E (retention time 740,6.16), whilst Figure 4.24b shows the mass spectrum of 1-methyl-3-propyl-1*H*-indene. This is proposed as the best match from the NIST library for E. Figure 4.25a shows the mass spectrum of F (retention time 908,6.92), with Figure 4.25b showing the mass spectrum of 3-butyl-1-methyl-1*H*-indene. This is suggested as the best match from the NIST library for F.

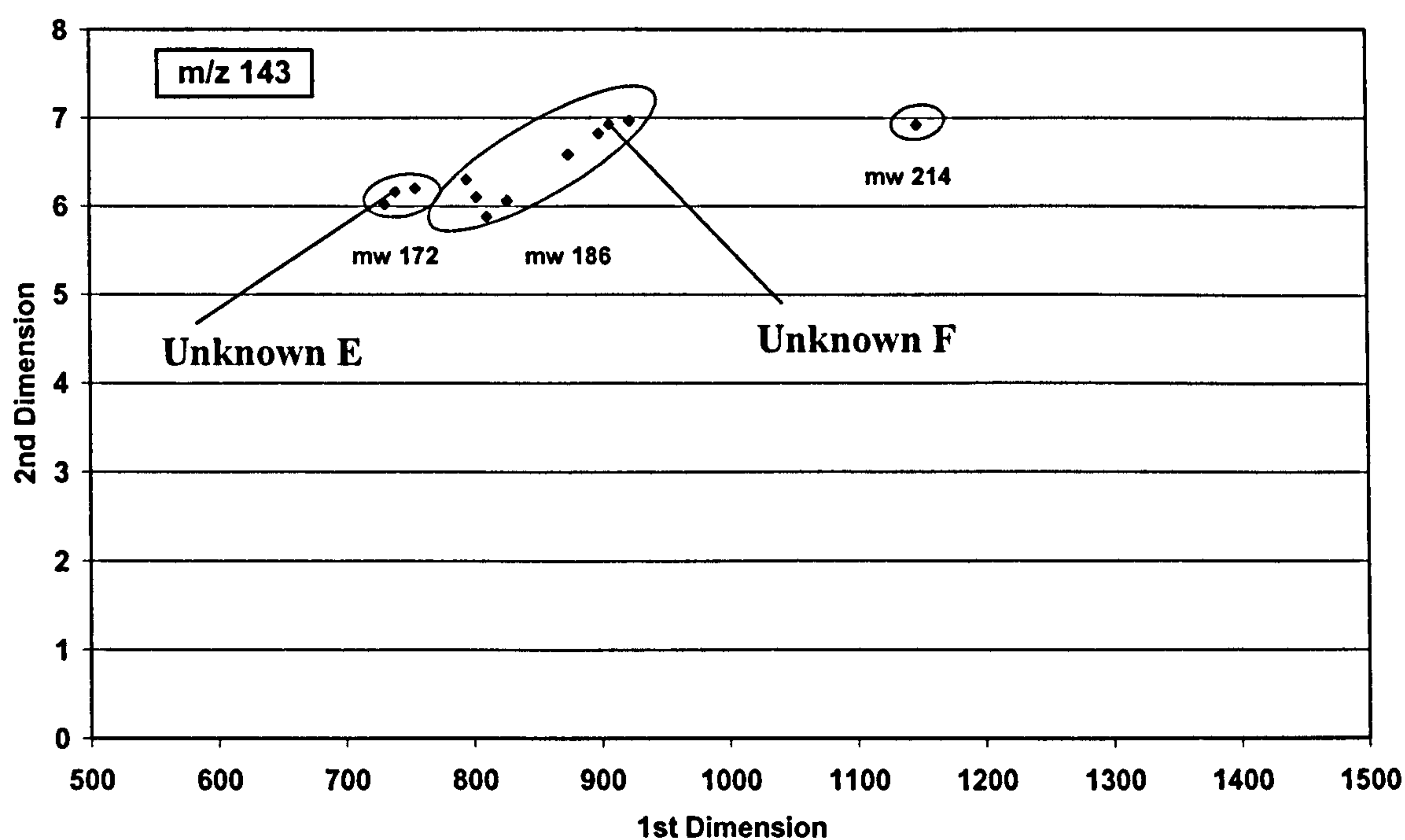


Figure 4.23 Peak marker plot of the compounds present in the water soluble monoaromatic UCM exhibiting a base ion of m/z 143. Those compounds displaying the same molecular weight are grouped together.

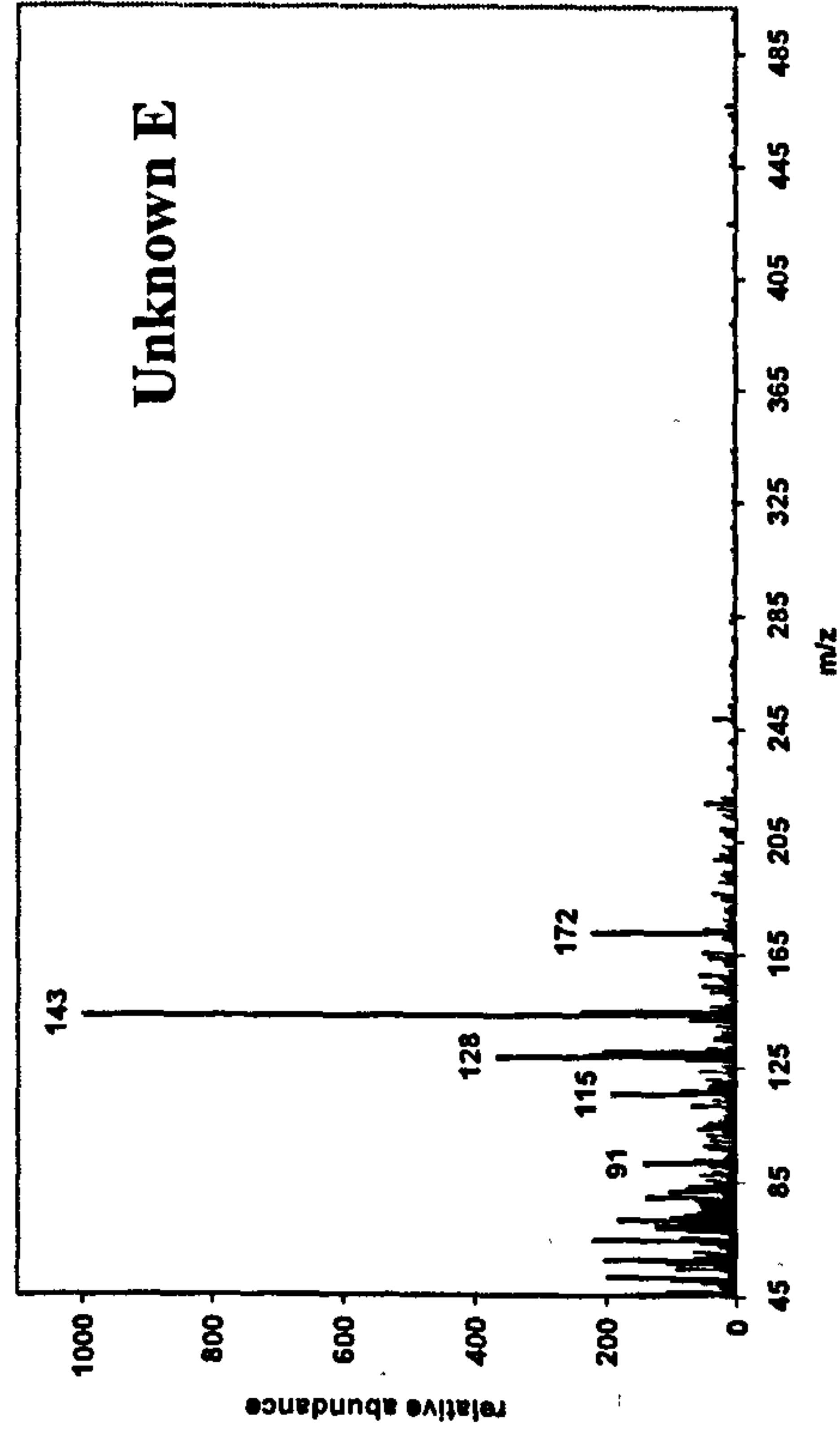


Figure 4.24a. Mass spectrum of the unknown compound E, which has a retention time of 740,6.16.

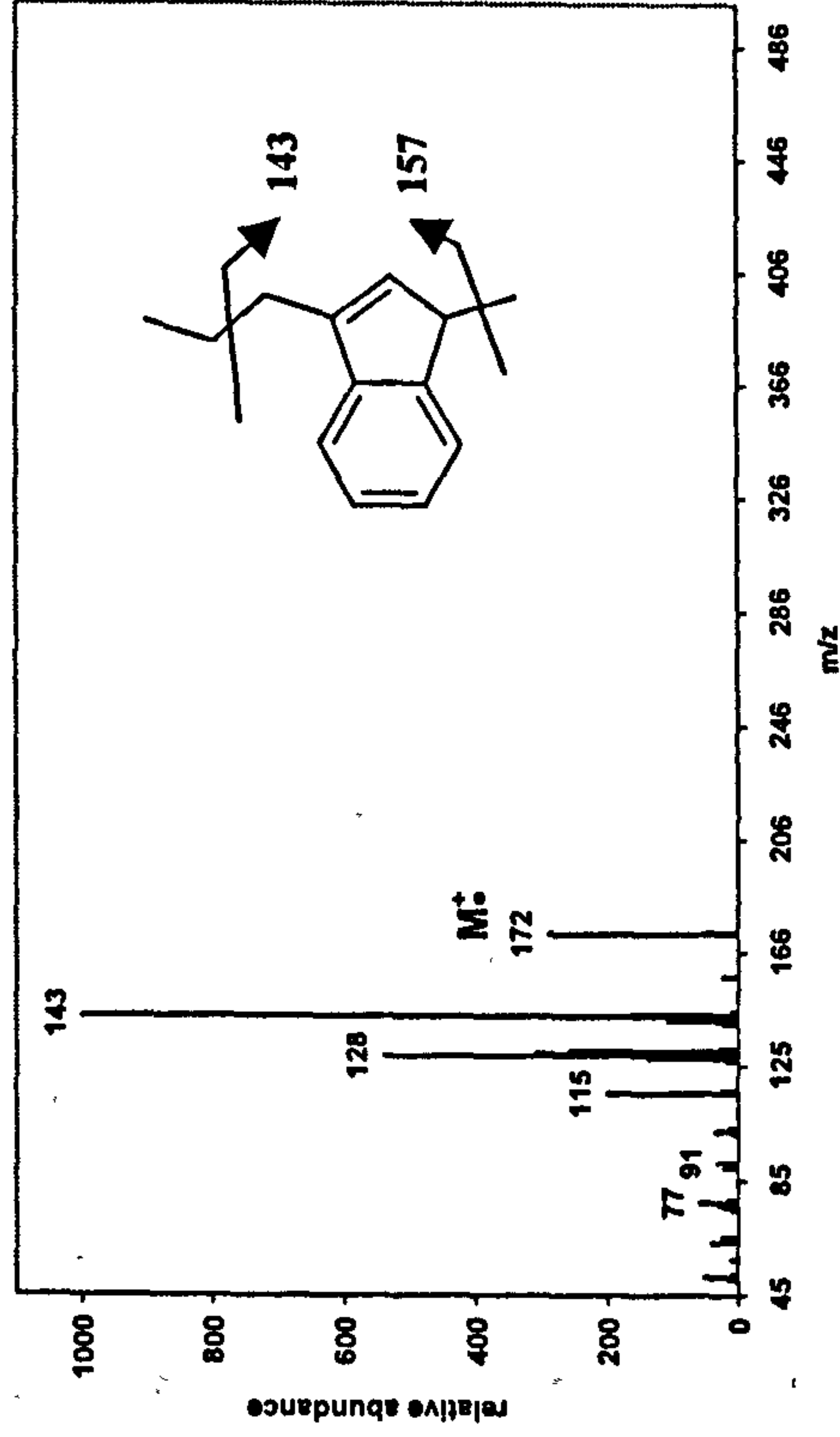


Figure 4.24b. Mass spectrum of the candidate NIST library compound, 1-methyl-3-propyl-1H-indene.

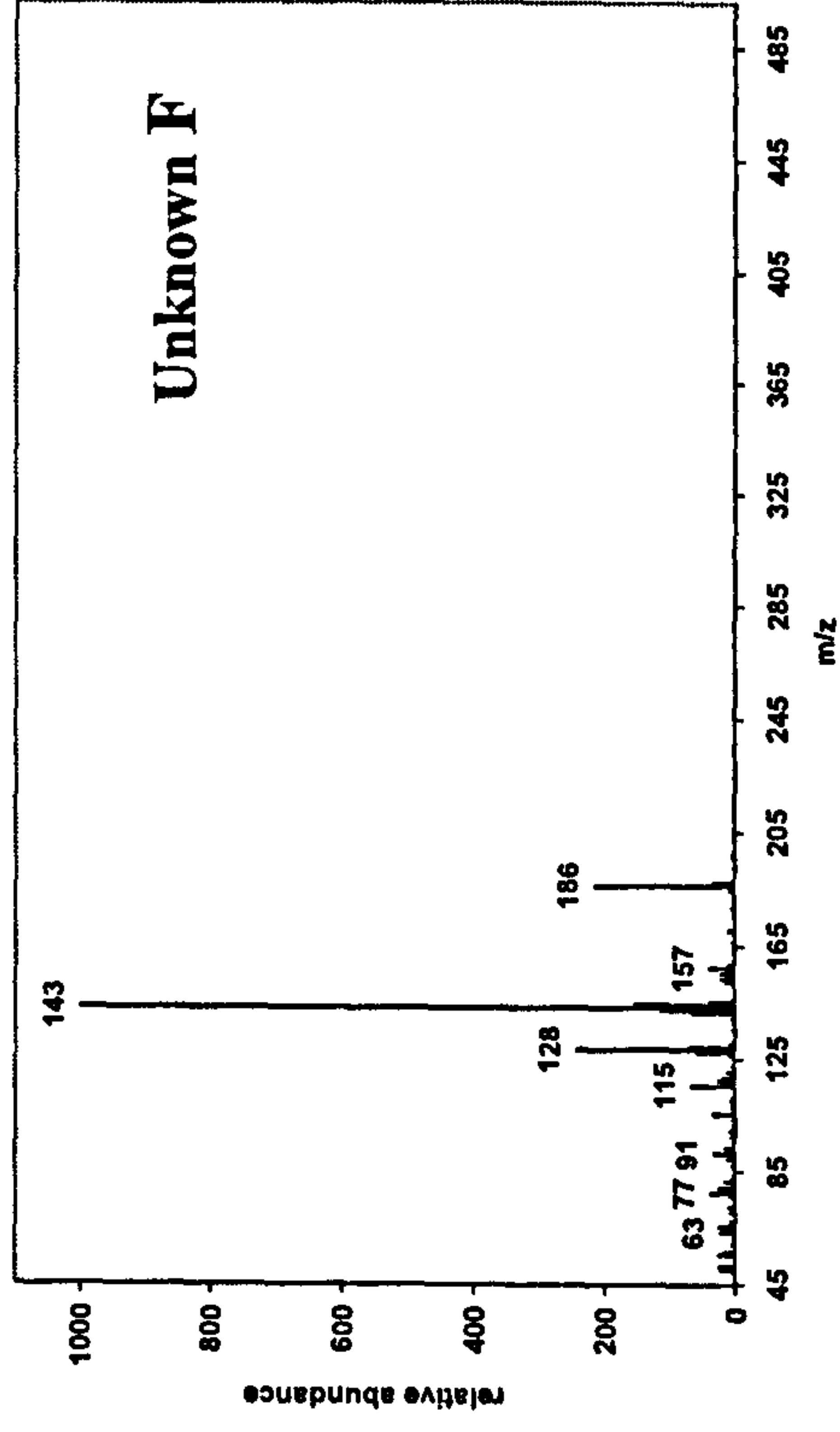


Figure 4.25a. Mass spectrum of the unknown compound F, which has a retention time of 908,6.92.

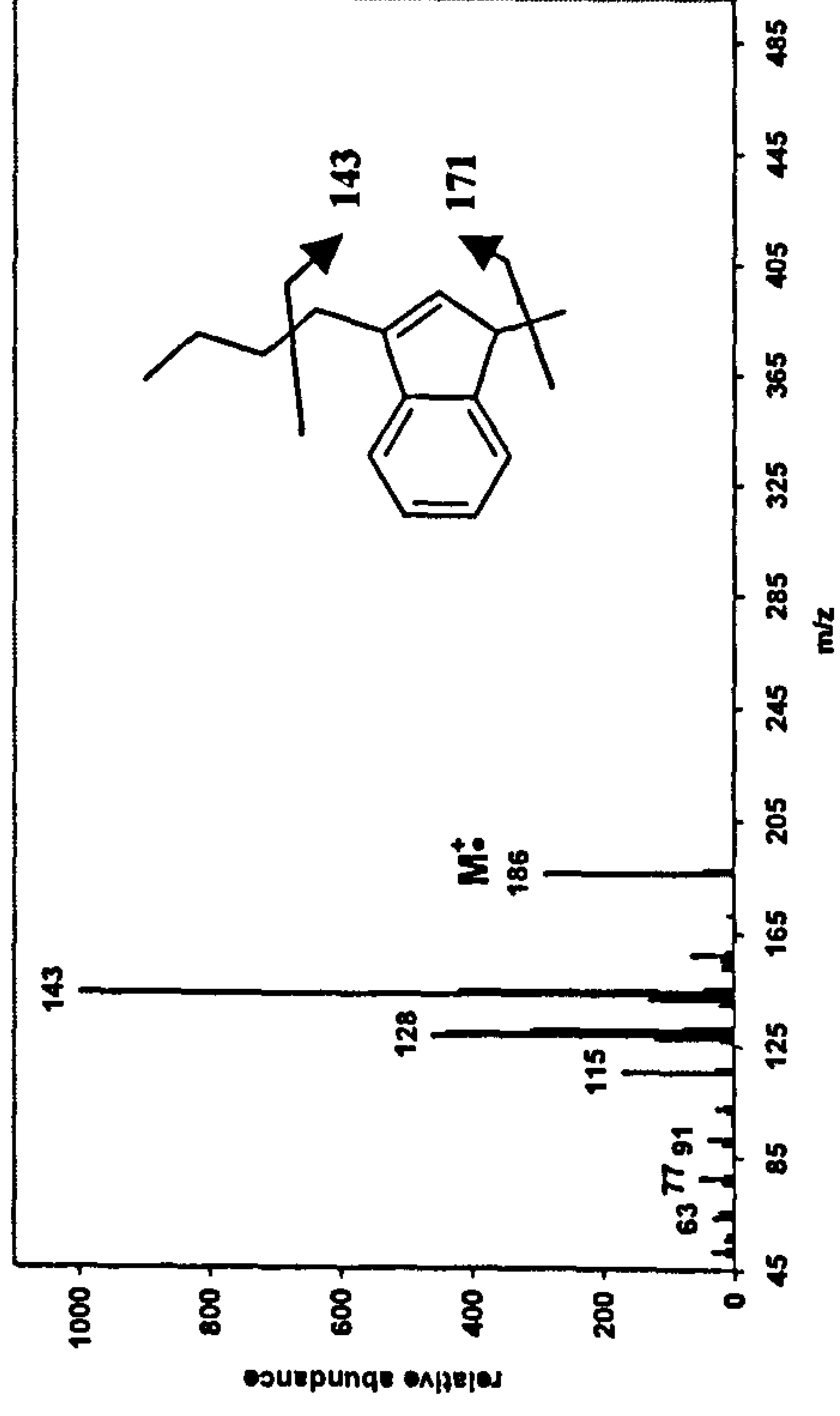


Figure 4.25b. Mass spectrum of the candidate NIST library compound, 3-butyl-1-methyl-1H-indene.

The mass spectra of the unknown compounds E and F exhibit very good matches to those suggested NIST library compounds. Such compounds may represent a homologous series of methyl-alkyl-indenes in the monoaromatic UCM. Although not widely reported, alkylindenes have been identified as constituents of crude oils (*e.g.* Matisova *et al.*, 1991; Robert *et al.*, 1994; Frysinger and Gaines 1999). Unfortunately, none of the studies reported mass spectra and assignment of the final structures was not possible in all cases.

Matisova *et al.* (1991) observed nine alkylindenes in a Russian crude oil, with the substitution ranging from C₁ to C₄. A C₄ substituted indene has a molecular weight of 172, identical to that of component E in the UCM. In fact, it is possible that unknown compound E is one of the indene isomers identified by Matisova *et al.* (1991). Frysinger and Gaines (1999) reported alkylindenes in a diesel fuel following analysis by GCxGC-MS. The authors observed longer retention times for the alkylindenes on the second-dimension than those observed for the alkylbenzenes. This is attributed to the presence of a second ring containing an additional double bond in the structure. These features increase polarity and thus, retention time. Although very similar in structure, the alkylindans also have shorter second-dimension retention times than the alkylindenes. This is caused by reduced polarity, resulting from the absence of the polar double bond seen in the indene structure. Thus the indene and indan compounds form separate groups/bands on the two-dimensional chromatogram (Frysinger and Gaines, 1999).

The ordered nature of compound class distributions across two-dimensional chromatograms can be applied in the present study. The proposed alkylindenes in the peak marker plot (Figure 4.23) also exhibit longer second-dimension retention times than the alkylbenzenes identified in Sections 4.3.1 and 4.3.2 (Figures 4.12 and 4.18). Their identification is further supported by comparison with the retention time of the alkylindans

in Section 4.3.4 (Figure 4.26). Expectedly, the alkyindans exhibit a retention time just less than that of the proposed alkyindenes, due to their reduced polarity.

Compounds E and F appear to be part of a homologous series of 1-methyl-3-alkyl-1*H*-indenes occurring in the monoaromatic UCM. The peak marker plot (Figure 4.23) indicates a large number of structural isomers, particularly for those compounds with a molecular weight of 186. Although branching of the C₃ chain on the methyl-propyl-1*H*-indenes is possible, it would prevent the loss of C₂H₅ (ethyl), required for generation of a *m/z* 143 base ion. Therefore, only linear methyl-propyl-1*H*-indenes isomers are suitable candidates, of which there are forty two. In contrast, branching of the alkyl chain on butyl-methyl-1*H*-indenes (isobutyl) does permit formation of a *m/z* 143 base ion *via* loss of C₃H₇ (propyl). The possibility of branched as well as linear substituents means there are 84 possible structural isomers of butyl-methyl-1*H*-indene. Whilst, substitution of the methyl and alkyl groups can be on either ring, it is not known how many of the possible isomers actually exhibit a *m/z* 143 base ion. As the number of possible isomers far exceeds that of the compounds in the peak marker plot (Figure 4.23), it is suggested that not all of the isomers exhibit a *m/z* 143 base ion.

There are many more compounds in the peak marker plot with a molecular weight of 186 than 172. Not only are there more compounds with a molecular weight of 186 possible but, due to branched isomers, such structures would probably be more resistant to biodegradation (*e.g.* Alexander, 1999). As the sample studied herein is derived from a biodegraded crude oil, a higher abundance of branched compounds over linear isomers would be expected.

A single compound, with a molecular weight of 214, is evident in the peak marker plot (Figure 4.23). Unfortunately the mass spectrum is not clear, and the NIST library does not

contain a reference compound with a molecular weight of 214 and a m/z 143 base ion to permit comparison. Given the ordered nature of two-dimensional chromatograms, the similarity of the second-dimension retention time (polarity) to that of the other components indicates all compounds are part of the same homologous series. From its molecular weight (214), the compound is suggested to be one of the hexyl-methyl-1H-indene isomers.

Interestingly, there appears to be a molecular weight 'cut-off' point, with only one compound exhibiting a greater molecular weight than 186. This may be indicative that compounds with longer alkyl chains were more accessible to bacteria and thus subject to increased biodegradation. More likely, this may represent a water solubility 'cut-off', where compounds in this homologous series, above the molecular weight of 186, were not solubilised by the generator column method.

4.3.4 Compounds with a base ion of m/z 159

A peak marker plot of the compounds exhibiting a m/z 159 base ion is presented in Figure 4.26. Figure 4.27a shows the mass spectrum of G (retention time 716,5.26), whilst Figure 4.27b shows the mass spectrum of 2,3-dihydro-1,1,5,6-tetramethyl-1*H*-indene. This is proposed as the best match from the NIST library for G. Figure 4.28a shows the mass spectrum of H (retention time 724,5.54), with Figure 4.28b showing the mass spectrum of 1,2,3,4-tetrahydro-1,6,8-trimethyl-naphthalene. This is suggested as the best match from the NIST library for H.

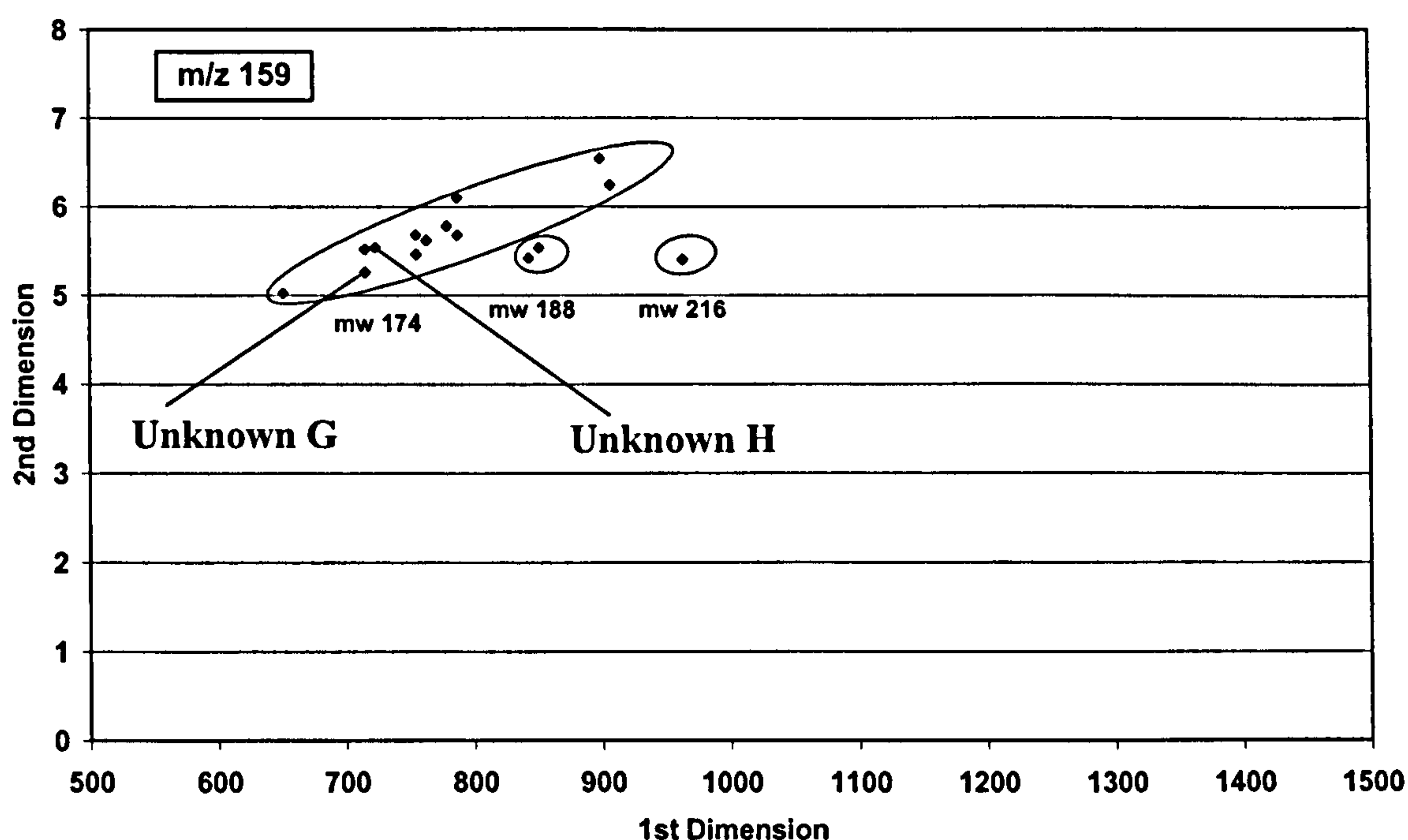


Figure 4.26. Peak marker plot of the compounds present in the water soluble monoaromatic UCM exhibiting a base ion of m/z 159. Those compounds displaying the same molecular weight are grouped together.

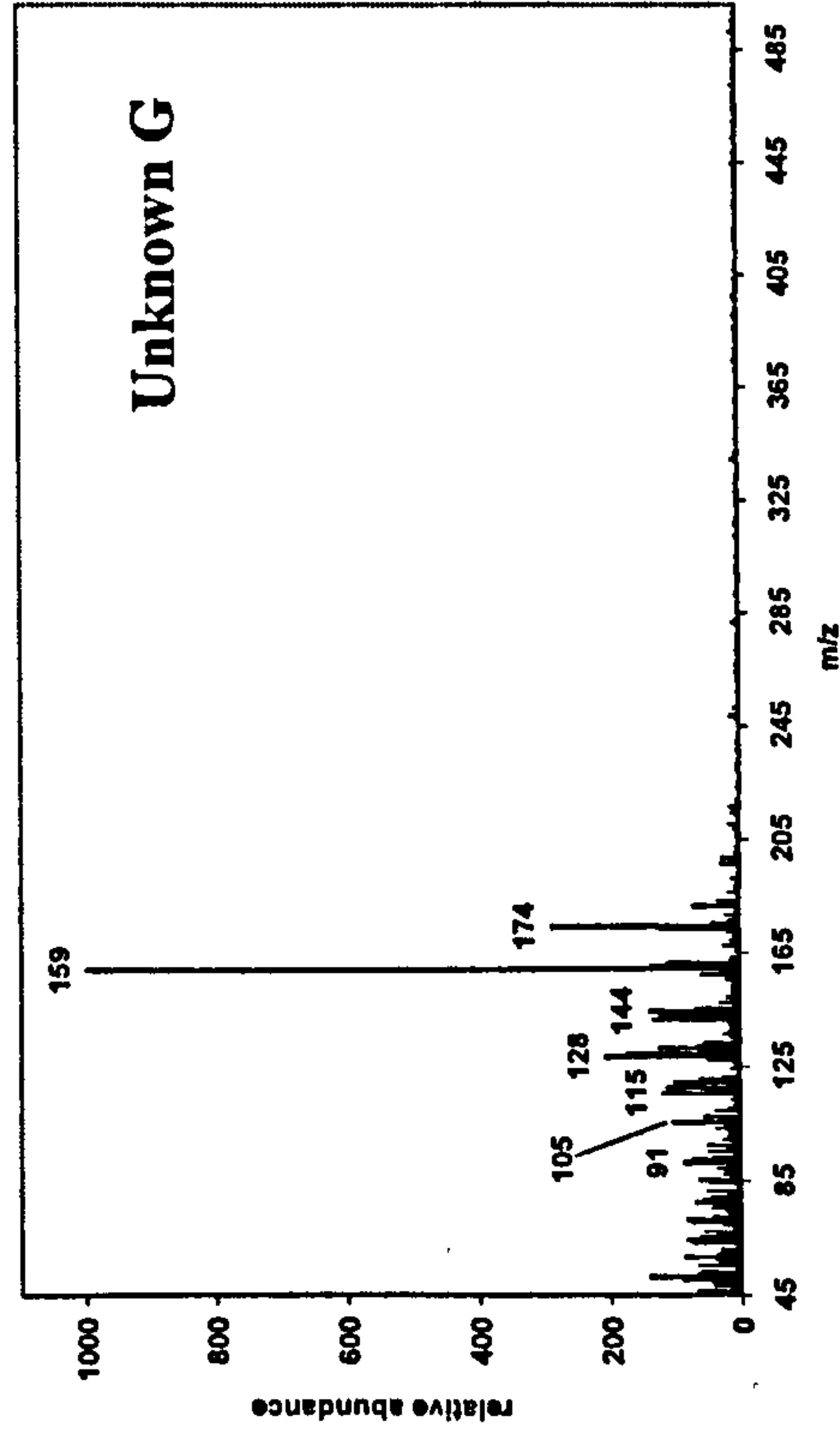


Figure 4.27a. Mass spectrum of the unknown compound G, which has a retention time of 716.5.26.

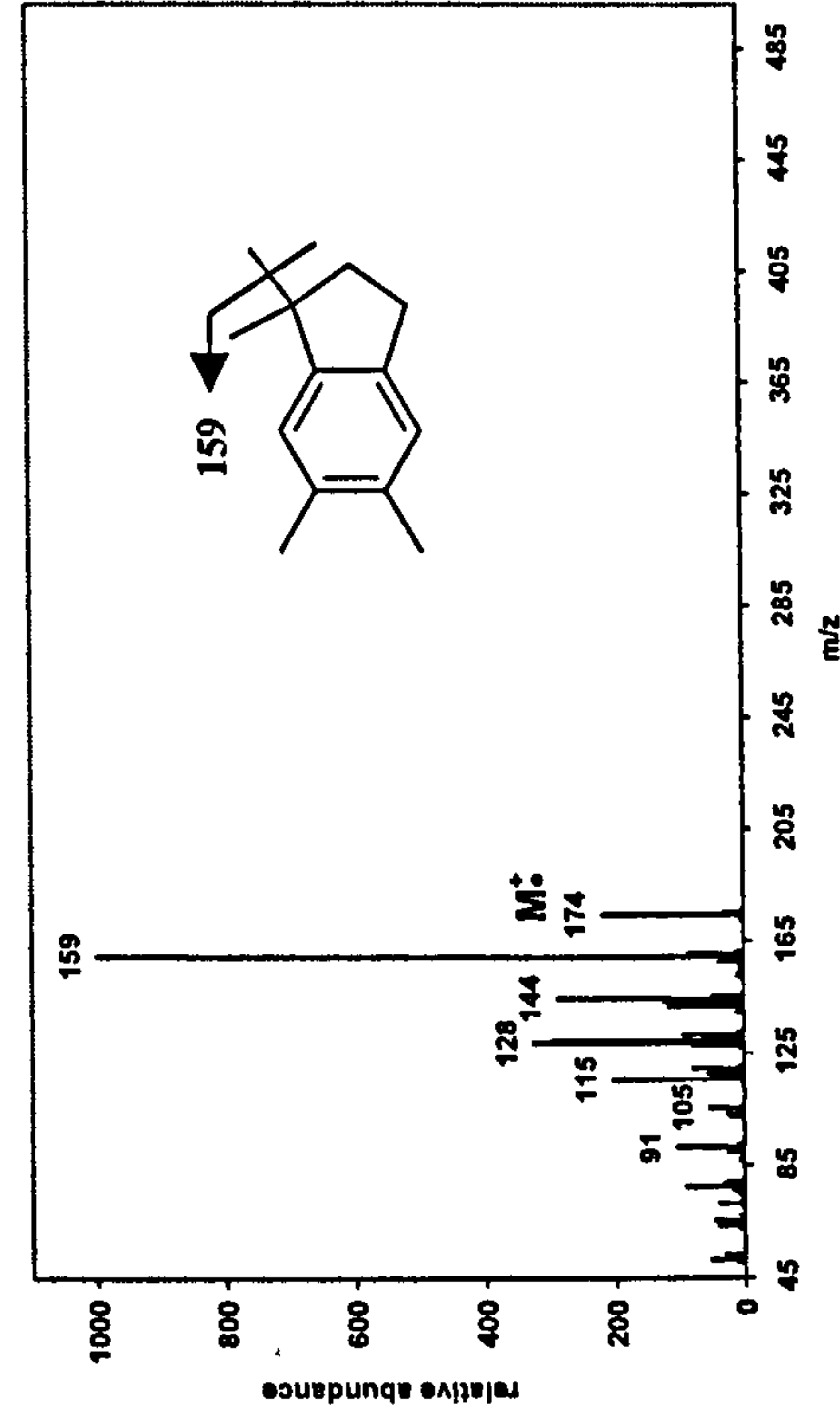


Figure 4.27b. Mass spectrum of the candidate NIST library compound, 2,3-dihydro-1,1,5,6-tetramethyl-1H-indene.

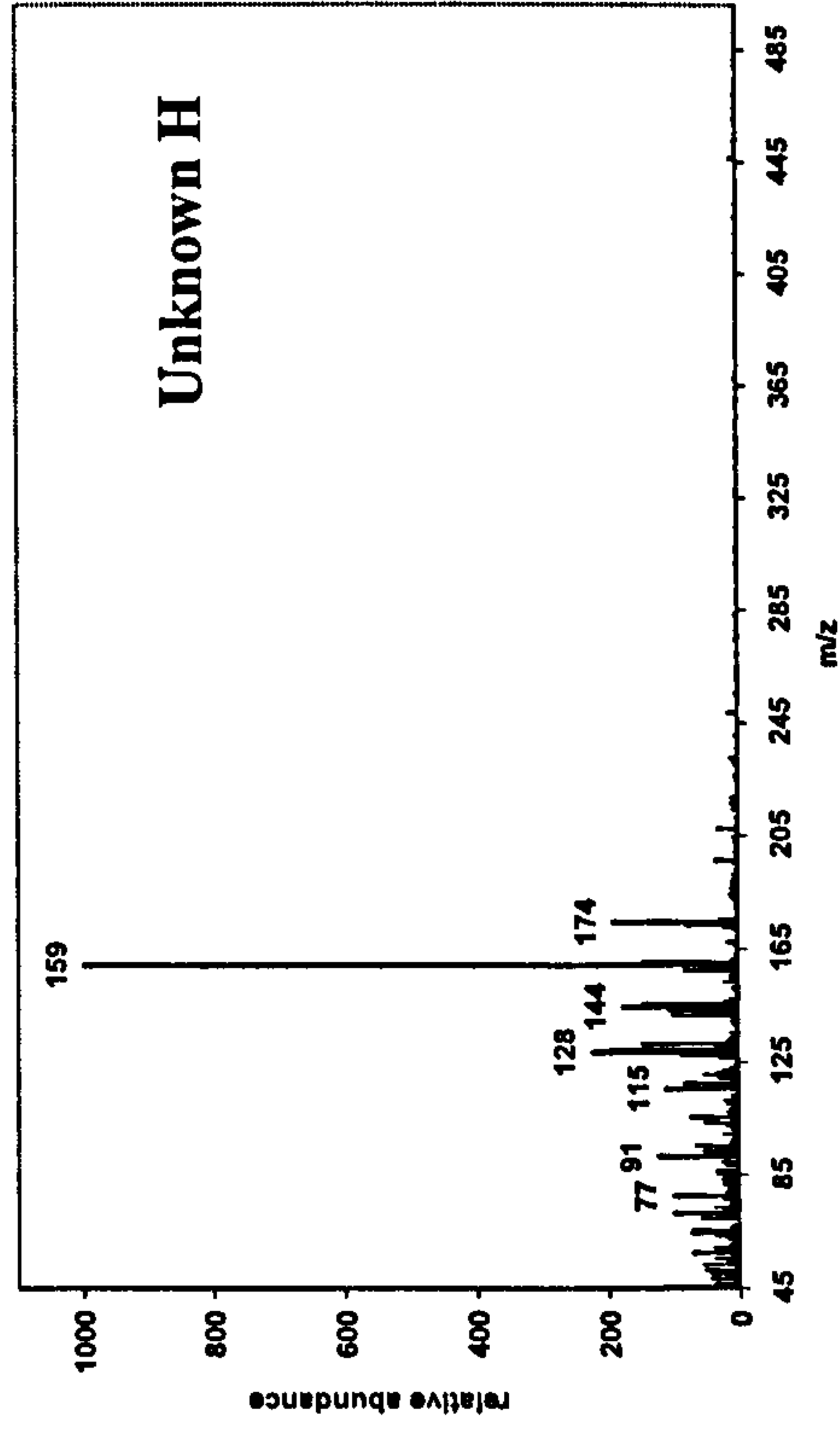


Figure 4.28a. Mass spectrum of the unknown compound H, which has a retention time of 724.5.54.

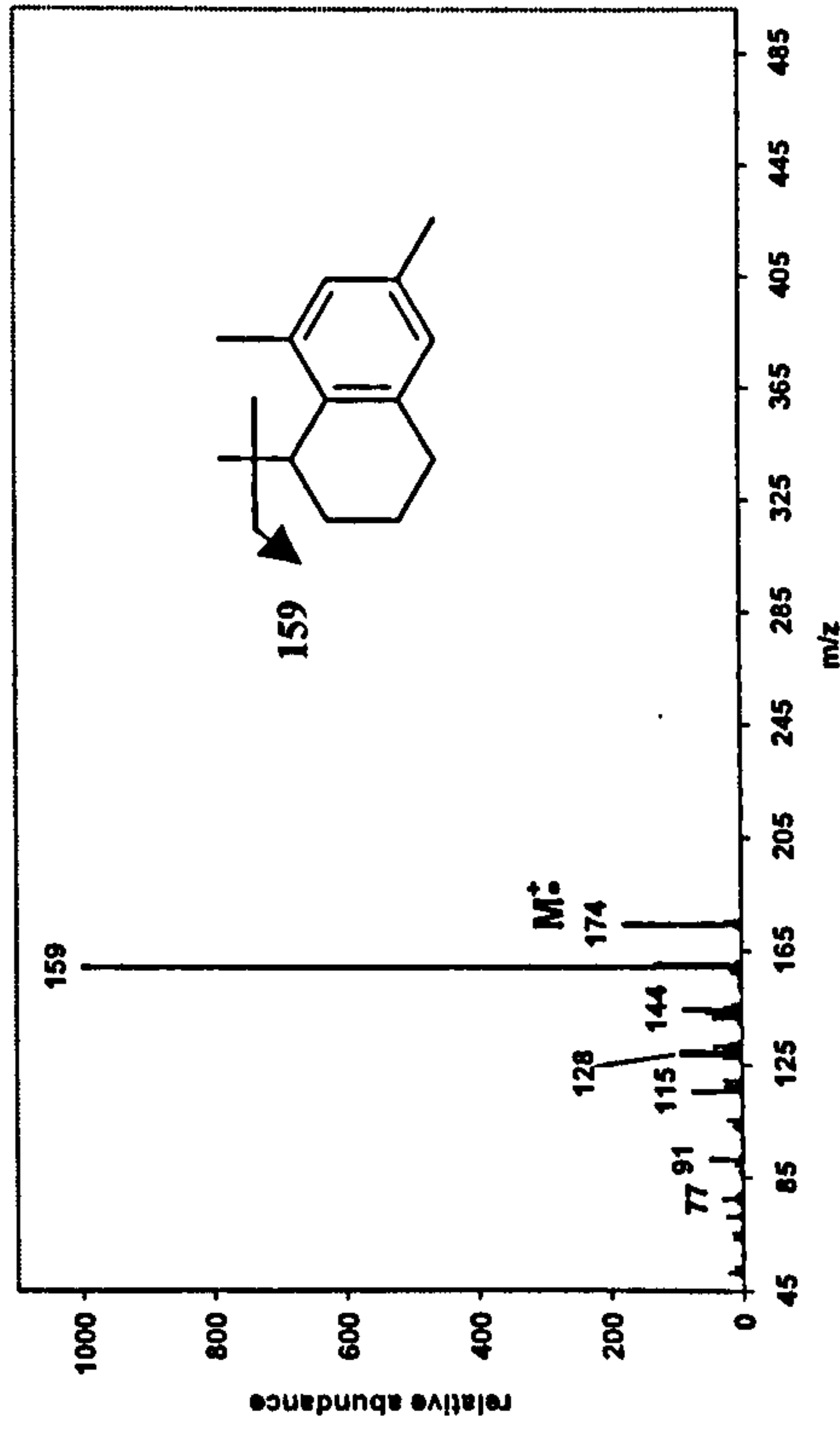


Figure 4.28b. Mass spectrum of the candidate NIST library compound, 1,2,3,4-tetrahydro-1,6,8-trimethyl-naphthalene.

The mass spectra of the unknown compounds **G** and **H** exhibit very close similarities to their suggested NIST library match compounds. However, the library matches indicate that compounds with a m/z 159 base ion may not represent a single homologous series. In this case, there is evidence for the presence of both 2,3-dihydro-tetramethyl-1*H*-indenes (indans) and tetrahydro-trimethyl-naphthalenes (tetralins) in the water soluble monoaromatic UCM sample. Alkylindans and alkyltetralins have been frequently identified in crude oil (*e.g.* Radke *et al.*, 1984; Matisova *et al.*, 1991; Robert *et al.*, 1994; Dixit and Ram 1996; Bastow *et al.*, 1998; Berthod *et al.*, 1998; Pal *et al.*, 1998; Gaines *et al.*, 1999). Sadly, none of the studies reported mass spectra and assignment of the final structures was not possible in most cases.

Generally mono- or dimethyl substituted indans and tetralins are more commonly reported (*e.g.* Williams *et al.*, 1988; Berthod *et al.*, 1998; Pal *et al.*, 1998) than the more highly substituted homologues proposed for **G** and **H** in this study. Bastow *et al.* (1998), who observed highly substituted pentamethyl-tetralins in crude oil, suggested that an increase in substituents would result in their reduced abundance in a crude oil. This low abundance may be the reason for the infrequent reports of such compounds. Furthermore, compounds that contribute to UCMs are generally present in low abundance within a crude oil, thus highly substituted indans and tetralins could be expected in the UCM sample studied herein. Indeed, Radke *et al.* (1984) observed di- and trimethyltetralins in very low abundance in chromatograms exhibiting dominant UCMs. Other isomers and higher homologues may be present but are 'lost' amongst the UCM due to their low abundance.

Matisova *et al.* (1991) identified 82 alkylindans and alkyltetralins in a Russian crude oil, with substitution ranging from C₁ to C₅ for the indans and from C₀ to C₄ for the tetralins. C₄ substituted indans and C₃ substituted tetralins both have molecular weights of 174. This is identical to components of the water soluble monoaromatic UCM. It is therefore possible

that unknown compounds **G** and **H** are two of the indan/tetralin isomers identified by Matisova *et al.* (1991).

Frysinger and Gaines (1999) have reported indans and tetralins in the GCxGC-MS analysis of diesel fuels. The ordered nature of the 2-dimensional chromatograms generated aided in compound class identification. The saturated ring in the indan and tetralin structures increases their polarity with respect to that of the alkylbenzenes. Thus, the alkylindans and alkyltetralins have longer second-dimension retention times than the alkylbenzenes (Frysinger and Gaines, 1999). In contrast, alkylindenes have longer retention times than alkylindans and alkyltetralins, as an additional double bond renders them more polar. In the current study, comparison of the peak marker plot (Figure 4.26) to that of the alkylindenes (Figure 4.23, Section 4.3.3) and alkylbenzenes (Figure 4.12, Section 4.3.1) reveals the same distribution. However, if the peak marker plot in Figure 4.26 contains a mixture of alkylindans and alkyltetralins there is little noticeable difference in their second-dimension retention time. This indicates that both types of compound exhibit very similar polarity.

Trimethyl-indans and tetramethyl-tetralins both exhibit a high degree of substitution, which increases resistance to biodegradation (*e.g.* Bayona *et al.*, 1986; Garrett *et al.*, 1999). Indeed, during a 28 day biodegradation study of Arabian crude oil, 1,1,3-trimethyl-indan was the least degraded (~9%) of all the components monitored (Del'Arco and Franca, 1999). Given the biodegraded nature of the sample studied, such bioresistant compounds are perhaps reasonable candidates as components of the monoaromatic UCM.

Compounds **G** and **H** seemingly correspond to a homologous series of tetramethyl-indans, a homologous series of trimethyl-tetralins or a mixture of both. The peak marker plot (Figure 4.26) indicates that a number of structural isomers are present, particularly for

those compounds with a molecular weight of 174. In the case of the C₄-indans, there are at least 119 structural isomers (including branched and linear), although it is not known how many of them exhibit a *m/z* 159 base ion following electron impact MS analysis. There are at least 70 C₃-tetralin structural isomers (including branched and linear). However, some of these, such as those with a linear C₃ chain (1-propyl-tetralin and 6-propyl-tetralin; NIST library) do not fragment to a *m/z* 159 base ion. Thus, in both homologous series of compounds, the total number of isomers with a molecular weight of 174 far exceeds the number of compounds observed in the peak marker plot (Figure 4.26). It is possible that either the C₃-tetralins or the C₄-indans, but most likely a combination of both, are present in this UCM sample given its complexity. The use of authentic reference compounds could perhaps confirm the characterisation of these compounds.

Only three compounds with molecular weights greater than 174 were observed (Figure 4.26). Their second-dimension retention times were similar to that of the other compounds, which indicates they are part of the same homologous series. A similar 'cut-off' point to that of the alkylindenes (Section 4.3.3) was also seen in this peak marker plot (Figure 4.26), although the molecular weight 'cut-off' point for the alkylindans/tetralins appeared to be slightly lower than that of the alkylindenes. Alkylindenes contain an additional double bond, which increases their polarity and this in turn would increase their water solubility compared to alkylindans/tetralins of a similar molecular weight. Hence they possess a higher 'cut-off' point.

4.3.5 Compounds with base ions of m/z 167 and m/z 181

A peak marker plot of the compounds exhibiting m/z 167 and m/z 181 base ions is presented in Figure 4.29. Figure 4.30a shows the mass spectrum of **I**, whilst Figure 4.30b shows the mass spectrum of 2,2'-dimethylbiphenyl, proposed as the best NIST library match. Figure 4.31a shows the mass spectrum of **J**, with Figure 4.31b showing the mass spectrum of 1,1'-(3,3-dimethylbutylidene) bis-benzene. This is suggested as the best match from the NIST library for **J**. Figure 4.32a shows the mass spectrum of **K** and Figure 4.32b the mass spectrum of 3,5-dimethyl-1-(phenylmethyl)-benzene from the NIST library. Figure 4.33a shows the mass spectrum of **L**, whilst Figure 4.33b shows the mass spectrum of 4-isopropyl-biphenyl, suggested as the best match from the NIST library.

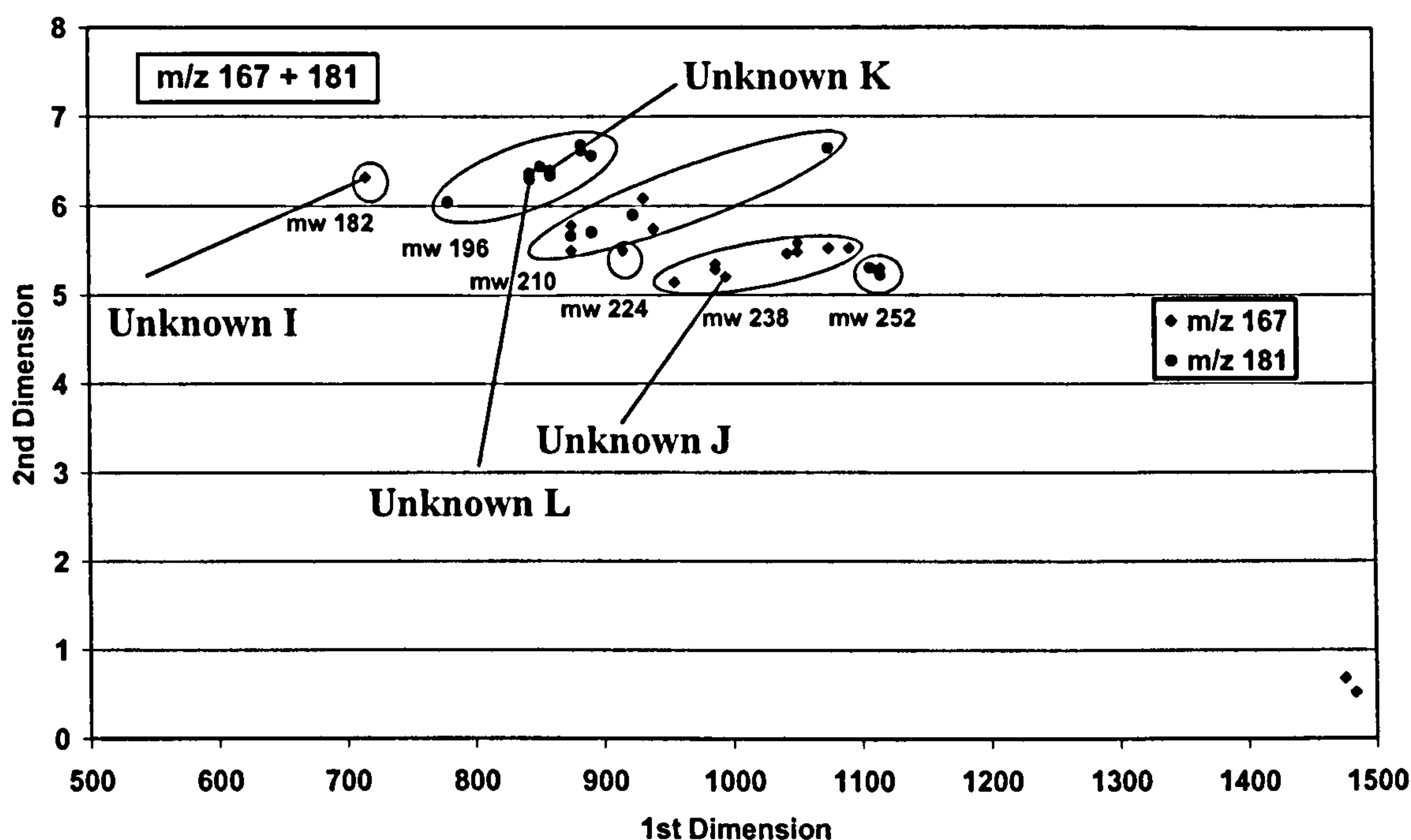


Figure 4.29. Peak marker plot of the compounds present in the water soluble monoaromatic UCM exhibiting base ions of m/z 167 and m/z 181. Those compounds displaying the same molecular weight are grouped together.

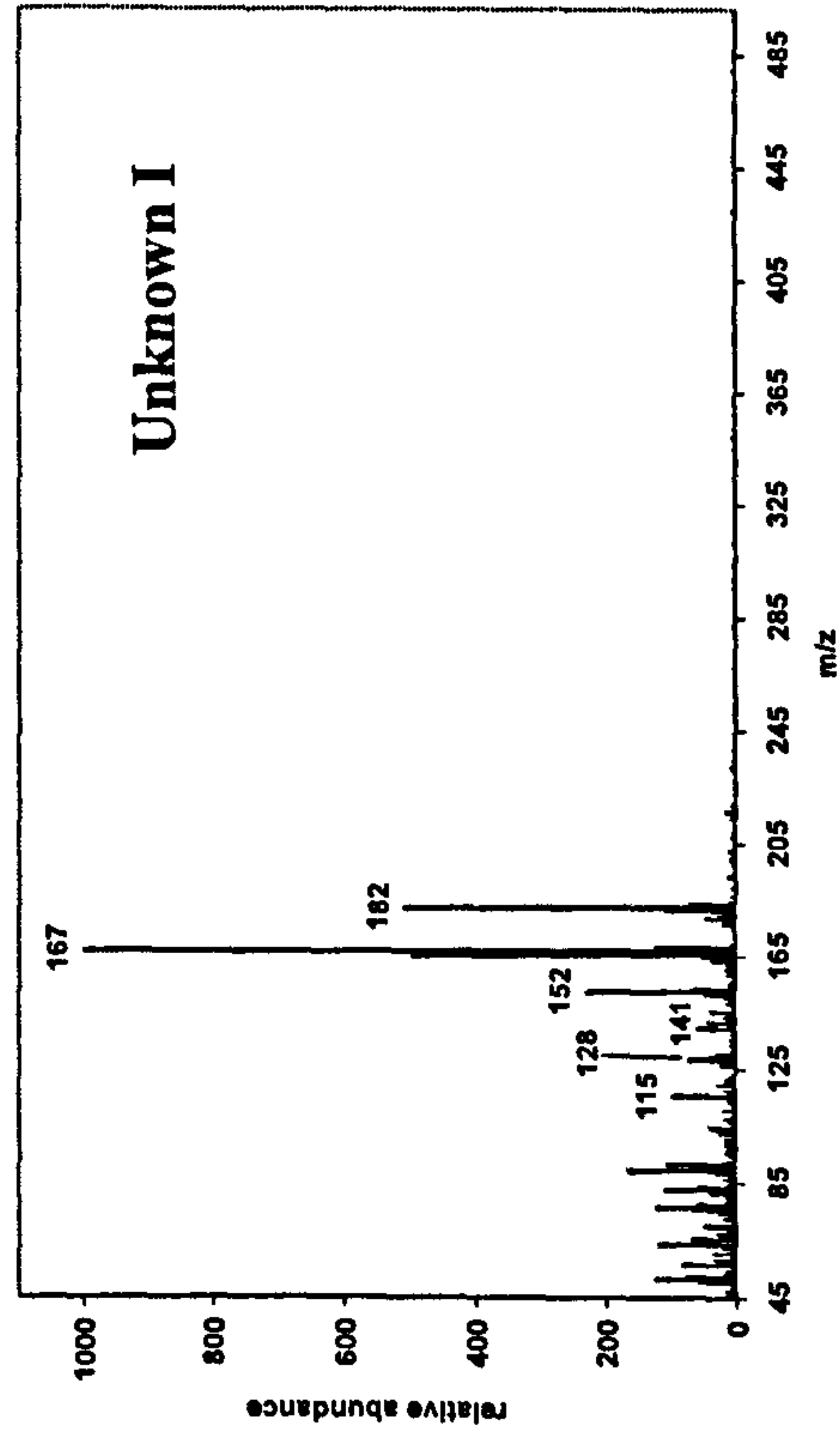


Figure 4.30a. Mass spectrum of the unknown compound **I**, which has a retention time of 716,6.32.

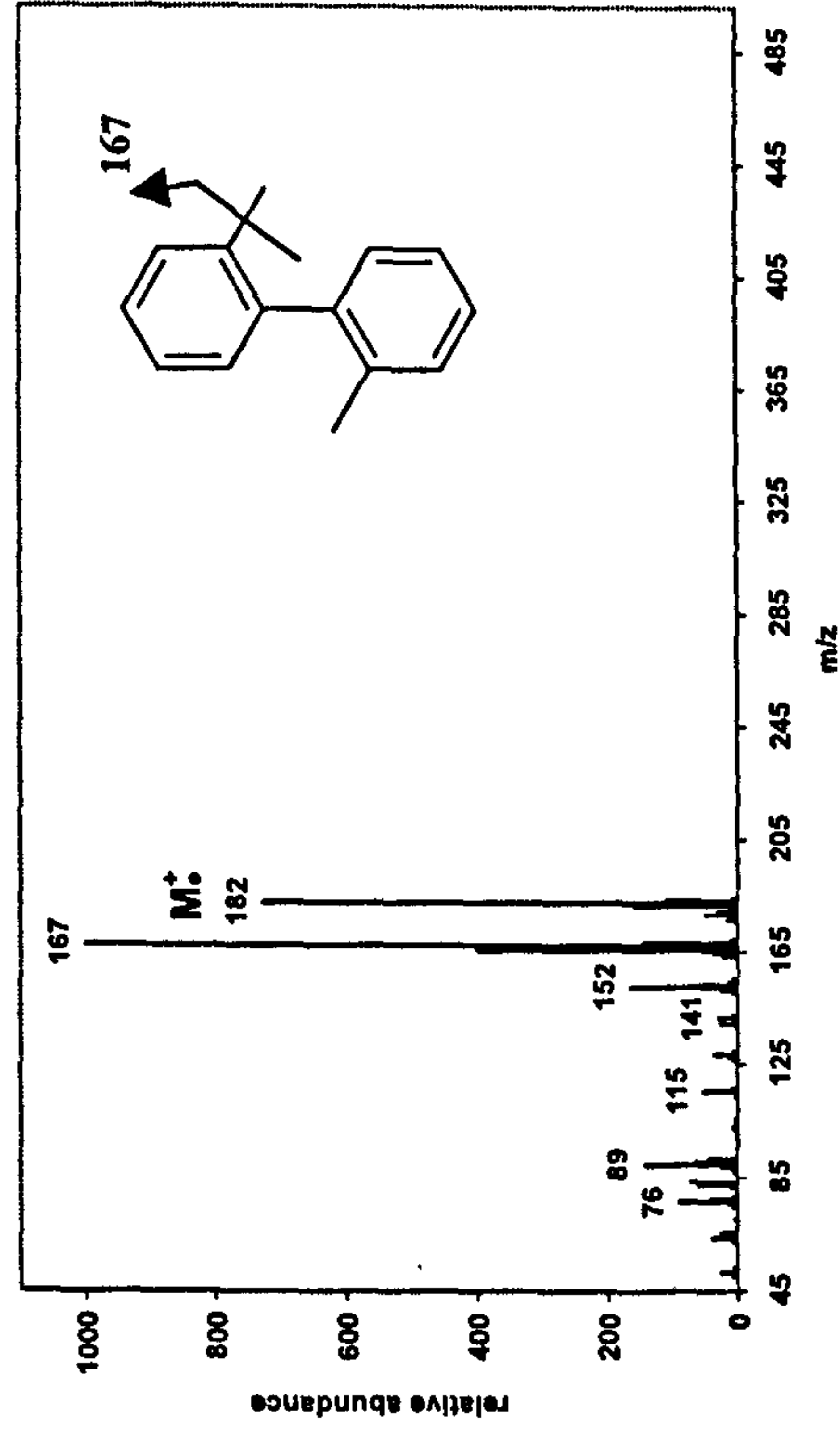


Figure 4.30b. Mass spectrum of the candidate NIST library compound, 2,2'-dimethylbiphenyl.

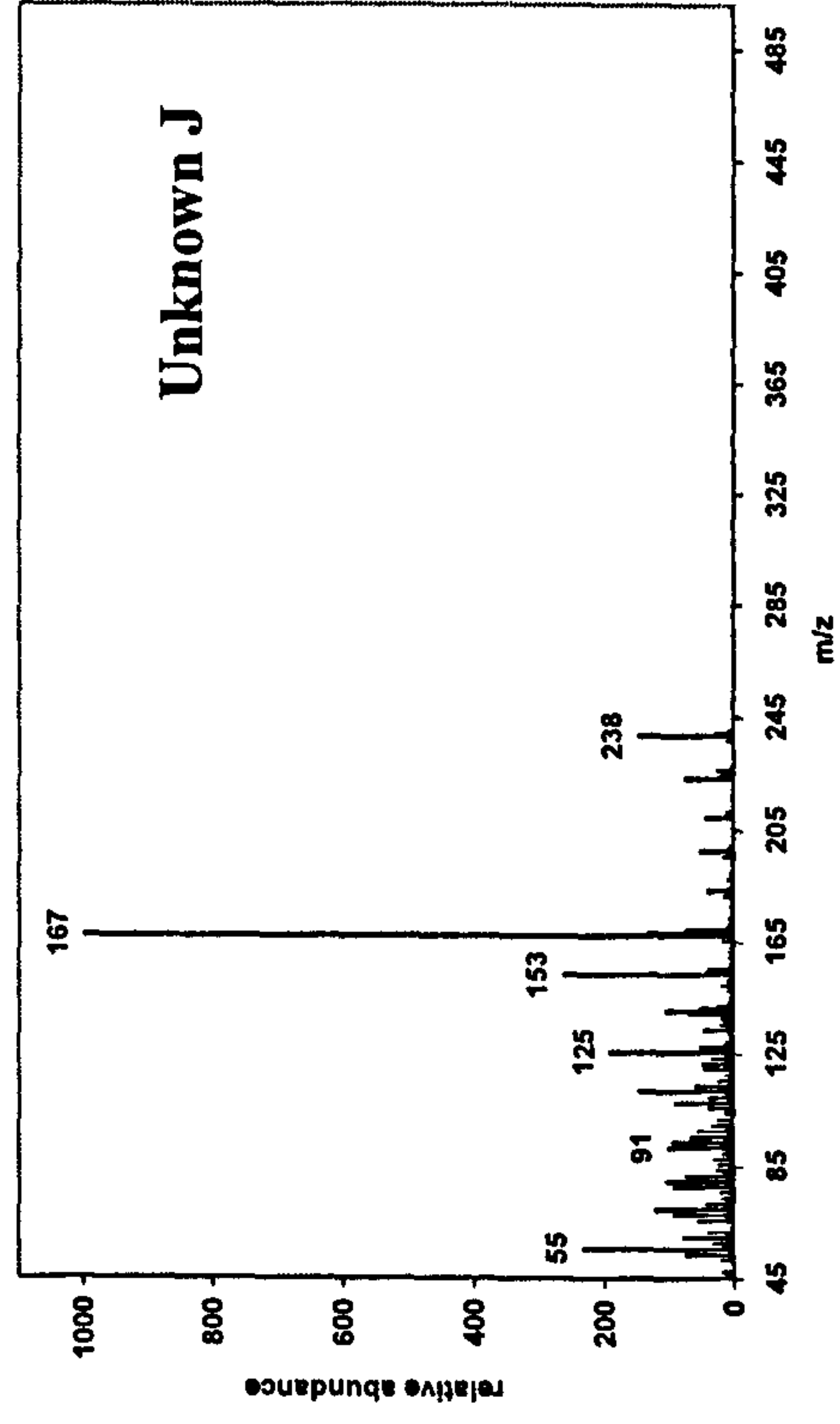


Figure 4.31a. Mass spectrum of the unknown compound **J**, which has a retention time of 996,5.2.

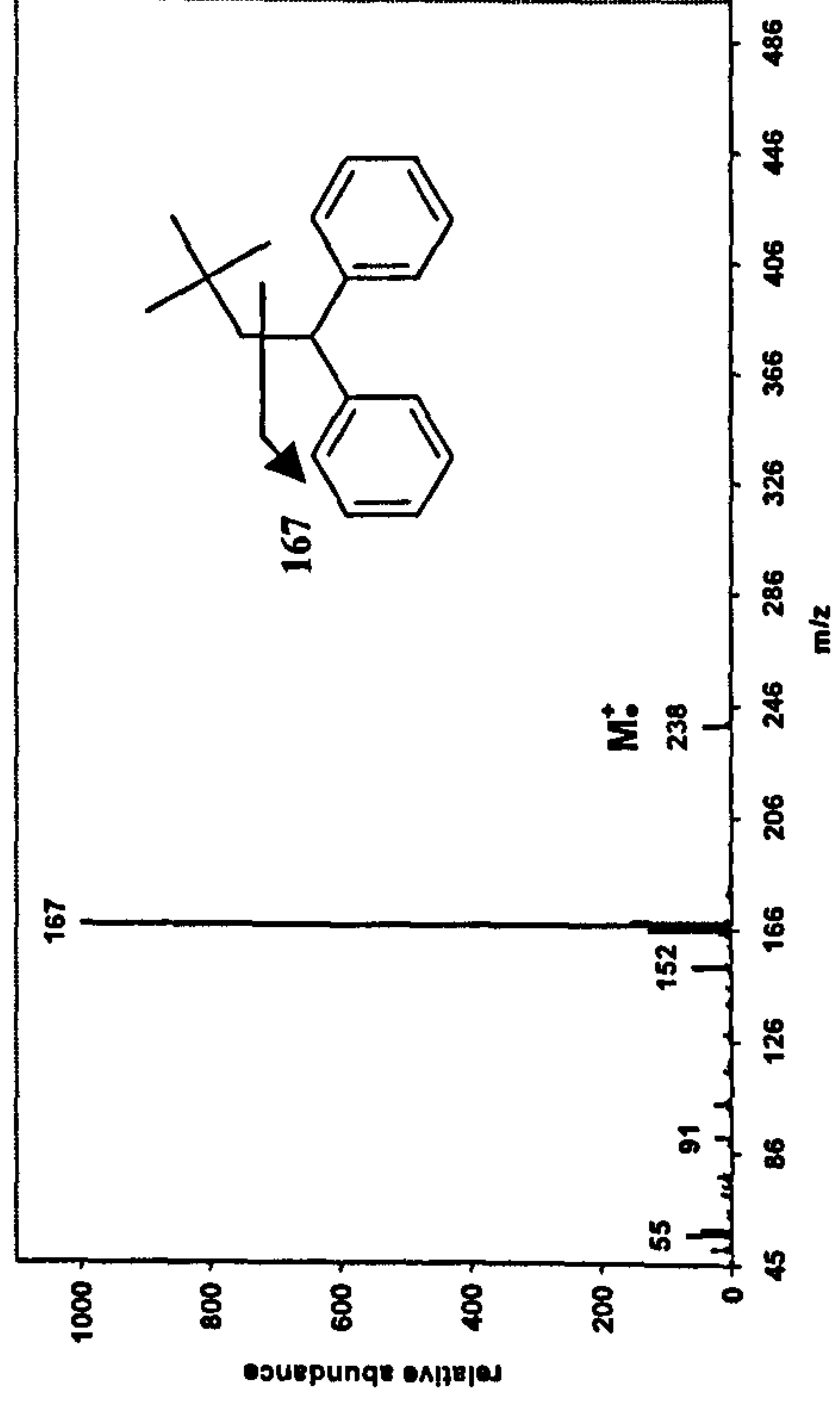


Figure 4.31b. Mass spectrum of the candidate NIST library compound, 1,1'-(3,3-dimethylbutylidene) bis-benzene.

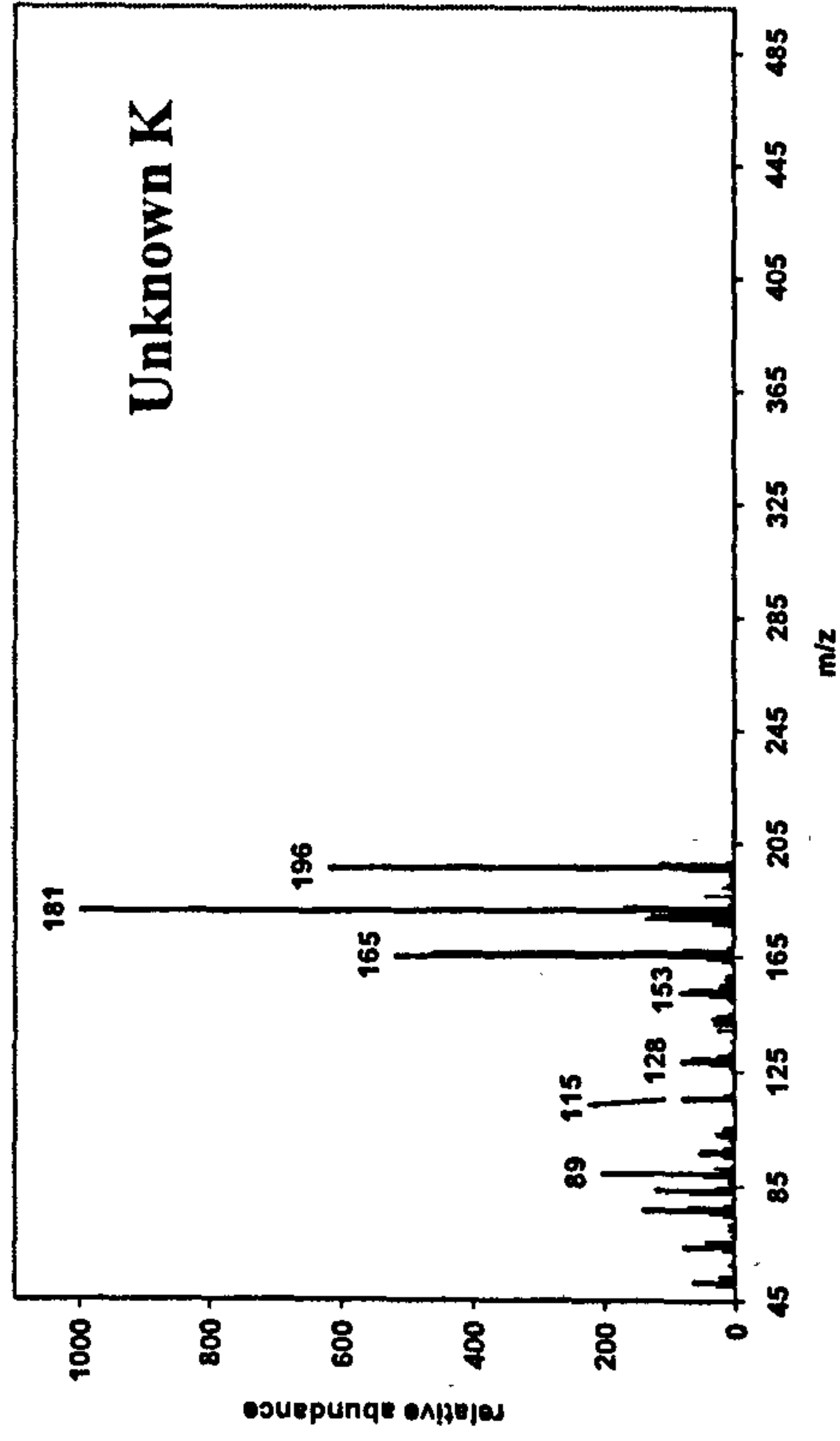


Figure 4.32a. Mass spectrum of the unknown compound K, which has a retention time of 860,6.34.

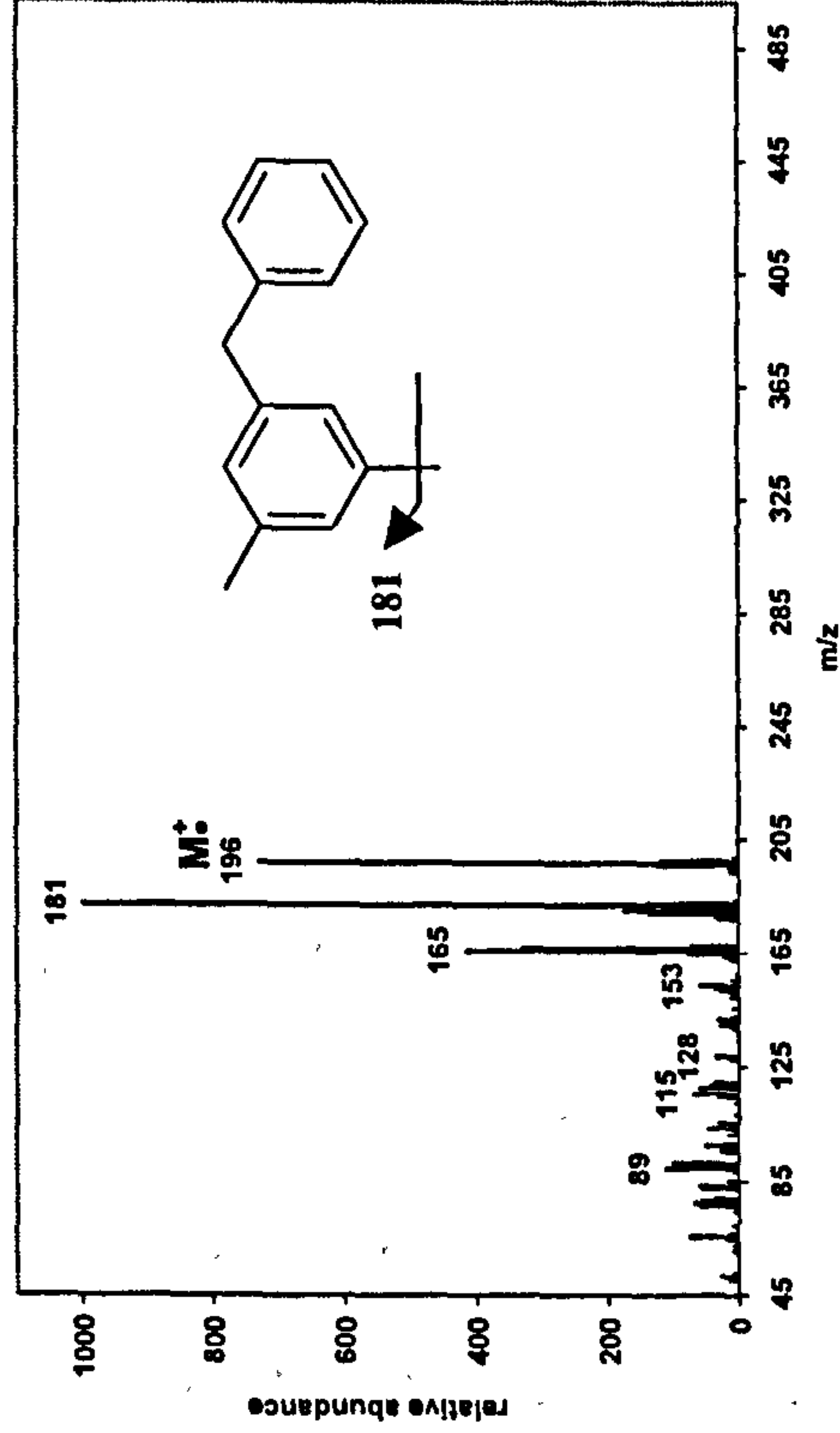


Figure 4.32b. Mass spectrum of the candidate NIST library compound, 3,5-dimethyl-1-1-(phenylmethyl)-benzene.

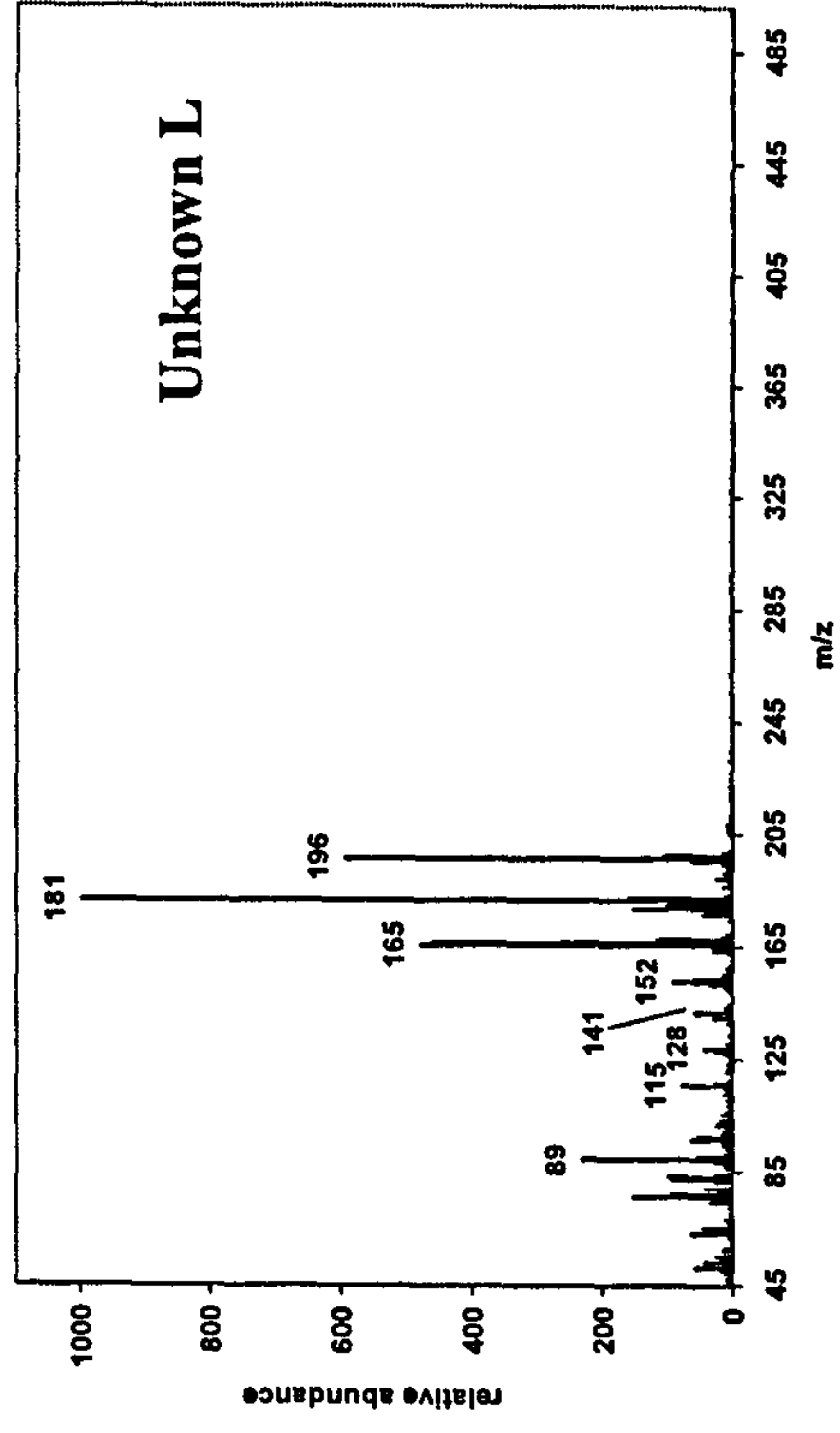


Figure 4.33a. Mass spectrum of the unknown compound L, which has a retention time of 844,6.3.

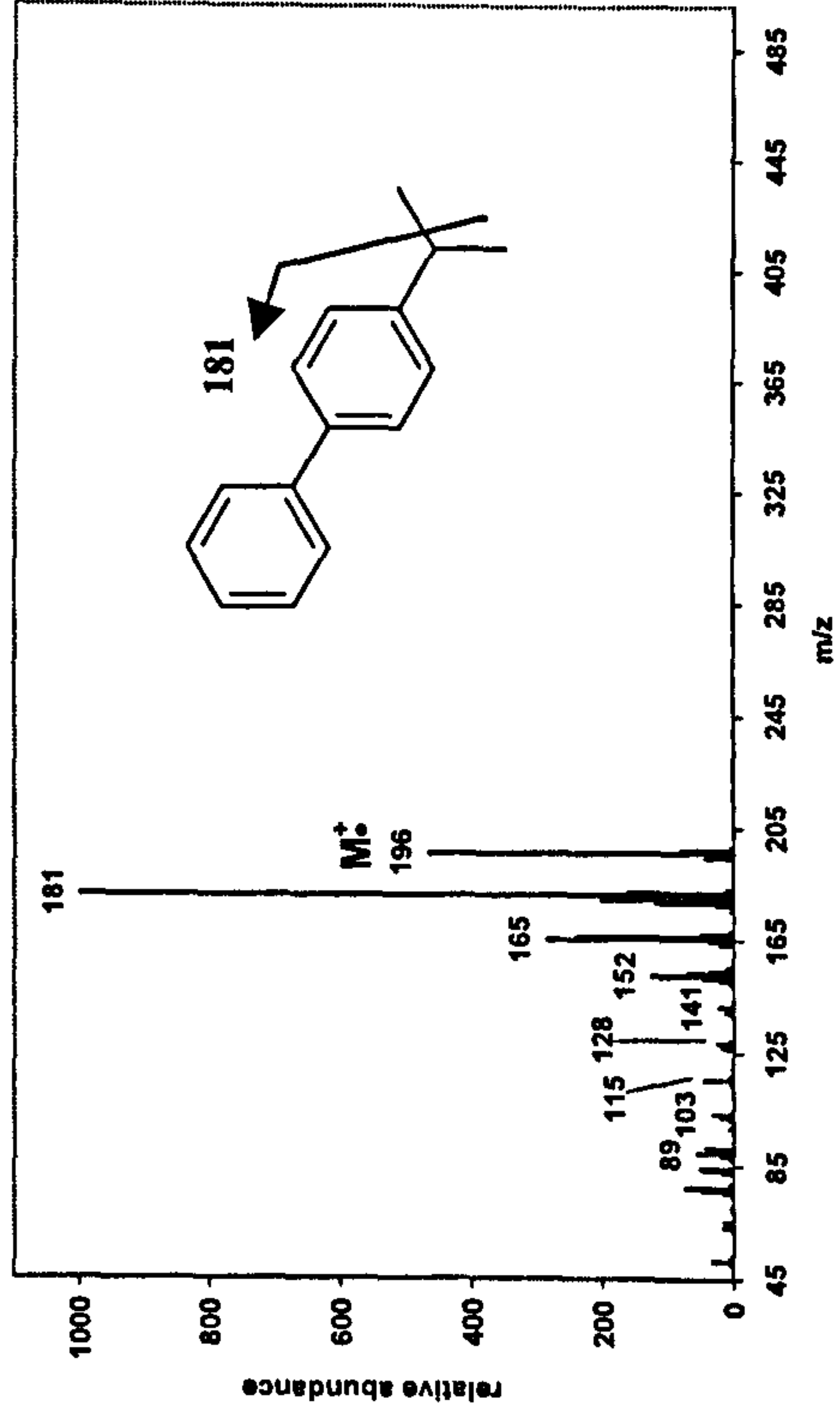


Figure 4.33b. Mass spectrum of the candidate NIST library compound, 4-isopropyl-biphenyl.

Those compounds with m/z 167 or m/z 181 base ions appear to correspond with compounds exhibiting very similar physicochemical properties, given their y -axis retention times on the peak marker plot (Figure 4.29). Indeed, when plotted together they form a band of compounds increasing in molecular weight by 14 mass units from 182 – 252. Thus, it was proposed that both groups corresponded to the same homologous sequence(s) of compounds. Comparison of unknown mass spectra (I – L) to NIST library references indicated that this was the likely scenario. Unknowns I – L exhibited to close mass spectral matches to two types of compound, alkylbiphenyls and alkyldiphenylmethanes. Library compounds 1,1'-(3,3-dimethylbutylidene) bis-benzene and 3,5-dimethyl-1-(phenylmethyl)-benzene are examples of alkyldiphenylmethanes.

Adams and Richardson (1953) were possibly the first to identify biphenyl and one of its methylated homologues (3-methylbiphenyl) in crude oil. Since then, low molecular weight alkylbiphenyls (*e.g.* C₁-C₃ substituted) have been frequently observed in crude oils (*e.g.* White and Lee, 1980; Alexander *et al.*, 1986; Blanco *et al.*, 1991; Alexander *et al.*, 1994; Lai and Song, 1995; Pal *et al.*, 1998; Jiang and Li, 2002). In contrast, higher substituted homologues are only rarely reported (*e.g.* Mair and Mayer, 1964; Yew and Mair, 1966; Trolio *et al.*, 1999). Unfortunately, very limited data regarding alkyldiphenylmethanes is available in the literature (*e.g.* Anders *et al.*, 1975; Lacotte *et al.*, 1996; Trolio *et al.*, 1996; Trolio *et al.*, 1999; Jiang and Li, 2002).

Whilst they have been suggested as common constituents of crude oil (Trolio *et al.*, 1996), diphenylmethane and its alkylated homologues generally occur in low abundance (*e.g.* Jiang and Li 2002). The concentration of alkylated biphenyls is also typically low in crude oils. For example, Yew and Mair (1966) determined a range of 0.0006-0.0063%, whilst Adams and Richardson (1953) estimated that the quantity of biphenyl in the crude oil was ~0.003%. Such low abundance may explain why alkylated biphenyls are present in the

UCM studied herein. Interestingly, Lacotte *et al.* (1996) observed small amounts of alkyl diphenylmethanes and biphenyls were released from the asphaltene matrix during biodegradation. These compounds appear to have been trapped in the asphaltene matrix prior to the degradation. In heavily biodegraded crude oils where UCMs predominate, this may be a source of UCM components.

Cumbers *et al.* (1986) identified all of the possible, methyl-, ethyl- and dimethyl-biphenyl isomers in a range of Australian crude oils. Isomers substituted at the *meta* position(s) were the most abundant, whilst *ortho*-substituted isomers the least. The order of stability for methyl-, ethyl- and dimethyl-biphenyls is reported as *meta*>*para*>*ortho* (Cumbers *et al.*, 1987). The authors suggest the thermodynamic stability of the individual isomers is responsible for this distribution pattern. A similar isomeric distribution for alkylbiphenyls has been reported in other studies investigating thermodynamic stability of crude oils (*e.g.* Alexander *et al.*, 1986; Cumbers *et al.*, 1987; Alexander *et al.*, 1994).

Trolio *et al.* (1999) studied the effect of crude oil biodegradation on the isomeric distribution of alkylbiphenyls and alkyldiphenylmethanes. C₁-C₂ biphenyls and C₁ diphenylmethanes with substitution at the *para*-position became more abundant with increasing biodegradation, indicating that they are the most bioresistant. The trimethyl-biphenyls are even more resistant to biodegradation due to their increased substitution, but the order of resistance between individual isomers was less obvious. However, the most sterically hindered conformations, suggested by Trolio *et al.* (1999) as those with *ortho*-substitution, were considered the least susceptible to biodegradation. This positional effect of the substituents upon the rate of microbial degradation has previously been observed for alkylated PAH (*e.g.* Budzinski *et al.*, 1998; Alexander, 1999; Holder *et al.*, 1999; Leblond *et al.*, 2001).

Trolio *et al.* (1999) observed that C₂-biphenyls and C₁-diphenylmethanes (mw 182) were absent in highly biodegraded crude oils, but that C₃-biphenyls (mw 196) were still present. A similar trend is apparent in the peak marker plot (Figure 4.29), where only one compound of molecular weight 182 but nine peak markers exhibiting molecular weights of 196 occur. There are 19 possible C₂-biphenyl and C₁-diphenylmethane (mw 182) isomers. In contrast, 68 C₂-diphenylmethane and C₃-biphenyl (mw 196) isomers are possible. The compounds in the peak marker plot (Figure 4.29) could correspond to isomers exhibiting substitution at the *ortho*-position, as they are the most bioresistant (Trolio *et al.*, 1999).

The number of alkylbiphenyl and alkyldiphenylmethane isomers far exceeds those seen in the peak marker plot (Figure 4.29). However, as the present sample is heavily biodegraded, removal or depletion of *meta*- and *para*-substituted isomers and preservation of the least abundant *ortho*-isomers could be expected to have occurred. This would result in those isomers which are abundant in non-biodegraded crude oils (*e.g.* Trolio *et al.*, 1999) being depleted or removed leaving only the bioresistant isomers initially present in low abundance. This provides the ideal conditions for the occurrence of a UCM. Thousands of compounds in low abundances, many of which are isomeric, all sharing very similar physicochemical properties. The peak marker plot (Figure 4.29) clearly indicates that only a few of the possible biphenyl and diphenylmethane isomers appear to remain in heavily biodegraded crude oils and UCMs.

It should be noted that not all of the alkylbiphenyl and alkyldiphenylmethane isomers present in the water soluble monoaromatic UCM analysed in the present study will be in the peak marker plot (Figure 4.29). It might be easy to assume that the mass spectra of structural isomers are very similar. However, mass spectral comparison of dimethylbiphenyl isomers in the NIST library indicates that whilst some isomers do exhibit almost identical mass spectra (*e.g.* 2,2' and 2,3') others show major differences. Therefore,

mass spectra can be diagnostic for individual dimethylbiphenyl isomers. Study of the NIST library mass spectra also indicated that not all dimethylbiphenyl isomers exhibit a base ion of m/z 167. In some cases, the molecular ion (182) is also the base ion, and these isomers would not appear in the peak marker plot (Figure 4.29). The trimethylbiphenyls can have base ions of m/z 181 or m/z 196 (also the molecular ion), although deviations from this can occur when substitution involves an ethyl or propyl group.

Hellou *et al.* (1994) monitored the bioaccumulation of crude oil in the mussel tissue of winter flounder. UV fluorescence and GC-MS analysis indicated the presence of C₂-biphenyls, but they were difficult to identify amongst the few resolved hydrocarbons in the sample. It was suggested that alkylbiphenyls occurred in the unsaturated UCM present in the mussel extract. Such components of crude oil appear to be bioavailable to marine organisms, and may contribute to the toxicity of UCMs reported by Rowland *et al.* (2001) and Donkin *et al.* (2003).

Those compounds present in the water soluble monoaromatic UCM with base ions of m/z 167 and m/z 181 appear to correspond to either a homologous series of alkylbiphenyls, alkyldiphenylmethanes, or a mixture of both. Reddy *et al.* (2002) and Frysinger *et al.* (2003) used GCxGC analysis to confirm the presence of alkylbiphenyls in a petroleum derived UCM, although characterisation of individual compounds was not possible. Trolie *et al.* (1996) suggest that methyldiphenylmethane isomers all have similar mass spectra, which in turn are similar to the C₂-biphenyls. Furthermore, the retention times of both compound types are very similar, making identification of individual compounds extremely difficult. Thus, the use of authentic reference compounds is essential for thorough characterisation.

4.3.6 Compounds with base ions of m/z 195 and m/z 210

A peak marker plot of the compounds exhibiting m/z 195 and m/z 210 base ions is presented in Figure 4.34. Figure 4.35a shows the mass spectrum of **M**, whilst Figure 4.35b shows the mass spectrum of 1,2,3-trimethyl-4*E*-propenyl-naphthalene, proposed as the best NIST library match. Figure 4.36a shows the mass spectrum of **N**, with Figure 4.36b showing the mass spectrum of 1,2,3,4-tetrahydro-9-propyl-anthracene. This is suggested as the best match from the NIST library for **N**. Figure 4.37a shows the mass spectrum of **O** and Figure 4.37b the mass spectrum of 3,4-diethyl-biphenyl from the NIST library. Figure 4.38a shows the mass spectrum of **P**, whilst Figure 4.38b shows the mass spectrum 1,2,3,4-tetrahydro-9,10-dimethyl-anthracene, suggested as the best match from the NIST library.

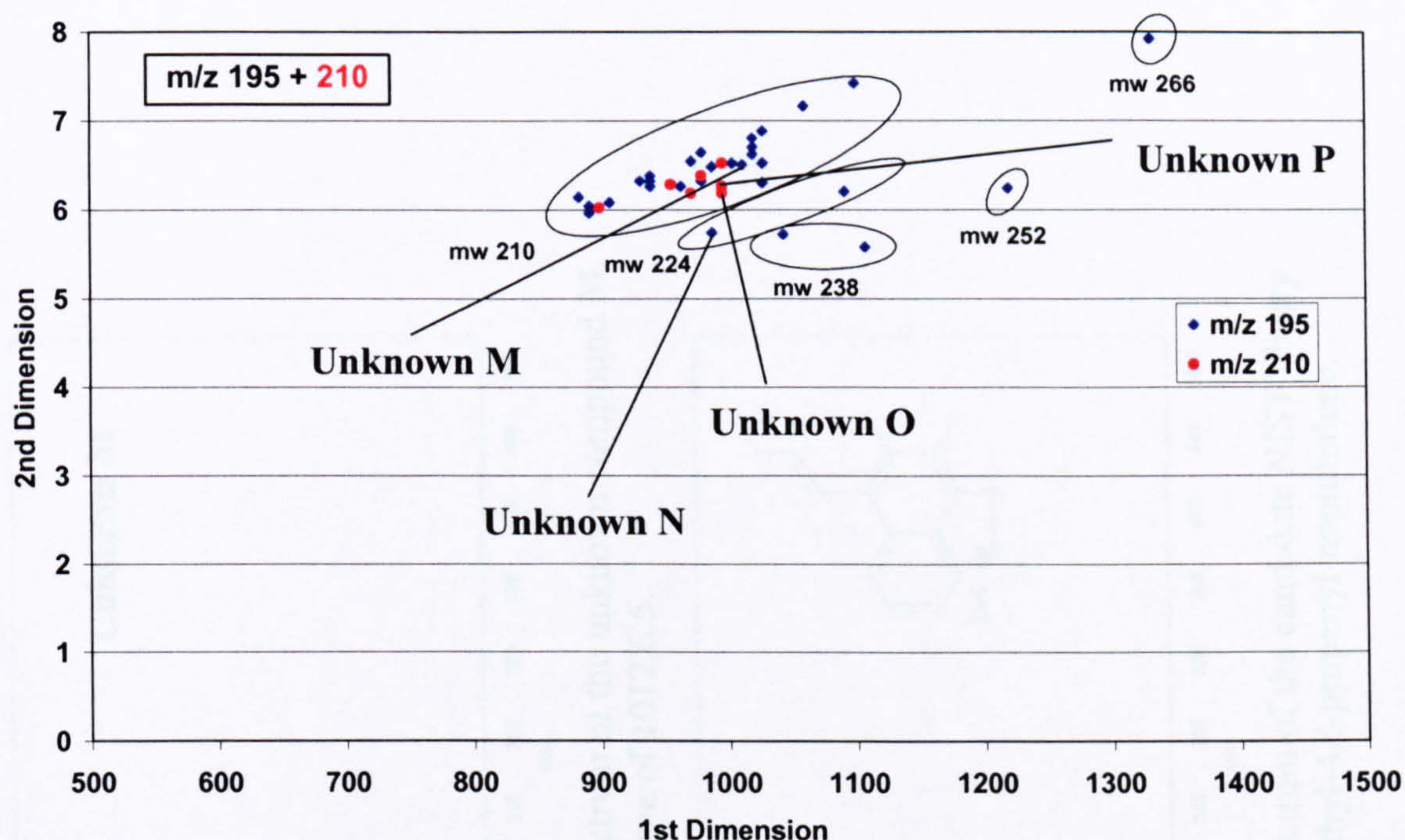


Figure 4.34. Peak marker plot of the compounds present in the water soluble monoaromatic UCM exhibiting base ions of m/z 195 and m/z 210. Those compounds displaying the same molecular weight are grouped together.

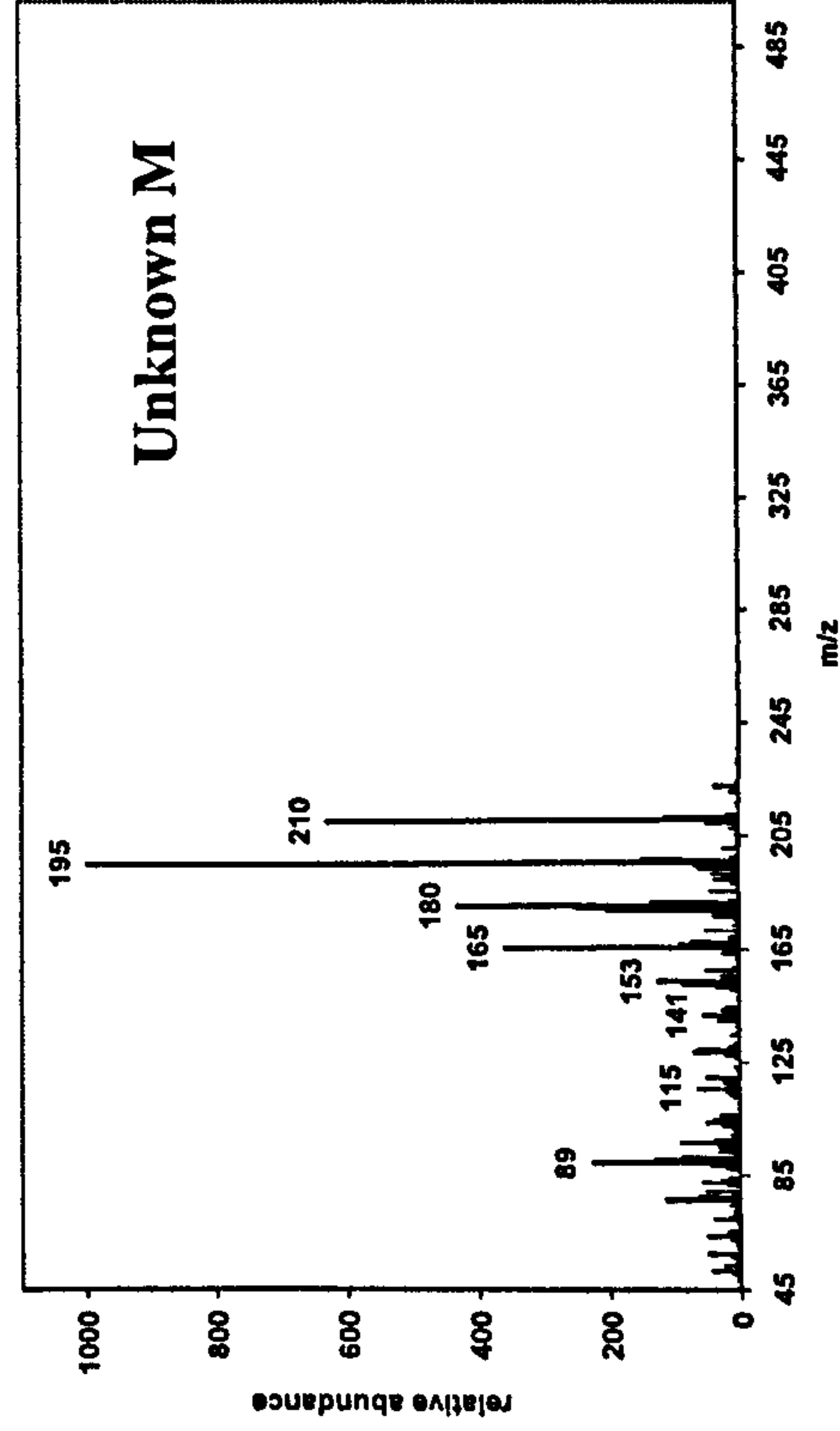


Figure 4.35a. Mass spectrum of the unknown compound M, which has a retention time of 1012,6.5.

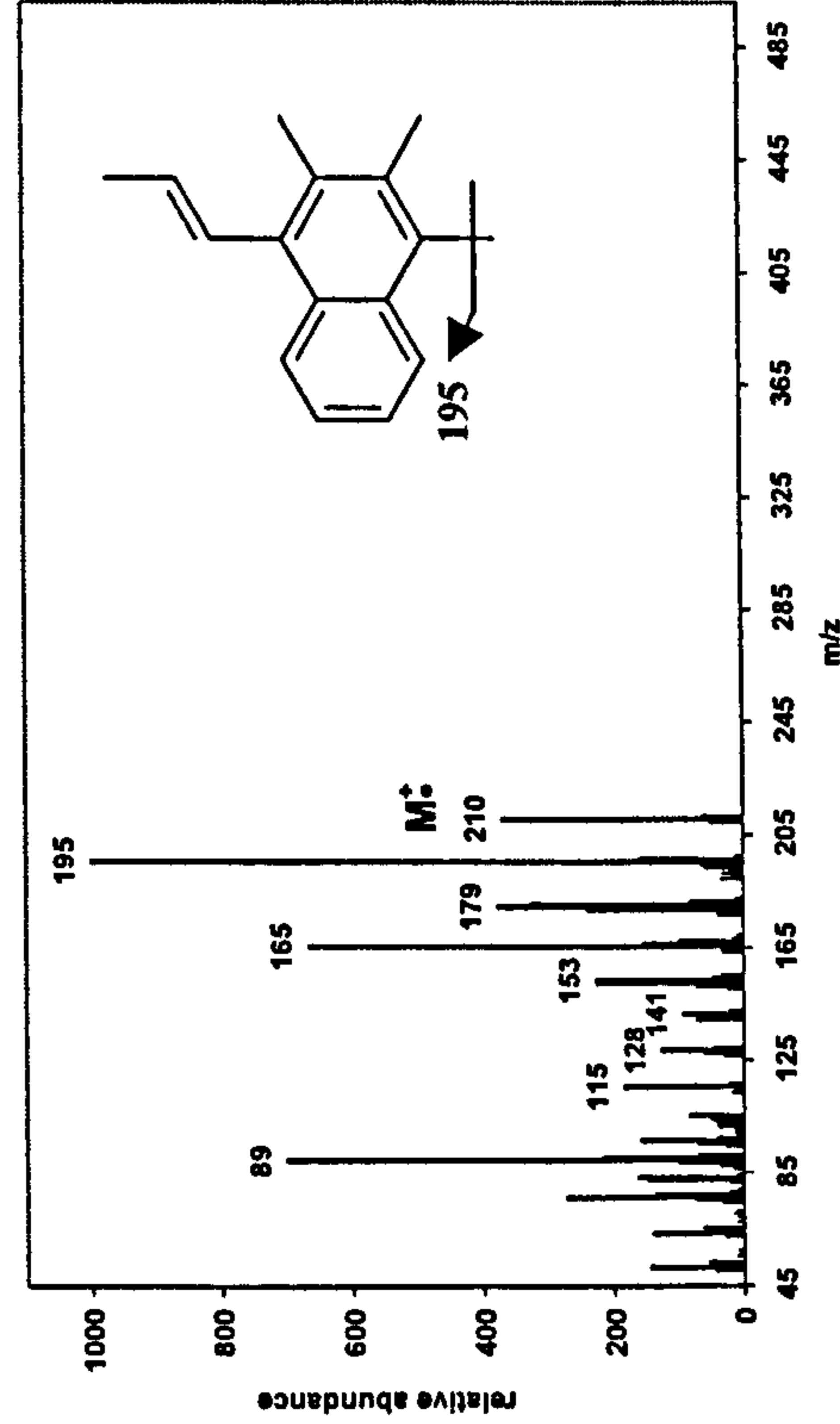


Figure 4.35b. Mass spectrum of the candidate NIST library compound, 1,2,3-trimethyl-4*E*-propenyl-naphthalene.

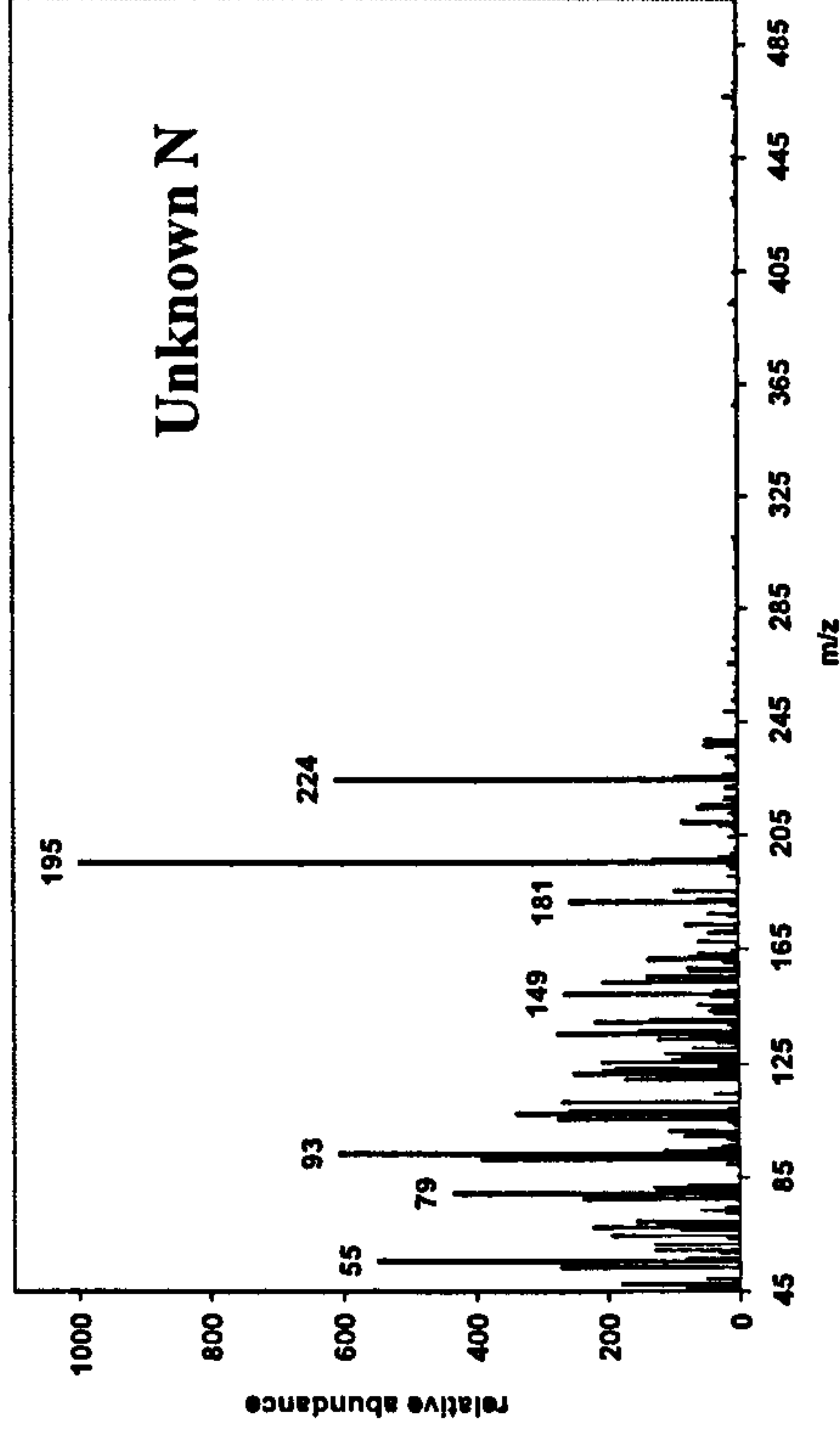


Figure 4.36a. Mass spectrum of the unknown compound N, which has a retention time of 988,5.74.

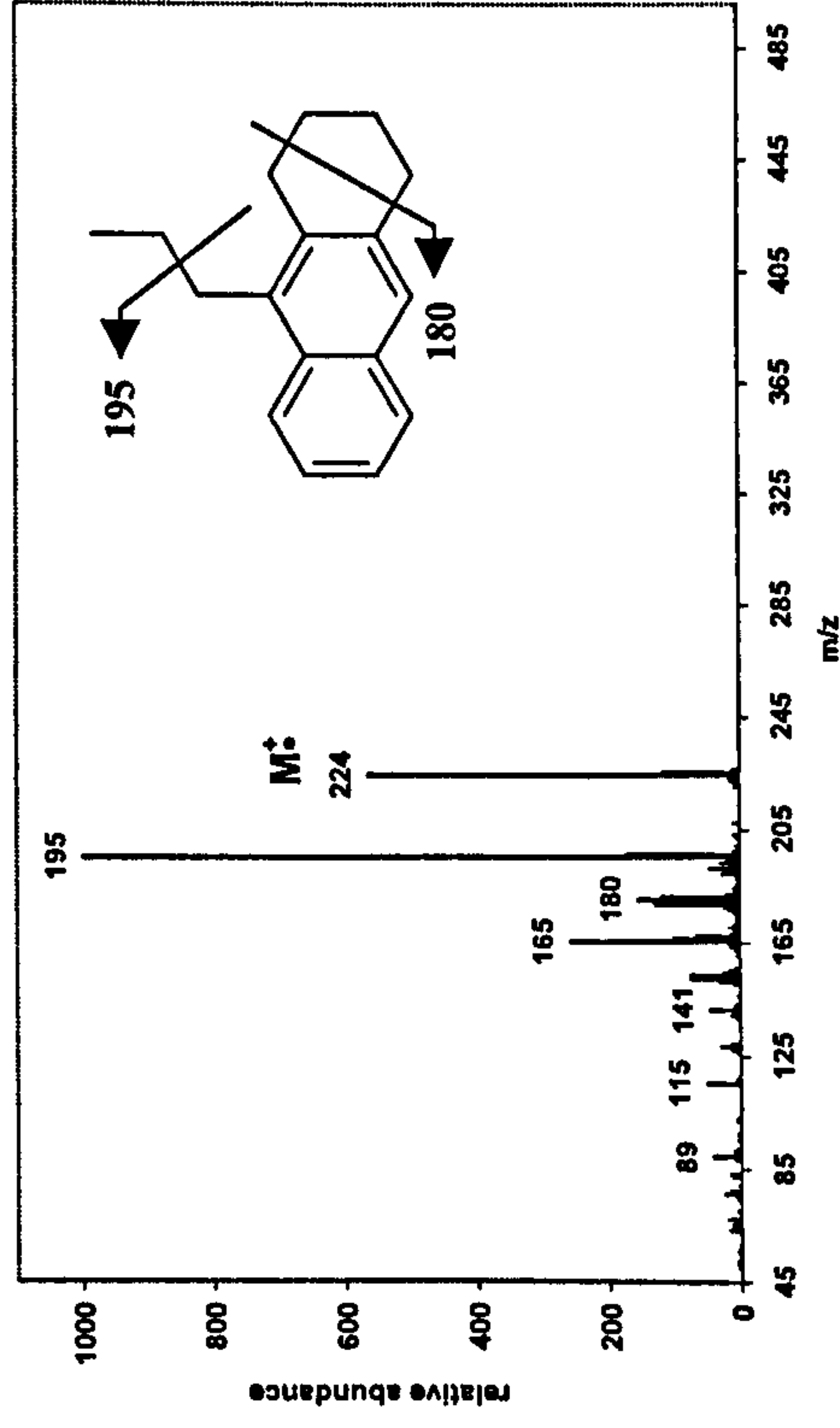


Figure 4.36b. Mass spectrum of the candidate NIST library compound, 1,2,3,4-tetrahydro-9-propyl-anthracene.

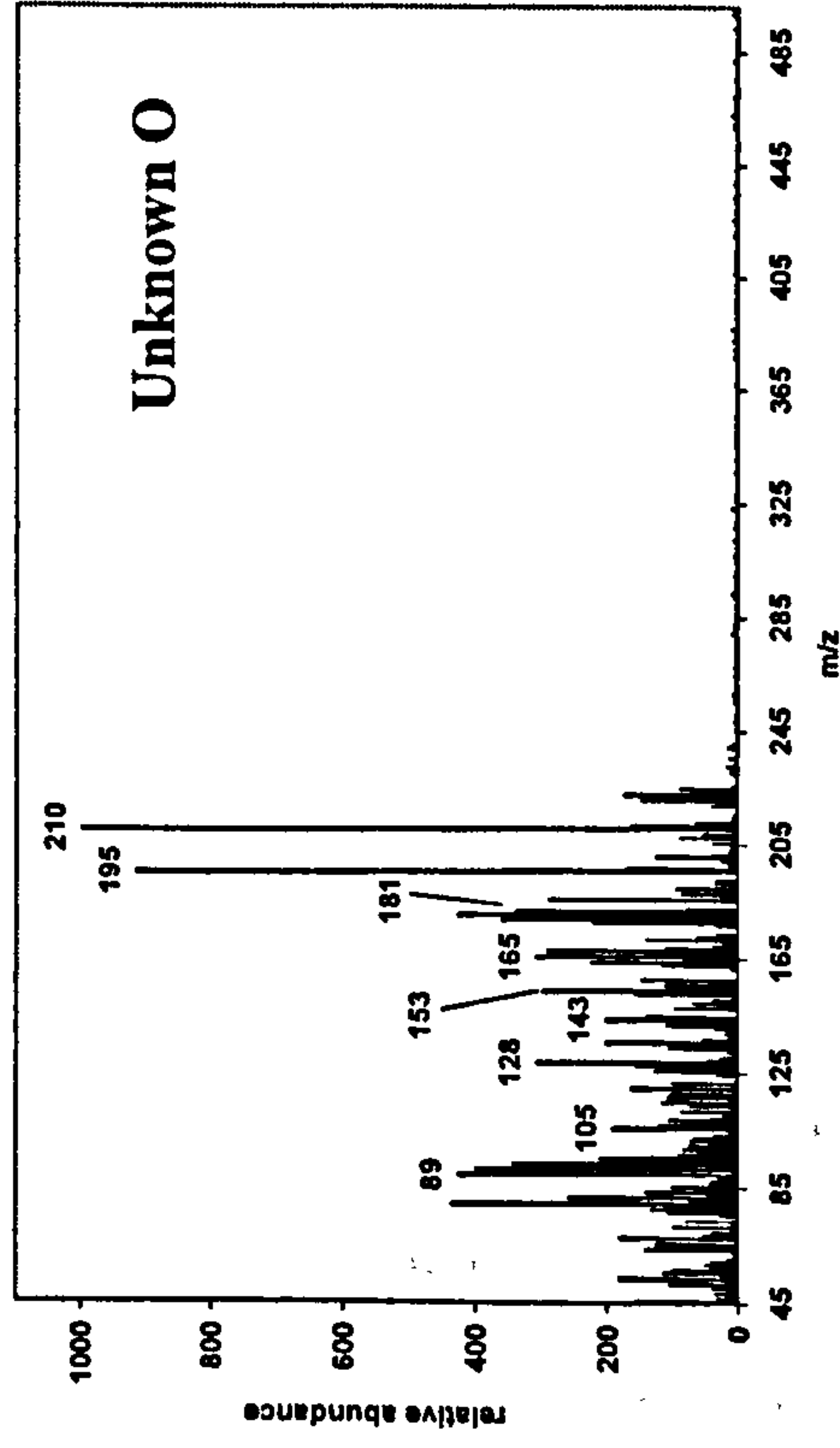


Figure 4.37a. Mass spectrum of the unknown compound **O**, which has a retention time of 996,6.18.

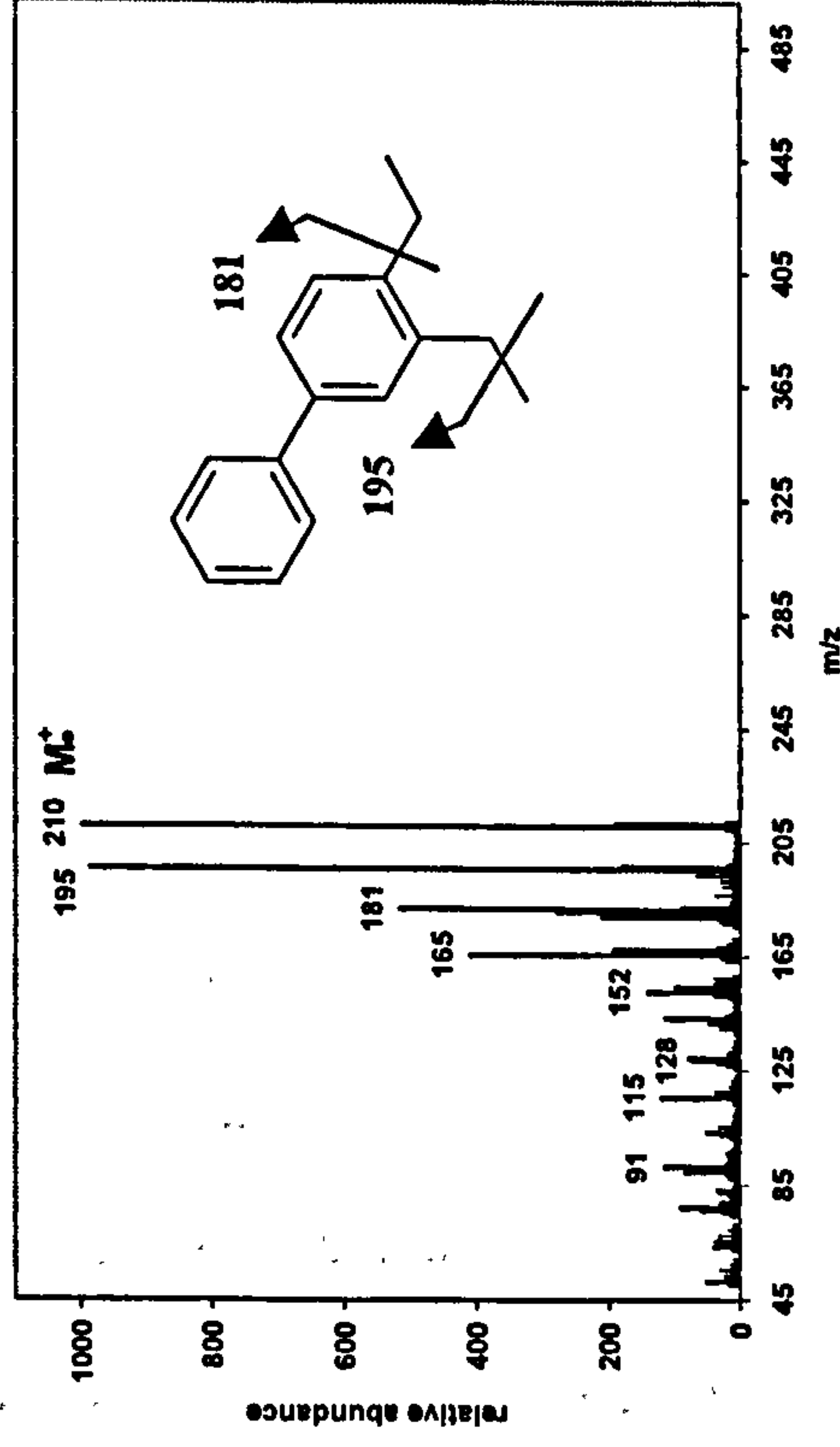


Figure 4.37b. Mass spectrum of the candidate NIST library compound, 3,4-diethyl-biphenyl.

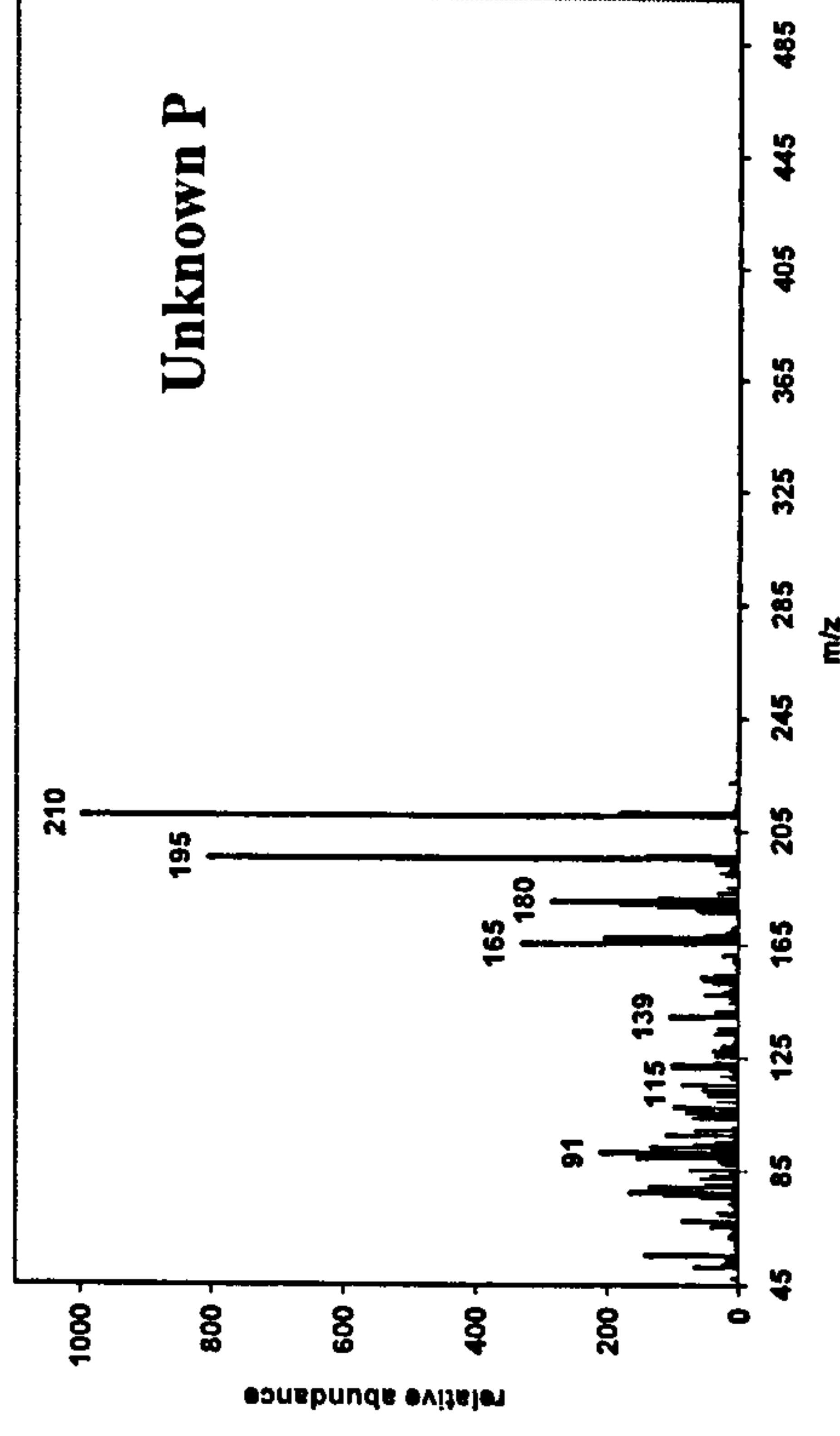


Figure 4.38a. Mass spectrum of the unknown compound **P**, which has a retention time of 996,6.26.

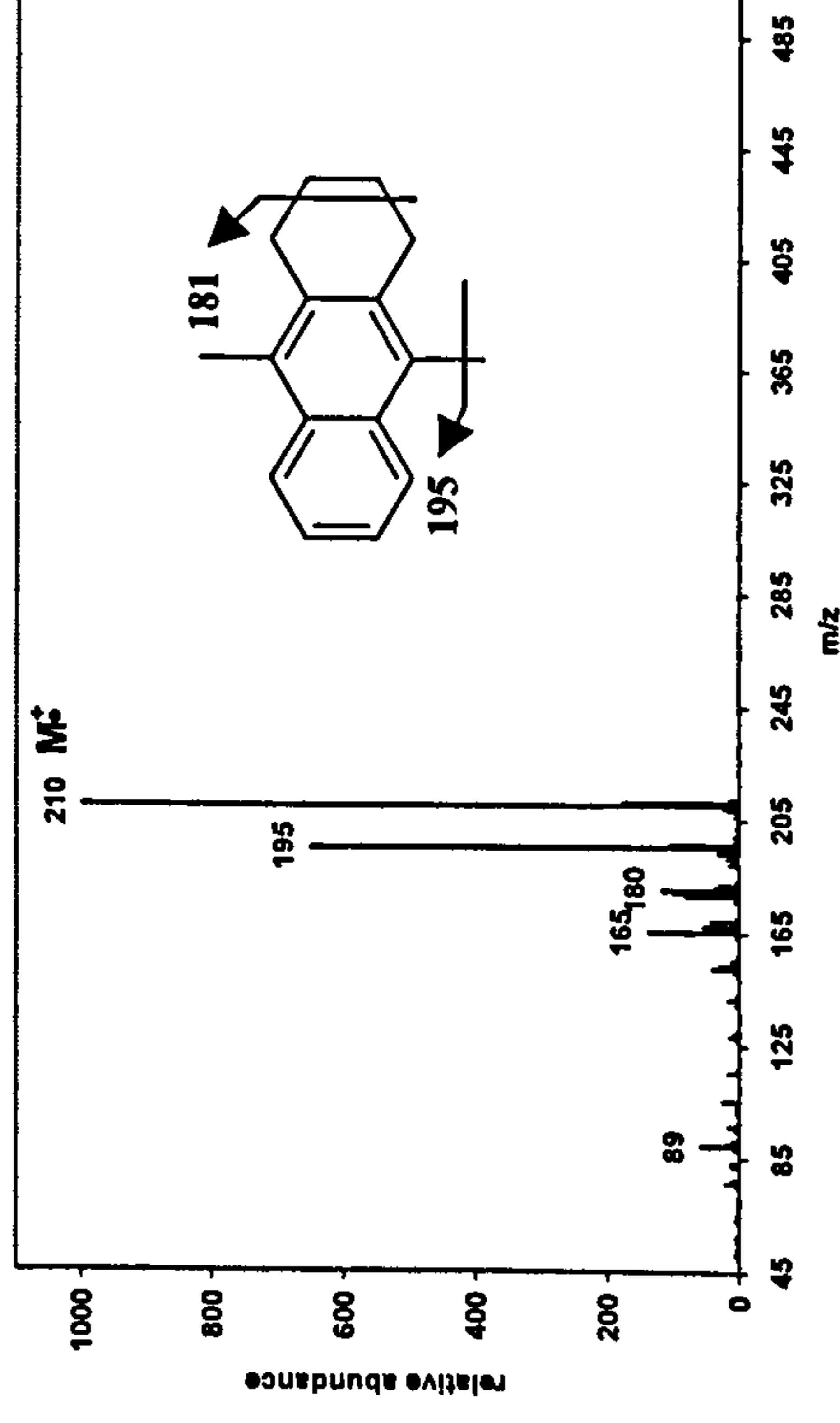


Figure 4.38b. Mass spectrum of the candidate NIST library compound, 1,2,3,4-tetrahydro-9,10-dimethyl-anthracene.

Those compounds exhibiting base ions of m/z 195 and m/z 210 have very similar y -axis retention times on the peak marker plot (Figure 4.34), indicative of compounds sharing similar physicochemical properties. For those compounds with a molecular and base ion of m/z 210, an abundant ion with a mass of 195 was also evident in the spectrum. When these two ions were plotted together they form a band across the x -axis of the peak marker plot (Figure 4.34), which increases in molecular weight by 14 mass units from 210 – 266. Both base ion groups appeared to be part of the same homologous series of compounds. Indeed, comparison of the unknown mass spectra **M** – **P** to those in the NIST library reference indicates that both groups exhibit a close match to alkylbiphenyls, alkyltetrahydroanthracenes and alkylnaphthalenes.

The only compound registered in the NIST library with a molecular weight of 210 and a base ion of m/z 195 is 1,2,3-trimethyl-4*E*-propenyl-naphthalene (Figure 4.35b). Whilst this compound is a good mass spectral match for component **M**, the double bond in the alkyl chain is unlikely to be representative of typical components in crude oil or of UCM compounds. The unsaturated bond is too unstable and readily biodegradable for such a compound to occur in a biodegraded crude oil. Rather, compound **M** is suggested to correspond to an isomer or homologue of one of the compounds proposed for **N** – **P**.

The alkylbiphenyls have been previously discussed in Section 4.3.5 with regard to their occurrence in the water soluble monoaromatic UCM. Only information specific to the particular examples given in this section will be discussed further. Tetrahydroanthracenes and the isomeric tetrahydrophenanthrenes, classed as hydroaromatics, have been reported in a wide variety of natural organic materials. This includes kerogen (Radke *et al.*, 1986), coal tar pitch (Blanco *et al.*, 1991), coal-derived oils (Marsh *et al.*, 1984), coal extracts (White and Lee, 1980) and oil shales (Borrego *et al.*, 1997). However, tetrahydroanthracenes and tetrahydrophenanthrenes are only rarely reported in crude oils

(*e.g.* Pankova *et al.*, 1977; Armstrong *et al.*, 1991; Killops, 1991) although other hydroaromatic compounds such as indans and tetralins are frequently identified in crude oils (Radke *et al.*, 1984; Matisova *et al.*, 1991; Bastow *et al.*, 1998; Gaines *et al.*, 1999). Therefore, the occurrence of tetrahydroanthracenes and tetrahydrophenanthrenes in crude oil is not necessarily unexpected. Perhaps their scarcity in the literature may be due to such compounds not typically being monitored or sought after in crude oil samples.

Alkylbiphenyls with a molecular weight of 210 possess a C₄-substitution. The two C₄-biphenyl mass spectra available in the NIST library demonstrate that a base ion of *m/z* 195 can be generated by the presence of both methyl- and ethyl-substituents. However, higher molecular weight compounds in the series (224, 238, 252) must contain longer alkyl chains rather than more substituents in order to preserve the *m/z* 195 base ion. Indeed, hexamethyl-biphenyl has a molecular weight of 238, but a base ion of *m/z* 223 rather than *m/z* 195 (NIST library). Nine compounds in the water soluble monoaromatic UCM sample studied exhibited a molecular weight of 238 and a base ion of *m/z* 223. From comparison to the NIST library, these compounds appear to correspond to C₆-biphenyls or C₅-diphenylmethanes (Section 4.3.5).

Whilst alkyltetrahydroanthracenes provide good matches in some cases, such compounds are unlikely to occur in crude oils. Not only is anthracene rather unstable (Killops and Killops, 1993), there is also a lack of suitable precursor molecules. Hence, anthracene and its alkylated homologues are hardly ever observed in crude oils. In contrast, phenanthrene is not only much more stable, but there are large numbers of biomarker precursors containing this structure *e.g.* steranes, diasteranes and terpanes (Radke *et al.*, 1986; Killops and Killops, 1993; Peters and Moldowan, 1993). Therefore, the compounds identified in the monoaromatic UCM studied are thought to correspond to alkyltetrahydrophenanthrenes (*e.g.* Figure 4.39) derived from biological sources. Indeed, Killops (1991) reports the

occurrence of a C₄-tetrahydrophenanthrene, in both crude oil and its source rock organic matter. However, the abundance of these compounds were significantly reduced in the oil compared to the source rock.

Owing to the limited literature regarding the occurrence of tetrahydrophenanthrenes in crude oil, discussion of their isomeric distribution is difficult, and analogies to other compounds must be drawn. Radke *et al.* (1982 and 1986) observed the 2- and 3-methylphenanthrene isomers (β -substituted) exhibited greater abundance than the 1- and 9-methylphenanthrene isomers (α -substituted). The two ethyl-tetrahydrophenanthrene isomers in Figure 4.39 (II and III) show substitution at both the β - and the α -positions.

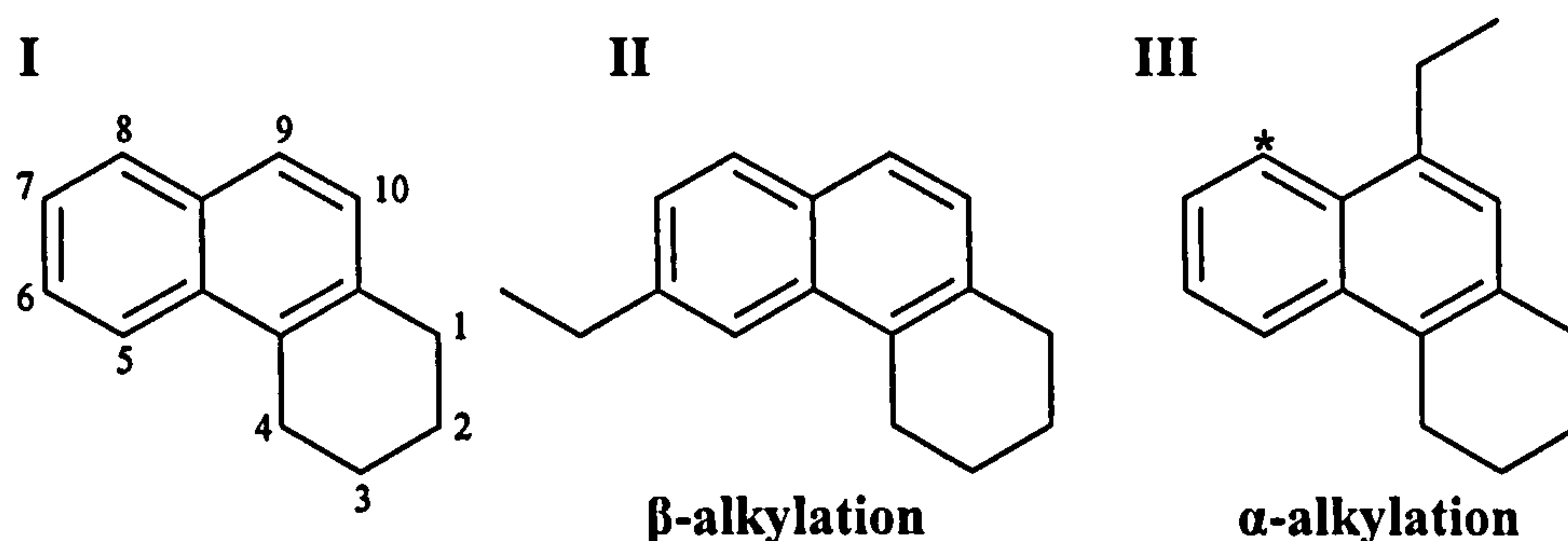


Figure 4.39. Possible substitution positions of tetrahydrophenanthrene (I), and two possible isomers of ethyl-1,2,3,4-tetrahydrophenanthrene, indicating both β (II) and α (III) substitution of the ethyl group.

α -substitution results in increased strain between the ethyl group and the substituent on the adjacent aromatic ring (*). The reduced strain in β -substitution results in higher thermal stability of these conformations and leads to their greater abundance (Radke *et al.*, 1986). For tetrahydrophenanthrene, positions 2, 3, 6 and 7 are β , whilst positions 1, 8, 9 and 10 are α . Positions 4 and 5 are even more sterically hindered (Figure 4.39). A similar distribution of α - and β -substituted isomers has been observed for alkylnaphthalenes (*e.g.* Alexander *et al.*, 1985; Alexander *et al.*, 1986; Strachan *et al.*, 1988; Bastow *et al.*, 1998).

Biodegradation also affects the isomeric distribution of compounds exhibiting α - and β -substitution. Volkman *et al.* (1984) observed that dimethylnaphthalenes exhibiting β -substitution were the most susceptible to biodegradation. This is in complete contrast to the pattern observed with thermal stability. Additionally, Volkman *et al.* (1984) observed that adjacent substitution (particularly methyl groups) on an aromatic ring is the most resistant to biodegradation. Thus increased steric hindrance appears to result in greater bioresistance. This preferential biodegradation of the abundant β -substituted isomers could lead to preservation and enrichment of minor α -substituted isomers. The resulting mixture could contain both isomeric conformations at low concentration. This process, therefore, may be one of the contributing factors in the enhancement of UCMs in weathered/biodegraded crude oils. It further supports the suggestion that tetrahydrophenanthrenes are components of the UCM studied herein.

There are 59 possible isomers of the C₂-tetrahydrophenanthrenes (mw 210), although those with substituents in the 4 and 5 positions are unlikely owing to severe steric hindrance. Assuming the presence of both α - and β -substituted compounds, this still leaves 40 feasible isomers, which can account for the 30 compounds with a molecular weight of 210 in the peak marker plot in (Figure 4.34). Additionally, C₄-biphenyls containing substituents no larger than methyl or ethyl groups can also contribute to those compounds with a molecular weight of 210 (Figure 4.34). Thus, both compounds easily account for the number of isomers observed. Alkylbiphenyls have already been proposed as components of the UCM sample studied (Section 4.3.5). It is suggested that the compounds in the peak marker plot (Figure 4.34) correspond to a mixture of both alkylbiphenyls and alkyltetrahydrophenanthrenes. However, the use of authentic reference compounds is required to confirm their occurrence, and characterise individual components.

4.4 Discussion

GCxGC-ToF-MS analysis (Section 5.10) has provided a more detailed, though still preliminary chromatographic characterisation of UCM compounds than previously possible and diagnostic mass spectral data have been obtained for these now resolved components. Sections 4.3.1 – 4.3.6 have provided evidence that alkyl- benzenes, indenenes, indans, tetralins, biphenyls, diphenylmethanes and tetrahydrophenanthrenes occur in the ‘water soluble’ UCM. Many of these compound classes might be regarded as biomarkers because the alicyclic parts of their structures resemble the sub-units of biological precursors (Killops and Killops, 1993; Peters and Moldowan, 1993; Gahm *et al.*, 1998). A significant number of the components identified in the present study appear to be previously unidentified isomers or homologues, with many appearing to be more highly substituted derivatives of known crude oil constituents. The number of potential isomers increases vastly with an increase in the complexity of hydrocarbon structures. Indeed, a rise in the number of isomers with greater substitution or chain length has been observed as an increase in the number and density of peaks in the later stages of the first dimension (Venkatramani and Phillips, 1993; Hamilton and Lewis, 2003). This may be one reason behind the occurrence of a UCM in a one-dimensional gas chromatogram of weathered crude oil.

GCxGC allowed the entire sample to undergo separation on two different phase columns. This resulted in greatly increased peak capacity compared to one-dimensional gas chromatography (Frysinger *et al.*, 1999; Dallüge, 2003; Hamilton and Lewis, 2003; Marriott *et al.*, 2003). The ordered nature of the chromatograms generated using the apolar/polar GCxGC column combination has aided in the identification of compound groups. However, this increased separation power enables the generation of many more high quality mass spectra when mass spectrometric detectors are coupled to the system (Dalluge *et al.*, 2002a). Thus, it is the power of the ToF-MS detector, which really provides

diagnostic data for each of the resolved compounds. This not only helps in confirming the presence of compound classes identified from the chromatogram, but goes some way to allowing characterisation of individual UCM components (*e.g.* Welthagen *et al.*, 2003). Identification of individual compounds was still reliant upon the availability of mass spectra listed in the NIST library and in the literature. For the majority of cases in the present study, sufficiently accurate library mass spectra matches were unavailable, resulting in misidentification occurring in the automated processing. Manual data processing and library searches helped to correct this but proved to be very time-consuming. Nonetheless, this was very worthwhile as the data may prove representative of crude oil UCMs in general. If so, this may have important regulatory considerations for the toxicological effects observed for UCMs (*e.g.* Rowland *et al.*, 2001; Donkin *et al.*, 2003).

Despite the instrument software being programmed to acquire 2500 peaks from the sample analysed, only 1860 peaks were identified. These included over 500 peaks which corresponded to bleed ions from the GC columns. Removal of these left 1252 peaks attributed as UCM hydrocarbons. The identification of only 1252 peaks suggests all the resolved sample components were detected. However, the water solubility study in Chapter 3 showed only a proportion of the compounds in the original monoaromatic UCM were sufficiently soluble for analytical detection.

A large number of the 1252 identified peaks exhibited mass spectra that appeared unsuitable for characterisation. In many cases, these spectra were 'noisy', appearing to be an amalgamation of abundant fragment ions from more than one compound (*e.g.* Figure 4.40a). This indicates that even with GCxGC, co-elution of compounds may still occur, and that the software is unable to deconvolute single mass spectra in all cases. Indeed, coelution of structural isomers has also been acknowledged in some GCxGC studies of petroleum (Gaines *et al.*, 1999). Furthermore, many chromatograms have fragmentation

patterns which do not appear to correspond to a single compound containing only carbon and hydrogen atoms (Figure 4.40b). In the case of Figure 4.40b abundant ions are present at masses of 159 and 167. This mass difference of 8 atomic units cannot be fragment ions from the same compound, providing further evidence for co-elution of UCM components. Removal of these non-diagnostic mass spectra resulted in 490 components deemed suitable for characterisation.

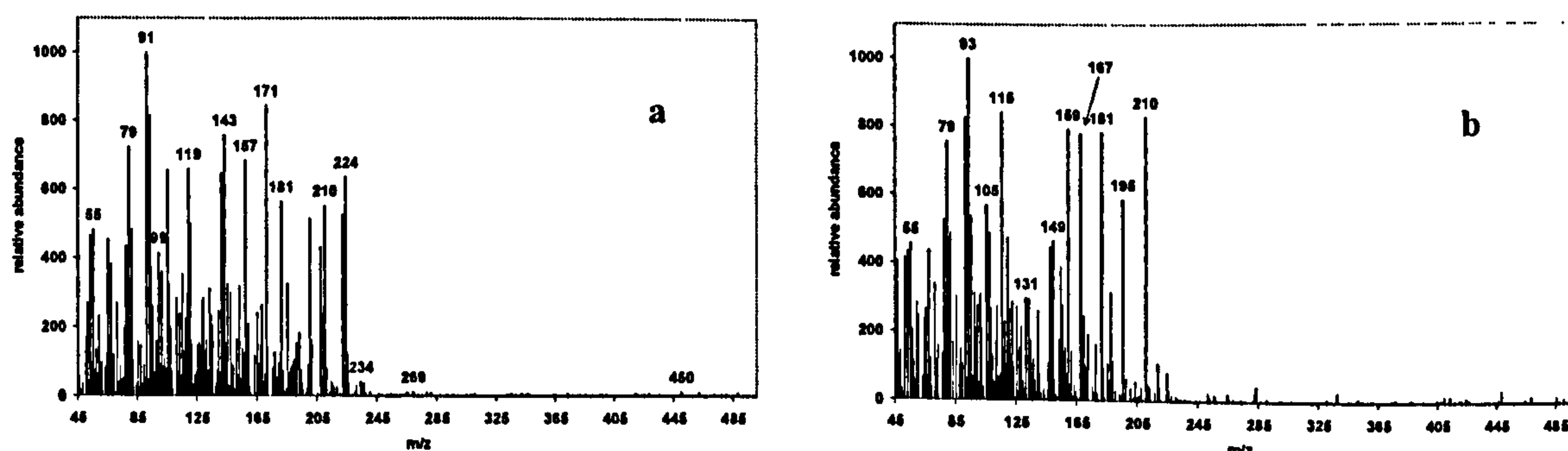


Figure 4.40a. Mass spectrum of the peak at a retention time of 988,6.14, which contains multiple abundant ions, indicating co-elution of compounds. Figure 4.40b. Mass spectrum of the peak at a retention time of 868,5.54, containing ions which cannot be accounted for by standard fragmentations of compounds comprised only of C and H atoms.

When the 490 peak markers were plotted (Figure 4.9), all of the components exhibited short first-dimension (volatility) retention times. In the case of hydrocarbons, those with the lowest molecular weight are generally the most soluble. Indeed, all hydrocarbons in the water soluble monoaromatic UCM elute before 1800 seconds in a chromatographic analysis that lasts 3500 seconds. Given the nature of the sample, it was no surprise to find that larger less soluble compounds, which are also less volatile, were absent from the sample. A GCxGC comparison of the original monoaromatic UCM (Sutton, unpublished) to that of the soluble fraction analysed in the current study highlights this absence of high molecular weight hydrocarbons (Figure 4.41).

Comparison of the second-dimension retention times for the two samples is effectively a measure of the polarity of a particular compound. Hydrocarbons can exhibit subtle differences in polarity depending upon their structure. For example, the presence of aromatic rings increases the polarity of a hydrocarbon with respect to those containing no double bonds (*i.e.* saturated). Therefore, compounds with higher polarities, such as aromatic hydrocarbons, are more soluble than saturated compounds (*e.g.* alkanes) of a comparable carbon number (McAuliffe 1966; Eganhouse and Calder, 1976; Siron *et al.*, 1991; Kuo, 1994; Schwarzenbach *et al.*, 2003).

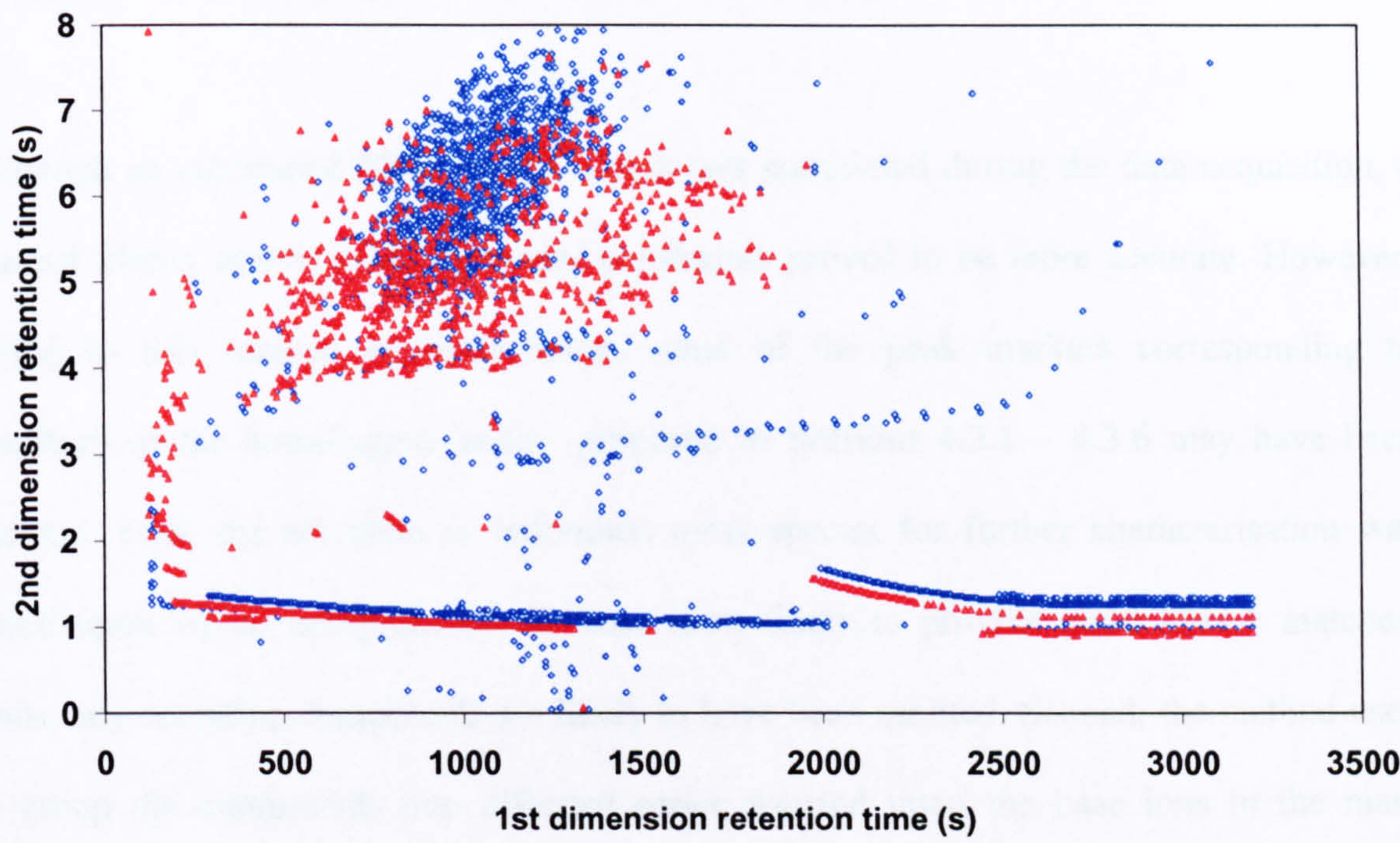


Figure 4.41. Comparison of the peak marker plots of the original monoaromatic UCM (Δ) and the water soluble fraction of the monoaromatic UCM (◇). Peak marker data for the original monoaromatic UCM provided by Sutton (unpublished data).

Figure 4.41 highlights differences in the retention times for the compounds present in the two samples. The water soluble monoaromatic UCM appears to be dominated by components which have longer retention times on the second axis than the original monoaromatic UCM. This indicates the water soluble monoaromatic UCM is dominated by the most polar hydrocarbons occurring in the original monoaromatic UCM. As the sample is considered to contain predominantly monoaromatic hydrocarbons, a narrow

range of polarities could be expected. However, the range of second-dimension retention times is quite broad, indicating that the sample actually contains a mixture of compounds with a wide range of polarities. Identification of compound classes in Sections 4.3.1 – 4.3.6 indeed showed that a wide range of both monoaromatic and diaromatic hydrocarbon classes were present in the sample, accounting for this broad polarity range. The *n*-alkanes in the water soluble monoaromatic UCM are thought to have been enriched compared to the original monoaromatic UCM as part of the dissolution process (Section 5.9). Additionally, the reduced number of compounds in the water soluble fraction may have permitted their detection by the GCxGC-ToF-MS system.

Although an automated NIST library search was completed during the data acquisition, a manual library search using elemental constraints proved to be more accurate. However, owing to this manual post-processing, some of the peak markers corresponding to members of the homologous series' proposed in Sections 4.3.1 – 4.3.6 may have been omitted. First, the selection of individual mass spectra for further characterisation was based upon visual interpretation of those most likely to provide good library matches. Thus, any coeluting compounds are likely to have been omitted. Second, the method used to group the compounds into different series focused upon the base ions in the mass spectra. During the study it became evident that not all compounds in a homologous series exhibited the same base ion (Sections 4.3.1 – 4.3.6). Some of the isomers or compounds from a particular series may still be present in the mixture, but due to differing base ions they will not occur in the peak marker plots (Sections 4.3.1 – 4.3.6).

A proportion of the data generated still remains not fully processed, and a large amount unreported due to limitations with both time and report length. Only a few of the base ion groups have been analysed and presented in detail (Sections 4.3.1 – 4.3.6). This limitation with time was the most significant, particularly because the data processing was conducted

manually rather than with computer software. Whilst processing software does exist, it was unavailable for use in the current study. Such software would certainly have increased the data processing efficiency and allowed the study of many other groups containing peaks and associated mass spectra that appear promising for group type identification and characterisation of individual compounds.

4.4.1 The molecular weight range of the water soluble monoaromatic UCM

The Lee index (aromatic) was used to determine a carbon number range of C₁₀ to C₁₇ for the water soluble monoaromatic UCM in Chapter 3. Figure 4.11 shows that the molecular weight of the 10 most abundant base ions ranges from 172 – 290 mass units. Whilst it is difficult to relate this directly to the Lee index retention data, calculated in Chapter 3, basic comparisons can be made. Calculations suggest a molecular weight of 172 will most probably correspond to a C₁₃ hydrocarbon, whilst a molecular weight of 290 more than likely represents a C₂₂ hydrocarbon. A definite difference between the two ranges is apparent, with the Lee index range (C₁₀-C₁₇) being lower than that estimated from the data in Figure 4.11 (C₁₃-C₂₂). As the Lee index is based on unsubstituted aromatic hydrocarbons, deviations will be expected for alkylated aromatic hydrocarbons, and this appears to be the case. Indeed, all compounds in the peak marker plots shown in Sections 4.3.1 - 4.3.6 are alkylated aromatics, with molecular weight ranges that fall within those determined in Figure 4.11. This allows a more accurate molecular weight range to be determined for the hydrocarbon components in the monoaromatic UCM.

From Figure 4.11 it is evident that many of the most abundant base ions exhibit significantly different molecular weight ranges. Some have broad molecular weight ranges whilst others are very limited. If the core structure for each of the compounds in a series remains the same, an increase in molecular weight is indicative of greater alkylation. Where the molecular weight range is only small, these abundant base ions are suggested to

correspond to: (i) a suite of different compound types all exhibiting the same base ion and molecular weight, or (ii) a suite of isomers of the same compound.

4.4.2 Modelling the biodegradation, water solubility and aquatic toxicity of the proposed monoaromatic UCM hydrocarbons

The Environment Protection Agency's (EPA) Office of Pollution Prevention Toxics (OPPT) and the Syracuse Research Corporation (SRC) developed the EPI (Estimation Programs Interface) Suite™. The EPI Suite™ is comprised of ten stand-alone programmes, which can be used to estimate a variety of environmentally important characteristics for individual chemicals. Those of interest to the present study allow estimation of the biodegradability (BIOWIN v 4.00), water solubility (WSKOWWIN v 1.40) and aquatic toxicity (ECOWIN v 0.99g). The BIOWIN program has been used and discussed in detail previously (Chapter 2, Section 2.3.3). The WSKOWWIN and ECOWIN programs are reviewed briefly below.

WSKOWWIN generates the log octanol-water partition coefficient (Kow) of an organic compound from its structure, and uses this to estimate the water solubility. A detailed description of the methodology used to calculate the water solubility is provided by Meylan *et al.* (1996) but is beyond the scope of the present study. The ECOWIN programme estimates the structure-activity relationships (SARs) of a chemical based on structure, and uses this to predict the aquatic toxicity. SARs are developed for chemical classes based on measured data sets. Toxicity values for submitted chemicals are calculated by inserting the estimated Kow into a regression equation. The ECOWIN program is a computerised version of the analytical procedures currently practised by the Office of Pollution Prevention and Toxics (OPPT).

The EPI Suite™ programs were used herein to estimate the biodegradation, water solubility and aquatic toxicity of the compounds proposed to be present in the UCM in Sections 4.3.1 – 4.3.6. The results are summarised in Table 4.2, and the compound structures (A-R) listed in Figure 4.42. The biodegradation estimates are based upon the Japanese MITI (Ministry of International Trade and Industry) test, where fragment constants were developed using multiple linear and non-linear regression analyses (Tunkel *et al.*, 2000). The estimated water solubility values correspond to the solubility of the hydrocarbon in distilled water at 25°C ($\mu\text{g L}^{-1}$). The estimated aquatic toxicity values represent the concentration ($\mu\text{g L}^{-1}$) that would cause a 50% mortality rate (LC50) in a test population of fish over a 14-day period. This is termed the baseline toxicity.

The compounds identified in Sections 4.3.1 – 4.3.6 should meet certain criteria if they are to be proposed consistent with the observed properties of water soluble monoaromatic UCM components. First, due to the biodegraded nature of the UCM sample, the compounds should be significantly resistant to biodegradation. Second, as the sample analysed is the water soluble fraction, the compounds should exhibit values comparable to that determined for the monoaromatic UCM (Chapter 3). Third, previous work (Rowland *et al.*, 2001; Donkin *et al.*, 2003) has shown monoaromatic UCMs are toxic to certain marine organisms (mussels). Thus, some of the compounds should be toxic to some aquatic organisms.

All of the compounds, except A and B, were deemed not readily biodegradable, confirming their candidacy with respect to this parameter. Compounds A and B are proposed as linear alkylbenzenes, and may be too susceptible to biodegradation to be considered as possible UCM components. It was suggested in Section 4.3.1 that branched alkylbenzene homologues may be more bioresistant and therefore more representative of UCM compounds. The same parameters were calculated for a branched alkylbenzene (Q, Table

4.2 and Figure 4.42) of comparable molecular mass to linear alkylbenzene B. The branched alkylbenzene (Q) was deemed not readily degradable. This provides further evidence for the occurrence of branched rather than linear alkylbenzenes in the UCM.

For comparison the same parameters were also calculated for the synthetic compound 6-cyclohexyltetralin (R, Table 4.2 and Figure 4.42). This compound has been previously proposed as an ‘average’ structure for components of the aromatic UCM (Thomas, 1995; Section 1.5), and has been used in previous UCM studies as a model compound (*e.g.* Rowland *et al.*, 2001; Smith, 2002). The present study has also employed this compound to study the biodegradation (Chapter 2) and the water solubility (Chapter 3) characteristics of an ‘average’ UCM compound. The calculated biodegradability has been previously discussed in Chapter 2, with the not readily biodegradable result being consistent with aromatic UCM component properties.

Compounds A-R exhibit a relatively broad range of estimated water solubility values. All compounds except the alkylbenzenes (A-D and Q) are above the value determined experimentally for the monoaromatic UCM ($57 \mu\text{g L}^{-1}$) under the same conditions (Chapter 3). The alkylbenzenes are approximately an order of magnitude less soluble than the monoaromatic UCM. However, the water solubility of the monoaromatic UCM is an average value based on the contribution of all the hydrocarbon components. Therefore some compounds will have higher individual solubilities and others will be lower. Despite their water solubility being lower than the average monoaromatic UCM value, the alkylbenzenes still appear sufficiently soluble to occur in the sample. Additionally, branched compounds of the same molecular weight are generally more soluble than linear homologues, due primarily to a reduction in compound surface area (Silla *et al.*, 1992; Tolls *et al.*, 2002; Schwarzenbach *et al.*, 2003). This provides further evidence for the occurrence of branched rather than linear alkylbenzenes.

Table 4.2. Summary of the biodegradability, water solubility and aquatic toxicity of the proposed water soluble monoaromatic UCM hydrocarbons, calculated using the programmes available in the EPI Suite TM.

		BIOWIN		WSKOWWIN		ECOWIN	
		v 4.00		v 1.40		v 0.99g	
Compound	ID Number	Base ion (m/z)	Molecular weight	MITI linear model prediction	MITI non-linear model prediction	Estimated water solubility (µg L ⁻¹)	LC50 in fish over 14 days (µg L ⁻¹)
1-propyloctyl-benzene	A	91	232	NRD	RD	12.82	6
1-propyldecyl-benzene	B	91	260	NRD	RD	0.8997	1.01
1-methylundecyl-benzene	C	105	246	NRD	RD	2.508	3
1-methyldodecyl-benzene	D	105	260	NRD	RD	0.6698	1.01
(1,5,9-trimethyl-decyl)-benzene*	Q	n/a	260	NRD	NRD	2.003	1.36
1-methyl-3-propyl-1H-indene	E	143	172	NRD	NRD	2181	378
3-butyl-1-methyl-1H-indene	F	143	186	NRD	NRD	709.8	153
2,3-dihydro-1,1,5,6-tetramethyl-1H-indene	G	159	174	NRD	NRD	1347	241
1,2,3,4-tetrahydro-1,6,8-trimethyl-naphthalene	H	159	174	NRD	NRD	1251	222

	2,2'-dimethylethyl-biphenyl	I	167	182	NRD	NRD	3860	806
	1,1'-(3,3-dimethylbutylidene) bis-benzene	J	167	238	NRD	NRD	204.7	105
	3,5-dimethyl-1-(phenylmethyl)-benzene	K	181	196	NRD	NRD	1976	515
	4-isopropyl-biphenyl	L	181	196	NRD	NRD	900.2	422
	1,2,3-trimethyl-4-propenyl-(E)-naphthalene	M	195	210	NRD	NRD	227.3	71
	1,2,3,4-tetrahydro-9-propyl-anthracene	N	195	224	NRD	NRD	66.61	26
29	3,4-diethyl-biphenyl	O	210	210	NRD	NRD	403.3	130
	1,2,3,4-tetrahydro-9,10-dimethyl-anthracene	P	210	210	NRD	NRD	185.8	58
	6-cyclohexyltetralin ⁺	R	214	214	NRD	NRD	60.45	20

NRD = Not readily degradable

RD = Readily degradable

* (1,5,9-trimethyl-decyl)-benzene is not a NIST library match compound, but its branched nature is suggested to be more representative of the alkylbenzene hydrocarbons (*m/z* 91 and 105) present in the water soluble monoaromatic UCM sample analysed.

+ 6-cyclohexyltetralin is not a NIST library match compound, but it is included as it is considered an ‘average’ aromatic UCM component (Thomas, 1995) for which there is experimentally determined biodegradation and water solubility data.

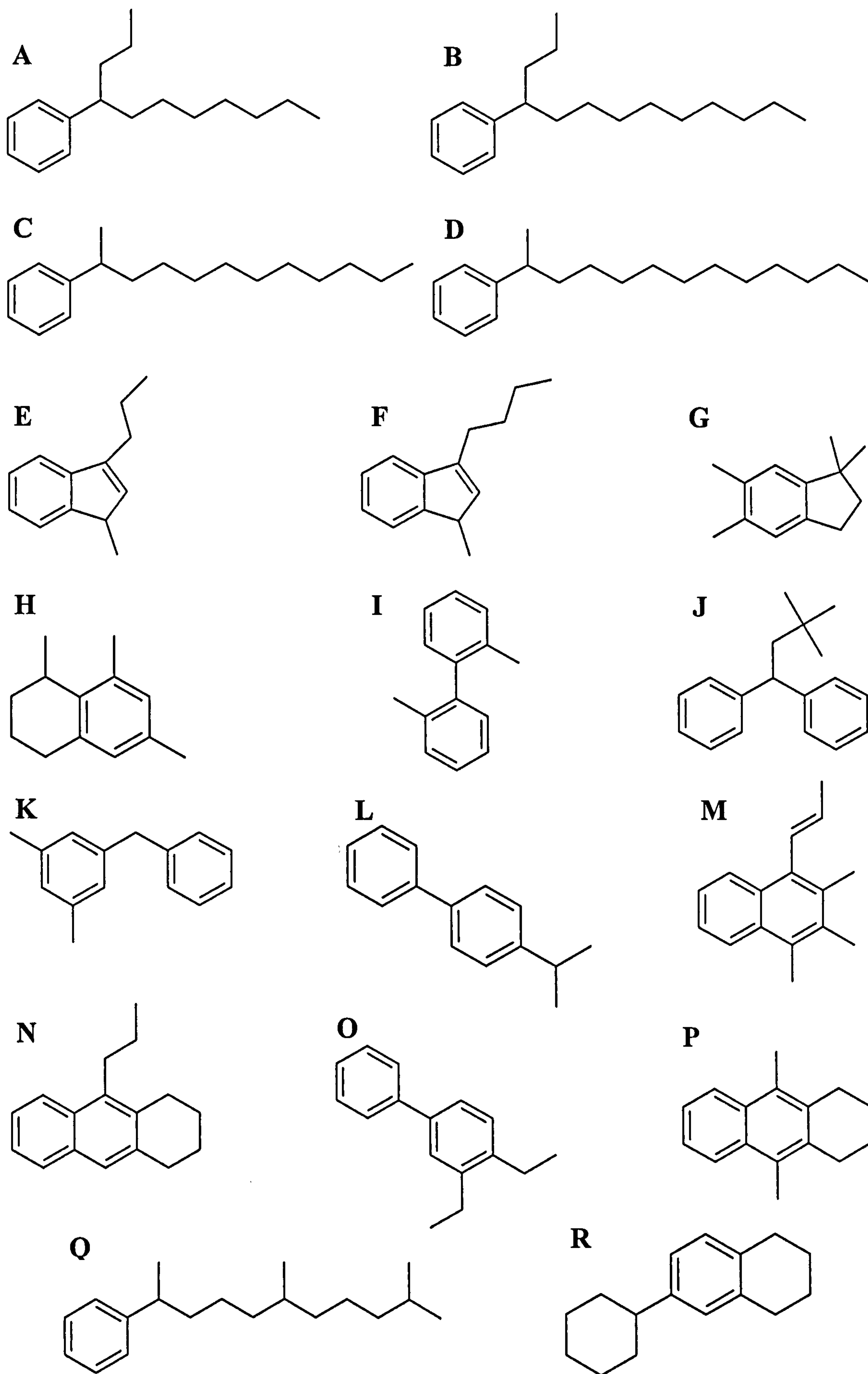


Figure 4.42. Summary of the chemical structures of hydrocarbons A-R proposed as water soluble monoaromatic UCM components. The full nomenclature for each of the compounds is given in Table 4.2.

Only a proportion of the monoaromatic UCM components have to be toxic for the mixture to elicit toxicological effects. The water solubility of alkylbenzenes B-D appears at the limit required to be toxic to aquatic organisms. However, toxicity studies of alkylbenzenes have been conducted, which indicate that such compounds do pose a toxicological risk to aquatic organisms (Hellou *et al.*, 1994; Fernandez *et al.*, 2002). All other compounds, including the branched alkylbenzene (Q) and 6-cyclohexyltetralin (R), appear sufficiently soluble to cause a 50% mortality rate in fish over a 14-day period.

The estimated water solubility of 6-cyclohexyltetralin (R) is $60.45 \mu\text{g L}^{-1}$ and that determined experimentally in Chapter 3 in the present study is $109 \mu\text{g L}^{-1}$. This indicates, in the case of this compound, the WSKOWWIN program is capable of accurately predicting the water solubility of compound to within less than an order of magnitude of the 'real' value. Thus, computer-based models can be very useful tools, but this study shows that there is no substitute for 'real' data. The predictive EPI Suite™ software used in this study provides complementary information to the data generated by the GCxGC-ToF-MS analysis. It provides an indication or estimate of some of the important parameters required for a positive identification of the component hydrocarbons in the monoaromatic UCM. Although helpful in this respect, it does not provide sufficiently reliable data to qualify as a substitute for experimentally generated results.

4.4.3 Considerations when using GCxGC-ToF-MS to analyse complex mixtures

The software is capable of identifying the 5000 most abundant peaks in a single analysis. However, this was restricted to 2500 to prevent inundation of too much data given the time constraints of the present study. Furthermore, the water soluble monoaromatic UCM contains only a small fraction of the compounds present in a whole oil, and 2500 peaks was deemed sufficient to provide a suitable separation of the sample. However, if a

detailed analysis of a complex mixture such as whole crude oil is required, a limit of 5000 peaks may not even be sufficient to identify all of the components within the sample. Those compounds present in lower abundance would not be recognised in the data processing and the quantity of data generated for each analysis would be very large. Therefore, complete resolution and identification of components and a manageable data set requires fractionation into simplified mixtures containing much fewer compounds. This approach was used in the present study, and appears to have been successful.

It is widely acknowledged that GCxGC using an apolar/polar column combination generates an ordered two-dimensional chromatogram which can help in the identification of compounds (*e.g.* Venkatramani and Phillips, 1993; Frysinger *et al.*, 1999; Gaines *et al.*, 1999; Phillips and Beens, 1999; Dallüge, 2003). However, this is not always a reliable method of characterising complex samples, and is mainly limited to the identification of compound classes rather than individual components. The sample is comprised predominantly of monoaromatic and some diaromatic compounds, which share very similar physicochemical properties, further limiting the utility of the ordered chromatograms. Thus, none of the clear ‘banding’ of compound classes observed with more chemically diverse samples (*e.g.* Frysinger and Gaines 1999; Phillips and Beens, 1999) is evident in the chromatogram (Figures 4.8 and 4.9). Indeed, identification of compound classes within the water soluble monoaromatic UCM would have been virtually impossible without the use of mass spectra. Ideally, a suite of authentic reference compounds would be analysed to confirm the presence of those compounds proposed in Sections 4.3.1 – 4.3.6. Unfortunately, time constraints, access to the GCxGC-ToF-MS instrument and availability of such reference compounds has prevented the opportunity to undertake this task. In many cases reference compounds would require laboratory synthesis and characterisation prior to analysis, which would also be time consuming.

The ‘wrapping over’ of the most polar compounds appears to have occurred. Owing to second-dimension retention times longer than the 8 s modulation period, these compounds elute very early in the subsequent second-dimension chromatogram (*e.g.* Phillips and Xu, 1995; Shellie *et al.*, 2001). For example, a compound with a retention time of 9 s will appear as a peak with an apparent retention time of 1 s on the next chromatogram. This situation presents a variety of potential problems. A loss of ordered chromatograms occurs, with polar compounds eluting early on the second-dimension axis. Furthermore, ‘wrap over’ could lead to coelution with apolar compounds (*e.g.* alkanes) which naturally elute in this region. Finally, in mixtures displaying a diverse array of compound types, ‘wrap over’ may not be identified unless mass spectral detection or reference compounds are used to accurately characterise these compounds.

Another issue to consider is the allocation of peak markers by the data processing software. It is likely that closely distributed peak markers correspond to isomers of the same compound. However, it is possible that more than one peak marker is being attributed to a single compound, especially as many of the peak markers are located extremely close to each other. If multiple allocation is occurring, it could lead to over estimation in the number of compounds present in the mixture.

The isolation and dissolution of a monoaromatic UCM has resulted in a mixture containing a limited selection of compounds, all of which share very similar physicochemical characteristics. This can be seen in the Figure 4.8, as a large proportion of the chromatographic space is not utilised. The absence of very low polarity saturate hydrocarbons means that only half of the second-dimension space is utilised. Furthermore, the absence of insoluble high molecular weight hydrocarbons results in only half of the first dimension being utilised. This means that separation of the sample components is not being optimised. However, the technique is not solely limited to the use of apolar and polar

column combinations (Marriott *et al.*, 2003). Whilst, a volatility separation on the first dimension is generally the best approach, the second dimension phase can be altered to optimise the two dimensional separation of the mixture. In fact different combinations of stationary phases will produce a different distribution of compounds in the same mixture (Focant *et al.*, 2003; Phillips and Xu, 1995; Phillips and Venkatramani, 1993). The second-dimension phase can therefore be selected based upon known characteristics of the mixture being studied. For instance, a polar phase will provide group type separation of components (Blomberg *et al.*, 1997). A chiral phase could be utilised for the separation of isomers *via* shape selectivity within a particular compound group (Frysinger *et al.*, 2003).

4.4.4 Limitations to characterisation and necessity of authentic reference compounds

Whilst comparison of unknown mass spectra to those in the NIST library has provided a tentative identification of some UCM components, this method of characterisation is subject to certain limitations. It appears that many of the components in the water soluble monoaromatic UCM have mass spectra that do not suitably match any of those listed in the NIST library, making identification of such compounds impossible. However, those compounds identifiable from library matches can provide an indication of unmatched compounds which exhibit similar fragmentation patterns, base ions and retention characteristics. Many compounds, including those from different compound classes can exhibit very similar chromatographic retention times and mass spectra. This makes confident or accurate characterisation of compounds very difficult. Even those components exhibiting good matches to library compounds cannot be identified with complete certainty based solely on mass spectral similarity. Finally, the current study employed a ToF-MS detector to generate the mass spectral data. Qualitative differences between the mass spectrum of a known compound analysed by ToF-MS and that reported in the NIST library

have been noted by Shellie *et al.* (2001). Mass discrimination between the ToF-MS and the library spectrum method was suggested as the reason for the differences. Sinha *et al.* (2003) suggest the difference in ionisation sources between the two MS systems also compounds the problem. As the majority of the spectra listed in the NIST library are likely to have been generated from traditional MS analysis, this must be considered in the identification of compounds. Thus, positive characterisation of an unknown compound is only achievable with the analysis of reference compounds on the GCxGC-ToF-MS instrument to generate two-dimensional retention data and mass spectra (*e.g.* Liu *et al.*, 1994; Schoenmakers *et al.*, 2000; Dallüge, 2003; Frysinger *et al.*, 2003). Indeed, Sinha *et al.* (2003) suggest designing a personalised library from compounds of interest to generate more suitable mass spectral matches.

4.4.5 Model monoaromatic and diaromatic UCM hydrocarbons

A suite of alkylcyclohexyltetralins, presented and discussed in Chapters 1, 2 and 3, were previously proposed as ‘average’ monoaromatic compounds containing structural features typical of some UCM components (Thomas, 1995; Wraige, 1997; Smith *et al.*, 2001; Smith, 2002). No mass spectral matches to the exact isomers of compounds (I-VI) were seen in the water soluble monoaromatic UCM sample. However, the alkyltetralins (Section 4.3.4), alkylbiphenyls (Section 4.3.5) and the alkyltetrahydrophenanthrenes (Section 4.3.6) all exhibit many of the structural features suggested previously (*ibid*). Not only do they all contain some degree of alkylation, but they are also of a comparable molecular weight. In addition, the combination of aromatic and saturated rings within the same structure is common to both the observed and ‘model’ hydrocarbons. Whilst none of the exact isomers of compounds I-VI were observed, it appears that the basic structures proposed by Thomas (1995) and developed by Wraige (1997) and Smith (2002) are certainly representative of

some of those compounds which have now been identified in a monoaromatic UCM by GCxGC-ToF-MS.

4.5 Conclusions

The GCxGC technique has allowed a detailed separation of a petroleum-derived unresolved complex mixture (UCM) of hydrocarbons isolated from Tia Juana Pesado crude oil and solubilised using a generator column technique. It is unlikely that even comprehensive two-dimensional chromatography has enabled complete resolution of the UCM sample. However, no analytical technique has previously provided the degree of separation of UCM hydrocarbons observed herein. The recent addition of a time-of-flight mass spectrometer (ToF-MS) to the system has provided a method for identifying these newly resolved UCM components. The increased separation provided by GCxGC has resulted in good quality mass spectra for complex mixture components. In the present study the technique was able to resolve 1252 compounds considered components of a water soluble monoaromatic UCM. Of these, 490 had associated mass spectra deemed suitable for characterisation. Sections 4.3.1 - 4.3.6 detail the characterisation of 16 of these 490 compounds. Importantly, the tentative assignment of many more peaks as isomers and homologues these compounds was possible, totalling 114 compounds.

Petroleum-derived UCMs are considered environmentally persistent as they are produced or enhanced during the biodegradation of crude oil. Rowland *et al.* (2001) indicated that the UCM was toxic to aquatic organisms, with the most toxic fraction appearing to comprise predominantly monoaromatic hydrocarbons (Donkin *et al.*, 2003). The water soluble monoaromatic UCM analysed in the present study is considered a better model of the bioavailable fraction of the monoaromatic UCM. Thus, it may be that compounds proposed in this study are typical of those contributing to the toxicity observed by Rowland

et al. (2001) and Donkin *et al.* (2003). Not only are they water soluble and therefore bioavailable to aquatic organisms, the highly substituted nature of these compounds is consistent with their bioresistant nature.

The identification of UCM hydrocarbons by comparison to NIST library mass spectra alone cannot be considered conclusive. Mass spectra listed in the NIST library will have been produced using standard MS instruments. Qualitative differences between library mass spectra and those generated by a ToF-MS instrument can lead to difficulties in accurately assigning compounds. Furthermore, close similarities between mass spectra of different compounds, or even from different compound classes can prevent precise identification of an individual component. These factors have highlighted the necessity in using authentic reference compounds to achieve reliable characterisation. Reference compounds, when analysed under the same conditions as the sample mixture, will provide a reference library. For each compound two independent retention times and mass spectra generated by ToF-MS will be recorded. This information should be sufficient to accurately identify UCM components.

Whilst GC x GC-ToF-MS provides an extremely powerful analytical technique, somewhat ironically its greatest disadvantage is the generation of vast quantities of data. The more complex the sample analysed, the more data is produced. In the case of petroleum-derived UCMs, which appear to contain many thousands of compounds, data processing has the potential to be very time consuming.

In conclusion, it is suggested that in GCxGC-ToF-MS, an analytical technique has finally been developed with the potential to enable complete characterisation of UCMs. Indeed, perhaps the term UCM, borne from the analysis of weathered crude oils by one-dimensional gas chromatography is no longer applicable. With comprehensive multi-

dimensional gas chromatography 'partially resolvable complex mixtures' (PRCMs) might be considered a more appropriate description. The task of complete characterisation may not be straightforward, but using GCxGC-ToF-MS and a library of reference compounds it could be achieved. Given the widespread distribution of UCMs in the environment, the implication of identifying representative members of the toxic UCM compounds are substantial.

Chapter 5

Experimental Procedures

This chapter describes the experimental procedures conducted and the analyses performed as part of this study.

5.1 General procedures

All glassware was pre-cleaned by soaking in 'Decon-90' (2%) overnight, rinsed with hot tap water, oven dried (120 °C, overnight) and finally rinsed with dichloromethane to remove any residual organics prior to use.

Solvents were obtained from Rathburn Chemicals Ltd., Scotland, and used once solvent purity had been determined by GC analysis. Typically, 100 mL of solvent was rotary evaporated, diluted in 1 mL dichloromethane, and then 1 µL was analysed by gas chromatography. The pass level was dependent upon the experimental procedure in use (*i.e.* the amount of solvent used), however, the solvent was rejected if impurities were of a concentration sufficient to be observable against a test mixture (see Sections 5.2.1 and 5.2.2).

Silica gel (SiO₂, Aldrich, grade 645, 60-100 mesh) and aluminium oxide (Al₂O₃, BDH, England; grade 1, neutral, 150 mesh) adsorbents used for open column chromatographic separations were soxhlet extracted (DCM, 24 hr) and oven dried (40 °C, overnight) before being stored in a desiccator. Preparation of the adsorbents was by activation (160 °C overnight), cooling in a desiccator, followed by deactivation with addition of Milli-Q grade water (Section 5.4) and homogenisation by mechanical shaker (1-2 hr).

Anhydrous sodium sulphate and cotton wool were pre-extracted (DCM, 24 hr), dried, and oven dried (160 °C, overnight) prior to use. Anhydrous sodium sulphate was stored in a desiccator.

5.2 General instrument details

5.2.1 Gas chromatography (GC)

Instrument: Hewlett Packard HP5890 series II gas chromatograph fitted with a Hewlett Packard auto-sampler and a flame ionisation detector (FID)

Column: HP-1 fused silica capillary column, 30 m x 0.25 mm id x 0.25 μ m film thickness

Injector: Autosplitless injection (250 °C), 1 μ L injection

Carrier gas: Helium 85 kPa head pressure

Detector: Flame ionisation detector (FID)

The oven temperature was typically programmed from 40-300 °C @ 10 °C min⁻¹ and held for 10 min. Data and chromatograms were monitored and recorded using Turbochrom Navigator (6.1.1.0.0: K20) software. Column performance was monitored with the use of a test mixture containing alkanes (pristane and heptadecane), aromatics (anthracene and phenanthrene) and an acid (octadecanoic acid methyl ester). The test mixture was analysed at 0.01 mg mL⁻¹, and the column deemed suitable for use if there was baseline resolution of the anthracene and phenanthrene isomers, and baseline resolution of the pristane and heptadecane compounds.

5.2.2 Gas chromatography-mass spectrometry (GC-MS)

Instrument: Hewlett Packard GC-MSD. Comprising a HP5890 series II gas chromatograph fitted with a Hewlett Packard HP7673 auto-sampler and HP5970 mass selective detector

Column: HP-1MS fused silica capillary column, 30 m x 0.25 mm id x 0.25 μ m film thickness

Injector: Autosplitless injection (250 °C), 1 μ L injection

Carrier gas: Helium (40 kPa head pressure)

The oven temperature was typically programmed from 40-300 °C @ 10 °C min⁻¹ and held for 10 min. Data and chromatograms were monitored and recorded using Hewlett Packard ChemStation (version B.02.05) software. Column performance was monitored with the use of a test mixture containing alkanes (pristane and heptadecane), aromatics (anthracene and phenanthrene) and an acid (octadecanoic acid methyl ester).

Mass spectrometer operating conditions:

Ion source temperature:	280 °C
Ionisation energy:	70 eV
Full Scan:	mass range 50 – 550 Daltons
Fragmentation:	Electron impact (EI)

5.3 Computer modelling of hydrocarbon structures

Two-dimensional structures of synthetic compounds I-VI (Figure 5.1) were drawn with CambridgeSoft ChemDraw[®] 4.0. These structures were then imported into the CambridgeSoft Chem3D[®] 4.0 software package where the energy of each molecule was minimised using an energy function. Energy functions are also called ‘force fields’ because the force acting on a molecule due to its conformation can be found by differentiating the energy function. In the present study the MM2 force field function (Allinger, 1977) was used to determine the minimum energy conformation of the synthetic compounds. This function, and modifications of it, have been commonly used to calculate the theoretical conformation of compounds based upon minimum energy (*e.g.* Broeker and Houk, 1991; Broeker *et al.*, 1991; Goodman *et al.*, 1994). The resulting structures were imported into the WebLab Viewerlite software package as individual MDL MolFiles. A ‘solvent’ surface was selected and applied to each of the molecules, and a suitable visual orientation was

determined. Individual molecules were then saved as JPEG images. The final image of 7-cyclohexyl-1-isoamyltetralin is presented in Chapter 3.

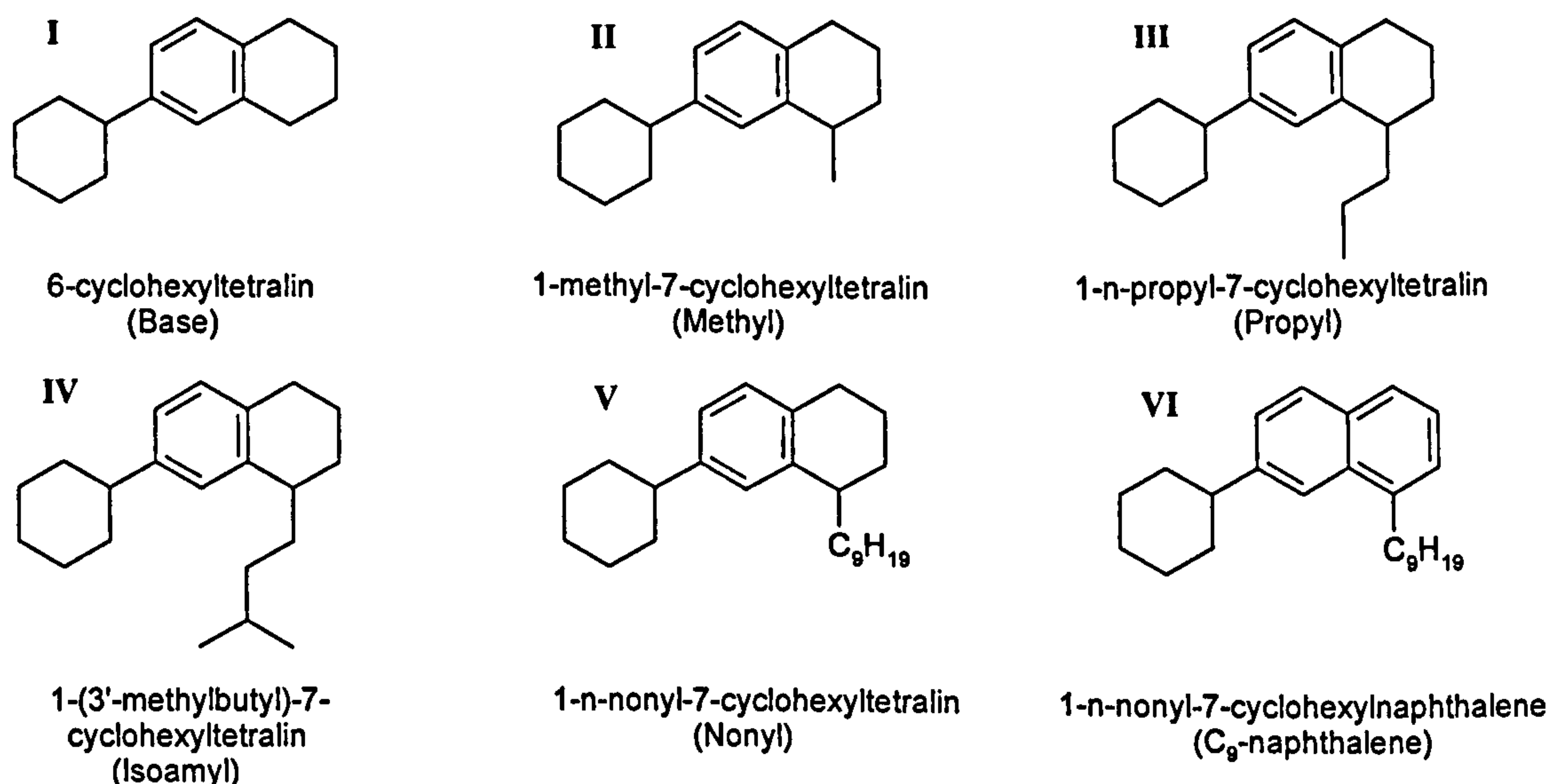


Figure 5.1. Two-dimensional structures of the synthetic alkyltetralins (I-VI) suggested as 'average' structures for some aromatic UCM components.

5.4 Isolation of an aromatic UCM hydrocarbon fraction

The aromatic UCM fraction was isolated from Tia Juana Pesado (TJP) crude oil (in-reservoir biodegraded, Venezuelan) using the method reported by Davies and Wolff (1990). Isolation of the aromatic fraction (TJPARO1) of TJP was *via* open column chromatography, which also yielded an aliphatic fraction (TJPALI) a heavy aromatic fraction (TJPARO2) and a polar fraction (POLAR) (Figure 5.2).

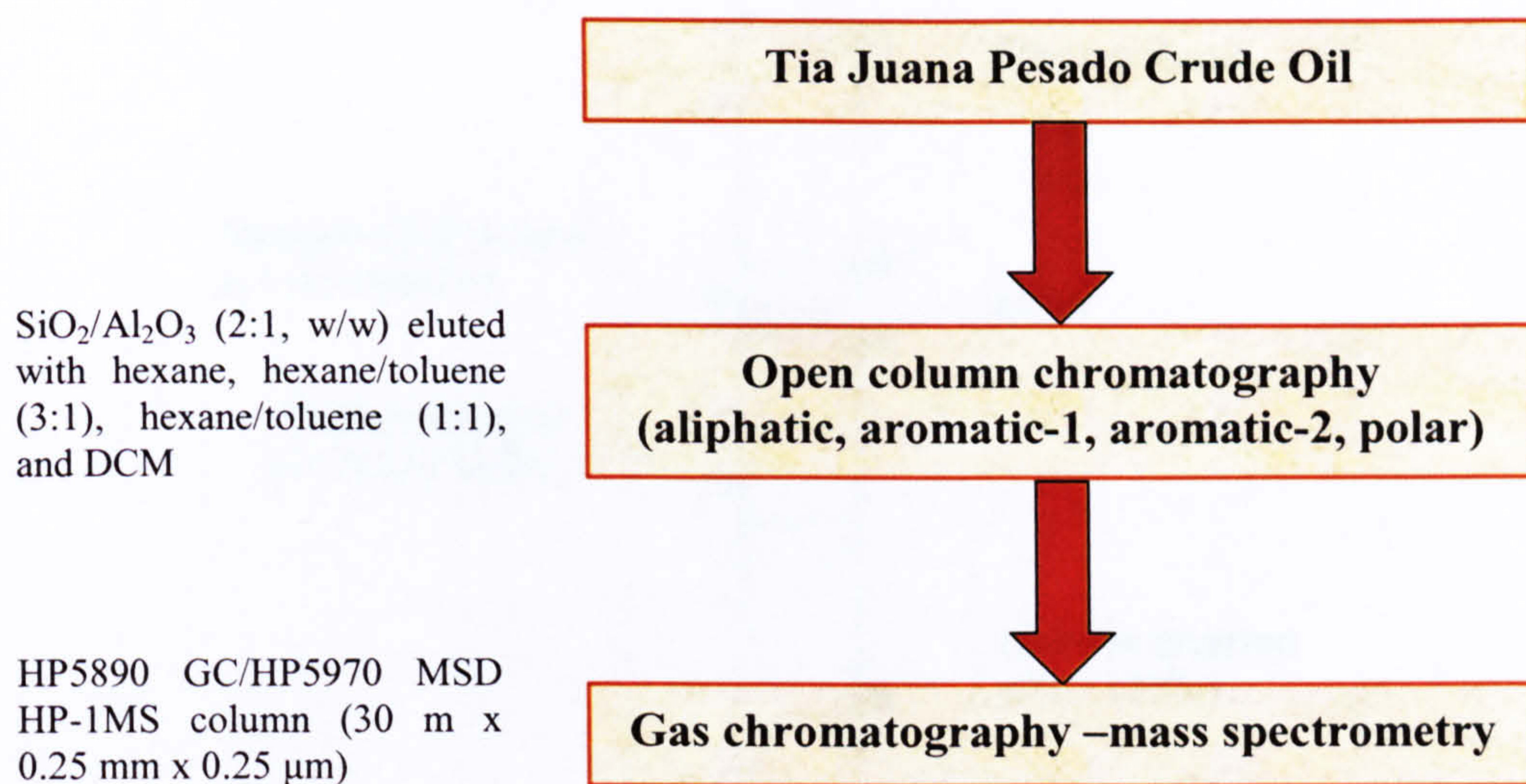


Figure 5.2. Schematic of experimental protocol - open column chromatography

Tia Juana Pesado crude oil (5 x *ca.* 1.0 g) was dissolved in hexane (~7 mL) and rotary evaporated (Buchi, 40 °C) to near dryness with x 10 w/w Al₂O₃ (BDH, England; grade 1, neutral, 150 mesh). Remaining solvent was allowed to evaporate at room temperature. The oil was loaded onto a sintered glass column silica/alumina (0.6 g cm⁻³ SiO₂ [Sigma-Aldrich Co. Ltd.; grade 645, 60 – 100 mesh], 5 % w/w H₂O deactivated; Al₂O₃, 1.5 % w/w H₂O deactivated; 50 % w/w Al₂O₃ : SiO₂). Deactivation was by addition of Milli-Q water and shaking for two hours (500 motions min⁻¹). The silica and alumina were each slurried using hexane and packed into the column, alumina above silica. The TJP on alumina was added to the top of the column (Figure 5.3) and eluted with three column volumes (330 cm³) each of hexane (TJPALI), hexane/toluene (3:1; TJPARO1), hexane/toluene (1:1; TJPARO2) and dichloromethane (POLAR) to provide fractions of the crude oil. The fractions were then rotary evaporated to near dryness (Buchi, 40 °C), and transferred to pre-weighed vials before gently blowing to dryness (N₂). The 3:1 hexane/toluene fraction was used as an 'aromatic' UCM for the biodegradation study.

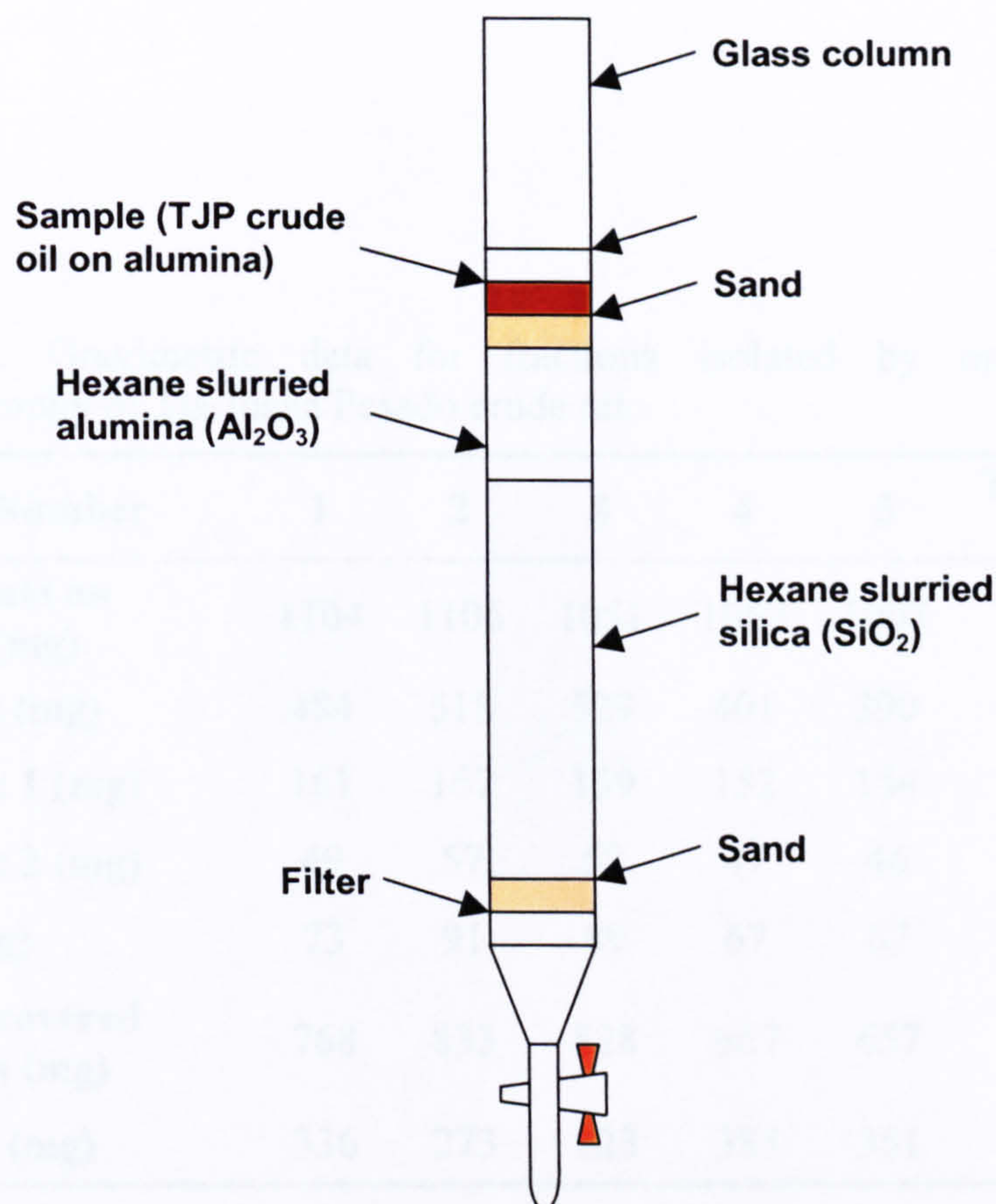


Figure 5.3. Apparatus used in open column chromatography Tia Juana Pesado crude oil fractionation.

Collected fractions were analysed using GC-MS (Section 5.2.2) with an Agilent Ultra-1 column (12.5 m x 0.20 mm x 0.33 μm) utilising a 72 min GC method (40-300 $^{\circ}\text{C}$ @ 5 $^{\circ}\text{C}$ min^{-1} , isothermal period of 10 min).

The gravimetric data from the fractionation of the Tia Juana Pesado crude oil by open column chromatography are summarised in Table 5.1. The residual fraction of the oil remaining on column was calculated by the difference between the mass of the total recovered fractions and initial on column mass of TJP crude oil. The gravimetric data reported in Table 5.1 are also summarised as percentages of the whole oil (Table 5.2, Figure 5.4). The TJPARO1 fractions from each of the columns were analysed individually using GC-MS to confirm their similarity before combining (Figure 5.5).

Table 5.1. Gravimetric data for fractions isolated by open column chromatography of Tia Juana Pesado crude oil.

Column Number	1	2	3	4	5	Total Mass (mg)
Initial Mass on Column (mg)	1104	1106	1051	1052	1007	5319
Aliphatic (mg)	484	518	529	401	390	2323
Aromatic 1 (mg)	161	167	159	152	154	793
Aromatic 2 (mg)	49	57	52	47	46	250
Polar (mg)	73	91	89	67	67	387
Total Recovered Fractions (mg)	768	833	828	667	657	n/a
Residual (mg)	336	273	223	385	351	1567

Table 5.2. Summary of the percentage composition of Tia Juana Pesado crude oil.

Fraction	Mean TJP Composition		
	mean % (n = 5)	Std dev	% rsd
Aliphatic	44	5	12
Aromatic 1	15	0	2
Aromatic 2	5	0	7
Polar	7	1	14
Total Recovered Fractions	71	7	9
Residual	30	7	22

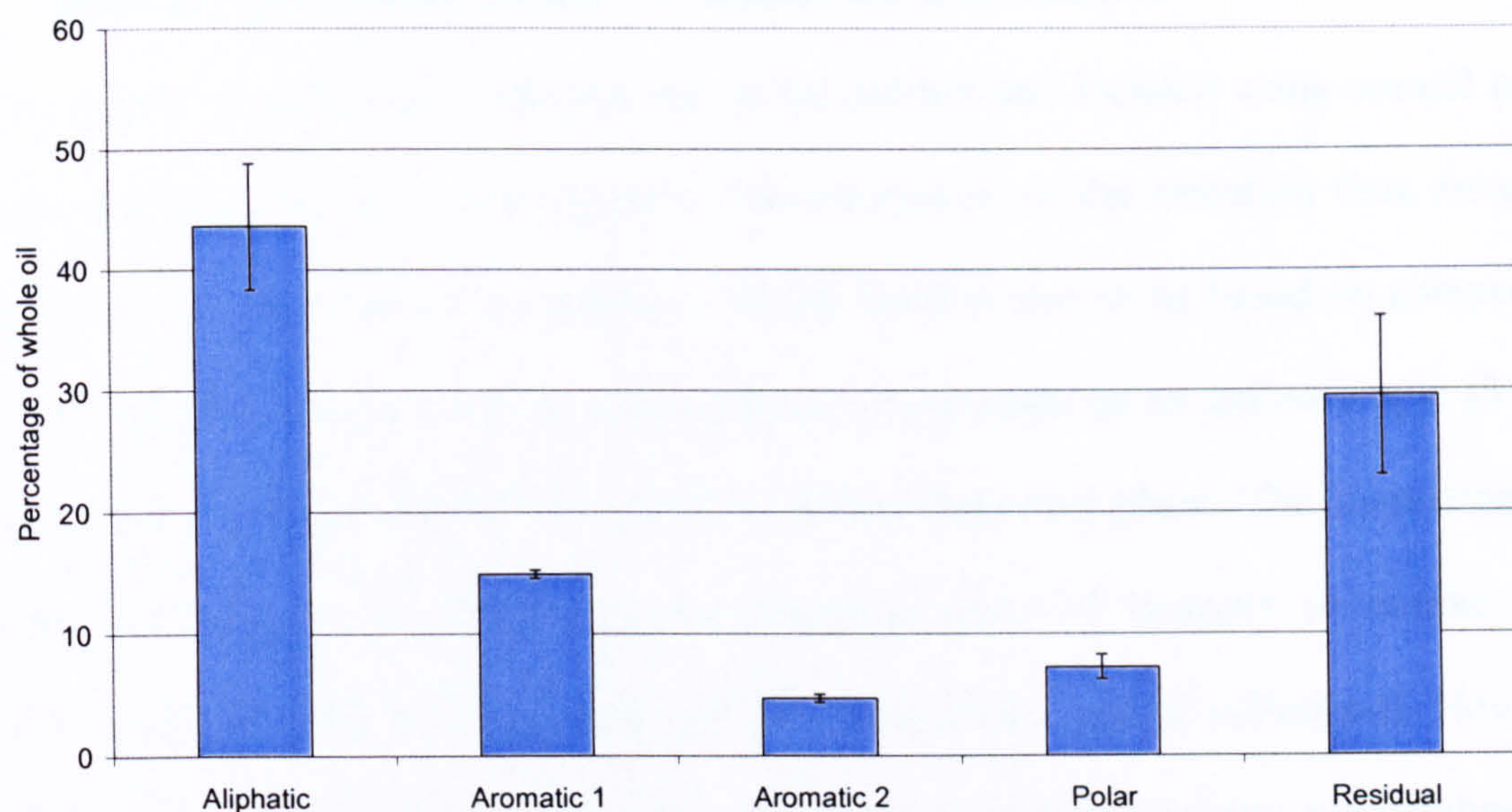


Figure 5.4. Relative proportion of each fraction of TJPARO1 whole oil from open column chromatography (n = 5).

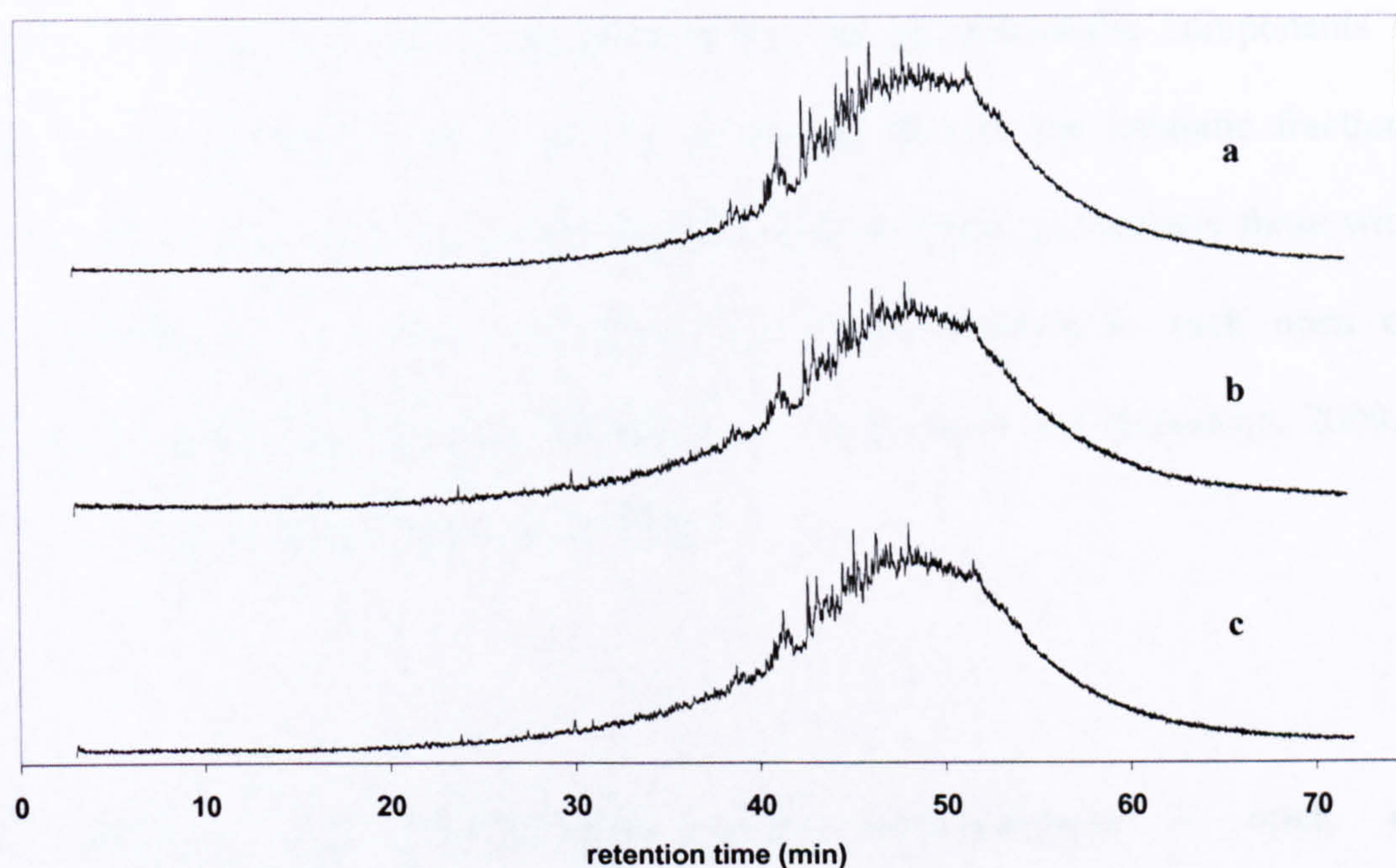


Figure 5.5. Examples of total ion current gas chromatograms of TJPARO1 isolated from (a) column 5, (b) column 2 and (c) the combined TJPARO1 fractions from all of the columns.

5.5 Isolation of a monoaromatic UCM hydrocarbon fraction

The so-called 'monoaromatic' fraction was to be defined and isolated using normal phase high performance liquid chromatography. Determination of the retention time envelope (beginning and end of fraction collection) of the fraction was to be based on comparison with the retention times of known monoaromatic compounds on an amino propyl (3 x 25 cm x 1cm, NH₂, 8µm Hypersil® HS APS-2 in series) stationary phase. The 'monoaromatic' fraction was thus to be defined by the retention times of benzene (4 double bond equivalents, DBE; start of collection) and indene (6 DBE; end of collection). However, when the TJPARO1 fraction isolated by open column chromatography was analysed by HPLC by the above method it was found not to contain the monoaromatic hydrocarbon components of the crude oil. Analysis of the saturate fraction collected during the open column chromatography procedure showed that the monoaromatic components of the crude oil had actually eluted in this fraction rather than in the aromatic fraction. The elution of the lower molecular weight aromatic hydrocarbons, particularly those with long alkyl side chains, in the saturate fraction is not uncommon in such open column chromatographic procedures (*e.g.* Robert *et al.*, 1994; Dutta and Harayama, 2000; Dutta and Harayama, 2001; Frysinger *et al.*, 2003).

5.5.1 Isolation of monoaromatic UCM hydrocarbons - open column chromatography

As the 'monoaromatic' fraction required for study was identified in the aliphatic (saturate) fraction of the crude oil, a further open column chromatography step was introduced to isolate these aromatic compounds. The chromatography was performed using 60 x 3 cm i.d. sintered glass columns. Columns were slurry packed with fully activated silica gel (60-100 mesh, Sigma Aldrich) in pentane, on a 50:1 mass ratio to the sample. A layer of washed sand was placed on top of the silica gel. The aliphatic oil fraction was dissolved in

a minimum amount of pentane and transferred carefully to the top of the column using a Pasteur pipette. The aliphatic compounds were then eluted from the column with three column volumes of pentane. The remaining aromatic compounds (TJPAROX) were eluted with three column volumes of dichloromethane. Fractions were collected in 500 mL round bottom flasks and solvent was removed by rotary evaporation (Buchi, 40 °C) until samples were almost dry. Samples were then transferred to pre-weighed 7 mL vials and the remaining solvent removed with a gentle stream of nitrogen.

The gravimetric data from the second open column chromatography step of the aliphatic fraction are summarised in Table 5.3. The residual fraction of the oil remaining on column was calculated by the difference between recovered fractions and initial on column mass of TJP. This data is summarised as percentage composition in Table 5.4.

Table 5.3. Masses of hydrocarbon fractions generated by open column chromatography of the aliphatic fraction of TJP crude oil. The mass of the residual fraction is determined by difference.

Column Number	1	2	Total mass (mg)
Initial mass on column (mg)	1040	796	1836
Aliphatic (mg)	823	589	1412
Aromatic (mg)	212	199	411
Residual (mg)	5	7	13

Table 5.4. Summary of the percentage composition of the aliphatic fraction of Tia Juana Pesado crude oil.

Mean composition			
Fraction	Mean (n = 2) percentage	Stdev	% rsd
Aliphatic	77	4	5
TJPAROX	23	3	14
Residual	1	0	43

5.5.2 Isolation of monoaromatic UCM hydrocarbons - HPLC

The separation and retention times of aromatic hydrocarbons on amino propyl (NH₂) HPLC columns, operated under the conditions used herein, is primarily based upon the double bond equivalent (DBE) value of an individual compound. In general, retention time increases with an increase in the DBE value of a series of compounds. However, the retention time can also be affected by the presence of substituent groups and further by the degree of substitution and the size of the substituents present on a compound. Generally, a more highly substituted aromatic compound will have its retention time shifted so that it elutes earlier than a more condensed form, *e.g.* benzene (condensed) will have a longer retention time than and nonyl benzene (substituted). It is therefore unlikely that the isolated 'monoaromatic' fraction will consist wholly of truly monoaromatic compounds. Highly substituted polyaromatic hydrocarbons (*e.g.* di-, tri-, and tetra-aromatic) will have reduced retention times (compared to unsubstituted homologues) which may result in their elution in the 'monoaromatic' fraction.

The double bond equivalent value for a compound was determined using the equation below. The start and end point of the fractions was defined by analysis of a suite of standards with different DBE values (Figure 5.6). Thus, the 'monoaromatic' fraction was defined as that material eluting between benzene (4 DBE) and indene (6 DBE). A 'diaromatic' fraction was defined as those hydrocarbons eluting between naphthalene (7 DBE) and fluorene (9 DBE) (Table 5.5).

$$\text{DBE} = (\text{no. carbon atoms} - (\text{no. hydrogen atoms} / 2)) + 1$$

$$\text{DBE} = (nC - (nH / 2)) + 1$$

$$\text{e.g. for benzene: } \text{DBE} = (6 - (6 / 2)) + 1$$

$$\text{DBE} = 4$$

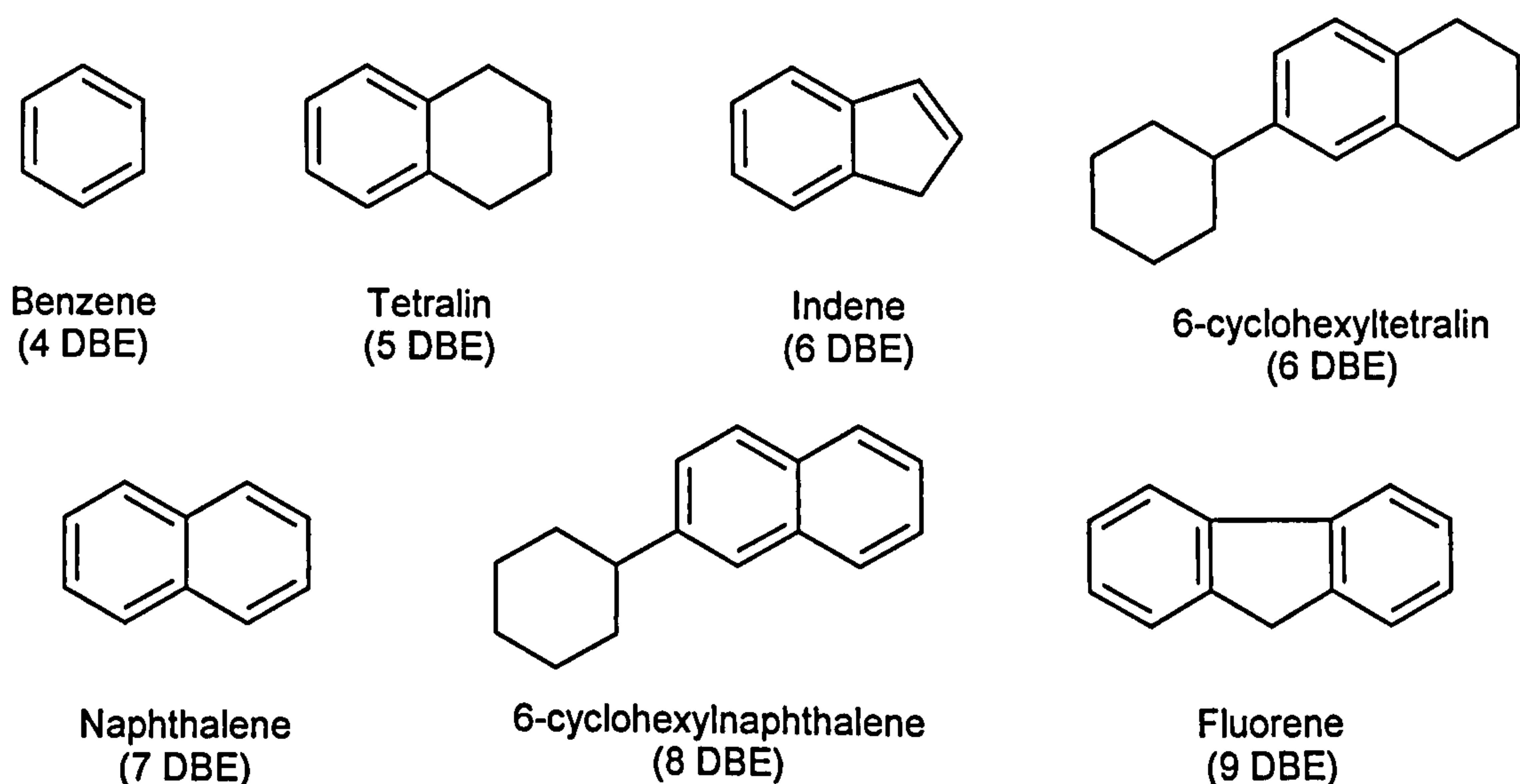


Figure 5.6. Molecular structure and 'double bond equivalent' (DBE) values of hydrocarbons used to determine the retention times of a 'monoaromatic' and 'diaromatic' fraction of TJP oil.

Table 5.5. Summary of retention times used to determine the 'mono-' and 'diaromatic' fractions of TJPAROX.

Fraction	Start time of cut (min)	End time of cut (min)
Monoaromatic	30.06	36.78
Diaromatic	36.78	54.95

5.5.2.1 Preparative HPLC conditions

Preparative HPLC isolation of the 'monoaromatic' fraction of TJP oil was performed using a Hewlett Packard high performance liquid chromatograph (1050 series), with 3 x amino propyl (Hypersil® HSAPS-2) stainless steel columns (25 cm x 10 mm) in series. TJPAROX samples were fractionated using a 100 min method (0-40min 100% hexane, 40-45 min 100% hexane – 100% DCM, 45-65 min 100% DCM, 65-70 min 100% DCM – 100% hexane, 70-100 min 100% hexane for column equilibration). Samples were dissolved in hexane and injected onto the column in amounts of approximately 30 mg per

analysis. Fractions were collected in round bottom flasks and solvent was removed *via* rotary evaporation (Buchi, 40 °C) until almost dry. Samples were then transferred to pre-weighed 7 mL vials and the remaining solvent removed using a gentle stream of nitrogen. A mixture of authentic aromatic hydrocarbons (Figure 5.6) was analysed periodically to monitor any changes in compound retention time.

The percentages of 'monoaromatic' and 'diaromatic' hydrocarbons in the TJPAROX fraction were determined *via* mass balance. Hydrocarbons with DBE values higher than 9 were collected as a residual fraction, and their mass was also determined (Table 5.6).

Table 5.6. Gravimetric summary of isolated fractions of TJPAROX. Also shown are the envelope/cutting times of the fractions as determined by the analysis of known compounds.

Fraction	Cut Time (min)	Mass (mg)	Percentage of TJPAROX	Percentage of whole oil
TJPALI	n/a	90	100	44
Monoaromatic	30.06 - 36.78	16	18	8
Diaromatic	36.78 - 54.95	23	26	11
Residue	20.00-30.06 / 54.95-75.00	40	44	19

Table 5.6 shows that the monoaromatic fraction isolated from the TJPAROX fraction of the crude oil by open column chromatography comprises approximately 18% of the aliphatic fraction, and therefore 8% of the whole oil by weight.

5.6 Synthesis of model monoaromatic UCM hydrocarbons

The cyclohexylalkyltetralins (I-V) and cyclohexylalkylnaphthalene (VI) were synthesised previously (Wraige, 1997; Sturt, 2000; Smith, 2002). However, some of these compounds were synthesised as mixtures that required further purification herein. The alkylnaphthalene (VI) was formed as a by-product in the synthesis of 7-cyclohexyl-1-nonyltetralin (V) and was isolated by column chromatography of the products from the Grignard reaction as described by Sturt (2000).

5.7 Purification of synthetic monoaromatic hydrocarbons by HPLC

The synthesis procedures used by Sturt (2000) and Smith (2002) for the alkylcyclohexyltetralins (I-V) and the alkylcyclohexylnaphthalene (VI) resulted in the presence of impurities in the final samples. Prior to use in experimental studies herein, compounds I-VI were individually purified using preparative HPLC.

Purification was performed using a Hewlett Packard high performance liquid chromatograph (1050 series), with a single amino propyl (Hypersil[®] HSAPS-2) stainless steel column (25 cm x 10 mm). The compounds I-VI were individually injected on to the column with separation of synthetic compounds and the impurities in the samples achieved by a 40 min method (0-40min 100% hexane). Samples were dissolved in hexane to a concentration of 50 mg mL⁻¹, and 100 µL (5 mg of each compound) was injected on column. All peaks were collected in individual vials. The largest component was assumed to be the target compound in each case. Purified samples were blown to dryness using a gentle stream of nitrogen, and the mass determined. Samples were then dissolved in dichloromethane to a concentration of 1 mg mL⁻¹, and diluted to 0.01 mg mL⁻¹ for analysis by GC-MS. Analysis by GC-MS (Section 5.2.2) confirmed that the target compounds (I-VI) had been isolated (Table 5.7).

Table 5.7. Results from HPLC purification of alkyltetralins (I-V) and alkylnaphthalene (VI).

Compound	Purity (%)
I (6-cyclohexyltetralin)	95.6
II (7-cyclohexyl-1-methyltetralin)	100
III (7-cyclohexyl-1-propyltetralin)	100
IV (7-cyclohexyl-1-isoamyltetralin)	87.3
V (7-cyclohexyl-1-nonyltetralin)	100
VI (7-cyclohexyl-1-nonylnaphthalene)	100

5.8 Biodegradation experimental procedures

The biodegradation experiments involved two main studies, one with a mono-culture, *Pseudomonas fluorescens* (Texaco) and one with a mixed culture of bacteria isolated from a natural site (Whitley Bay, Tyne and Wear, UK).

5.8.1 *Pseudomonas fluorescens* mono-culture studies

The biodegradation experiments were based on the methods of Gough *et al.* (1992) and Heath *et al.* (1997). All glassware was sterilised by autoclave (121 °C, 20 min) or dry heat (160 °C, 60 min). Following sterilisation, all glassware was rinsed with dichloromethane, to remove any residual hydrocarbons. The bacterium *Pseudomonas fluorescens* (Texaco) was originally isolated from a metal working fluid waste by Beech and Gaylarde (1989). A pure culture of the bacterium was grown from stock used previously (Gough, 1989; Heath *et al.*, 1997). The culture was grown in a nutrient broth (13 g OXOID in 1 litre de-ionised water) for 24 h at 37 °C.

5.8.1.1 Reagents and inoculum

A minimal salt solution was prepared by dissolving the following salts (w/v); 0.5% NH_4Cl ; 0.3% K_2HPO_4 ; 0.2% Na_2SO_4 ; 0.1% NH_4NO_3 ; 0.1% KH_2PO_4 and 0.01% $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ in 1000 mL deionised (Milli-Q) water and sterilised by autoclave (121 °C, 20 min). 10 mL of the sterilised minimal salts solution was added to each of 60 conical flasks (25 mL). This number of flasks allowed for triplicate samples to be taken at each sampling time. An approximately equimolar mixture of the individual hydrocarbons (I-VI, or *n*-pentacosane) or crude oil aromatic UCM, was dissolved in hexane to a concentration of 1 mg mL⁻¹ (aromatic UCM 10 mg mL⁻¹) and 100 µL was injected into each culture flask. Lastly, 0.5 mL of bacterial inoculum was then added, and the flasks sealed with sterile non-absorbent cotton wool. Incubation (up to 50 d) was performed aerobically on a shaking water bath maintained at a minimum of 22 °C and covered with foil to prevent any action caused by light. Control flasks containing minimal salts solution and the hydrocarbon substrate but no bacterial inoculum, were incubated under the same conditions to monitor any abiological losses.

5.8.1.2 Measurement of bacterial viability and monitoring bacterial growth

Two biotic cultures were set aside and used solely to monitor bacterial viability and monitor bacterial growth. On each day of sampling, using sterile techniques, a solution sample was streaked onto a nutrient agar plate. This was incubated for 24 h at 30 °C and any bacterial growth observed was taken as evidence of viability.

Estimating the turbidity is a practical way of monitoring bacterial growth. As bacteria multiply in a liquid medium, the medium becomes turbid, or cloudy with cells (Tortora *et al.*, 1998). Turbidimetry is the term given to any procedure in which the concentration of cells in a suspension is estimated by passing a beam of light through the suspension and

comparing the intensity of the transmitted light with that obtained with a cell-free control. Light transmitted by a suspension is of lower intensity than that of the control due mainly to light scattering (Singleton and Sainsbury, 2001). Turbidimeters (*e.g.* colorimeters and absorptimeters acting as spectrophotometers) consist essentially of a colourless, transparent sample holder (cuvette), a means of passing a narrow beam of monochromatic light through the cuvette, and a photoelectric cell to measure the intensity of the transmitted light (Koch, 1994; Isaac and Jennings, 1995; Singleton and Sainsbury, 2001). A measure of the decrease in transmitted light (turbidity) is the optical density (OD) or absorbance – given by $\log(I_0/I)$ where I_0 is the intensity of the incident light, and I the intensity of the transmitted light (Koch, 1994; Isaac and Jennings, 1995; Tortora *et al.*, 1998; Singleton and Sainsbury, 2001). When the bacteria are in logarithmic growth or decline, a graph of absorbance versus time will form an approximately straight line (Tortora *et al.*, 1998).

In the present study the optical density of the solutions was measured using two different spectrophotometers (a) Cecil CE 1010 (1000 series) and (b) Unicam Helios Epsilon to monitor any bacterial growth. Samples containing only the minimal salts solution and the test substrates but no bacteria were used as blanks to determine a ‘zero’ value. All samples were analysed in 1 cm³ quartz cuvettes.

5.8.1.3 Hydrocarbon Extraction

Pentacosane (n -C₂₅) was used as an internal standard during extraction and analysis procedures in order to monitor any losses of the synthetic compounds. The n -C₂₅ was dissolved in hexane to a concentration of 1 mg mL⁻¹ and 100 µL was injected into each conical flask immediately after bacterial exposure prior to extraction. For the study of the degradation of n -C₂₅, n -hexacosane (n -C₂₆) was used as the internal standard.

Dichloromethane (10 mL) was added to each flask and left to stand for 5 minutes to kill the bacteria and also to extract the hydrocarbons from the minimal salts solution. Flask contents were transferred to glassware suitable for centrifugation, and extracts were recovered by centrifugation (2500 rpm, 20 min). The hydrocarbon extraction procedure was repeated twice more and the extracts combined. After drying (anhyd. Na₂SO₄; 30 min), extracts were evaporated to near-dryness (Buchi, 30 °C), transferred to vials and gently blown down to dryness (N₂). Samples were made up in dichloromethane (1 mL) and diluted 10 x prior to analysis by gas chromatography (synthetic compounds only diluted, Section 5.8.1.3).

5.8.1.4 Gas chromatography

Extracts from the *P. fluorescens* biodegradation study were examined by GC (Section 5.2.1, except HP-1 fused silica capillary column 12 m x 0.2 mm id x 0.33 µm film thickness and oven temperature was programmed from 40-300 @ 10°C min⁻¹ and held for 10 min). Quantification was made by comparison of peak areas with calibration data and internal standard recoveries. The aromatic UCM samples were measured relative to a series of solutions of known UCM concentration (*cf.* Gough *et al.*, 1992).

5.8.2 Whitley Bay consortium mixed culture studies

The biodegradation experiments were based on the method of Watson *et al.* (2002) and performed on compounds I, II, IV and VI. These compounds were supplied by the author to C. Aitken (University of Newcastle) where the experiments were conducted. All data analysis and processing was completed by the author (A. Booth) at the University of Plymouth. Culturing of the bacterial inoculum has been described previously (Watson *et al.*, 2002). Briefly, seawater was collected from St. Mary's Island (Whitley Bay, Tyne and

Wear, UK) and a marine sediment ('clean' sand) was collected from Cullercoats beach (Tyne and Wear, UK). Both were stored at 4 °C in the dark for 24h prior to use.

5.8.2.1 Reagents and inoculum

A total of thirteen (Flasks 1-13) 250 mL Erlenmeyer flasks were used for the experiment. All flasks contained 10 g marine sediment, 30 mL seawater, and a supply of nutrients as reported previously (Watson *et al.*, 2002). Flasks 1 – 4, 9 and 10 contained 45 mg North Sea oil, and test compounds I (45 µg), II (34 µg), IV (59 µg) and VI (39 µg). Flasks 9 and 10 were sterilised by autoclaving and the addition of 500 µg mL⁻¹ sodium azide (Watson *et al.*, 2002). Flasks 5 – 8, 11 and 12 contained 39 mg *n*-hexadecane and test compounds I (45 µg), II (34 µg), IV (59 µg) and VI (39 µg). Flasks 11 and 12 were sterilised by autoclaving and the addition of 500 µg mL⁻¹ sodium azide (Watson *et al.*, 2002). Flask 13 contained only sediment, seawater and nutrients. The flasks were stoppered with non-absorbent cotton wool and incubated under aerobic conditions in the dark at room temperature on an orbital shaker (80 rev/min). The total contents of each of the flasks were extracted and analysed after 0 (Flask 13), 28 (Flasks 1, 2, 5, 6, 9, 11) or 119 days (Flasks 3, 4, 7, 8, 10, 12). Thus Flasks 9 and 11 acted as 28 day sterilised controls and Flasks 10 and 12 as 119 day sterilised controls. After removal from the incubator the flasks were placed in the freezer until extraction and analysis could be completed for all samples simultaneously. Flask 13 containing only the sand, seawater and nutrients, was plugged with non-absorbent cotton wool and placed in the freezer straight away.

5.8.2.2 Hydrocarbon extraction

The samples in each of the flasks were removed from the freezer and allowed to thaw before spiking with squalane (*ca* 200 µg, Aldrich, UK) as a recovery standard (R.S.) prior

to extraction. The contents of each flask were then transferred to a 250 mL round bottom flask and each sample hydrolysed by reflux (1M KOH, 1 hr; Watson *et al.*, 2002). Samples were then allowed to cool and the contents transferred to separating funnels, acidified (HCl) to pH 2 and extracted (x 3) with aliquots (50 mL) of DCM. Extracts were rotary evaporated to ~5 mL, dried with anhydrous sodium sulphate and then divided equally into two portions. One portion was retained for analysis of acid biodegradation products (Aitken, 2003) and is not discussed further here. The other was used for hydrocarbon analysis as described herein. Prior to analysis by gas chromatography (Section 5.8.2.3), *n*-C₂₅ (100 µg) was added to this portion as an internal standard (I.S.).

5.8.2.3 Gas chromatography

Extracts from the bacterial consortium biodegradation study were examined using GC (Section 5.2.1, except HP-5MS fused silica capillary column 30 m x 0.25 mm id x 0.25 µm film thickness; oven temperature programmed at 50 °C for 2 minutes then from 50-300 °C at 4 °C min⁻¹ and held for 20 min). Quantification was made by comparison of peak areas with calibration data and internal standard recoveries. GC-MS analyses were performed as previously described (Section 5.2.2, except oven temperature programme was the same as above) Analyses were carried out in both selected ion monitoring and (selected samples only) full scan mode.

5.9 Water solubility behaviour of isolated aromatic UCM fractions and a synthetic hydrocarbon

5.9.1 Test materials

6-cyclohexyltetralin, previously synthesised in good yield (Wraige, 1997) and purified to > 98 % by Smith (2002) was used as a test substrate. The 'aromatic' UCM was isolated from Tia Juana Pesado (Venezuela) crude oil as described in Section 5.4, and a 'monoaromatic' sub-fraction isolated as described in Section 5.5.

5.9.2 Experimental procedure

A generator column technique (Figure 5.7), based upon that originally devised by May *et al.* (1978), and used by Smith *et al.* (2001) was employed to determine the water solubility of the test substrates. Individual generator columns consisted of stainless steel HPLC columns (25 cm x 4.6 mm i.d. x ¼' o.d.) with stainless steel frits at either end. These were then dry-packed with glass beads (size 60-80 mesh; Alltech Associates), coated with the test compound or UCM fraction. Prior to coating, the beads were washed in a soxhlet thimble (3 x 100 mL of dichloromethane; hexane; acetone; and Milli-Q water) and dried in an oven (120°C overnight). The glass beads were then stored in a dessicator until required. All test substrates (35 mg) were dissolved in 50 mL of hexane to which 7 g of the glass beads were added. The solvent was removed by gentle rotary evaporation and left to dry overnight in a fume cupboard. This resulted in a 0.005 % coating of the test materials on the beads. After the beads were dry packed into the generator column it was attached to a HPLC pump (Waters Associates, Milford, Massachusetts, isocratic chromatography pump, model 590) using Teflon tubing attachments, connected to a Milli-Q water reservoir.

The system was maintained at 25 °C by means of a water bath, which contained the submersed generator column and the Milli-Q water reservoir. The temperature of the water bath was monitored using a Squirrel datalogger (SQ32-4U, Grant Instruments) equipped with two probes. The water reservoir comprised 2 x 2 L sealed glass bottles, containing Milli-Q water which was topped-up as required.

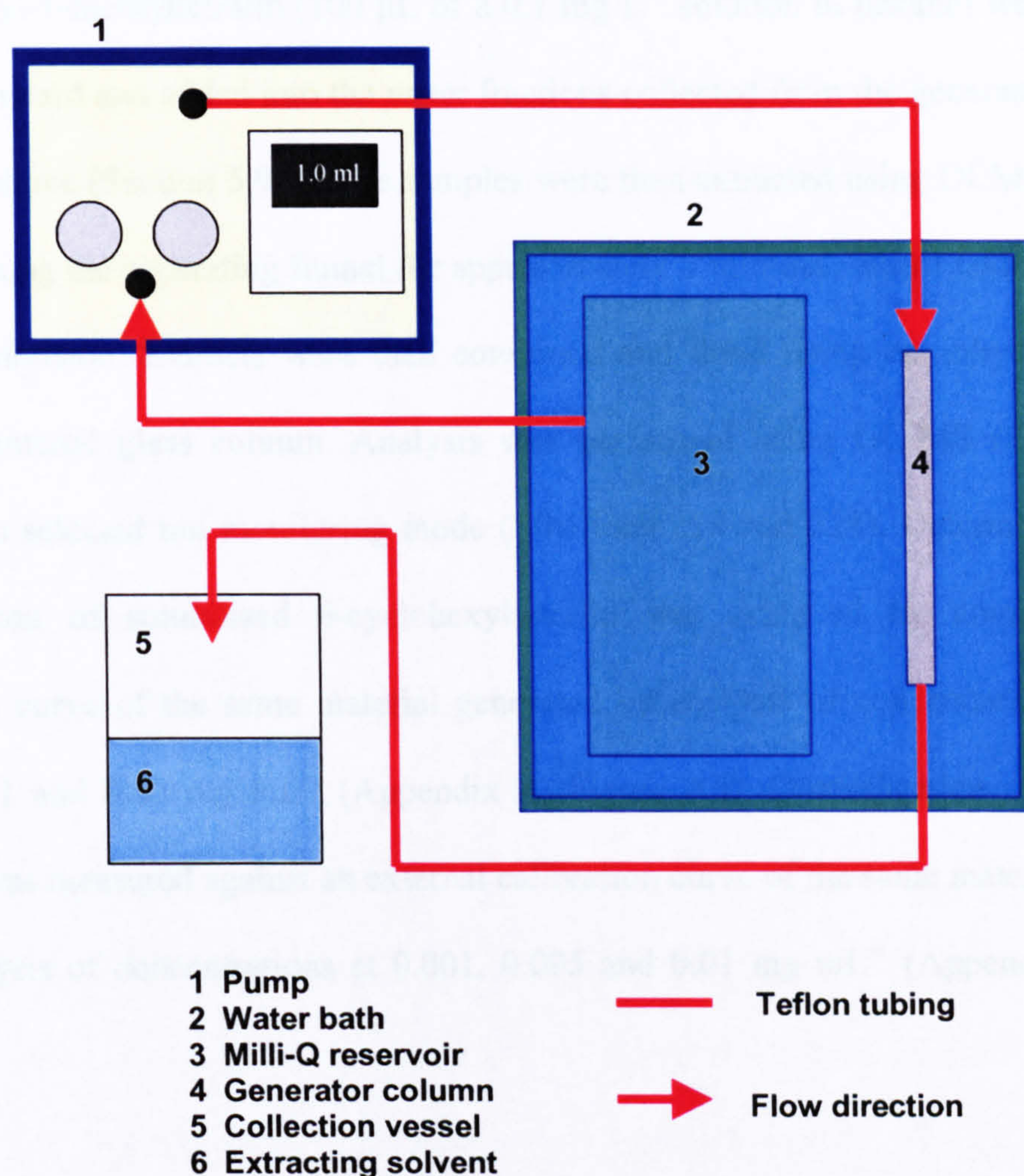


Figure 5.7. Schematic of the generator column apparatus used in the determination of water solubility.

5.9.3 Generation of aqueous solutions

Once connected to the system, the generator column was initially flushed with 500 mL of water to allow equilibration of the system before measurement. Water was pumped through the generator column at a rate of 1 mL min⁻¹. Generated solutions were collected at

either 50 minute (50 mL) or 500 minute (500 mL) intervals, with the former being collected in 100 mL separating funnels and the latter in 500 mL separating funnels. Ten consecutive samples were collected in each case.

5.9.4 Extraction of aqueous solutions of 6-cyclohexyltetralin

7-cyclohexyl-1-methyltetralin (100 μL of a 0.1 mg L^{-1} solution in hexane) was used as an internal standard and added into the water fractions collected from the generator column as described above (Section 5.9.3). The samples were then extracted using DCM (3x 25 mL), gently shaking the separating funnel for approximately 5 minutes, whilst taking care not to form an emulsion. Extracts were then combined and dried using an anhydrous sodium sulphate sintered glass column. Analysis was performed using GC-MS (Section 5.2.2) operated in selected ion monitoring mode (SIM ions 214 and 228). Determination of the concentration of solubilised 6-cyclohexyltetralin was achieved by comparison to a calibration curve of the same material generated by analysis of concentrations at 0.001, 0.005, 0.01 and 0.05 mg mL^{-1} (Appendix A, Figure A3). Quantification of the internal standard was measured against an external calibration curve of the same material generated from analysis of concentrations at 0.001, 0.005 and 0.01 mg mL^{-1} (Appendix A, Figure A4).

5.9.5 Extraction of aqueous solutions of aromatic and monoaromatic UCM fractions

For determination of the water solubility of the isolated aromatic and monoaromatic UCMs it was not possible to accurately use an internal standard in the extraction procedure. The inherent nature of the UCMs used in this study *i.e.* that they could not be chromatographically resolved using the gas chromatographic techniques employed (Sections 5.2.1 and 5.2.2), meant that addition of an internal standard would result in its

co-elution with other compounds present naturally within the UCM. Instead of an internal standard, results were quantified using an external calibration. The generator column was removed from the system and 500 mL of Milli-Q water was collected in a separating funnel. To this 1 mg of either the aromatic or 'monoaromatic' UCM was added (dissolved in 100 μ L of hexane). The external calibration sample was extracted in the same manner and analysed using GC-MS (Section 5.2.2). Quantification was determined against a calibration curve of the same material generated from the analysis of triplicate samples of concentrations (0.1, 0.25, 0.5, 0.75, and 1.0 mg mL⁻¹; Appendix A, Figure A1 and A2). Samples containing the test substrates were extracted as reported in Section 5.9.4 and analysis performed using GC-MS operated in full scan mode (Section 5.2.2.).

5.10 Comprehensive gas chromatography (GCxGC) coupled to time-of-flight (ToF)

Mass Spectrometry (MS)

The material collected in the solubility experiments conducted as part of this research was studied in order to gain a detailed understanding of the hydrocarbon composition of the water soluble monoaromatic UCM. Owing to its complex nature, the material was analysed by the latest chromatographic and mass spectrometric methods available; gas chromatography x gas chromatography-time of flight mass spectrometry (GCxGC-ToF-MS). The low concentration of the water soluble monoaromatic UCM ($\sim 57 \mu\text{g L}^{-1}$) meant each 500 mL aliquot collected contained on average $\sim 30 \mu\text{g}$ of organic material. A concentration of 10 mg mL⁻¹ was required for analysis of a complex mixture by GCxGC-ToF-MS. Since individual aliquots did not contain sufficient material to achieve suitable analysis, five of the aliquots which contained the most UCM hydrocarbons were pooled together to create a composite sample, to give an approximate amount of 150 μg (0.15 mg) of UCM hydrocarbons. Dilution of this material in 10 μL of solvent (hexane) gave a concentration of approximately 15 mg mL⁻¹, which was sufficient for analysis by GCxGC-

ToF-MS. Details of the individual water soluble monoaromatic UCM aliquots combined to create the sample are reported in Table 5.8.

Table 5.8. Summary of the water soluble monoaromatic UCM composite sample, created from the 500 mL aqueous aliquots for analysis by GCxGC-ToF-MS. Amounts of material in each extract were determined by external calibration of a monoaromatic UCM (Section 5.9.5).

Water soluble monoaromatic UCM aliquot	Concentration of UCM hydrocarbons in the fraction ($\mu\text{g L}^{-1}$)	Amount of UCM hydrocarbons in the sample (μg)
1000-1500 mL	76	38
1500-2000 mL	39	19.5
2000-2500 mL	83	41.5
2500-3000 mL	32	16.5
3500-4000 mL	64	32
Composite sample	n/a	147.5

The composite sample of the water soluble monoaromatic UCM material was analysed on a Pegasus 4D (Leco Corporation, USA) GCxGC-ToF-MS system, based on a HP 6890 Gas Chromatograph (Agilent Technologies, Wilmington, DE, USA) interfaced to a Pegasus III time-of-flight mass spectrometer (LECO, St Joseph, MI, USA). The system used the following parameters: injector 250 °C; transfer line 300°C, ion source 280 °C, EM 1900 V. The first-dimension column was a 10 m x 180 μm x 0.18 μm DB-5 (apolar; J&W Scientific, Folsom, CA, USA), and the second-dimension column was a 1.9 m x 100 μm x 0.1 μm DB-17 (polar). The first-dimension oven was programmed at 70 °C for 0.5 min, then raised from 70-285 °C at 5 °C min⁻¹ and held at this temperature for 10 min. The second-dimension oven was programmed at 100 °C for 0.5 min, then raised from 100-300 °C at 5 °C min⁻¹ and held at this temperature for 12 min. The modulator temperature was offset by 30 °C, hot pulse time 1.0 s, cool time between stages 3.0 s; EPC control in constant flow 1 mL min⁻¹. The carrier gas was helium. Prior to analysis the sample was

dissolved in 10 μl of hexane. 1 μl of the sample was injected (splitless) into the GCxGC-ToF-MS system *via* an Agilent Technologies 7863 Series Autosampler.

The ToF-MS system was used to produce mass spectral data, and operated at a spectrum storage rate of 50 Hz (50 spectra s^{-1}). The mass range monitored was from 45-500 Daltons. The automated data processing was achieved using LECO[®] ChromaToF[™] software (version 2.01, Leco Inc., USA). The software completes the peak finding routine, the deconvolution of mass spectra from partially coeluting compounds and a preliminary NIST library search.

Identification of unknown compounds within the sample was based on NIST library mass spectral matches. Whilst this could be achieved using system computer software, it was found in the present study that a manual approach to identifying library matches proved more effective but was time consuming. The common use of library mass spectra to identify unknown compounds in samples is now being applied to samples analysed by GCxGC-ToF-MS (*e.g.* Shellie *et al.*, 2001).

Chapter 6

Conclusions and future work

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

6.1 Conclusions

Unresolved complex mixtures (UCMs) of hydrocarbons, comprised of environmentally persistent compounds (Seymour and Geyer, 1992; Readman *et al.*, 1996; Gogou *et al.*, 2000; Schwarzenbach *et al.*, 2003), are widespread, occurring in sediments, aerosols, the water column and most poignantly in marine organisms such as molluscs and fish (*e.g.* Requejo and Boehm 1985; Preston *et al.*, 1992; Fowler *et al.*, 1993; Maldonado *et al.*, 1999; Meniconi *et al.*, 2002; Reddy *et al.*, 2002). Whilst bioaccumulative, aliphatic UCMs are not considered detrimental to the health of marine organisms (*e.g.* Thomas, 1995). In contrast, aromatic UCMs, which can also bioaccumulate to high concentrations in marine organisms (*e.g.* Neff *et al.*, 2000; Page *et al.*, 2002) exhibit non-specific narcotic toxicity. Recent studies (*e.g.* Rowland *et al.*, 2001; Donkin *et al.*, 2003) have linked the monoaromatic hydrocarbon UCM fraction with observed impaired health in mussels. Despite growing environmental concerns, little is known about the composition of aromatic UCMs. The overall aims of the present study were to obtain more information about the environmental behaviour and the chemical composition of these seemingly persistent compounds.

The specific objectives of the current study were therefore:

- to determine the environmental persistence of an aromatic UCM and synthetic aromatic hydrocarbons by conducting biodegradation experiments
- to generate and compare aqueous solutions of aromatic and monoaromatic UCMs, to provide an indication of their bioavailability
- to use a state-of-the-art GCxGC-ToF-MS instrument to attempt to better resolve an aromatic UCM and to better characterise the component hydrocarbons.

In the first assay, an aromatic UCM isolated from Tia Juana Pesado (TJP) crude oil (Venezuela), and a suite of synthetic alkylcyclohexyltetralins and an

alkylcyclohexylnaphthalene were exposed to the known hydrocarbon degrading bacterium, *Pseudomonas fluorescens* (Texaco). After an exposure period of 50 days, biodegradation had not exceeded 20% for any of the model compounds. The most resistant compounds (~2% biodegradation) appeared to be those with alkyl chains of C₃-C₅ length, particularly those which were branched. In the case of the aromatic UCM an exposure period of 41 days resulted in negligible biodegradation (~2%), comparable to that of some of the synthetic compounds.

The second assay used a natural consortium of aerobic bacteria isolated from a hydrocarbon-polluted environment (Whitley Bay, Tyne and Wear, UK). In these studies, four of the synthetic compounds were exposed to the consortium with both North Sea oil and *n*-hexadecane as co-substrates. The synthetic compounds exhibited 15-80% degradation after 28 days. By 119 days, two were completely degraded, but 60% of the branched chain C₅ homologue still remained. Under these severe conditions the North Sea crude oil co-substrate was extensively degraded with even the highly bioresistant pentacyclic hopanes undergoing some degradation. Thus, some of the alkylcyclohexyltetralins are resistant to all but heavy biodegradation on accepted scales (Peters and Moldowan, 1993), and this further supports their candidacy as structural representatives of some bioresistant components of aromatic UCMs. However, the data generated for the control samples in the biodegradation experiments using the Whitley Bay consortium should be treated with some caution. Additionally, a more frequent sampling regime is necessary for accurate degradation rates to be assigned to the study compounds.

For compounds to be bioavailable to filtering aquatic organisms, they are generally dissolved in the surrounding water (*i.e.* water soluble). Therefore, the water solubility (at 25 °C) of an aromatic and monoaromatic UCM was determined, using a generator column. In both cases, a secondary UCM was clearly evident in the aqueous phase extracts but the

method was rather irreproducible. Nonetheless, this indicates that under some conditions large numbers of compounds in the original aromatic and monoaromatic UCMs exhibit similar 'dissolution' characteristics. Their comparable solubilities imply that the compounds have analogous physicochemical properties and mole fractions within the parent UCMs. Some of the synthetic UCM compounds (alkylcyclohexyltetralins) had GC retention times that were within the range exhibited by the 'soluble' monoaromatic UCM gas chromatographic distribution.

However, not all compounds in the aromatic and monoaromatic UCMs were present in the aqueous phase. The higher molecular weight hydrocarbons in each of the UCMs were absent from the generated solutions, indicating a solubility cut-off point probably based predominantly on molecular weight. Additionally, different molecular weight distributions were evident for the water 'soluble' aromatic and monoaromatic UCMs. The lower molecular weight hydrocarbons, which dominated the water 'soluble' monoaromatic UCM, appeared to be absent from the water 'soluble' aromatic UCM. However, these compounds must have been present in the original aromatic UCM, as it is from this that the monoaromatic UCM was isolated. The isolation of the monoaromatic hydrocarbons from the aromatic UCM produced a mixture containing a much narrower range of compound types. This fractionation procedure is suggested to have caused a significant increase in the mole fractions of the isolated monoaromatic UCM components. An increase in mole fraction, according to Raoult's law, results in a relative increase in water solubility. The differences in hydrocarbon composition of the aromatic and monoaromatic UCM are suggested to cause the disparity observed in their aqueous distributions. The aromatic UCM appeared to contain di- or polyaromatic hydrocarbons with high mole fractions, which were not present in the monoaromatic UCM. Thus, the dissolution of hydrocarbons from a complex mixture appears to be a dynamic process which is explicable by the application of Raoult's Law.

A composite sample of the water soluble monoaromatic UCMs produced with the generator column was analysed by GCxGC-ToF-MS. GCxGC achieved the separation of 1252 compounds. The ToF-MS analysis of these components resulted in 490 good quality mass spectra deemed suitable for compound identification. A detailed characterisation of 16 compounds is reported based upon mass spectral matches to compounds listed in the NIST library. The identities of many other peaks were inferred as isomers and homologues of these compounds making 114 in total. The compounds characterised include highly alkylated homologues of benzene, indene, indan, tetralin, biphenyl, diphenylmethane and tetrahydrophenanthrene. Although many of these compound classes are commonly observed in crude oils, such highly alkylated versions are rarely, if ever, reported. The compounds identified by the GCxGC-ToF-MS analysis of a monoaromatic UCM appear to be the geochemically minor isomers and analogues of known major crude oil constituents. As narcotic toxicity of petroleum hydrocarbons is an additive effect (Deneer *et al.*, 1988; Peterson, 1994) this finding is extremely important. As UCMs in total, generally account for a larger proportion of crude oil mass than the major resolved components, these persistent residues may also contribute significantly to the observed narcotic toxicity of crude oil.

In conclusion, the present study has reported on the behaviour of aromatic and monoaromatic UCM hydrocarbons in the environment in terms of biodegradability (persistence) and water solubility (bioavailability). This has been achieved by the study of model monoaromatic UCM hydrocarbons and isolated aromatic and monoaromatic UCMs. Weathered crude oil residues are characterised by dominant UCMs, considered resistant to biodegradation, and therefore persistent in the environment. The biodegradation studies confirm that UCMs appear considerably resistant to further alteration by micro-organisms, except under severe conditions. Large numbers of hydrocarbons in aromatic and

monoaromatic UCMs appear sufficiently water soluble to be bioavailable to marine organisms. The monoaromatic UCM is of particular relevance as this has been shown to elicit toxicological responses in marine organisms. The water soluble monoaromatic UCM analysed by GCxGC-ToF-MS may be most representative of the bioavailable fraction of the monoaromatic UCM. The compounds identified from their mass spectra appear to be highly substituted, consistent with resistance to biodegradation. Furthermore, these compounds can be considered typical of those contributing to the monoaromatic UCM toxicity observed by Rowland *et al.* (2001). Thus it appears the term UCM is no longer accurate, and that further research in the characterisation of potentially resolvable complex mixtures (PRCMs) is warranted.

6.2 Future work

In addition to the processes of biodegradation and dissolution, photodegradation provides another mechanism with the potential to degrade, alter or facilitate the removal of crude oil hydrocarbons released into the environment. Many studies have highlighted the ability of high-energy wavelengths (*e.g.* UV) to degrade or alter organic compounds, perhaps influencing their toxicity (*e.g.* Nicodem *et al.*, 1997; Ziolli and Jardim, 2002). Previous work by Smith (2002) has shown that the synthetic model monoaromatic UCM compounds used in this study are susceptible to photodegradation. It was hoped that the effect of photodegradation on the composition of water soluble aromatic and monoaromatic UCMs could be conducted as part of this study.

Unfortunately, the low water solubility of the UCM fractions combined with the limited sample volume capacity of the photodegradation apparatus meant the study was not feasible. Individual samples of 50 mL could be accommodated by the apparatus, which corresponded to <30µg of organic material prior to any effects caused by exposure to UV radiation. This amount of material is only just sufficient for analytical detection. Following irradiation, the samples would require extraction and fractionation into polar and hydrocarbon groups which would be virtually impossible with such small amounts of organic material. Furthermore, it was considered that the analytical instruments available would be unable to monitor any change in UCM composition caused by photolysis. It is therefore suggested that future work should address this important environmental process, and focus on the development of a technique that can monitor the effects of photodegradation at such low concentrations. Ideally, a method whereby large samples of water (*e.g.* 1000 mL) containing dissolved UCM components can be subject to controlled UV radiation should be developed. The amounts of organic material associated with samples of this size would permit fractionation and detailed analysis of the organic composition.

A review of the available methods for production of aqueous solutions of organic compounds was undertaken, with the most suitable deemed the generator column technique. This has proven to be reliable for the generation of aqueous solutions of individual compounds, as evidenced by the synthetic UCM hydrocarbon (6-cyclohexyltetralin) used in the present study. However, when applied to aromatic and monoaromatic UCM fractions the current study has indicated this method is not capable of generating reproducible data for highly complex mixtures. Future work, should therefore consider a more gradual transition from the use of single compounds, up to the study of highly complex mixtures such as UCMs. Further studies might also consider experimental trials of the available dissolution methods to determine which is the most reproducible and environmentally realistic in the case of complex mixtures. The fractionation of a UCM into compound classes (*e.g.* aromatics) will alter the mole fractions of the mixture components and this, in turn, has been shown to significantly effect the composition of the generated solution, often increasing dissolution. Thus, the use fractions isolated from complex mixtures may lead to environmentally unrealistic dissolution behaviour. Future research involving the production of aqueous solutions of organic compounds should therefore focus on the dissolution of the whole mixture as it would enter the environment. It is suggested that the fractionation and analysis of the organic material could be performed after the dissolution process. Such a method would require large sample volumes to ensure sufficient organic material is available for this procedure. However, solid phase extraction (SPE) presents a method that could be more amenable to the extraction of small amounts of organic material, and may permit fractionation of the sample at the same time.

An important issue raised in the present study concerns the fractionation procedures and definitions used for petroleum-derived UCMs. Previous UCM research has reported a variety of techniques together with varying definitions of what constitutes individual

isolated UCM fractions (*e.g.* aromatic or monoaromatic UCMs). Thus, it is suggested that a standardised approach or protocol for the fractionation and measurement of petroleum UCM isolates is essential. This would permit the inter-laboratory comparison of data and provide a much clearer view of the effects of UCMs in the environment.

GCxGC-ToF-MS is already proving to be a powerful analytical technique for the study of complex mixtures such as petroleum-derived UCMs. However, it is still in its infancy and optimisation of the technique will be an essential part of any future work employing this instrument. For example, Phillips and Xu (1995) and Phillips and Venkatramani (1993) have shown that particular combinations of stationary phases will produce different distributions of compounds in the same mixture. Therefore, specific combinations can be utilised depending on the type of separation required from individual samples. Furthermore, this and other studies (*e.g.* Gaines *et al.*, 1999) have indicated that the technique is still not capable of completely resolving such complex mixtures (even when fractionated), as some coelution of structural isomers still occurs. Although some degree of sample fractionation appears necessary, there are other options to further increase the separation of components in a mixture. These include maximising the use of the chromatographic space available in order to optimise the separation of components or perhaps even the addition of a third-dimension chromatographic step using another stationary phase (*e.g.* a chiral phase).

The current study has shown that identification of unknown UCM hydrocarbons by comparison to NIST library mass spectra alone, cannot be considered conclusive. There is a clear need for comparison to a library of authentic reference compounds if reliable characterisation is to be achieved. Ideally, a suite of compounds including isomers and homologues of the proposed components should be used. Many of the compounds identified in the current study are not commercially available and will require synthesis. If

analysed by the same GCxGC-ToF-MS technique, this would provide a library containing mass spectra and two-dimensional retention times for each individual compound. The mass spectra should be produced with the ToF-MS detector, effectively removing the qualitative differences observed between conventional MS and ToF-MS instruments. Comparison of such information should then be sufficient to identify unknown components of the UCM. However, further development of more efficient automated data processing software will be essential in the analysis of the huge quantities of data generated by GCxGC-ToF-MS. Quantitation of individual components within a mixture is also possible using the GCxGC-ToF-MS technique. Although it was not undertaken in the present study, future work should consider the determination of single component concentrations within UCMs. Such information will be required if the technique is to be used in the fingerprinting of crude oil residues.

Another area for future work is continuation of the toxicity studies using mussels which have already bioaccumulated weathered petroleum UCMs. Studies could either use mussels exhibiting impaired health which have been collected from petroleum impacted sites (*in vivo*), or 'clean' mussels which are exposed to a weathered crude oil or an isolated aromatic UCM in the laboratory (*in vitro*). The affects of UCM bioaccumulation on the health of mussels can then be monitored as in previous studies (*e.g.* Rowland *et al.*, 2001; Smith, 2002; Donkin *et al.*, 2003). Following exposure/collection the bioaccumulated UCM compounds can be extracted from the mussel tissue and, following fractionation of the extract, can then be analysed by GCxGC-ToF-MS. This should provide the first data identifying the UCM components accumulated by aquatic organisms that are eliciting the observed toxicological effects.

Whilst predictive software (*e.g.* EPI Suite™) can be a useful tool for estimating physicochemical and toxicological parameters for individual compounds, there is no

substitute for 'real' data. Therefore, future work may also consider studying the biodegradation, dissolution, photodegradation and toxicity of the aromatic UCM hydrocarbons identified in the current study. The creation of a simple mixture containing such compounds may help in the understanding of how these processes affect more complex mixtures such as weathered crude oil UCMs. Synthesis of the compounds identified in the water soluble monoaromatic UCM would be useful for their chromatographic and mass spectral information. Additionally, future research could replace the alkylcyclohexyltetralins, currently being used as "model" UCM compounds with genuine UCM hydrocarbons.

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Appendix A

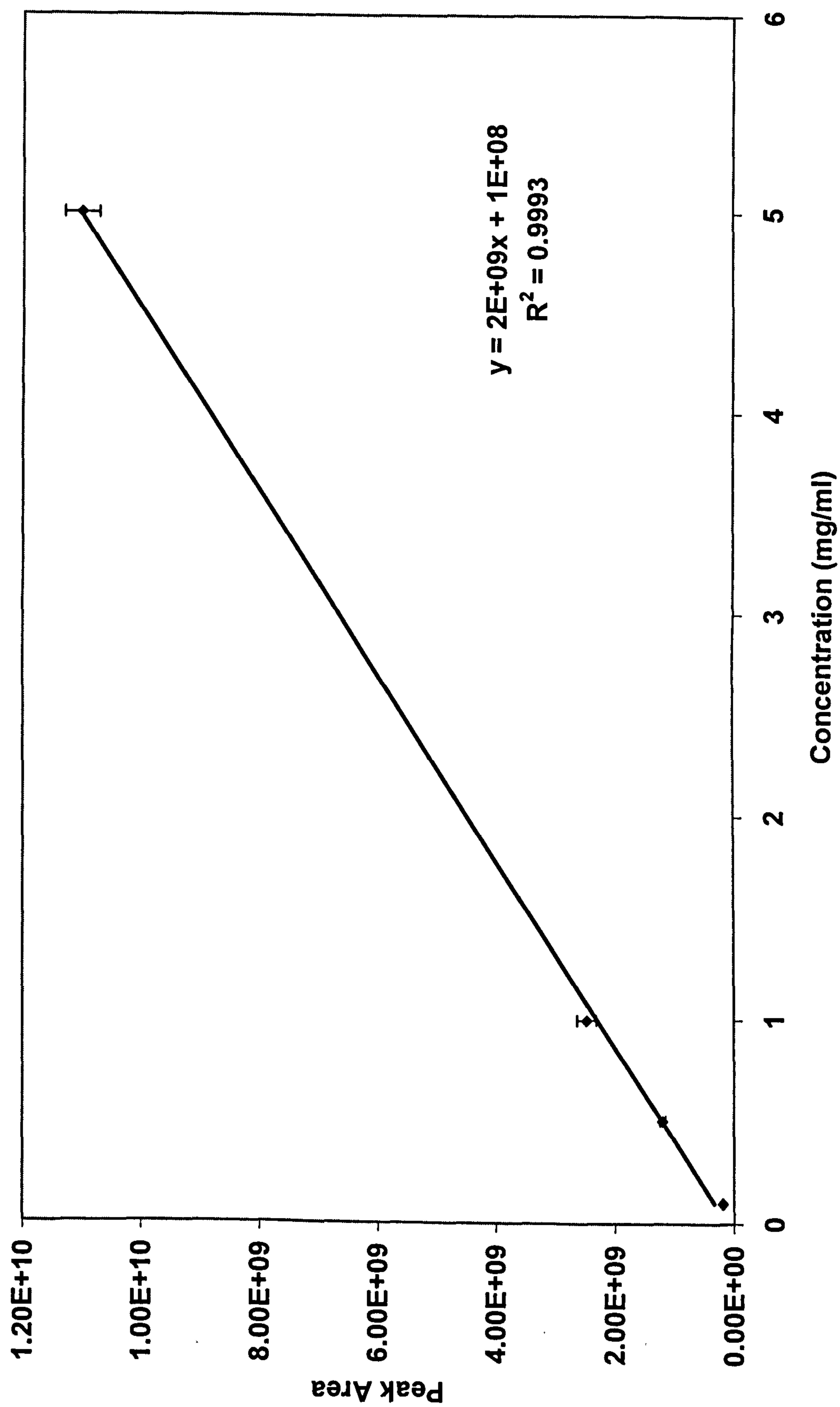


Figure A1. External calibration curve for the aromatic UCM (n=3).

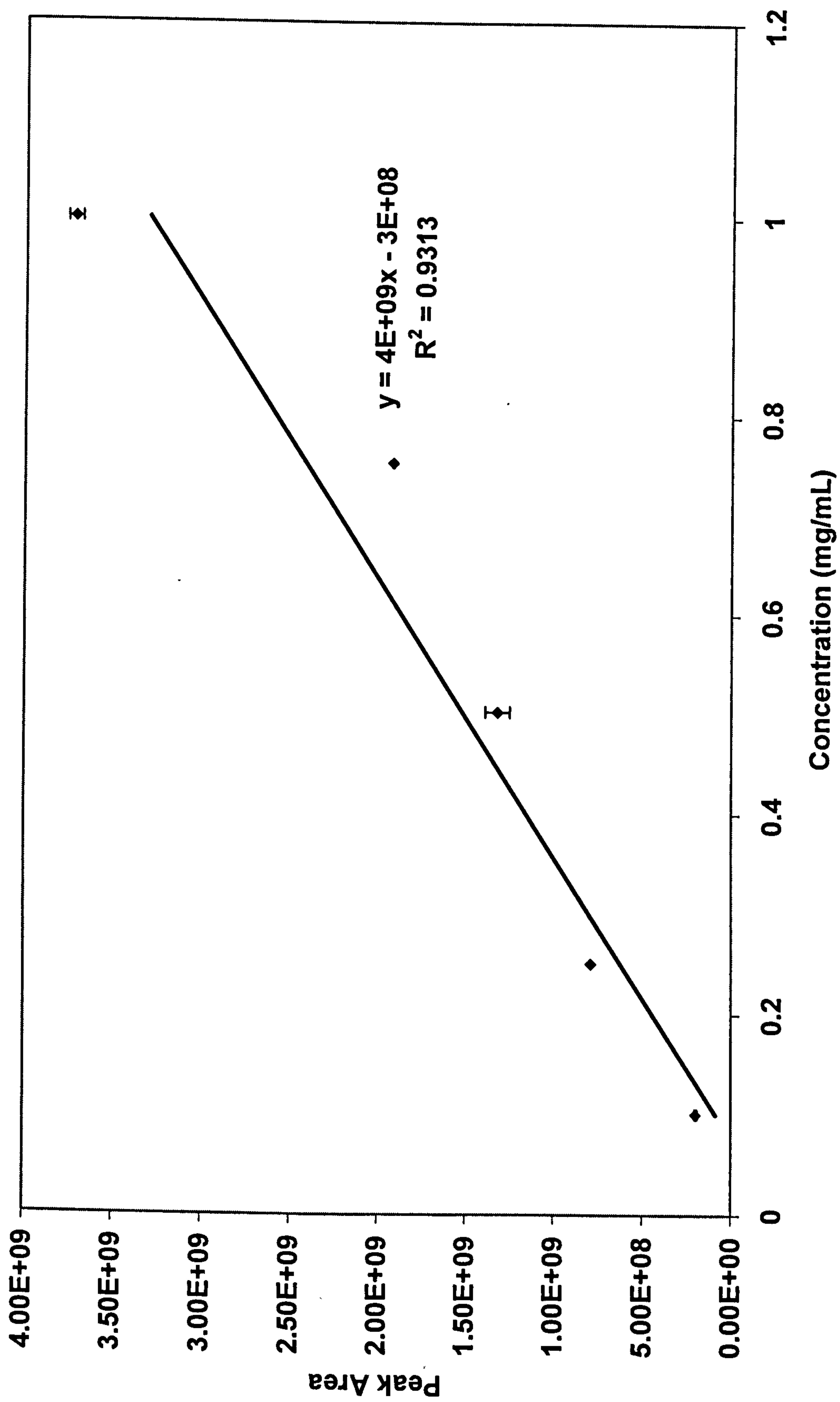


Figure A2. External calibration curve for the 'monoaromatic' UCM (n=3).

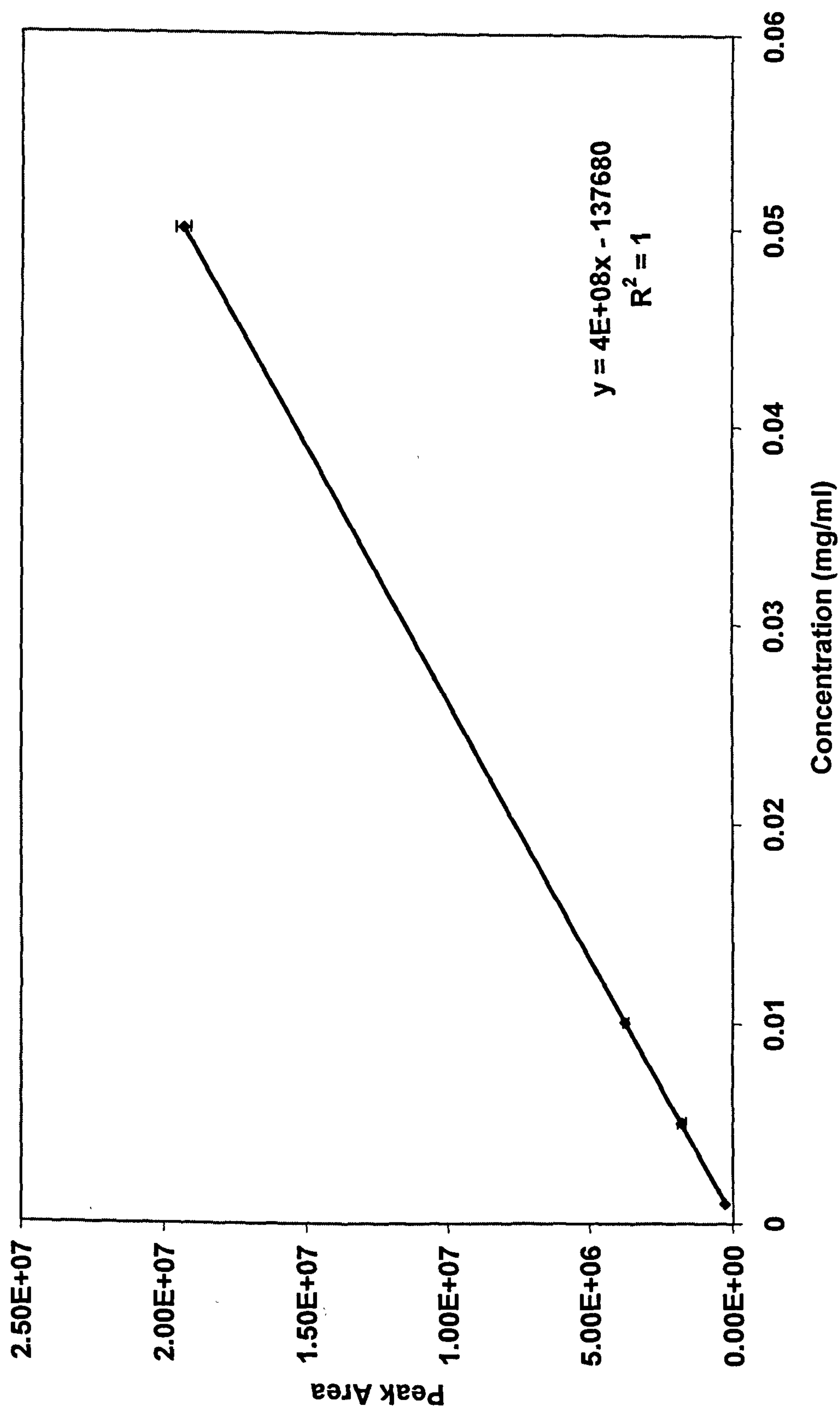


Figure A3. Calibration curve for 6-cyclohexyltetralin ($n=3$), analysis was performed in SIM mode (214). Calibration curve allowed concentration determinations to be made on generated aqueous soluble fractions.

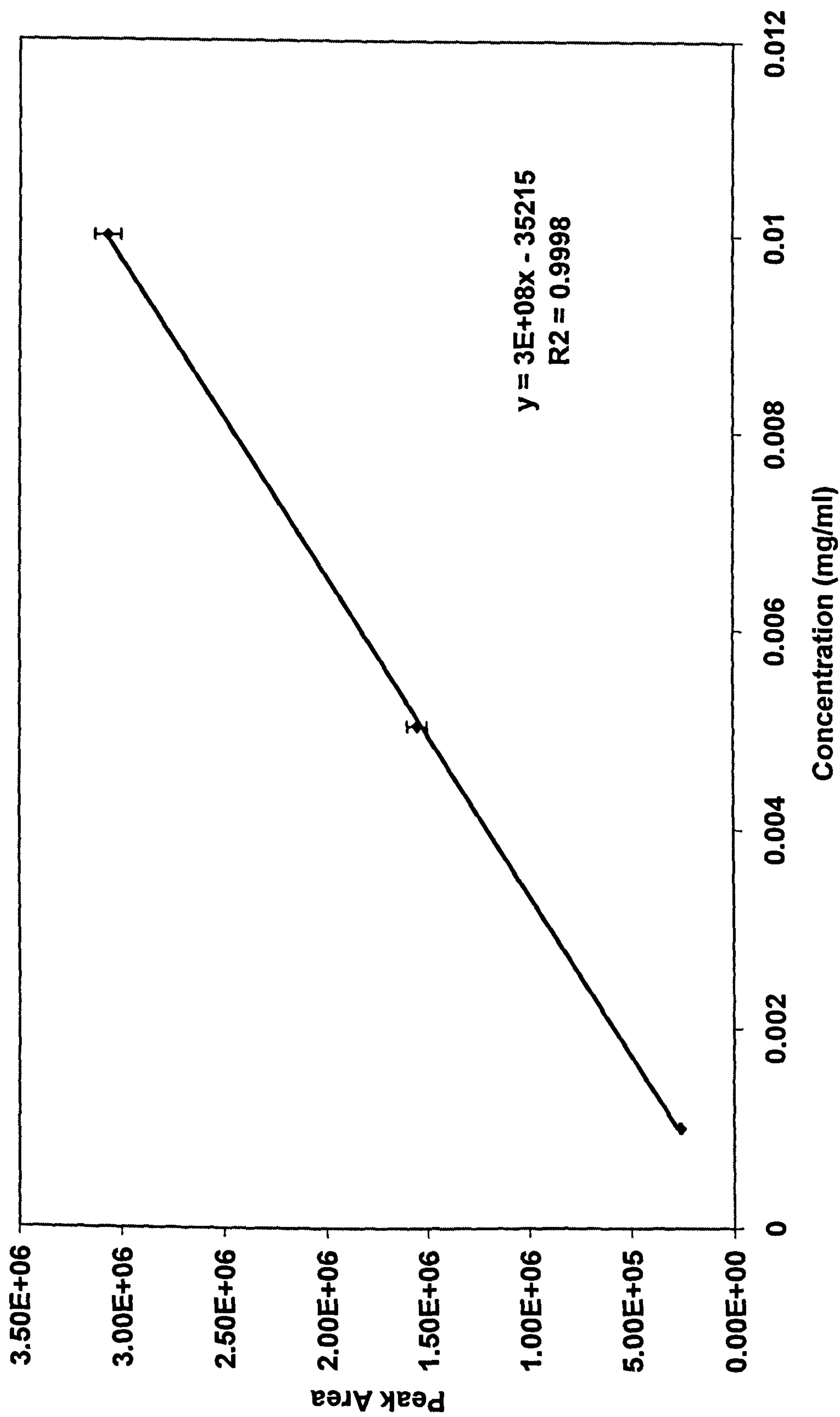


Figure A4. Internal standard calibration curve for 7-cyclohexyl-1-methyltetralin ($n=3$), analysis was performed in SIM mode (228).

Appendix B

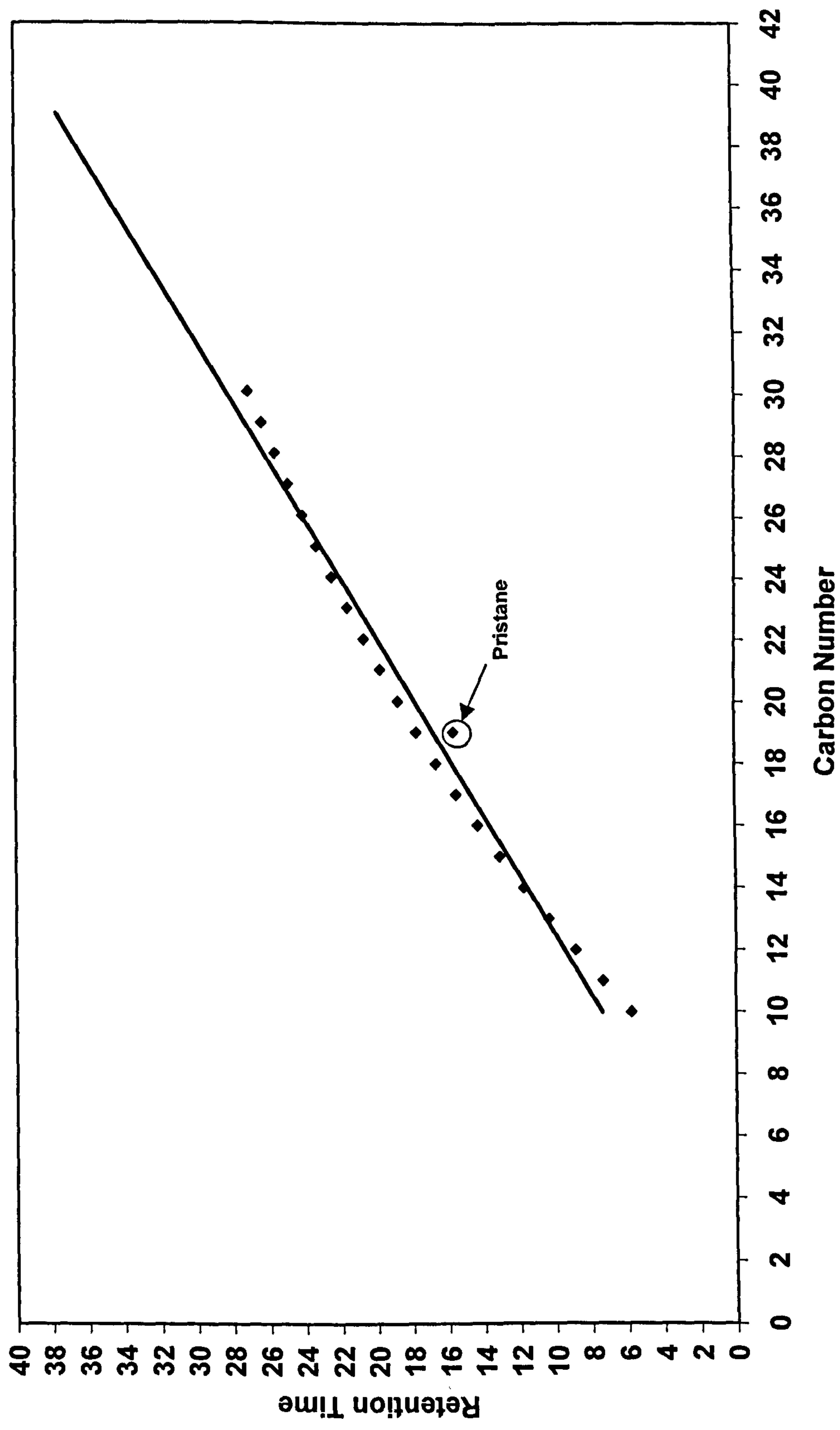


Figure B1. Kovats retention index determined using C₁₀-C₃₀ alkanes. Analysis program was 40-300°C at 10° /min, isothermal period of 10 min. Total analysis time of 36 min. Column - HP-1MS, 30 m x 0.25 mm I.D. x 0.25 um film thickness, polysiloxane.

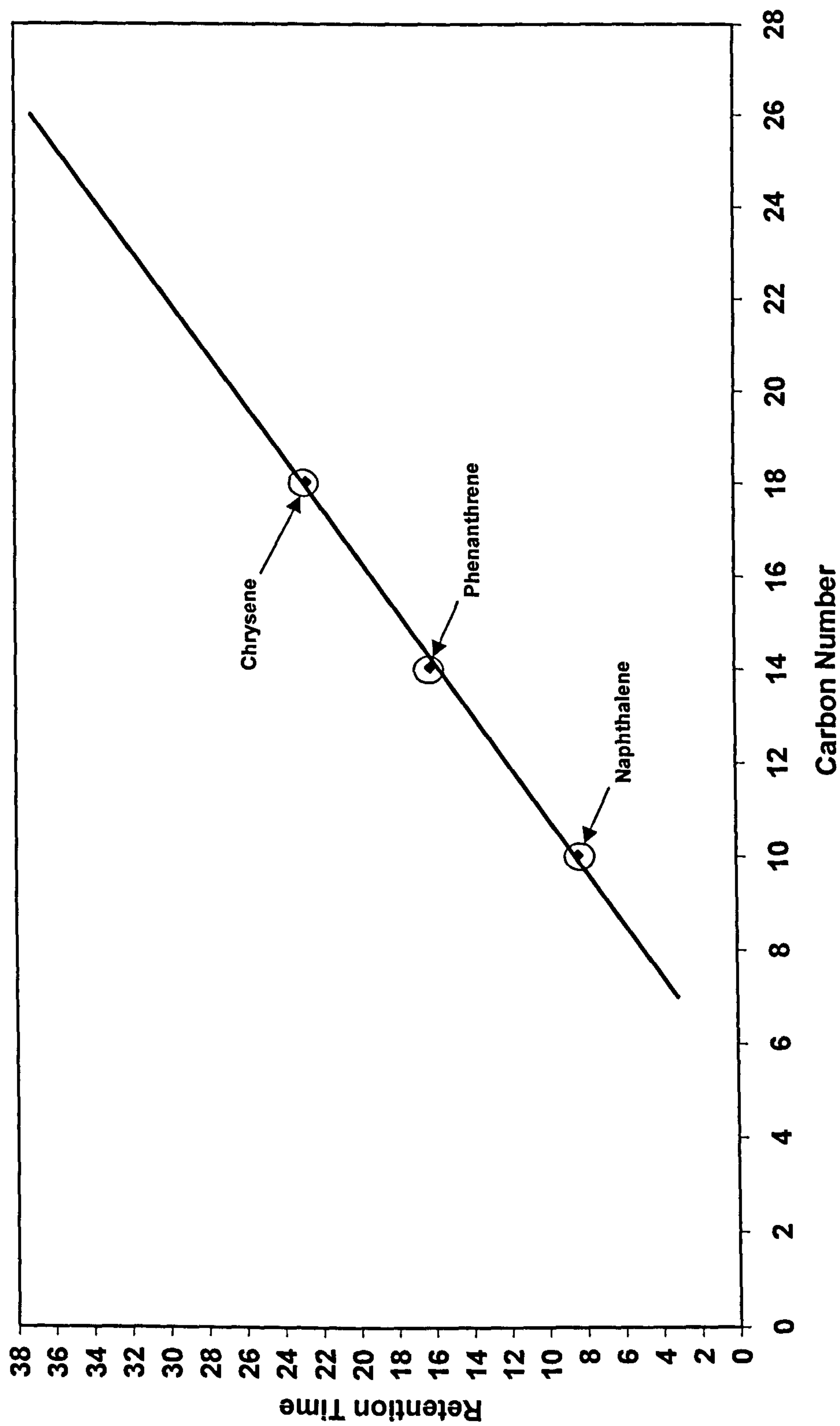


Figure B2. Lee retention index determined using naphthalene (C_{10}), phenanthrene (C_{14}), and chrysene (C_{18}). Analysis program was 40-300°C at 10° /min, isothermal period of 10 min. Total analysis time of 36 min. Column - HP-1MS, 30 m x 0.25 mm I.D. x 0.25 μ m film thickness, polysiloxane.

Appendix C

Table C.1. Summary of the diagnostic base ions used to identify those chromatographic peak markers corresponding to column bleed and alkane impurities.

Impurity	Diagnostic mass spectral base ions
Column bleed ions	56, 57, 73, 135, 207, 253, 281
Alkanes	57, 71

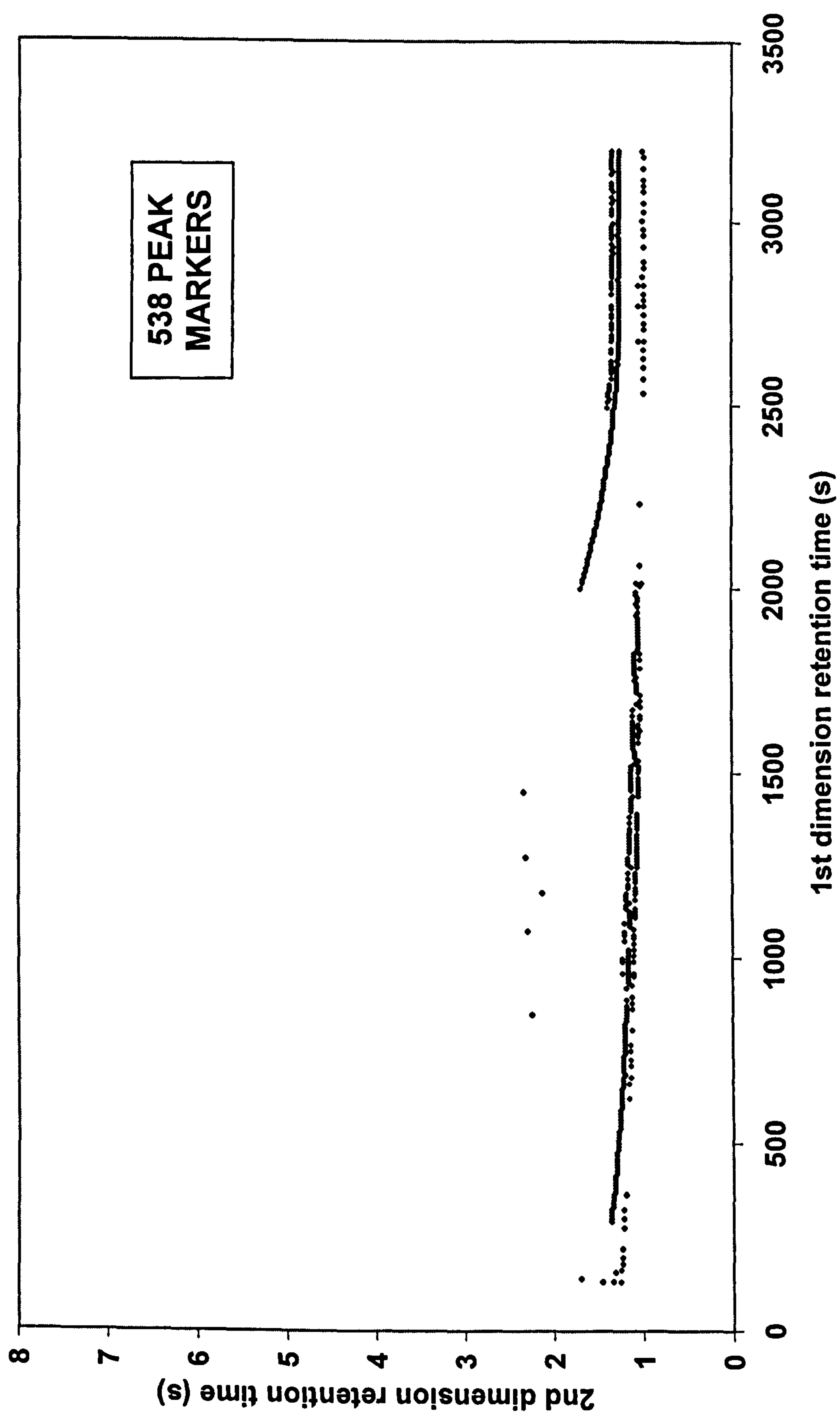


Figure C.1. Peak marker plot of the GC column bleed ions.

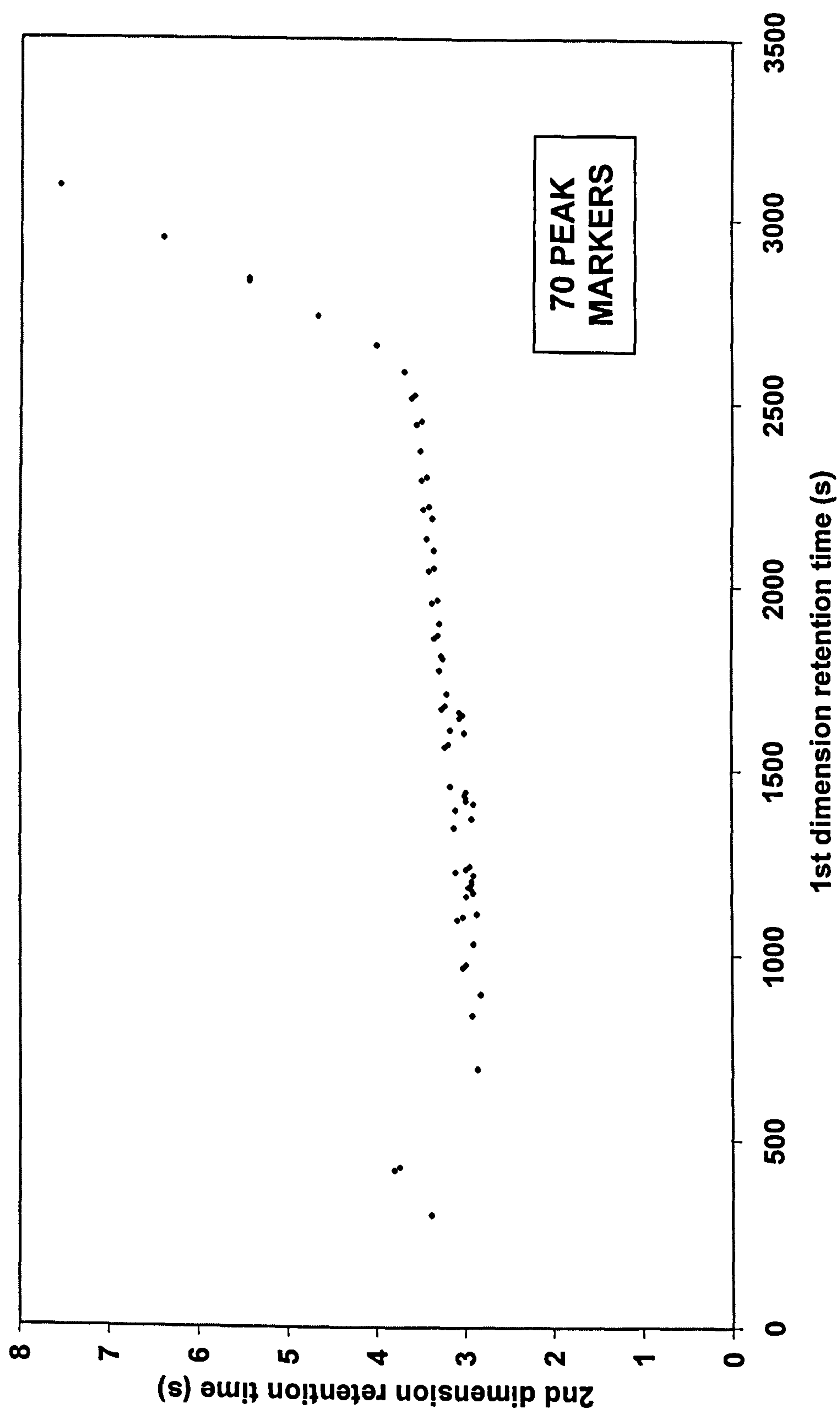


Figure C.2. Peak marker plot of the alkane impurity compounds.