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# Determination of odour compounds in surface waters

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# **DETERMINATION OF ODOUR COMPOUNDS IN SURFACE WATERS**

A thesis submitted in fulfilment  
of the requirements for the award of the degree of

**DOCTOR OF PHILOSOPHY**

from

**THE UNIVERSITY OF WOLLONGONG**

**BY**

**WOLFGANG KORTH, B.Sc.**

**DEPARTMENT OF CHEMISTRY**

**1992**

I hereby certify that, except for elemental analyses and recording of NMR and IR spectra, the experimental work described in this thesis was performed by me and has not been submitted previously for a higher degree at this or any other university.

Wolfgang Korth

30/7/92

## **Dedication**

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This thesis is dedicated to my wife, my two lovely daughters and to my parents.

To Sue for her encouragement, inexhaustible moral support and for having to endure endless evenings on her own, to Kristen and Michelle for letting dad take their favourite games off the computer during the writing up stage of this thesis and to mum and dad for their reassurance and unwavering belief in me.

## ABSTRACT

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Deuterated geosmin [*trans*-1,10- $^{2}\text{H}_3$ ]-dimethyl-9 $\alpha$ -decalol] and deuterated 2-methylisoborneol [2-*exo*-hydroxy-2- $^{2}\text{H}_3$ ]-methylbornane] have been synthesised and evaluated as internal standards in the determination of geosmin and 2-methylisoborneol (MIB) in water by closed loop stripping analysis (CLSA) followed by gas chromatography/mass spectrometry (GC-MS) in either the full scan or multiple ion detection mode (MID).

The labelled standards were compared with chloroalkanes added as internal standards either at the time of sampling or immediately before closed loop stripping. When added at sampling time, the new standards enabled accurate determination of the geosmin and MIB present initially, even when the samples were analysed as much as three weeks later. The new standards gave better precision and accuracy than the chloroalkanes and overcame the underestimation of analyte concentration which usually results from losses of analyte through adsorption, volatilisation, biodegradation etc. during sample transport and storage. When added immediately before closed loop stripping, the labelled standards negated the need for reanalysing samples or frequent recalibration as they are insensitive to changes in CLSA parameters such as, flow rate through the carbon filter, stripping time or temperature, size or shape of stripping bottle, salt concentration, sparging rate and air leaks.

Geosmin had a limit of detection of <0.1 ng/L and 1 ng/L was determined with a coefficient of variance (CV) of 1.2% (n=5). MIB was determined at 1 ng/L with a CV of 3.5% (n=5).

Geosmin and MIB were stored (at room temperature and  $-15^{\circ}\text{C}$ ) as dilute solutions in methanol, ethanol, hexane, carbon disulphide and acetone for two years without deterioration. Dichloromethane caused substantial decomposition of MIB (but not geosmin) when stored at room temperature. However, neither compound deteriorated when stored ( $\sim 2$  years) at  $-15^{\circ}\text{C}$ . The labelled compounds were effective internal standards for the determination of other volatile odorous metabolites such as the carbonyl compounds  $\beta$ -cyclocitral,  $\beta$ -ionone, geranylacetone and 6-methylhept-5-en-2-one.

An enantioselective GC technique was developed which enabled the use, for the first time, of (+)-geosmin or (+)-geosmin- $\text{d}_3$  (from ( $\pm$ )-geosmin or ( $\pm$ )-geosmin- $\text{d}_3$ ) as the internal standard for the determination of (-)-geosmin, with detection being either by flame ionisation detection (FID) or MID. When ( $\pm$ )-geosmin- $\text{d}_3$  is added at the time of sampling, rather than at the time of analysis, the (-)-labelled enantiomer compensated perfectly for losses of natural (-)-geosmin by biodegradation or physical and chemical processes during sample transport and storage.

Several gas chromatographic techniques were evaluated using nonchiral and chiral capillary columns combined with FID and MID detection. The order of precision and accuracy of the various methods is: enantioselective GC-MID (labelled ( $\pm$ )-standard) > GC-MID (labelled ( $\pm$ )-standard) > enantioselective GC-FID (unlabelled ( $\pm$ )-standard) > enantioselective GC-FID (labelled ( $\pm$ )-standard).

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## ABBREVIATIONS USED IN TEXT

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<b>1-ClC<sub>8</sub></b>	1-chlorooctane
<b>1-ClC<sub>10</sub></b>	1-chlorodecane
<b>1-ClC<sub>12</sub></b>	1-chlorododecane
<b>2-IB-3-MP</b>	2-isobutyl-3-methoxypyrazine
<b>2-IP-3-MP</b>	2-isopropyl-3-methoxypyrazine
<b>BSA</b>	bovine serum albumin
<b>CD<sub>3</sub>I</b>	deuterated methyl iodide
<b>CLSA</b>	closed loop stripping analysis
<b>CSP</b>	chiral stationary phase
<b>CS<sub>2</sub></b>	carbon disulphide
<b>CV</b>	coefficient of variance
<b>DBP</b>	disinfection by-product
<b>DCBR</b>	differential column batch reactor
<b>DCM</b>	dichloromethane
<b>DFHSDM</b>	dispersed-flow homogeneous surface diffusion
<b>DNP</b>	dinitrophenylhydrazine
<b>EDTA</b>	ethylenediaminetetraacetic acid
<b>ELISA</b>	enzyme-linked immunosorbent assay
<b>FID</b>	flame ionisation detection
<b>GAC</b>	granular activated carbon
<b>GC</b>	gas chromatography
<b>GC-MS</b>	gas chromatography-mass spectrometry
<b>HPLC</b>	high-performance liquid chromatograph
<b>IR</b>	infrared
<b>J&amp;W</b>	J&W Scientific, USA
<b>LAD</b>	lithium aluminium deuteride
<b>LAH</b>	lithium aluminium hydride
<b>MCGP</b>	microwave cooking gas purging
<b>MCPBA</b>	metachloroperbenzoic acid

<b>MDGC</b>	multidimensional gas chromatography
<b>MIB</b>	2-methylisoborneol
<b>MID</b>	multiple ion detection
<b>MSD</b>	mass selective detector
<b>MWD</b>	Metropolitan Water District of Southern California
<b>NMR</b>	nuclear magnetic resonance
<b>NTU</b>	nephelometric turbidometric unit
<b>PAC</b>	powdered activated carbon
<b>PAH</b>	polycyclic aromatic hydrocarbon
<b>PMR</b>	proton magnetic resonance
<b>ppt</b>	part per trillion i.e. ng/L or ( $10^{-12}$ )
<b>P&amp;T</b>	purge and trap
<b>RHS</b>	right hand side
<b>SCP</b>	sodium carbonate peroxyhydrate
<b>SDE</b>	simultaneous distillation and extraction
<b>SE</b>	solvent extraction
<b>SGE</b>	Scientific Glass Engineering, Australia
<b>SIM</b>	single ion monitoring
<b>TCA</b>	trichloroanisole
<b>THM</b>	trihalomethane
<b>TMS</b>	trimethylsilane
<b>US EPA</b>	United States Environmental Protection Agency
<b>UV</b>	ultraviolet
<b>VOC</b>	volatile organic compound
<b>VOE</b>	vegetable oil extraction

# 1 INTRODUCTION

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## 1.1 GENERAL

The ability of water to dissolve a large number of organic and inorganic compounds as it interacts with the environment is one of its unique properties. Often referred to as the "universal solvent" water contains solutes derived from such sources as the erosion and weathering of rocks and the products of biological activity. Consequently, freshwater from even the most pristine sources will be contaminated to some degree with salts, dissolved gases, organic carbon compounds and suspended solids. If this water subsequently flows through urban, agricultural or industrial areas it may be used and re-used many times adding an ever increasing number of chemical substances to the contamination load. As the water becomes more and more eutrophic, biological activity escalates, algal blooms invariably occur and taste and odour problems become evident.

The significance of the presence of taste and odour compounds to the perceived aesthetic and health qualities of drinking water has been the subject of research for many years. Ancient philosophers and physicians considered ways of removing bad tastes and odours and Aristotle was probably the first to associate the taste and odour of water to its hygienic suitability. He wrote "water in its own nature has no flavour" while Hippocrates advocated searching out only the most health-giving source of supply (Mallevalle and Suffet, 1987, pp. 1,6; Baker, 1981, pp. 4,5). The perception of an odour may be the first indication the consumer has regarding the possible health risk of drinking water.

The presence of an odour implies that the water is contaminated with one or more chemical substances and that these substances may be toxic. It is fortunate that most toxic substances can be detected in contaminated water by their odour well before any acute toxic effect is sensed by the consumer. A literature survey of 97 chemical substances, normally present in drinking water, found that none would produce an acute toxic effect in humans before it could be detected by its odour (Piet von, *et al.*, 1976). However, the lack of an odour in drinking water does not guarantee its safety. The inability to detect an odour may be because the compound present has no odour or because the consumer is simply not sensitive to it. Testing of odour thresholds conducted by Amoore (1986) has demonstrated that normal olfactory sensitivities of a population vary widely and the occurrence of specific anosmia, or "smell-blindness" is common. Conversely the presence of an odour does not indicate that the water is toxic in every case. Nevertheless consumers quite correctly reject water because of its odour and water managers continue to strive to provide clean water that is safe to drink.

*"To be smelled, a substance must be volatile and able to reach the olfactory nerve receptors in the uppermost recesses of the nasal cavities" ( Amoore, 1986) .*

The volatile organic compounds (VOC's) that cause odours can enter a water regime from natural or man made sources. VOC's from man made sources include those added directly from industrial waste; formed during water and waste water treatment processes; or leached from water pipes and storage reservoirs. Natural VOC's include those produced by microorganisms (such as algae or bacteria) in both ground water and



surface water supplies or in pipes and storage reservoirs (Piet von, *et al.*, 1976; Wajon, *et al.*, 1986; Anselme, *et al.*, 1985; Mallevalle and Suffet, 1987; Bartels, *et al.*, 1989). Although it is not possible to list all volatile organic compounds that may be present in a water supply, those identified as causing odour problems and that occur frequently in drinking water are shown in the following tables.

**Table 1**                      Compounds formed by microorganisms in pipes or storage reservoirs with odour thresholds of 10 µg/L or less

Compound	Odour Threshold (µg/L)	Reference
dimethyltrisulphide	0.010	Wajon, <i>et al.</i> , 1986
2,3,6-trichloroanisole	0.0072 0.0070	Guadagni and Buttery, 1978 Amoore, 1986
1-octene	0.50	Zoeteman, <i>et al.</i> , 1977
2-methylisoborneol	0.020	
geosmin	0.015	
heptanal	3.0	
octanal	0.70	
nonanal	1.0	
decanal	2.0	
heptan-3-one	7.5	

**Table 2**                      Compounds formed during chlorination of drinking water, their odour thresholds and likely precursors

Likely Precursor	Odour Threshold (µg/L) x 10 <sup>3</sup>	Chlorinated Compound	Odour Threshold (µg/L)
aniline	70	dichloroaniline	50
benzene	10	monochlorobenzene	100
		dichlorobenzene	10
		trichlorobenzene	10
		tetrachlorobenzene	50
		pentachlorobenzene	60
cyclohexane	200	monochlorocyclohexane	50
		dichlorocyclohexane	20
dichloroethane	2	terachloroethane	200
heptane	50	tetrachloroheptane	2.5
nonane	10	tetrachlorononane	3
phenol	3	<i>o</i> -chlorophenol	3
		2,4-dichlorophenol	3
		trichlorophenol	0.4
		pentachlorophenol	300
toluene	1	dichlorotoluene	50
undecane	10	tetrachloroundecane	7

Ref. Piet von, *et al.*, 1976

**Table 3** Odour compounds of industrial origin with odour thresholds 10 µg/L or less

Compound	Odour Threshold (µg/L)	References
dibenzofuran	3.3	Alexander, <i>et al.</i> , 1986
chlorpyrifos	1.2	
diethylbenzene	1.2	
ethylbenzene	2.4	
Number 2 fuel oil	0.82	
2-hydroxyethyl acrylate	10	
styrene	3.6	
trichlorobenzene	10	
vinyltoluene	5	
<i>p</i> -dichlorobenzene	0.3	Zoeteman <i>et al.</i> , 1977
1,3,5-trimethylbenzene	3.0	
2-chloroaniline	3.0	
1,2,4-trichlorobenzene	5.0	
5-chloro-2-toluidine	5.0	
γ-hexachlorocyclohexane	0.3	
hexachlorobutadiene	6.0	
3,4-dichloroaniline	3.0	
<i>o</i> -dichlorobenzene	10	

**Table 4** Compounds in drinking water with odour thresholds of 10 µg/L or less (for earthy/musty odour compounds see later)

Compound	Odour Threshold (µg/L)	Compound	Odour Threshold (µg/L)
β-ionone*	0.007	butanal	9.0
cadinene	1.0	butyric acid	1.0
indene	1.0	chlordan	0.5
naphthalene	1.0	6-chloro-methylphenol	0.08
limonene	4.0	2-chlorophenol	0.2
octanal	0.70	4-chlorophenol	0.5
nonanal	1.0	1,4-dichlorobenzene	0.3
propanal	4.0	2,4-dichlorobenzene	2.0
undecanal	5.0	2,6-dichlorobenzene	3.0
oct-1-ene	0.50	dichloropropane	1.4
decanal	0.10	dodecanal	2.0
hexanal	5.0	3-methylbutanal	0.15
heptanal	3.0	2-methylpropanal	0.9

Ref. Piet von *et al.*, 1976

\* the author quotes an odour threshold of 0.007 µg/L, this seems to be too low given that some of the most odorous compounds known, the earthy/musty odour compounds, have odour thresholds ranging between 0.002 and 0.02 µg/L. The low threshold value could be due to a typographical error and should have read mg/L not µg/L. Note: An odour threshold exactly 1000 times higher was quoted by Stahl, 1973.

**Table 5** Earthy/musty odour compounds with odour thresholds of 1.0 µg/L or less

Compound	Odour Threshold (µg/L)	Reference
2-methylisoborneol	0.035	Persson, 1979
	0.020	Piet von <i>et al.</i> , 1976
	0.100	Rosen <i>et al.</i> , 1970
	0.029	Amoore, 1986
	0.100	Medsker <i>et al.</i> , 1969
	0.200	Rosen <i>et al.</i> , 1968
		Persson, 1979
		Persson, 1980
		Gerber, 1979
	0.0085	Persson, 1983
geosmin	0.015	Persson, 1979
	0.015	Piet von <i>et al.</i> , 1976
	0.010	Amoore, 1986
	0.021	Bowmer <i>et al.</i> , 1992
	0.050	Medsker <i>et al.</i> , 1968
	0.200	Safferman <i>et al.</i> , 1967
	0.200	Rosen <i>et al.</i> , 1968
	0.100	Gerber, 1974
		Tyler <i>et al.</i> , 1978
	0.0040	Persson, 1980
2-IP-3-MP		Tuorila <i>et al.</i> , 1980
	0.002	Amoore, 1986
		Seifert <i>et al.</i> , 1970
2-IB-3-MP		Gerber, 1977
	0.002	Amoore, 1986
		Seifert <i>et al.</i> , 1970
2,3,6-TCA	0.007	Amoore, 1986
	0.0000003*	Curtis, <i>et al.</i> , 1972
	0.0074-0.0245	Guadagni and Buttery, 1978
2,4,6-TCA	0.00003	Curtis <i>et al.</i> , 1972
2,3,4,6-TCA	0.004	Curtis, <i>et al.</i> , 1972

\* the value reported by Curtis *et al.*, 1972 for 2,3,6-TCA seems extremely low given the odour thresholds published for the other earthy/musty compounds. This fact was also noted by Guadagni and Buttery who in 1978 determined the odour threshold for water solutions of 2,3,6-TCA to be 7.4 and 24.5 ng/L. The authors presented evidence which seriously questioned the low odour thresholds claimed by Curtis *et al.* 1972. In light of their findings (i.e. Guadagni and Buttery, 1978) one must question the values quoted by Curtis *et al.*, 1972 for 2,4,6- and 2,3,4,6-TCA as well.

Of the large number of volatile organic compounds that can be present in water the compounds that impart an earthy/musty taste and odour to drinking water are the most troublesome. These compounds are of particular concern to water managers because they are difficult to remove during normal water treatment processes, can be perceived by the general public at very low concentrations (typically at 10 ppt) and require expensive water treatment options to remove them. The two compounds most often found to cause these taste and odour problems in the USA, Europe, Japan and Australia are 2-methylisoborneol and geosmin.

## 1.2 Discovery of MIB and Geosmin

The occurrence of compounds in drinking water that cause water to smell and taste earthy and/or musty has been known for quite some time (Mallevalle and Suffet, 1987, pp. 57). However the process of identifying the actual chemical compound responsible for a specific odour proved to be more difficult. Actinomycetes were the first organisms identified as odour producers in the late 19th century. In 1895, Rullman obtained an earthy/musty odour from a culture of an organism he called *Cladothrix odorifera* (Romano and Safferman, 1963). From the description of the organism Romano and Safferman (1963) believed that Rullman was describing an actinomycete. The production of earthy/musty odours in drinking water was first investigated scientifically by Adams (Romano and Safferman, 1963) who suggested that the intermittent odours that occurred in the River Nile in 1929 were the result of volatile products produced by actinomycetes. Romano and Safferman (1963) remarked that the view that actinomycetes were responsible for the earthy/musty taste and odour in drinking water was not shared by other workers. They cite Burger and Thomas who in 1934 believed

that the population of actinomycetes in the water was too small and therefore suggested that the problem was the result of a mixture of organisms. They continued by citing Thaysen who in 1936 investigated a salmon-rich river in the British Isles for the origin of the earthy taint in fish and could correlate the objectionable odour with the occurrence of large numbers of actinomycetes in the muddy river banks that the fish were exposed to. As the concentration of actinomycetes in the water was very low Thaysen concluded that the taint in the fish was due to odour compounds produced by actinomycetes in the river banks being washed into the water. This view was shared by several others working in the field; Issachenko and Egorova who investigated the Moscow River; and Ferramola who studied the River Plate in Argentina (Romano and Safferman, 1963).

Collins and Bean (1963) extracted volatile compounds from a culture of *Chlamydomonas globosa* and later (Collins and Kalnins, 1965) from *Syanura petersenii* in an attempt to identify the compounds associated with odour episodes. They managed to isolate and identify a number of aldehydes, ketones, alcohols, esters and acids and concluded that these compounds could contribute to taste and odour episodes.

Gerber and Lechevalier (1965) were the first to show that a single compound isolated from several actinomycetes was responsible for the earthy taste and odour. They named the compound geosmin (from the Greek "ge" = earth and "osme" = odour) and also demonstrated that the compound could be transformed by acid catalysed dehydration to another compound without an odour. This compound was named argosmin (from the Greek "argos" = inactive and "osme" = odour). Although Gerber and Lechevalier were

able to obtain milligram quantities of geosmin from a culture of *Streptomyces griseus* they were not able to elucidate its structure. They did however establish that geosmin had an approximate boiling point of 270°C, was neutral and contained hydrogen and carbon but no nitrogen. They also established that geosmin reacted with acid to form argosmin which had a boiling point of 230°C, was neutral, has no odour and contained only carbon and hydrogen. They determined the optical rotation  $[\alpha]_D^{25}$  of geosmin and argosmin to be  $-16.5^\circ$  and  $+25^\circ$  respectively.

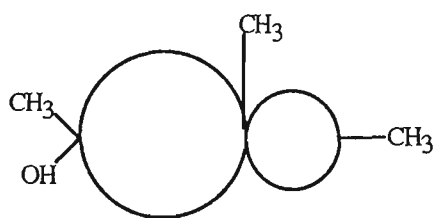
Collins and Kalnins (1966) isolated the carbonyl compounds (methanal, ethanal, propanone, pentanal and heptanal) produced by *Cryptomonas ovata* and concluded that although these compounds had odours of their own none was responsible for the odour of "violets" which was attributed to *Cryptomonas ovata*. They suggested that the compound responsible for the "violet" odour must be present at very low concentrations and that it is necessary to work with bigger cultures if the identification of this compound was possible. They suspected that the compound was most likely neutral and that it could be  $\beta$ -ionone.

In 1966 Dougherty *et al.*, believed that they had isolated a compound from cultures of certain actinomycetes that was responsible for causing earthy/musty taste and odour episodes in the Cedar River in Iowa. Using steam distillation followed by solvent extraction of the distillate they were able to isolate a colourless oil that had the characteristic musty odour. GC of the oil showed that seven peaks were detectable but that by peak areas one peak represented  $\sim 95\%$  of the total. They were able to establish an empirical formula of  $(C_6H_9O)_n$  for the major peak, and from NMR data

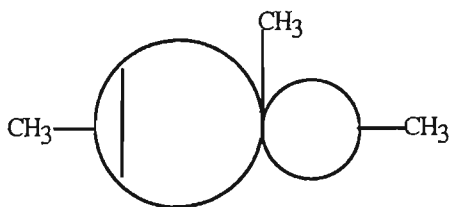


established that 18 protons were present and hence a formula of  $C_{12}H_{18}O_2$  was suggested. The molecular weight of 194 was confirmed by mass spectrometry. From UV, IR, NMR, mass spectral and wet chemistry data they deduced that the compound was an ester or lactone and suggested some partial structures. The compound was named "mucidone" and later identified as 6-ethyl-3-isobutyl-2-pyrone (Sipma *et al.*, 1972). However, Lechevalier (1974) showed that pure mucidone had no odour and concluded that the musty odour detected by Dougherty and co-workers must have been due to a contamination of the extract with geosmin present at very low levels (it was most likely one of the other 6 peaks in their chromatogram).

In 1967 Gerber suggested a tentative structure for geosmin and argosmin (Fig. 1) which accounted for all of the physical measurements made other than the lack of an O-H absorption in the IR.



geosmin (proposed structure)



argosmin (proposed structure)

**Figure 1** Tentative structure of geosmin and argosmin as proposed by Gerber (1967)

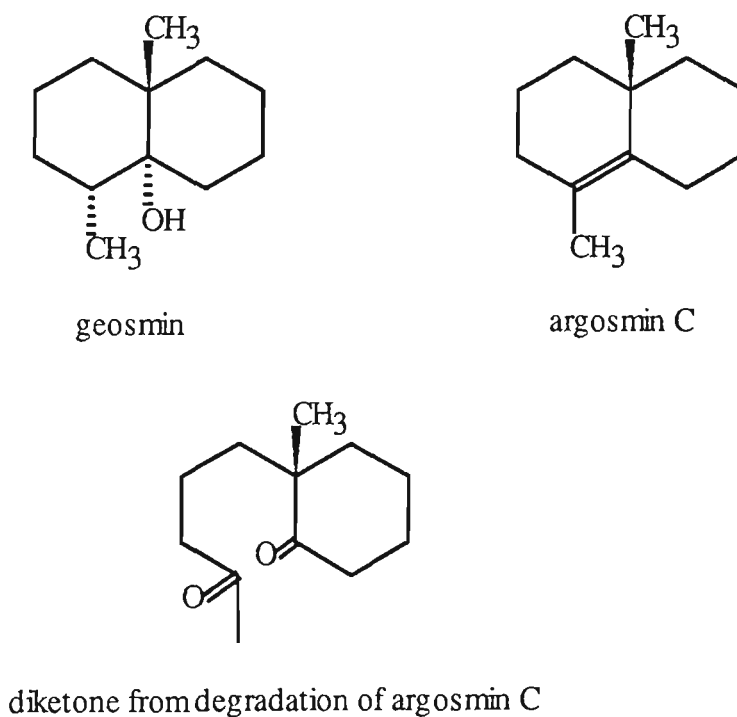
In 1967 Safferman *et al.*, were the first to report that geosmin was found to be produced by something other than actinomycetes. They showed that the alga *Symploca moscorum* (strain **IU 617**) produced geosmin at a yield of 0.6 mg/L of pooled algal culture (24 L). Similar to Gerber (1967) they noted the absence of an O-H absorbance in the IR spectrum when they analysed a solution of geosmin. They also noted that when geosmin was treated with acid the odour intensity was reduced 2000 fold as geosmin was being transformed into argosmin.

Rosen *et al.*, (1968) isolated and identified the major odour producing substance of *Streptomyces griseoluteus* **IM 3718**. They pointed out that although Gerber and Lechevalier had earlier identified geosmin as the substance responsible for producing earthy odours isolated from several actinomycetes (Gerber and Lechevalier, 1965) they were not able to find geosmin in the products of *Streptomyces griseoluteus* **IM 3718**. A partially purified extract of *S. griseoluteus* was compared, by Rosen *et al.*, with an authentic sample of geosmin he obtained from Gerber. As a result of identical GC and odour characteristics of their extract compared to geosmin and the fact that the GC peak for geosmin as well as its odour was destroyed when acidified, Rosen concluded that the earthy smelling substance produced by *S. griseoluteus* was geosmin. Rosen *et al.*, also noted that when their extract (or geosmin) was acidified 5 argosmins and not one, as Gerber and Lechevalier suggested (Gerber and Lechevalier, 1965), were produced. By varying the starting concentration of geosmin or the contact time with aqueous acid, Rosen *et al.*, observed that the ratios of the 5 peaks relative to each other would change but the overall area did not. They suggested that the transformation of geosmin to argosmin was more complex than previously reported.

Medsker, *et al.*, (1968) commented on Gerber's earlier work (Gerber, 1967), in which she presented a partial structure for geosmin, and suggested that certain discrepancies existed between their work on the identical compound to that of Gerber's. When geosmin was acidified, Medsker and co-workers found 9 argosmins were produced and not one as reported by Gerber. They also noticed that a tenth peak with a higher retention time was also seen and suggest that under strong acid (HCl) conditions geosmin dehydrates to form a series of nine isomeric hydrocarbons accompanied by some substitution of Cl at the hydroxyl group. When the acidified solution was in turn treated with a drop of 1% bromine in dichloromethane and again chromatographed then a new set of seven peaks replaced the previous nine argosmin peaks. Interestingly, when geosmin was treated with acid (HCl in hexane) and the reaction mixture was hydrogenated with 30 cm of hydrogen pressure over Adam's catalyst the nine peaks were replaced by a single peak with an identical retention time and odour to geosmin. However, unlike geosmin this compound gave a negative Lucas test eliminating the possibility that residual or regenerated geosmin caused the peak.

They presented further evidence that only two methyl groups were present in geosmin and not three as Gerber suggested (Gerber, 1967) and they also found a sharp absorbance at  $3630\text{ cm}^{-1}$  in the IR spectrum indicating a sterically hindered OH group was present (something Gerber deduced but could not show spectroscopically). Medsker *et al.* (1968) postulated that "little positive can be said about the nature of the ring system" and suggested that it resembled that of  $\alpha$ -pinene and that the decalin structure is unlikely from comparing PMR spectra of geosmin and several monomethyl decalins.

Gerber (1968) compared mucidone and geosmin and found that the two compounds were different in type of odour, odour threshold and GC retention time. She was able to obtain an O-H IR absorbance for neat geosmin which had not been observed earlier (Gerber, 1967) for a carbon tetrachloride solution of geosmin. She also pointed out that on an extremely sensitive GC, argosmin was shown to be a mixture of five isomers. The three main peaks she called argosmin A, B and C in order of increasing retention time on a non-polar column. From NMR spectra and derivatisation with DNP of the argosmin C degradation product, they deduced that argosmin C was 1,10-dimethyl-1(9)-octalin and confirmed this by comparison with an authentic compound supplied to them by Dr. Marshall. The IR spectrum of geosmin was sent to Dr. Marshall for comparison with the four isomeric 1,10-dimethyl-9-decalols he had synthesised. It matched that of *trans*-1,10-dimethyl-*trans*-9-decalol. The two compounds had identical NMR, IR and GC characteristics and produced the same argosmins when acidified. The structure of geosmin and argosmin C as well as the argosmin degradation product are given (Fig. 2).



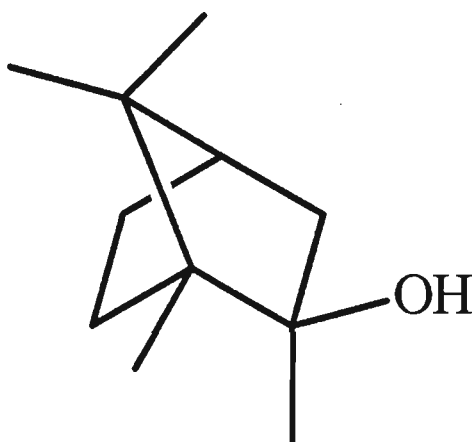
**Figure 2** Confirmed structures of geosmin, argosmin and the argosmin degradation product (Gerber, 1968)

Medsker *et al.*, (1969) reported that a camphor-smelling compound had been isolated and identified as the major odour component of three actinomycete species (*S. antibioticus* **5324**, *S. praecox* **ATCC 3374** and *S. griseus* **ATCC 10137**) of 28 actinomycetes surveyed. They noted that the compound was an extremely volatile white solid with an odour threshold of 0.10 µg/L and a m.p. 158 to 160°C . They identified the compound as 2-*exo*-hydroxy-2-methylbornane (later to be abbreviated to MIB, Fig. 3) on the basis of NMR, GC-MS, IR and wet chemistry data. A mass spectral fragmentation pattern, and the relevant structures of each fragment for this compound, was also given.

The similarity in structure and odour between geosmin and MIB was discussed and Medsker *et al.* noted that both compounds had a tertiary and highly sterically hindered OH group and a saturated ring systems with methyl substitution. They suggested that other compounds with similar structural shape may have the same odour characteristics as geosmin and MIB.

Gerber (1969) reported that although they had found geosmin to be the odorous volatile component found in twenty different actinomycetes, in four of them a white crystalline solid with a strong camphor-menthol-like smell was isolated as the major volatile component. From NMR data they deduced that the compound was 2-methyl-isoborneol or 1,2,7,7-tetramethyl-2-norbornanol (Fig. 3). The identity was proven by synthesising an authentic sample from camphor and methylmagnesium iodide as described by Toivonen (1968). Gerber confirmed that the identification of MIB as the major component of *S. antibioticus* **5324**, *S. praecox* **ATCC 3374** and *S. griseus* **ATCC 10137**

as reported by others (Medsker *et al.*, 1969) is correct. Gerber noted that an eleven membered carbon compound in nature is rare and suggested that MIB might be derived from either a smaller or larger normal terpenoid by gain or loss of carbon. She gave a possible biosynthetic pathway for MIB from a sesquiterpene precursor but added that it was just as likely that MIB was formed biosynthetically by addition of a methyl group to a monoterpene such as borneol.



**Figure 3**      Structure of 2-methylisoborneol, 1,2,7,7-tetramethyl-2-norbornanol, 2-exo-hydroxy-2-methylbornane or MIB

As a result of the successful isolation and structural elucidation of geosmin and MIB (Gerber and Lechevalier, 1965; Gerber, 1968; Gerber, 1969 and Medsker *et al.*, 1969) those two compounds were in great demand. During a five year period (1967-1972) Gerber received twenty three requests, from the US EPA and other researchers in the field, for the supply of authentic geosmin. As her supplies of geosmin were exhausted Gerber undertook a large scale research project (Gerber, 1974) to supply purified geosmin and MIB in milligram quantities to the US EPA for distribution to other researchers and for use in US EPA water treatment development studies. Gerber used

cultures of *Streptomyces* sp. CWW3 for the microbial production of geosmin and MIB. As the yield of geosmin was ~1 mg/L a one-year target output of 480 mg of geosmin was forecast by Gerber, however, increased yields during the year meant that geosmin (709 mg) as well as MIB (16 mg) and a mixture of MIB and geosmin (71 mg) was actually supplied. The yield of geosmin was further improve to ~6 mg/L by growing *Streptomyces griseus* LP-16 in fermentors followed by extraction and purification using an ion exchange resin (Gerber and Lechevalier, 1977).

*"Once geosmin standards were available from fermentation it was possible to prove that geosmin was produced by other microorganisms and that odors and taints due to geosmin were world-wide" (Gerber, 1979).*

Table 6		List of microorganisms that have been shown to produce geosmin (g) and/or MIB (m)		
ALGAE			REFERENCE	
<i>Anabaena</i>	<i>circinalis</i>	<i>g</i>	Persson, 1983; Bowmer <i>et al.</i> , 1992	
	<i>macrospora</i>	<i>g</i>	Yagi <i>et al.</i> , 1983	
	<i>scherevieti</i>	<i>g</i>	Persson, 1983	
<i>Aphanizomenon</i>	<i>gracile</i>	<i>g</i>	Jüttner, 1984	
<i>Lyngbya</i>	<i>cf. aestuarii</i>	<i>g</i>	Tabachek and Yurkowski, 1976	
	<i>cf. martensiana</i>	<i>g</i>	Tabachek and Yurkowski, 1976	
	<i>cryptovaginata</i>	<i>m</i>	Tabachek and Yurkowski, 1976	
<i>Oscillatoria</i>	<i>cf. acutissima</i>	<i>g</i>	Tabachek and Yurkowski, 1976	
	<i>agardhii</i>	<i>g</i>	Persson, 1983	
			Tabachek and Yurkowski, 1976	
	<i>animalis</i>	<i>g</i>	Yagi <i>et al.</i> , 1983	
	<i>autumnalis</i>	<i>g</i>	Yagi <i>et al.</i> , 1983	
	<i>bornetti</i>	<i>g,m</i>	Berglind <i>et al.</i> , 1983	

	<i>bornetti</i>	<i>g</i>	Persson, 1983
	<i>brevis</i>	<i>g,m</i>	Berglind <i>et al.</i> , 1983
	<i>brevis</i>	<i>g</i>	Naes <i>et al.</i> , 1985
	<i>sp.</i>	<i>g</i>	Burlingame <i>et al.</i> , 1986
	<i>cortiana</i>	<i>g</i>	Tabachek and Yurkowski, 1976
	<i>curvicepts</i>	<i>m</i>	Persson, 1983
	<i>prolifca</i>	<i>g</i>	Tabachek and Yurkowski, 1976
	<i>salina</i>	<i>g</i>	Tabachek and Yurkowski, 1976
	<i>simplicissima</i>	<i>g</i>	Persson, 1983
	<i>splendida</i>	<i>g</i>	Tabachek and Yurkowski, 1976
	<i>tenuis</i>	<i>g,m</i>	Tabachek and Yurkowski, 1976
			Persson, 1983
	<i>variabilis</i>	<i>g</i>	Tabachek and Yurkowski, 1976
<i>Phormidium</i>	<i>sp.</i>	<i>g,m</i>	Berglind <i>et al.</i> , 1983
	<i>tenue</i>	<i>m</i>	Yagi <i>et al.</i> , 1983
<i>Schizothrix</i>	<i>sp.</i>	<i>g</i>	Burlingame <i>et al.</i> , 1986
	<i>muelleri</i>	<i>g</i>	Persson, 1983
	<i>muelleri</i>	<i>g</i>	Yagi <i>et al.</i> , 1983
<i>Symploca</i>	<i>cf. muscorum</i>	<i>g</i>	Tabachek and Yurkowski, 1976
	<i>muscorum</i>	<i>g</i>	Persson, 1983

## ACTINOMYCES

<i>Streptomyces</i>	<i>antibioticus</i>	<i>m</i>	Gerber, 1979
	<i>odorifer</i>	<i>m</i>	Gerber, 1979
	<i>griseus</i>	<i>m</i>	Gerber, 1979
	<i>praecox</i>	<i>m</i>	Gerber, 1979
	<i>lavendulae</i>	<i>m</i>	Gerber, 1979
<i>Actinomadura</i>	<i>sp. 1-15</i>	<i>m</i>	Gerber, 1979
<i>Nocradiopsis</i>	<i>dassonvillei</i>	<i>m</i>	Gerber, 1979
<i>Microbisphora</i>	<i>rosea</i>	<i>g</i>	Gerber, 1979
<i>Streptomyces</i>	<i>fradiae</i>	<i>g</i>	Gerber, 1979
	<i>antibioticus</i>	<i>g</i>	Gerber, 1979
	<i>griseus</i>	<i>g</i>	Gerber, 1979
	<i>odorifer</i>	<i>g</i>	Gerber, 1979
	<i>alboniger</i>	<i>g</i>	Gerber, 1979
	<i>lavendulae</i>	<i>g</i>	Gerber, 1979
	<i>viridochromogenes</i>	<i>g</i>	Gerber, 1979
<i>Nocardia</i>	<i>sp. 1-15</i>	<i>g</i>	Gerber, 1979



MYXOBACTERIA

*Nannocystis exedens* g Trowitzsch *et al.*, 1981

FUNGI

*Chaetomium globosum* g Kikuschi *et al.*, 1981

AMOEBAS

*Gymnamoeba vannellidae*\* g Hayes *et al.*, 1991

\* may also be produced by symbiotic bacteria, Hayes *et al.*, 1991

Table 7 Foods in which geosmin (g) and/or MIB (m) have been observed

FOOD		REFERENCE
Table beets	g	Acree <i>et al.</i> , 1976
Sugar beets	g	Maga, 1987
Sugar	g	Maga, 1987
Swiss chard	g	Maga, 1987
Navy beans	g	Maga, 1987
Sweet corn	g	Maga, 1987
Brie & camembert cheese (fresh)	g	Maga, 1987
Freshwater fish	g/m	Maga, 1987
Bream	g/m	Persson, 1981
Canned champignons	m	Whitfield <i>et al.</i> , 1983
Drinking water	g/m	Maga, 1987
Processed dry beans	g/m	Swanson and Hernandez, 1984
Catfish	g	Dupuy <i>et al.</i> , 1986
	g	Martin <i>et al.</i> , 1988
Trout	g	Tabachek and Yurkowski, 1976
Clam	g	Tanchotikul, 1990
Wheat flour	g	Whitfield <i>et al.</i> , 1991

Gerber continued to work with actinomycetes and algae in an effort to isolate and identify more volatile compounds produced by these organisms which may have a

detrimental effect on water quality. She was particularly interested in terpenoid compounds and/or compounds that were very odorous.

An extremely odorous compound with an odour threshold approximately ten times lower than MIB and geosmin was isolated by Gerber from an actinomycetes species she received from M. Taylor of Apopke, Florida (Gerber, 1977). The compound had an intense odour and like the odour of geosmin tended to persist and cling to any piece of equipment or person it came into contact with. The compound was identified as 2-isopropyl-3-methoxypyrazine (abbr. 2-IP-3-MP) with an odour threshold of 0.002 µg/L (see Table 5). The odour of 2-IP-3-MP was described as "musty", "like roots" and "potato-like". Gerber believed that this compound was responsible for the musty odour attributed to mucidone by Dougherty *et al.* in 1966. She pointed out that mucidone which was later identified as 3-isobutyl-6-ethyl-2-pyrone (Sipma *et al.*, 1972) and found to have a fruity odour (Lechevalier, 1974) could not have been responsible for the musty odour reported. She suspected that 2-IP-3-MP was present at a very low concentration and that it was the cause of the perceived odour. She concluded that it was unlikely that the odour was caused by either MIB or geosmin as the odour was destroyed by treatment with potassium permanganate whereas the odours of MIB and geosmin were not.

Gerber (1971) observed that chromatograms of several whole broth extracts of actinomycetes with either geosmin and/or MIB present usually showed one or more peaks at higher retention times. She investigated the major peak of an extract of *Streptomyces* sp. (strain B-7) and found that it exhibited a mass spectral fragmentation

pattern typical of sesquiterpene alcohols. Gerber used a combination of spectral and wet chemical techniques to identify the compound as cadin-4-ene-1-ol. Two cadin-4-ene-1-ols were known from commercial cubeb oil, cubenol and epicubenol. By a direct comparison with the known cadineols she was able to establish that her compound was an enantiomer of epicubenol. It had a "woody" or "earthy" odour whereas epicubenol had a "sweet-spicy" and more intense odour. Gerber noted that to find sesquiterpenes in these extracts was novel because they were usually regarded as products of higher plants. However, when they were present the optical rotation as well as the odour characteristics of the microbially produced compound was found to be opposite to the sesquiterpenes produced by higher plants (Gerber, 1979). This was also true for a second sesquiterpene alcohol (selina-1(14),7(11)-diene-9-ol) which was produced by *S. fradiae* 3535 and had the opposite rotation to the natural diene isolated from vetiver oil (Gerber, 1979).

Gerber isolated and identified a great number of volatile compounds from actinomycetes during her research including MIB, geosmin, mucidone, 2-isopropyl-3-methoxypyrazine, 5-methyl-3-heptanone, furfural, 1-phenyl-2-propanone, 2-phenylethanol, cadin-4-ene-1-ol, salina-1(14),7(11)-diene-9-ol and a number of volatile lactones (Gerber, 1979). She pointed out that of all the compounds identified from odorous actinomycetes so far, only three substances had odour thresholds low enough to cause water quality problems at very high dilutions. The three compounds were MIB, geosmin and 2-IP-3-MP, however, as the latter is removed during normal water treatment processes Gerber believed that MIB and geosmin remained as the two most troublesome compounds water managers needed to contend with.

### 1.3 Other Volatile Organic Compounds

Once researchers began looking for MIB and geosmin in water supplies as well as algal, fungal and actinomycetes cultures an ever increasing number of other volatile compounds were identified. These included: nor-carotenoids, terpeneoids, alcohols, alkanes, alkenes, aldehydes, esters, thioesters, dialkyl-sulphides, fatty acids, O- and S-esters, alkyl-di and -tri sulphides, benzofuranones, nitriles, furans, sulphur heterocyclics, isothiocyanates, ectocarpene and dictyopterene (Jüttner, 1976; Vanhaelen *et al.*, 1978; Bechard and Rayburn, 1979; Jüttner, 1979; Anthoni *et al.*, 1980; McInnes *et al.* 1980; Kikushi *et al.*, 1981; Jüttner, 1981 (a); Jüttner, 1981 (b); Jüttner and Hahne, 1981; Yashuhara and Fuwa, 1982; Jüttner *et al.*, 1982; Slater and Block, 1983; Henatsch and Jüttner, 1983; Jüttner, 1983; Jüttner, 1984; Jüttner and Wurster, 1984; Hofbauer and Jüttner, 1988; Wajon *et al.*, 1986; Hayes and Burch, 1989; Jüttner, 1992).

Although not all compounds identified were odorous, or if they were their odour thresholds were usually significantly higher than those of both MIB or geosmin, they provided important information about the organism producing them. Correlation of the suite of volatile excretion products and the organism which produced them provided a way of identifying the organism by its volatile chemical "finger-print". The taxonomic assignment of an algal genus down to species level, based on morphological differences, was difficult and often not possible because it relied on recognition of subtle differences in physical features or on subjective judgements as to the three dimensional shape of the organisms when viewed under a microscope. To further complicate matters the appearance of algal colonies could change when taken from the field and kept for prolonged periods in laboratory unialgal or axenic cultures. However, in some cases

volatile chemical markers could be used not only to identify a particular algal genus but also to distinguish between the different strains of the same genus.

e.g.

- i) microorganisms capable of producing geosmin and MIB (see Table 6)
- ii) Jüttner *et al.* (1986) monitoring an algal bloom for an entire season were able to establish the following correlations:  $\beta$ -cyclocitral and *Microcystis*; heptadec-*cis*-5-ene and *Oscillatoria redekei*; hepta-*trans,cis*-2,4-dienal, deca-*trans,cis*-2,4-dienal and *Dinobryon*; octa-*trans,cis*-1,3,5-triene and *Asterionella formosa*.
- iii) Hofbauer and Jüttner (1988) again showed that the occurrence of  $\beta$ -cyclocitral could be used to identify the presence of the algal genus *Microcystis* in a water body and, more importantly, that the ability to produce isopropylthio compounds could be used to distinguish *Microcystis flos-aquae* from other strains of this genus such as *Microcystis aeruginosa*. They pointed out that to be able to distinguish between these two strains had very important implications for water managers because *Microcystis aeruginosa* was also known to produce algal toxins whereas *Microcystis flos-aquae* was not.

Whenever a natural water body was monitored for volatile organic compounds it was necessary to distinguish between the compounds biogenically produced and pollutants. Jüttner, (1983) pointed out that a great variety of organic volatile compounds have been

identified from the natural aquatic environment and that demonstration of the role of microalgae in their production was difficult but absolutely necessary. Artefacts produced during the analytical procedure, pollutants and compounds produced by bacteria associated with an alga need to be eliminated from the total volatiles identified. Strategies suggested by Jüttner (1983) to determine the origin of the volatile compounds were as follows:

- i) if grown under the same conditions different algae should produce different compounds. If the same compounds appear in all cultures they should be suspected as being contaminants.
- ii) the kinetics of formation of excretion products should be different for different growth stages of the organism. A steady increase of a compound during the steady increase of the organism would indicate that it was a true algal excretion product.
- iii) it should be possible to assign a compound to a group of compounds derived from a common precursor. e.g. the structural relationship between  $\beta$ -cyclocitral and  $\beta$ -ionone and their likely precursor  $\beta$ -carotene; the position of double bonds and oxygen containing substituents of the degradation products of unsaturated fatty acids such as  $C_5$  and  $C_8$  alkenes, alcohols, aldehydes and ketones; presence of alcohols with one less carbon than leucine, isoleucine and valine after decarboxylation of those amino acids; if oxidation and esterification follows then formation of methyl, ethyl and thiomethyl esters would be expected.

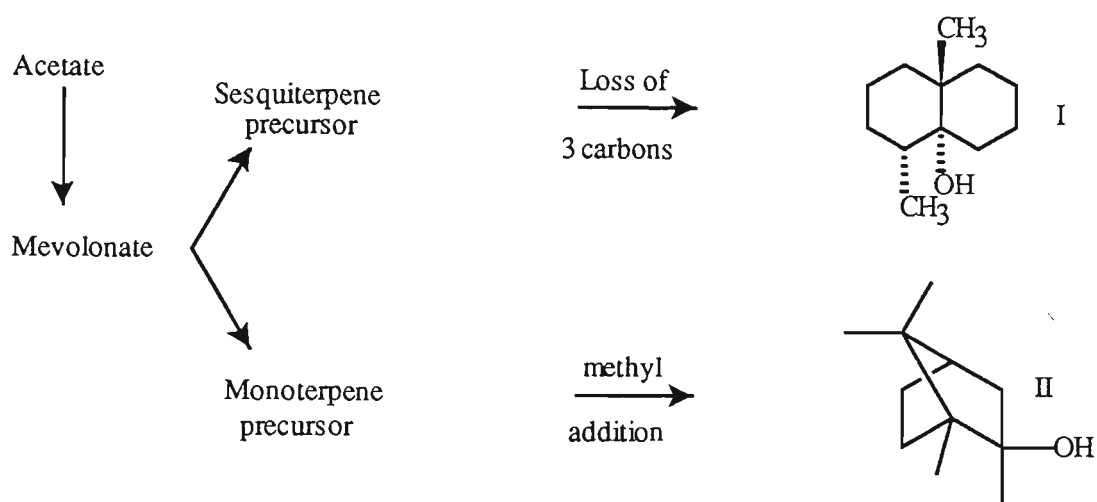
- iv) isomers that were easily converted into a more stable compound were likely to be of biological origin. This would be demonstrated by the formation of a more stable isomer (such as *trans,trans*-2,4-decadienal) at the expense of a less stable one (such as *trans,cis*-2,4-decadienal).

#### 1.4 Metabolic Pathways Leading to Production of MIB and Geosmin

As well as identifying these odour compounds researchers attempted to explain the reason for and factors that influence their production. Experiments were conducted looking at the metabolic pathways to their (especially MIB and geosmin) production and whether or not the compounds themselves exhibited any biological activity.

Gerber (1969) suggested that the precursor for the production of MIB was a larger molecule such as a sesquiterpene. She pointed out that, although MIB appeared to resemble a monoterpene with one extra carbon atom, true monoterpenes of microbiological production were rare. An example of a possible biosynthetic pathway for the production of MIB was given. In 1979 Gerber also suggested that geosmin was produced from a sesquiterpene precursor followed by the loss of an isopropyl group.

However, Bently and Meganathan (1981) using [2-<sup>14</sup>C]-acetate, [1-<sup>14</sup>C]-acetate and *L*-[methyl-<sup>14</sup>C]-methionine suggested that MIB was produced from a monoterpene precursor along the isoprenoid pathway followed by transfer of the additional methyl group from S-adenosylmethionine. They also gave evidence that geosmin was derived from a sesquiterpene precursor followed by loss of an isopropyl group (see Fig 4).



**Figure 4** Proposed biosynthetic pathway for geosmin (I) and MIB (II). Bently and Meganathan, 1981

Croteau *et al.* (1981) demonstrated that in garden soil the monoterpene ketone *d*-camphor was the likely precursor of MIB. They postulated that condensation of camphor with acetate to form MIB after decarboxylation of the intermediate carboxymethylisoborneol was the likely biochemical pathway. This was supported when the intermediate carboxymethylisoborneol was found in soil extracts containing the acidic metabolites and also when radio labelled MIB was produced after incubation of soil to which radio labelled carboxymethylisoborneol had been added.

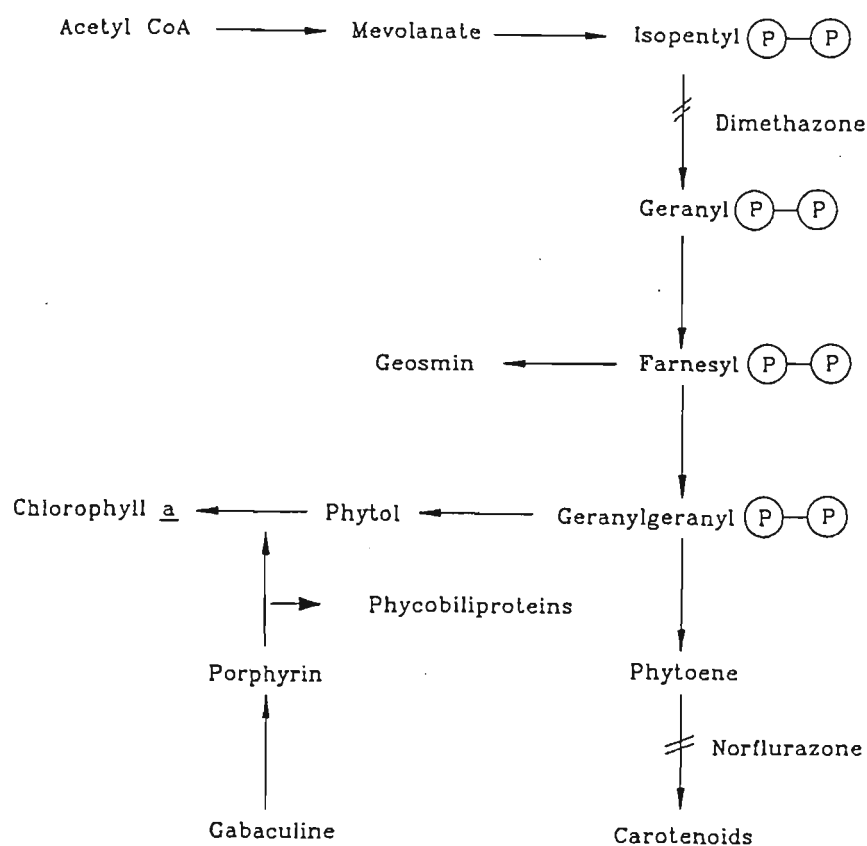
Wood *et al.* (1983) studied the factors that influence geosmin production by *Streptomyces albidoflavus* in five large reservoirs at Longridge, Lancashire in north west England. To identify the likely source of geosmin they looked at the water body, sediment, plant debris and soil run-off. They concluded the following:



- i) reservoir water was not the source of geosmin as the nutrient levels found in the water were always lower than the lowest levels required for supporting geosmin production. Experiments using reservoir water and varying concentrations of nutrients showed that the minimum amount required for supporting geosmin production was: C =  $5.6 \times 10^{-3}$  M (added as glucose); N =  $1.0 \times 10^{-4}$  M (added as  $\text{NH}_4\text{Cl}$ ) and P =  $5.7 \times 10^{-5}$  M (added as  $\text{K}_2\text{HPO}_4$ ). Nutrient levels measured in reservoir water between 1976 and 1981 were significantly lower than this with the highest concentrations found as follows: C =  $6.9 \times 10^{-6}$  M (as total dissolved organic carbon), N =  $1.3 \times 10^{-5}$  M (as total soluble nitrogen) and P =  $6.0 \times 10^{-8}$  M (as total phosphate).
- ii) sediment and plant debris which accumulates between loose stones at the side of the reservoirs and support geosmin production may cause taste and odour problems due to run-off or leaching through reservoir walls.

The effect of light on the production of geosmin was investigated by Naes *et al.*, (1985, 1988) and Naes and Post, 1988. They compared the relationship between geosmin and chlorophyll-*a* content of *Oscillatoria brevis* when grown under various light regimes and concluded that both compounds appear to be synthesised via the same isoprenoid pathway as suggested for actinomycetes (Bently and Meganathan, 1981). An inverse relationship between light intensity and geosmin and chlorophyll-*a* concentration was also demonstrated. Naes *et al.* (1989) further investigated the relationship between geosmin and pigment production by using specific inhibitors of the isoprenoid pathway

(norflurazone and dimethazone) as well as gabaculine which inhibits porphyrin production (see Fig 5).



**Figure 5** Schematic representation of the biosynthetic pathway of geosmin, chlorophyll *a* and carotenoids with the sites of action for the inhibitors dimethazone, norflurazone and gabaculine (Naes *et al.*, 1989).

The three inhibitors were assessed under light and nitrogen limiting conditions and the production of geosmin,  $\beta$ -carotene, chlorophyll-*a* and phycobiliproteins were monitored. Evidence was presented that isoprenoid precursors are channelled towards geosmin production in times of low or limited pigment production. They concluded that although their data does not categorically prove that geosmin is produced via the isoprenoid pathway it strongly supports the hypothesis.

Wu and Jüttner (1988) investigated the relationship between geosmin and chlorophyll-*a* production in axenic cultures of *Fischerella musicola* (ATCC 29114). To obtain reliable total geosmin concentrations a methanol desorption step prior to closed loop stripping was required because > 90 % of total geosmin was bound in the cells of this organism and could not be removed by the normal stripping technique. They observed that increased geosmin production occurred in the lag-phase of the culture whereas a decrease in production occurred during optimum growth conditions. They also demonstrated that geosmin production changed under varying temperature regimes with the highest geosmin production occurring at the lower and upper temperature limits while the lowest production of geosmin was observed at the optimum temperature of 30°C. Their observations led them to conclude that the biosynthesis of geosmin and chlorophyll-*a* must be regulated separately as the ratio of the two changed during cultivation.

Experiments to ascertain the effect of chelating agents on geosmin and chlorophyll-*a* production as well as growth rates of *Anabaena macrospora* (Miwa and Morizane, 1988) showed that chelating agents were very important for the healthy growth of that organism. Cultures grown minus a chelating agent (either EDTA or humic acid) resulted in a 90 % reduction of cell numbers (measured as cell/mL), a 75 % reduction in chlorophyll-*a* content (measured as µg/cell) and the appearance of the culture changed from green to yellow. However, a constant concentration of geosmin ( $\sim 1-4 \times 10^{-5}$  ng/cell) was maintained although the ratio of extracellular geosmin to total geosmin increased 2-3 fold. The authors concluded that the physiological state of *Anabaena macrospora* deteriorated with the lack of a chelating agent and that the ratio of

extracellular geosmin increased as a result.

Aoyama, 1990 studied the effect of *L*-methionine and folic acid addition to *Streptomyces griseofuscus* on the production of both MIB and geosmin. Aoyama expected a linear increase in MIB production with addition of *L*-methionine as earlier work using C<sup>14</sup> labelled *L*-methionine (Bently and Meganathan, 1981) showed that the label was taken up by MIB but not geosmin. However, the concentration of both odour compounds increased with addition of *L*-methionine and folic acid and the variation of their production was similar for all experimental conditions. Aoyama confirmed that label incorporation was not evident in geosmin when C<sup>14</sup> labelled *L*-methionine was used and concluded that the label was lost during the formation of geosmin from a labelled intermediate precursor. MIB and geosmin production increased for all concentrations of *L*-methionine added (10-1000 mg/L) whereas addition of 1000 mg/L of folic acid was required before any increase in MIB or geosmin could be observed. The author concluded that there was no doubt that *L*-methionine was involved in both MIB and geosmin production and that the production was independent of incubation time.

Recently, Dionigi *et al.*, (1990) investigated the effect of mixed function oxidase inhibitors on the production of geosmin by *Streptomyces tendae*. The premise for this work was that because geosmin contained a hydroxyl group a hydroxylation reaction must have occurred at some point in the biosynthetic pathway to its production. The link between pigment production and geosmin production established by earlier workers (Naes *et al.*, 1985; Bently and Meganathan, 1981) provided evidence that both were produced via the isoprenoid pathway. Mixed function oxidase inhibitors (piperonyl

butoxide and MGK-264) known to inhibit hydroxylation and pigment production were chosen for this work. Although dry matter production was not affected by either compound both piperonyl butoxide (300  $\mu$ M) and MGK-264 (300  $\mu$ M) decreased pigment production. Interestingly the two inhibitors had different effects on the biosynthesis of geosmin ranging from a 400 % increase (when exposed to piperonyl butoxide) to a 40 % decrease (when exposed to MGK-264) compared to untreated controls. The authors cite this as evidence opposing the hypothesis that geosmin was as an overflow product synthesised when the organisms pigment production was restricted or totally inhibited as suggested by Naes *et al.*, (1985). Dionigi *et al.*, (1990) suggested that the observed increase in geosmin production by addition of piperonyl butoxide might have been due to induced mixed function oxidase activity which resulted from repeated exposure to the inhibitor. They concluded that although the exact mechanisms of geosmin synthesis were not known the production of geosmin by *S. tendae* was not inhibited by piperonyl butoxide. The authors also noted that geosmin production and spore formation was observed for *S. tendae* grown on solid media whereas neither geosmin nor spore formation was observed for the organism grown on the equivalent liquid media. The reason for those observed differences were not given. However, the fact that geosmin production and spore formation coincided was put forward as evidence that geosmin was involved in propagation.

Dionigi *et al.*, (1991) investigated the use of the sesquiterpene alcohol farnesol and geosmin itself as inhibitors of biomass and geosmin production by *S. tendae*. They reasoned that because farnesyl pyrophosphate, regarded as the universal precursor of sesquiterpene derived metabolites, was easily phosphohydrolysed to farnesol and that

sesquiterpene alcohols showed antimicrobial activity then accumulation of farnesol should harm the organism. However, if farnesol or farnesyl pyrophosphate was removed by conversion to a less toxic metabolite the toxic threat would be avoided. Addition of farnesol (300  $\mu\text{M}$ ) and geosmin (300  $\mu\text{M}$ ) to separate cultures of *S. tendae* showed that farnesol does reduce biomass, optical density, geosmin and metabolic heat production compared with controls whereas the addition of geosmin does not. The authors pointed out that the presence of geosmin could indicate the presence of farnesyl moieties and organisms capable of converting these compounds would be less susceptible to farnesol toxicity than those that could not. However, as the concentration of farnesol was increased both biomass and geosmin production declined. At 100  $\mu\text{M}$  farnesol addition the biomass production of *S. tendae* was reduced by 20 % whereas geosmin production was reduced by 40 %. Higher concentrations of farnesol (> 200  $\mu\text{M}$ ) showed > 90 % reduction of both biomass and geosmin. As normal water treatment options for the removal of geosmin usually resulted in killing the organism responsible for its production an initial increase in the odour compound was usually observed as the geosmin stored in the cells was also released into the surrounding water body. Dionigi *et al.*, (1991) suggested that because of these difficulties any management practice that resulted in reducing the production of geosmin without killing the organism was a good one. They concluded that farnesol might have a place as a selective inhibitor of geosmin production.

Clearly the biosynthesis of MIB and geosmin is very complex and may vary depending on the organism under investigation. Research to date seems to provide conflicting explanations with Naes suggesting (Naes *et al.*, 1985, 1988; Naes and Post, 1988) that

geosmin is a co-metabolite of chlorophyll-*a* synthesis and its production increased as the production of pigments is decreased whereas Wu and Jüttner (1988) held the opinion that geosmin and chlorophyll-*a* biosynthesis was regulated separately. This view was supported by experiments using two mixed function oxidase inhibitors (Dionigi *et al.*, 1990) which showed that although both inhibitors reduced pigment production relative to controls the concentration of geosmin increased or decreased dependent on the inhibitor used.

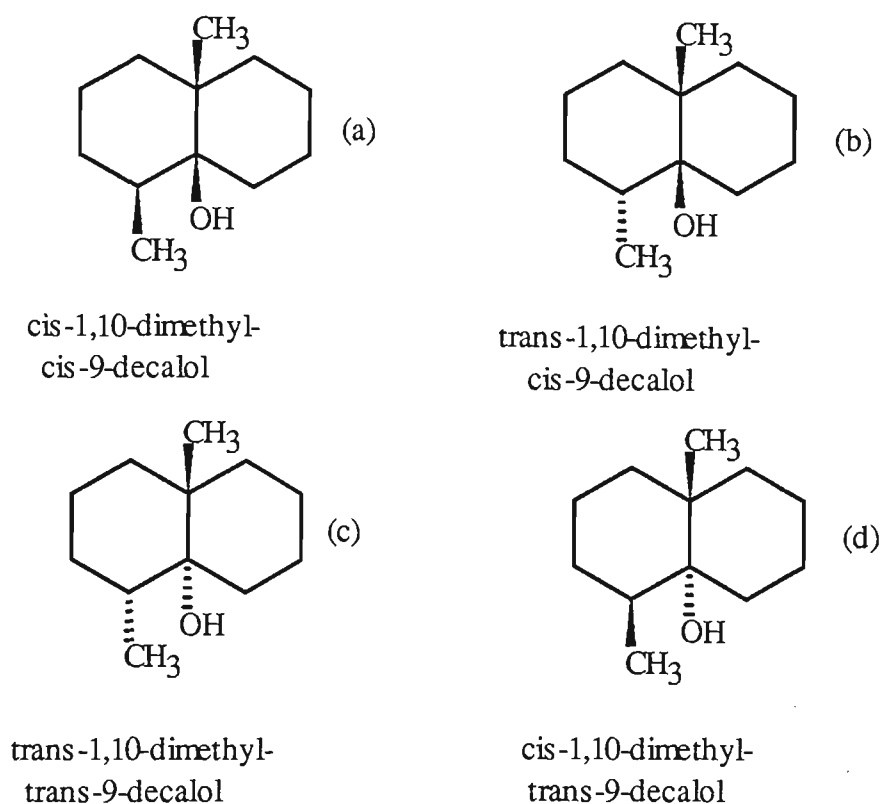
Although knowledge of the exact biological pathway to the synthesis of both MIB and geosmin would help understand the reason for the production of these odour compounds, and may help to stop or minimise their synthesis, a complete understanding is a long way off. Water managers must live with the fact that these compounds exist and they will contaminate their water supply now and for a long time in the future.

### 1.5 Structure/Odour Relationships for Geosmin, MIB and Related Compounds

Structural and odour similarities of the earthy/musty odour compounds geosmin and MIB were first noticed by Medsker *et al.* in 1969 when they observed that both compounds contained a tertiary and highly sterically hindered OH group and a saturated ring system with methyl substitution. They suggested that compounds with similar structural features might have the same odour characteristics as geosmin and MIB. This was certainly true for the four isomers of ( $\pm$ )-1,10-dimethyl-9-decalol synthesised in 1968 by Marshall and Hochstetler (Fig 6). All shared the characteristic earthy odour of geosmin (Fig. 6, c) although the *cis*-fused decalol isomers (a) and (b) had fragrances reminiscent of camphor and cedar whereas the corresponding *trans* isomers (c) and (d) were more pungent.

Polak *et al.* (1978) investigated seven pure cyclic compounds to determine the common structural features which contributed to their earthy odour. Three geosmin isomers (Fig. 6; b, c, and d), three dimethylcyclohexanol isomers and 2-ethylfenchol (Fig. 7) were evaluated. The three geosmin isomers as well as those with a partial geosmin structure, *cis-cis* 2,6-dimethylcyclohexanol and *exo*-2-ethylfenchol (Fig. 7; a and b), all had earthy odours whereas the remaining dimethylcyclohexanol isomers had odours that resembled their aromatic precursor 2,4-dimethylphenol. Polak *et al.* (1978) pointed out that even at concentrations 1000 times higher than the lowest concentration tested (low = 0.0015 ppm) the two *trans* geosmin isomers and 2-ethylfenchol retained their earthy odour. However, the *cis* geosmin isomer and *cis-cis* 2,6-dimethylcyclohexanol were perceived to change odour characteristics from earthy at low concentrations to camphoraceous at



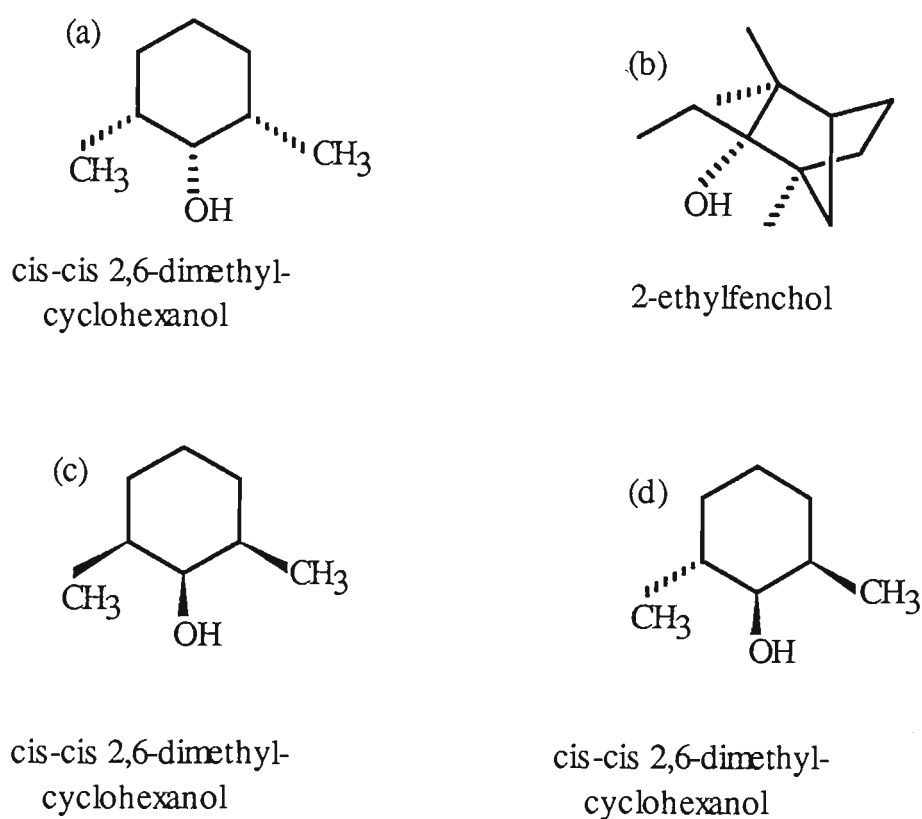


**Figure 6** The four ( $\pm$ )-decalol isomers synthesised by Marshall and Hochstetler (1968), (c) = ( $\pm$ )-geosmin

higher concentrations. The key structural features of the five earthy compounds were suggested to be:

- \* an axial or semi-axial hydroxy group and
- \* a partial carbon skeleton consisting of a five or six carbon ring with alpha methyls or methylenes on both sides of the hydroxy group

However, those structural features were not present in all five earthy/musty odour compounds (Fig. 8) tested by Amoore (1986) as part of his investigations into specific



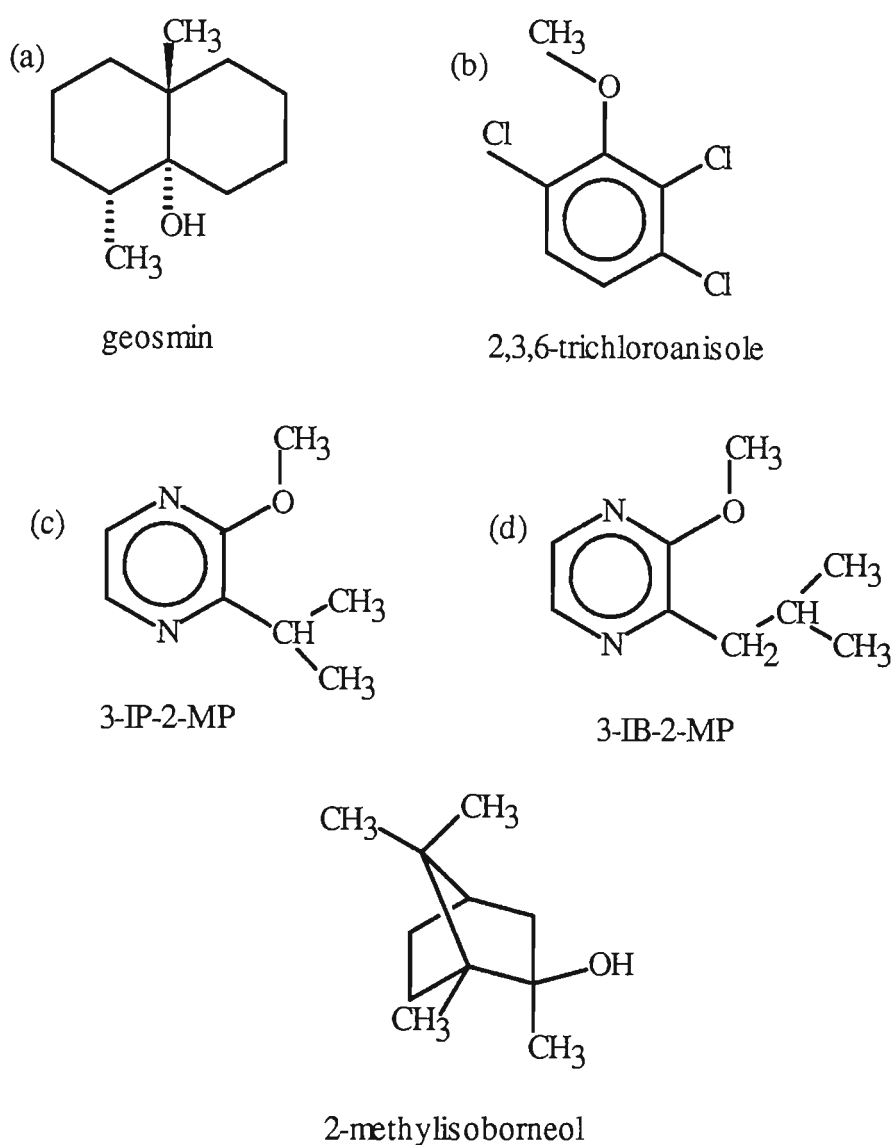
**Figure 7** Two additional cyclic alcohols with "partial" geosmin structures, Polak *et al.* (1978)

anosmia or "smell blindness" and the concept of "primary odours". Primary odours were suggested to be analogous to primary tastes (sweet, sour, salt and bitter) or primary colours (red, blue and yellow). The odours an individual perceived were therefore due to a combination of one or more discrete primary odours, each of which could be present at different concentrations, which gave rise to a tremendous range of odour sensations. Eight primary odours (camphor, minty, musky, fishy, sweaty, urinous, spermy and malty), for which the sense of smell was particularly sensitive, were identified. Because of the extremely low odour thresholds of the five earthy/musty compounds (Fig. 8) and their similar odour characteristic Amoore suggested that they belonged to the same primary odour group. He pointed out that although they

represented four different chemical families the compounds had about the same molecular size and very similar shape. Each compound had, on one side of the molecule, either a methoxy group or a methyl and a hydroxy group very close together.

However, quite subtle differences in molecular structure can make a big difference to the perceived odour of a compound. Griffiths (1974) assessed the odour characteristics of anisole and nineteen different chloroanisoles, from monochloro- to pentachloroanisole, and observed that the position of chlorine substitution had a greater effect on the odour threshold and character of the compound than the anisole skeleton. Anisole and monochloroanisole had high odour thresholds and exhibited no musty odour character. However, once anisole was more than monochloro substituted, compounds with substitution in the 2,6 position were the most odorous and most musty, the exceptions were pentachloroanisole and 2,3,5,6-tetrachloroanisole. The author suggested that an additional requirement for low odour threshold and musty odour character may be an unsubstituted five position in addition to 2,6 chloro substitution.

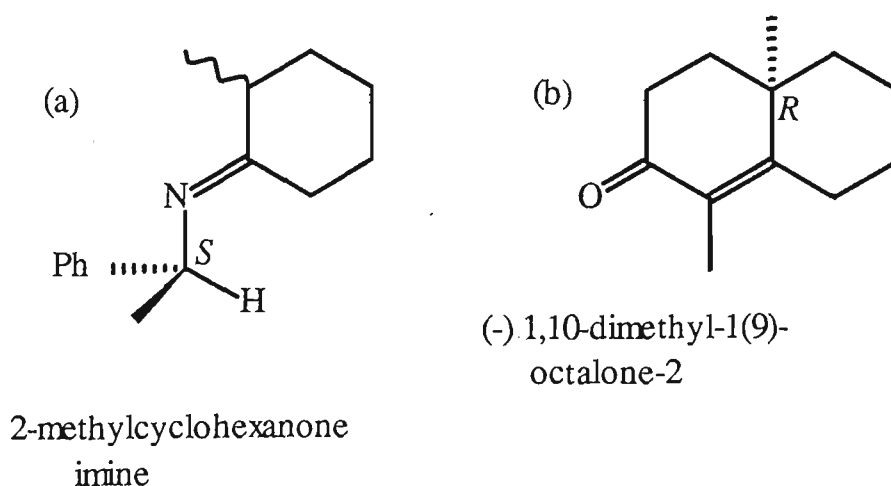
Individual enantiomers could conceivably have different odour nuances and Tyler *et al.* (1978) synthesised and compared (-) and (+) MIB and (-) and (+) methylborneol. Using a GC sniffing technique they reported an absence of earthy odour in both cases; instead the odour was described as camphor-like, both at aqueous concentrations of 1 part per thousand and 1 part per million. The methyl borneols exhibited rubbery overtones. Evidently they had exceeded the very low concentrations needed to register an earthy odour, which is certainly present for ppb-ppt concentrations of MIB in water.



**Figure 8** five earthy/musty odour compounds of the same odour group, Amoore (1986)

Reviel was interested to see if the individual enantiomers of geosmin had different odours and in 1989 described an enantioselective synthesis of (+)-geosmin and (-)-geosmin. Racemic 2-methylcyclohexanone was reacted with a chiral amine ((*S*)-(-)- $\alpha$ -methylbenzylamine).

The resultant imine (Fig. 9, a) was condensed stereoselectively with ethylvinylketone using sodium methoxide as catalyst. (Both (+) and (-) forms of this amine are available commercially). Very high stereoselectivity was achieved and the optical purity was increased further by crystallisation of the chiral ketone (Fig. 9, b) from methanol at sub-ambient temperatures. This was then converted to (-)-geosmin by the method of Gosselin *et al.* (1989). Similarly, by using (*R*)-(+)- $\alpha$ -methylbenzylamine he obtained optically pure (+)-geosmin. The odours of the two enantiomers were suggested to be very similar. ( $\pm$ )-geosmin is a patented additive to ambergris-type perfumes (Escher *et al.* 1981).

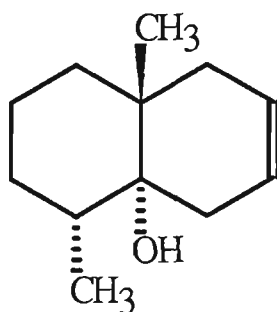


**Figure 9** (a) 2-methylcyclohexanone imine and (b) (-)-1,10-dimethyl-1(9)-octalone-2

A novel natural compound, identified as dehydrogeosmin (Kaiser and Nussbaumer 1990), was recently isolated from various species of *Cactaceae* and found to be responsible for the earthy/musty scent of their flowers.

The olfactory properties of this compound (Fig. 10) were characterised by the earthy/musty odour of geosmin with an additional camphoraceous aspect. The authors noted that the flowers of species that exhibited the distinct odour were all yellow. They also found it strange that representatives of the *Cactaceae* family normally growing under extremely dry and hot conditions were olfactorily dominated by a compound of earthy/musty character which is usually associated with damp/moist places. They suggested that dehydrogeosmin might play a significant role in the pollination biology of such *Cataceae*.

Interestingly the odour threshold of dehydrogeosmin was reported to be ten times higher than that of geosmin ( $2 \times 10^{-11}$  g/L air vs  $2 \times 10^{-12}$  g/L air) which indicates that the three dimensional shape of the molecule may influence the intensity of the odour perceived whereas the position and type of functional groups may determine the odour characteristic.



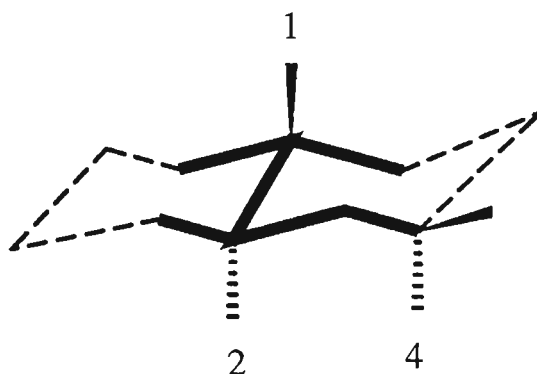
Dehydrogeosmin

**Figure 10**

A novel natural compound isolated from *Cactaceae* species with an earthy/musty odour, dehydrogeosmin

One could speculate that the OH group was essential for developing the earthy/musty odour character of geosmin as was clearly demonstrated by Gerber (1968) when she acidified geosmin to produce the dehydration product argosmin which had little or no odour. However, this was not the case for the dehydration products of MIB (2-methylenebornane and 2-methylbornene) which had similar musty odour characteristics to the parent compound (Martin *et al.* (1988). Similarly no substantial change in the earthy/musty odour of geosmin was observed by omission of the secondary methyl group of geosmin (Ohloff 1971). As stated earlier geosmin and its optical as well as diastereo isomers are used as perfume ingredient to impart an ambergris odour to the product. Interestingly, although both optical isomers of geosmin exhibit an earthy/musty character, their odour thresholds differ. The (+) enantiomer has an odour threshold eleven times higher than the natural (-) compound (Polak and Provasi, 1992).

Ohloff (1971) pointed out that the ambergris odour was composed of six distinguishable qualities which arose exclusively in compounds which had a decalin ring system of strictly determined stereochemistry (Fig. 11). As long as a 1,2,4-triaxial arrangement of the substituents, one being an oxygen function, in the decalin ring system was maintained an ambergris odour was generated. He suggested that the position of the oxygen functional group within the triaxial system determined which of the ambergris odours would be sensed and that the chemical nature of the oxygen substitution and the surroundings of the odour centre seemed to be unimportant.



**Figure 11** 1,2,4-triaxial arrangement of the substituents in the decalin ring system, Ohloff (1971)

## 1.6 Water Treatment Practices

Although early water treatment strategies revolved around procedures designed to remove bad taste and odour from water, efforts to identify either the source of the taste and odour problem, or the specific compounds responsible, were not intensified until the late 19th and early 20th centuries. Once the sources of the odour compounds were identified, particularly those responsible for the production of earthy/musty odours such as actinomycetes and cyanobacteria (Romano and Safferman, 1963; Safferman *et al.*, 1967), water managers focused on treatment strategies which prevented or minimised their production (Montiel, 1983). The identification of the actual compounds responsible for the earthy/musty odours, such as MIB and geosmin (Gerber and Lechevalier, 1965; Medsker *et al.*, 1969; Gerber, 1969), meant that those compounds could be targeted with a water treatment procedure specifically tailored for their reduction or removal.



### **1.6.1 Water Treatment Methods for MIB and Geosmin**

Generally water treatment methods, to control taste and odour problems due to the earth/musty compounds geosmin and MIB, fall into two categories:

- i) preventing the compounds entering the water and
- ii) removing the compounds once present

Some examples of both methods are given in the following section and are presented as an overview to indicate the complexity of the problem and the diversity of treatment methods water managers have tried in an effort to provide odour free water that is safe to drink.

#### **1.6.1.1 prevention of taste and odour problems**

Bartholomew (1957) controlled the growth of odour producing organisms in Dry Canyon Reservoir (40 miles north of Los Angeles) and supply channels with residual copper (~0.2 ppm). He had found from earlier experiments that treatment with residual copper throughout the season was more successful at controlling pond weeds and plankton populations in the water than intermittent treatment with high doses of copper. The procedure was continued for the following seven years and was also used to treat Lower Hollywood reservoir (1953), Bouquet Reservoir (1955) and Haiwee Reservoir (1957) successfully.

Safferman and Morris (1964) investigated the use of viruses to control algal blooms. They pointed out that treatment methods commonly used relied on copper sulphate or

chlorine to control algal growth and that both were nonspecific. Rather than the nuisance organisms being destroyed the entire algal population was often annihilated and the aquatic environment was seriously disturbed. The authors suggested that this could be avoided if an algicidal agent could be found that would specifically target nuisance organisms so that only those were destroyed and subsequently succeeded by a more desirable algal species. Safferman and Morris believed that such an agent was discovered when they successfully isolated a virus (LPP-1) that could infect and destroy the freshwater cyanobacteria (= blue-green algae) *Lyngbya sp.* (2), *Phormidium sp.* (4) and *Plectomonas sp.* (8) while a further forty nine algal strains tested as well as bacteria, actinomycetes, fungi and kidney tissue cells from monkeys were not susceptible to the virus.

The biological control of algal blooms was investigated further by Martin *et al.* (1978) and Philips *et al.* (1990) who worked with cyanophages (= cyanobacterial viruses). The authors described the discovery of new cyanophages which infected and killed problematic cyanobacteria such as *Microcystis aeruginosa*, *Anabaena flos-aquae*, *Anabaena circinalis*, *Plectonema notatum*, *Synechococcus cedrorum*, *Synechococcus elongatus* and the benthic *Lyngbya birgei*. Philips *et al.* (1990) pointed out that while the use of cyanophages had not gained universal acceptance, their unique ability to kill specific nuisance organisms without damaging other more desirable ones should make biocontrol a viable alternative in the future.

The use of *Bacillus* species to rapidly degrade geosmin was investigated by Narayan and Nunez (1974). The degradation rate of geosmin (1.6 ppm) was assessed by measuring

(manometrically) the oxygen consumption for each organism using a differential respirometer. These results were later disputed and criticised by MacDonald et al. (1987) who evaluated the use of the same *Bacillus* species to reduce the geosmin contamination of their municipal water supply. No significant change in the concentration of geosmin (from either synthetic or natural sources) was found for any of their trials. The authors doubted that geosmin could be degraded by *Bacillus* species.

In an effort to avoid taste and odour problems before they occurred the Metropolitan Water District of Southern California (MWD) in 1981 devised an early warning system to detect the odour compound MIB (produced by the benthic alga *Oscillatoria curviceps*) at a very early stage (McGuire *et al.*, 1983). Levels of MIB were monitored in samples taken by scuba divers from 30 cm above the algal beds. Once the level of MIB reached ~25 ng/L or more, action to destroy the alga was effected. Using this system an early warning period of one month was achieved and levels of MIB were kept below 20 ng/L at the lakes outlet throughout the season.

Once the water was treated it was supplied by MWD to their member agencies where it was stored in open reservoirs for further distribution. The open storage reservoirs however, were the weak link in the distribution system because during storage, residual chlorine was depleted, nutrient levels increased (from bird droppings etc.) and biological activity (algal blooms) with the associated taste and odour problems reappeared (Krasner and Means, 1986). In order to avoid those problems the MWD covered two of their open storage reservoirs (Garvey and Orange County) with floating

black rubber mats. The mats were attached to the side walls of the reservoir and moved up and down as the water level in them fluctuated. After an initial holding period, to allow for the leaching of xylenes from the liner, the reservoirs were put back into service and vastly improved microbiological water quality was achieved and maintained.

#### **1.6.1.2 removal of taste and odour compounds**

Cherry (1962) described the use of potassium permanganate to supplement activated carbon in the treatment of odour problems caused by a severe algal bloom on the Cedar River, Iowa. Jar tests showed that a satisfactory reduction of odour using activated carbon would require thirty one-ton cars of carbon to be delivered to the treatment plant every three days at an estimated cost for July of US\$ 50,000. Clearly increasing the carbon dose was neither practical nor economically feasible and an alternative to carbon treatment was sought. Several oxidising agents were tested to find one which would reduce the odour problem in a cost effective way. Potassium permanganate provided the best results because it was a good oxidising agent for most organics and a local supply was available. Although the permanganate-carbon treatment process was successful at reducing the odour problem and at a lower cost than carbon alone the odours were not remove totally and a disagreeable earthy/musty odour problem persisted.

Dougherty and Morris (1967) used activated carbon, chlorine and potassium permanganate to remove actinomycete musty taste and odour problems from water. They worked with aqueous solutions of a pure actinomycete musty compound mucidone, a compound they believed to be an extremely odorous metabolite but one which was

latter shown not to be odorous at all (Lechevalier 1974), and found that it was easily oxidised by both chlorine and permanganate. However, they noticed that even though the concentration of mucidone was reduced by more than ninety percent the threshold odour did not vary. The only way effective odour removal was achieved was with activated carbon. This was not surprising given that the odour problem was most likely caused by either MIB or geosmin present at low levels (both resistant to oxidation) and not mucidone which was present at high concentration but was not responsible for the odour.

An intensive study to determine how MIB and geosmin adsorb on powdered activated carbon (PAC) and granular activated carbon (GAC) in the presence of high natural dissolved organic matter was commenced by Herzing *et al.* in 1977. The authors wanted to determine whether adsorption, biological degradation or some other mechanism was responsible for the removal of those odour compounds. Biological activity was ruled out as a contributing factor in the removal of MIB and geosmin. Humic substances interfered with the amount of both compounds adsorbed by GAC filters, with the effect being more pronounced for geosmin. However, once the compounds were adsorbed they were not easily displaced by other compounds even when all adsorptive capacity of the GAC filter was exhausted. Thus unloading of MIB or geosmin from the filter bed by displacement with humic acid was unlikely.

A pilot plant study for MIB and geosmin removal using ozone and/or GAC was conducted by Terashima in 1988. Their results show that ozone was an effective oxidant for the removal of both MIB and geosmin at dose rates of 2 mg/L and above.

They obtained removal rates for MIB (55-250 ng/L initial concentration) of > 70 % (2 mg/L O<sub>3</sub>) and > 90 % (5 mg/L O<sub>3</sub>) and for geosmin (33-89 ng/L initial concentration) of > 80% (2 mg/L O<sub>3</sub>) and > 90% (5 mg/L O<sub>3</sub>). This seems to be in contrast to the relatively low removal efficiency of ozone found by Lalezary *et al.* (1986) and points to the care that must be taken when extrapolating results obtained for a small benchtop system to a large scale pilot plant or eventually to a full scale treatment facility. Terashima also demonstrated that good removal of both MIB and geosmin was obtained using GAC and that the efficiency of GAC was reduced if the water was pre-chlorinated.

Lalezary-Craig *et al.* (1988) optimised the removal of MIB and geosmin, at environmentally realistic levels (10-100 ng/L), in bench and pilot plant trials using PAC. The optimisation procedure involved studying the effects of initial PAC dose rate (5, 10, 15 and 23 mg/L), initial concentration of odour compounds (5, 10, 20, 30, 40, 60, 100 and 150 ng/L), residual chlorine and chloramines (2 mg as Cl<sub>2</sub>/L), background organics (humics), filtration rate and contact time on PAC efficiency. A comparison of the results obtained from bench-scale and pilot-scale trials was also made.

Their results showed that the optimum dose rate of PAC required to remove MIB and geosmin depended on the initial concentration of those compounds. If the concentration of MIB and geosmin was kept below ~20 ng/L then a PAC dose rate of 5 mg/L was sufficient to reduce their concentration to < 1 ng/L. Lalezary-Craig *et al.* (1988) also showed that chlorine, chloramines and the presence of background humic acid had a detrimental effect on the adsorption efficiency of PAC whereas filtration rate and PAC contact time had little effect.

Ozonation as a treatment option for the removal of taste and odour compounds from surface and drinking water was investigated by Tuorila *et al.* (1980). They found it to be effective in removing earthy odours caused by geosmin in raw water (at 4.7 mg/L ozone) and tap water (at 1.7 mg/L ozone) but noted that new odour compounds were created during the process. Although no attempt was made to identify the new compounds; which were most likely low molecular weight aldehydes, ketones and carboxylic acids; they described their odours as "*sweet or buttery*" and "*pungent, waxy, rancid, bitter, empty and chemical-like*". The overall sensory quality of the water was claimed to have been improved by ozonation because ozonated raw water rated slightly better than conventionally treated earthy-smelling tap water and the latter, when treated with ozone, generated the least odorous drinking water.

Lalezary *et al.* (1986) evaluated several commonly used techniques for the removal of taste and odour compounds in drinking water. The compounds they used in their trial were MIB, geosmin, TCA, 2-IP-3-MP and 2-IB-3-MP and the treatment processes investigated were oxidation using ozone (O<sub>3</sub>), chlorine (Cl<sub>2</sub>), chlorine dioxide (ClO<sub>2</sub>) and potassium permanganate (KMnO<sub>4</sub>). They deliberately worked with odour compounds at the extremely low concentrations at which they appear in the environment (~100-200 ng/L) and their results are summarised as follows:

OXIDANT		OBSERVATION	
i)	Cl <sub>2</sub>	*	dependent on dose rate and contact time
		*	not effective at removing MIB, geosmin or TCA even under extreme conditions

		* effective removal of 2-IB-3-MP and 2-IP-3-MP was only achieved using unrealistic dose rates and contact times
		* economically inefficient particularly for removal of MIB and geosmin
ii)	ClO <sub>2</sub>	* dependent on dose rate and contact time
		* greater than 50 % removal of TCA, 2-IP-3-MP and 2-IB-3-MP at realistic dose rates and contact times but < 30 % removal of MIB and geosmin
		* no additional removal at ClO <sub>2</sub> concentrations above 4 mg as Cl <sub>2</sub> /L
iii)	KMnO <sub>4</sub>	* KMnO <sub>4</sub> , not efficient for any compound
	& MnO <sub>2</sub>	* MnO <sub>2</sub> , efficient removal of TCA, 2-IB-3-MP and 2-IP-3-MP but not MIB or geosmin
		* main mechanism for removal of organics by KMnO <sub>4</sub> treatment appears to be by adsorption and not oxidation. However, dose rates were much higher than would be used in practice
iv)	O <sub>3</sub>	* efficiency dependent on contact time and dose rate
		* at realistic dose rates TCA, 2-IP-3-MP and 2-IB-3-MP were removed efficiently (> 70 %) but MIB and geosmin were not (< 30 %)
		* efficiency of MIB and geosmin removal dropped at concentrations above 4 mg/L O <sub>3</sub> however, the authors explained that this could have been due to "analytical imprecision"

Baker *et al.* (1987) evaluated several water treatment methods such as coagulation, oxidation (chlorine, chlorine dioxide, chloramines or potassium permanganate) and activated carbon. The compounds of biological origin were the same as those investigated by Lalezary *et al.* (1986) (i.e. MIB, geosmin, TCA, 2-IB-3-MP and 2-IP-3-MP) except at concentrations approximately one thousand times higher (µg/L rather than ng/L) however, the concentrations of treatment chemicals were similar (2-50 mg/L). After a one year monitoring period of a full-scale water treatment plant at Mosang-sur-



Sein in Paris, France the authors concluded that: chlorine, chlorine dioxide, chloramine and potassium permanganate were inefficient at removing the taste and odour compounds; ozone removed most odour compounds but produced other compounds with sweet/fruity odours; chlorination was effective at removing fishy odours but increased the occurrence of perceived earthy/musty odours as detected by flavour profile analysis and coagulation was totally ineffective but PAC and GAC removed muddy taste and odours.

Ashitani *et al.* (1988) studied the effect of pre-chlorination of raw water as part of the conventional treatment process which included coagulation, sedimentation, rapid sand filtration and post-chlorination. Coagulation and sedimentation without pre-chlorination reduced the taste and odour problem markedly because cyanobacterial cells, with their store of the odour compounds, were removed intact. Biodegradation of MIB and geosmin on rapid sand filters, no pre-chlorination, was observed and found to be very effective for geosmin removal but less so for MIB removal. The authors also suggested that geosmin, and to a lesser extent MIB, were photochemically degraded in the presence of free residual chlorine.

Glaze *et al.* (1990) evaluated nine oxidation procedures ( $\text{Cl}_2$ ,  $\text{ClO}_2$ , chloramines,  $\text{KMnO}_4$ ,  $\text{H}_2\text{O}_2$ ,  $\text{O}_3$ ,  $\text{O}_3:\text{H}_2\text{O}_2$ ,  $\text{O}_3:\text{UV}$  and  $\text{H}_2\text{O}_2:\text{UV}$ ) for their removal efficiency of six odour compounds (1-hexanal, 1-heptanal, 2,4-decadienal, dimethyltrisulphide, MIB and geosmin). They showed that conventional oxidation procedures such as  $\text{Cl}_2$ ,  $\text{ClO}_2$ , chloramines,  $\text{KMnO}_4$  or  $\text{H}_2\text{O}_2$  are ineffective at removing the odour compounds MIB and geosmin. Advanced oxidation techniques that rely on the formation of OH radicals such

as  $O_3:H_2O_2$ ,  $O_3:UV$  and  $H_2O_2:UV$  were much more successful. Interestingly ozone alone (i.e. without  $H_2O_2$  or UV) was found to be effective at oxidising MIB and geosmin. This was suggested to be due to natural compounds found in the source water, such as dissolved iron,  $H_2O_2$  and humics, which could react with ozone to produce OH radicals.

The effectiveness of a combined ozone/hydrogen peroxide (PEROXONE) treatment process was assessed at MWD by Ferguson *et al.* (1990) in a pilot plant study. Production of disinfection by-products (DBPs), due to secondary disinfection by chloramine after the initial ozone or PEROXONE treatment, and the ability of the treatments to inactivate indicator microorganisms such as *E. coli* were also monitored. The pilot plant study showed that peroxone was much more effective at removing both MIB and geosmin (initial concentration 100 ng/L each) than ozone alone although, MIB was consistently more difficult to oxidise than geosmin.

The optimum ratio of ozone to hydrogen peroxide varied depending on the source water and the authors attributed this either to the presence of natural OH radical promoters in one source water or lower levels of OH radical scavengers, such as bicarbonate, in the other source water (Glaze *et al.* 1990). Contact time (6-12 min) had little effect on the removal rates of the odour compounds. Levels of trihalomethanes (THM's) and DBP's were kept acceptably low by treatment of raw water with ozone or PEROXONE followed by disinfection with chloramines. However, when chlorine was used as the disinfectant THM levels were unacceptably high. The inactivation of indicator microorganisms such as *E. coli* was similar for both ozone and PEROXONE treatments.

Lalezary *et al.* (1984) evaluated air stripping as a management option for the removal of five extremely odorous compounds from drinking water. The compounds tested were geosmin, MIB, 2-isobutyl-3-methoxypyrazine (2-IB-3-MP), 2-isopropyl-3-methoxypyrazine (2-IP-3-MP) and 2,3,6-trichloroanisole (TCA). They concluded that the Henry's Law constants for 2-IB-3-MP, 2-IP-3-MP, geosmin and MIB were too small to make removal economically practical. TCA however, was considered to be a compound which could be removed by air stripping.

Sävenhed *et al.* (1987) contrasted the efficiency of artificial groundwater recharge with alum coagulation/sand filtration for the removal of several off-flavour compounds which included geosmin, MIB, 2-IP-3-MP, TCA, 1-octen-3-one, 1-nonen-3-one, dimethyl trisulphide and a number of unidentified muddy or musty odour compounds. They showed that artificial groundwater recharge was much more efficient at removing the odour compounds than conventional alum flocculation/sand filtration treatment. Geosmin and MIB were both removed to levels well below their analytical detection limits even though the initial concentrations were more than fifty times higher than that. In addition to the excellent removal efficiency, particularly for MIB and geosmin, Sävenhed *et al.* pointed out that artificial groundwater recharge was a one-step process and that formation of other odorous compounds was not observed.

The use of ozone and biologically active sand filters was investigated by Lundgren *et al.* (1988) for the removal of several off-flavour compounds including geosmin, MIB, TCA, 1-octen-3-one and dimethyl trisulphide. Their results showed that effective removal of odour compounds was achieved with fully aerated sand filters (50 cm depth)

and with a residence time of two hours. They maintained that good oxygen supply was essential for prolonged filter life and good odour removal rates. The ozonation of raw water prior to biologically active sand filtration was found to be detrimental. Even though some odour compounds were removed during the ozonation process other odour compounds were formed and some of those were less responsive to biodegradation than the odour compounds initially removed. The authors suggested that pre-ozonation, in certain circumstances, may have provided some benefit when used with biologically active filters not because of the oxidising power of ozone but rather by ensuring that the bio-active filter was not oxygen depleted.

#### 1.6.1.3 recent developments

The latest treatment methods for the abatement of taste and odour problems in drinking water were presented at a recent conference on "off-flavours in the aquatic environment" in Los Angeles, March 3-8, 1991. A physical treatment that was successfully used in Holland to reduce the population of a benthic geosmin producing *Oscillatoria sp.* involved disturbing the bottom of the reservoir by dragging a steel frame, to which several long lengths of chain were attached, across it (Breemen van *et al.* 1992). The biodegradation of geosmin and MIB on filters which supported a series of microbes was presented by Egashira *et al.* (1992) and involved treatment of Lake Biwa drinking water in a pilot plant study (50,000 cu-m/d capacity). The microbes *Pseudomonas aeruginosa*, *Candida lipolytica* and *Flavobacterium multivorum* were identified as the organisms responsible for the removal of MIB (80 %) and it was noted that the activity of the microbes was pH and temperature dependent.

Similar work but relating to MIB removal from drinking water of Lake Kasumigaura due to biodegradation by a gram-positive, rod shaped bacterium was presented by Ishida and Miyaji (1992). Their work showed that a biofilm reactor inoculated with the bacterium could degrade MIB at ng/L levels and consistent removal rates of 90 % (500 ng/L MIB) were achieved. Sumitomo (1992) isolated bacteria from natural sludges capable of degrading MIB, such as *Pseudomonas fluorescence* and *Pseudomonas putrefaciens*, and studied the by-products of MIB degradation. They also showed that enzymes extracted from those bacteria could breakdown MIB in the same way as the bacteria themselves.

The use of sodium carbonate peroxyhydrate (SCP) was investigate with respect to off-flavour compound production in commercial catfish ponds by Martin *et al.* (1992). Seven ponds were treated with two doses of SCP at 50 lbs/surface acre which resulted in a hydrogen peroxide concentration of 3-5 ppm with a half-life of 0.5-1.0 hours at 30°C. In four out of the seven ponds the off-flavour compounds decreased to acceptable levels five days after treatment. Of the remaining three ponds MIB was present in pond one at 40-60 ng/L however, the fish were on-flavour 7-10 days after treatment. Ponds two had a severe *Oscillatoria* bloom and the treatment was not effective whereas pond three showed high concentration of MIB dehydration products which required twentyone days to clear. The authors concluded that the treatment appears to work when the off-flavour is not chronic.

Pirbazari *et al.* (1992) presented a protocol they had developed for the engineering, design and operation of a fixed-bed granular activated carbon (GAC) adsorber. The

adsorber was specifically designed for the removal of MIB and geosmin and the adsorber dynamics, for the target compounds, were predicted using a dispersed-flow homogeneous surface diffusion model (DFHSDM). A differential column batch reactor (DCBR) was used for rate studies. The authors concluded that the DFHSDM model could be used to accurately predict or simulate adsorber dynamics which they believed to be essential when extrapolating bench-scale results to a full-scale model. Suffet and Mallevalle (1991) emphasised the important role played by sensory and instrumental evaluation of water quality throughout the treatment process and distribution systems. Successful taste and odour control could be achieved if unit operations of a treatment process were correctly sequenced, especially with respect to the use of activated carbon.

Ando *et al.* (1992) pointed out that because a high percentage of MIB and geosmin was retained in algal cells, a treatment option which removed those cells intact, such as coagulation and sedimentation without breakpoint pre-chlorination, was a viable one. PAC was suggested as being effective at removing aqueous MIB and geosmin, however, at high concentrations the removal efficiency suffered and ozonation followed by activated carbon treatment was required.

## 1.7 Instrumental Analytical Methods

Because of the extremely low odour threshold of both MIB and geosmin it is possible to perceive the presence of these compounds in drinking water at levels thousands of times lower than the detection limit of the best analytical instruments. Consequently, a sensitive analytical procedure, including an extremely efficient concentration step, is required for the quantification of these compounds at environmental levels (ng/L).

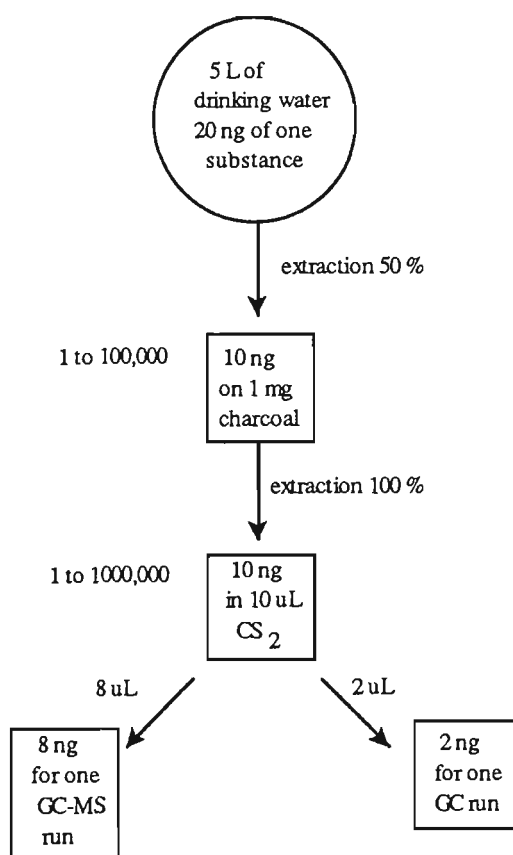
The analytical techniques available and in common use at the time the odour compounds were identified (mid 1960's) usually involved steam distillation followed by solvent extraction of the distillate and concentration using evaporation or vacuum distillation (Gerber, 1969; Medsker, *et al.*, 1969). In order to obtain enough of the compounds for identification and structural elucidation it was necessary to extract large volumes of whole broth cultures, usually *Actinomyces* *sp.* and in particular *Streptomyces* *sp.*. However, obtaining enough of the compounds from a natural water sample proved to be much more difficult. Rosen *et al.* (1970) was the first to show that MIB and geosmin occurred and caused taste and odour problems in natural waters. To do this they extracted the organic compounds of extremely large water samples, from the Warbash River and Grand Lake, Ohio, by passing 3000 L batches of water through carbon filters. Each filter was extracted with chloroform, steam distilled and the distillate salted-out (20 % NaCl) and extracted with ether. The ether was concentrated to a final volume of 1 mL and analysed using GC-FID (1.8 m x 3.2 mm column packed with 20 % SE-30 on 90/100 mesh Celite; nitrogen carrier gas at 30 mL/min; temp. prog. 70 to 230°C at 6°C/min).

Although they were able to identify and verify the presence of MIB and geosmin in river and lake samples they were not able to quantify them directly. Instead an estimate of the % odour contribution of MIB or geosmin to the total odour of the steam volatile fraction was determined by stream splitting the GC effluent (10:1 trap:FID) and trapping the organic matter of each GC peak on silica gel. The contents of each silica gel trap was then placed into 500 mL of odour-free water, for quantitative transfer of the odour compounds to the water, and submitted to an odour panel for assessment. In this way MIB was shown to be a minor component (6 % by weight) of the total steam volatiles collected from a Grand Lake extract, however, it was the major odour component contributing 68 % of the total odour. Mass spectral confirmation of the odour compound was obtained by submitting the contents of a silica gel trap for solid probe mass spectrometry.

Quantification of volatiles present in natural water samples at parts per trillion (ppt = 1 in  $10^{12}$  w/w) was not achieved routinely until the development and implementation of the Closed Loop Stripping system (Grob, 1973, Grob and Grob, 1974, Grob *et al.*, 1975, Grob and Zürcher, 1976). Grob (1973) developed the closed loop stripping technique to extract and concentrate only the volatile components of a water sample without requiring additional concentration of the extracting solvent. Traditional techniques such as steam distillation, solvent extraction or solid phase adsorption had the disadvantage that a great number of semi- and/or non-volatile organic compounds were also extracted and all compounds ended up in a large volume of solvent. In order to detect and quantify the volatiles at ppt levels it was necessary to concentrate the final solvent ~ 1,000 fold (100 mL to ~ 100  $\mu$ L) which resulted in the simultaneous concentration of



contaminants in the solvent and the loss of volatile compounds through evaporation. Grob (1973) overcame those problems by using the closed loop stripping system and taking advantage of the fact that the compounds of interest were volatile. The original system involved the transfer of volatile organic compounds from a water sample (5 L) to a small activated carbon filter (~1 mg) by sparging the sample with air in a closed loop system. The organic compounds were then eluted from the carbon filter with carbon disulphide (10  $\mu$ L) and a solute to solvent ratio of 1:1,000,000 was achieved (see below), without concentration of the solvent (or its inevitable contaminants) or loss of volatile compounds.

**Figure 12**

Schematic showing extraction efficiency and concentration factors achieved (Grob, 1973)

Grob (1973) opted for a closed loop system to transfer volatiles to a carbon filter, as opposed to an open loop system using an inert gas, for the following reasons:

- \* small amounts of organic substances normally lost because of incomplete retention on the carbon filter, due to unfavourable particle distribution or because they by-passed the filter (filter holder provides ~ 95-98 % seal), are recovered because they are automatically recycled and re-presented to the carbon filter
- \* in an open loop system contaminants in the gas stream were also adsorbed by the carbon filter. A procedure for ultra trace or less volatile organic substances required long stripping times (20 h at 2 L/min) and large volumes of gas (2400 litres) would pass through the carbon filter. However, in a closed loop system this problem did not exist as the same volume (2400 L) of gas was passed through the carbon filter by recycling 0.5 L continuously

The closed loop stripping system was primarily developed for analysis of hydrocarbon contamination in Lake Zürich as well as spring, ground and tap water. Semi-quantification was achieved by adding hexamethylbenzene as an internal standard to the carbon filter after the stripping procedure but prior to extraction with carbon disulphide. The resultant extract was analysed, the compounds of interest were identified and their ratio to the internal standard was noted. A known concentration of those compounds was then added to pure water, concentrated as a normal sample, and again hexamethylbenzene was added to the filter after stripping but prior to carbon disulphide extraction. The unknown concentration of each compound of interest was then

calculated by comparison of their ratios to hexamethylbenzene and the hexamethylbenzene ratios of the same compound of known concentration, added to pure water. Grob (1973) pointed out that the major drawback with the procedure was caused by accidental co-elution of a compound of interest and the internal standard.

Grob and Grob (1974) improved the earlier version of the stripping apparatus by eliminating or minimising air leaks, improving the carbon disulphide extraction procedure and changing the internal standard to 1-chlorodecane, although it was still added to the carbon filter after the sample was stripped and before it was extracted. A large number of hydrocarbons from diesel oil and other industrial contaminants as well as compounds from natural sources such as terpenes, C<sub>15</sub> and C<sub>17</sub> alkanes and alkenes, sulphur compounds and some unidentifiable substances were detected. However, Grob and Grob (1974) pointed out that numerous organic pollutants which occurred in large amounts (e.g. detergents, many kinds of acids such as humic acids and amino acids), carbohydrates and urea) were not detected with their procedure because the vapour pressure of those strongly polar substances was too low.

Grob *et al.* (1975) modified and optimised the procedure further by improving the flow and adsorptive characteristics of the carbon filter. Best results were achieved by using specially selected activated carbon (1.5 mg) of particle size 0.05-0.1 mm and assembling this in a cylindrical disc of 2.5 mm diameter and 0.8-1.2 mm thick. The more efficient carbon filter allowed them to reduce the size of the sample bottle from 5 L to 1 L. The sample bottle (1 L) was completely immersed in a water bath, which reduced the possibility of air leaks, and resulted in a reduction of stripping times from 12-24 h to

2 h. The reduced stripping times also eliminated sample alteration which occurred, probably due to biological activity, during extended sample preparation.

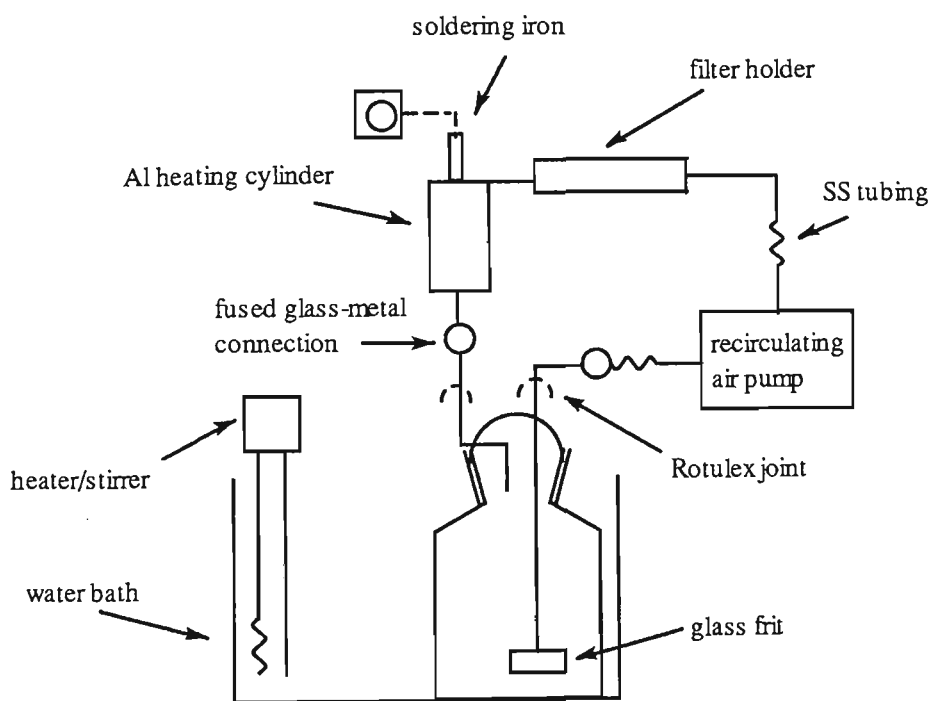
Grob *et al.* (1975) evaluated the stripping procedure and contrasted it to a rapid solvent extraction technique they had also developed. To avoid the problems of solvent (and its contaminants) concentration they devised a method which extracted 1 L of water with 0.6 mL of pentane recovering a final solvent volume of 200  $\mu$ L for analysis. Turbidity was a major problem with this technique and samples with even trace amounts of suspended solids were required to be filtered prior to solvent extraction whereas the same samples could be stripped immediately. The two methods were compared with respect to recovery and sensitivity for a number of compounds representing seven different classes (alkanes, aromatics, chlorinated hydrocarbons, ketones, alcohols, pyridines and phenols). The stripping technique was assessed to be much more sensitive than rapid solvent extraction for more volatile substances, up to  $\sim C_{20}$ , after which the solvent extraction procedure was more sensitive (the two methods were equivalent for eicosane, dimethylnaphthalene, pentadecanone and decanol). Overall the stripping procedure was judged to be more suitable for routine work, quantitatively more reproducible, less effected by turbidity and less severe on GC columns as a cleaner extract was obtained. Internal standards used during the assessment procedure were a series of 1-chloroalkanes ( $C_6$  to  $C_{18}$ ) added to the sample before extraction for the pentane extraction technique however, it is unclear how the internal standards were used in the stripping procedure (i.e. added to the water prior to stripping or to the filter after stripping).

As a result of several laboratories adopting the stripping procedure for analysis of water samples for trace organics Grob and Zürcher (1976) described their closed loop stripping procedure in detail to make it easier for laboratories wishing to introduce the technique, to do so. They pointed out that the stripping procedure was based on a set of independent parameters, changing a single parameter necessitated corresponding modification throughout the entire set and could eventually result in modification of the equipment. As a result of years of study under a wide range of conditions they recommended the following:

* water sample	0.5-2.0 L (immersed in a water bath)
* water bath temp.	30°C
* internal standards	1-chloroalkanes, C <sub>6</sub> , C <sub>10</sub> , C <sub>14</sub> and C <sub>18</sub>
* adsorbent filter	pure wood charcoal, heat activated (1.5 mg, particle size 0.05-0.1 mm, disc 2.7 mm x 0.7 mm)
* stripping flow rate	1.0-2.5 L/min (1-3 h)
* Temp. gas entering filter	40°C
* extracting solvent	carbon disulphide, 5-15 µL

They emphasised that both stripping equipment (Fig. 13) and procedure must be designed to eliminate errors and uncertainty and that most problems were caused by: leakage in the closed loop system; adsorptive material (charcoal dust, plastic material) in the pumping circuit; carbon particles irregularly distributed in the filter disc; water droplets from bubbling carried to the filter disc and condensation of water in the pores of filter particles. Quantification was achieved by adding a series of 1-chloroalkanes to the water prior to stripping and comparing the ratios of the internal standard to the compound of interest and the same compound to the same internal standard stripped

from spiked pure water. A series of chloroalkanes were chosen as internal standards because they were not found in natural water, represented a wide range of substances with respect to their stripping and extraction behaviour, were commercially available in pure form and because a homologous series was easier to identify in a complex GC trace. However, it was pointed out that the determination of extracted organics was influenced by; the efficiency of stripping from the water sample, the efficiency of adsorption on the filter, the efficiency of extraction from the filter, the fraction of the total extract transferred to the GC and the sensitivity of the GC detector for an individual substance.



**Figure 13** Closed-loop stripping apparatus (Grob, 1973)

The stripping procedure developed by Grob (1973) was successfully used, albeit in a modified form, for the extraction and analysis of volatile organic compounds (VOC's) in culture and water samples (Jüttner and Wurster, 1979; Jüttner, 1984; Jüttner, 1988). To extract the VOC's from a water sample NaCl (2.4 kg, oven dried at 240°C for 24 h) was added to raw water (10 L) and magnetically stirred (5 min). 1,3-dichlorobenzene (200 ng) was added as an internal standard to the sample which was placed into a thermostatically controlled water bath (20°C) and stripped in a closed loop system (1 h). The volatiles however, were trapped on Tenax TA (500 mg, 60-80 mesh) rather than activated carbon (1.5 mg). Transfer of the organic compounds from the Tenax tube to the GC column was achieved by thermal desorption (300°C/ 3 min) under a stream of helium while holding the capillary column at 0°C with liquid nitrogen. In this way solvent dilution was avoided and maximum transfer of VOC's to the analytical column was achieved although, the entire sample extract was used up in a single chromatographic run. Although an internal standard was used quantification was generally achieved by adding the compounds of interest to purified water at levels that, after stripping and extracting, would generate peak heights similar to those of the unknown compounds.

Krasner *et al.* (1981, 1983) adopted the closed loop stripping analysis (CLSA) procedure for the determination of five earthy/musty odour compounds (geosmin, MIB, 2-IB-3-MP, 2-IP-3-MP and 2,3,6-TCA) in drinking water, sediments and culture samples. The main alterations to the CLSA method proposed by Grob and Zürcher (1976) were that the rotulex glass connectors were replaced by stainless steel quick-connectors and the stripping bottle was replaced with a "tall form" bottle (effective height-to-diameter ratio

was increased from 1.7 to 3.3). The tall form bottle increased the stripping efficiency of the method because a greater volume of water was exposed to the air bubbles as they moved through the water column. This improved the sensitivity of the technique as more of each compound was transferred to the carbon filter. 1-chlorooctane and 1-chlorododecane (200 ng/L each) were added as internal standards prior to stripping. Identification of compounds was achieved using GC-MS in full scan mode whereas quantification was by GC-MS in multiple ion detection mode. Detection limits for the five odour compounds ranged between 2 and 5 ng/L with a coefficient of variance of ~20 %. The detection limits of the technique were lowered for all five compounds (0.8 ng/L), the precision improved (CV 15%) and the stripping time reduced (1.5 h) by addition of salt ( $\text{Na}_2\text{SO}_4$ , 80g /L) to the tall form bottle prior to stripping (Hwang *et al.*, 1984). Various salt concentrations from 0 to 300 g/L were assessed. Salt concentrations much above 100 g/L became awkward to handle due to poor rates of dissolution and problems of clogging the stripping apparatus, especially the carbon filter, while 300 g/L was totally unmanageable. The "salted-CLSA" stripping technique was optimised with respect to stripping time and salt concentration and a combination of 1.5 h stripping time and a salt concentration of 80 g/L was suggested to be the best. The method was so successful and well accepted as a technique for the routine analysis of water for volatile odour compounds (particularly geosmin and MIB) that it is now an official Standard Method (APHA, 1985).

An open loop stripping method (Boéren *et al.*, 1982; Sävenhed *et al.*, 1983) was described for the analysis of odorous compounds in drinking water. The authors pointed out that the open loop system gave them greater sensitivity, blank levels were reduced



and higher stripping temperatures could be used. Nitrogen was used as the stripping gas and needed to be purified, using an activated carbon filter, to minimise contamination and achieve low background levels. A series of 1-chloroalkanes were used as internal standards for quantification. Naes and Post (1988) used this method to analyse cyanobacterial cultures for the presence of geosmin. Approximately 5-10 mL of the culture was added to a tall form stripping bottle and the volume was adjusted with distilled water to ~ 1 L. Internal standards (a series of 1-chloroalkanes) were added (100 ng/L). The sample was then purged with stripping gas for 1.5 h at 60°C. She pointed out that the open loop system had the advantage that at elevated temperatures (60°C) total geosmin (i.e. geosmin in the water and in the algal cells) was determined. The closed loop system on the other hand, when used at 25°C with or without salt, only allowed the geosmin in the aqueous phase to be determined. However, when cells were lysed prior to closed loop stripping the same result as open loop stripping was achieved.

While Grob and others were refining both the closed- and open-loop stripping techniques other researchers were investigating alternate ways of extracting trace volatile organics from water. Cees *et al.* (1974) developed a continuous liquid-liquid extraction apparatus with which they extracted 1-2 m<sup>3</sup> of water per day. Quantification was achieved by batch extractions of 1-50 L samples of water in the second stage of the procedure. Exact details of the method used for this were not given.

Yasuhara and Fuwa (1979) used a salting-out technique (NaCl, 300 g/L) followed by solvent extraction (50 mL dichloromethane) and concentration of the extract using Kuderna-Danish concentrators. Extracts were analysed using GC with computer-

controlled mass fragmentography. Quantification was achieved by using *n*-butylbenzoate as an internal standard and preparing a calibration curve using the ratios of the peak areas of geosmin ( $m/z$  112 ion) and *n*-butylbenzoate ( $m/z$  105 ion) added to dichloromethane. The internal standard was chosen because the GC retention time was similar to that of geosmin and the mass spectral base peak at  $m/z$  105 was close to geosmin ( $m/z$  112). A reliable detection value of 50 ng/mL (50,000 ng/L) was quoted with a coefficient of variance of 18 %. However, as a footnote, an example of an application was given which claimed a detection of 31 and 34 ng/L of geosmin for water collected from the Hanamuro River. It is not clear how or with what degree of precision or accuracy this thousand fold improvement in detection limit was achieved.

Yagi *et al.* (1983) described a purge and trap method for the determination of MIB and geosmin using gas chromatography and mass fragmentography. The procedure involved purging a water sample (100 mL) to which salt (NaCl 10 g) was added with nitrogen (100 mL/min) for 10 min. Volatiles were trapped on Tenax-GC and transferred to the chromatograph by heating the Tenax trap to 200°C. The sample was analysed by temperature programming the GC and detecting the compounds of interest by mass-fragmentography. Ions monitored were  $m/z$  95 for MIB and  $m/z$  112 for geosmin. A detection limit in the nanogram per litre range was quoted with an analysis time of less than half an hour for a 100 mL sample.

An inexpensive, low technology, method for the routine determination of geosmin and MIB in large numbers of samples was developed by Johnsen and Kuan (1987). Pond water or culture samples were filtered and added to a separating funnel (500 mL, or

made up to that volume with milli-Q water) internal standards (2-undecanone, 1-dodecanol and 1-naphthalene) and dichloromethane (50 mL) were also added. The samples were shaken gently for three minutes, the dichloromethane layer was removed and centrifuged to break any emulsion formed. The organic layer was dried over sodium sulphate, evaporated to ~25-100  $\mu$ L with nitrogen (at room temperature) and the extract analysed using GC-FID. Six compounds were evaluated as internal standards and 2-undecanone and 1-dodecanol were suggested to be the best. Detection limits of 19.23 ng/L (MIB,  $\pm$  8-10 %) and 9.61 ng/L (geosmin,  $\pm$  3-5 %) were reported.

Similar to Johnsen and Kuan (1987) a simple and convenient method for the determination of MIB and geosmin in large numbers of samples was developed by Brownlee *et al.* (1988). The sample was added to a volumetric flask (2 L), so that it was ~ half way up the neck of the flask, hexane or pentane (5 mL) was added as the extractant. The solution was stoppered, magnetically stirred (2 h) and the organic layer was removed after phase separation (0.25-1 h). The hexane layer was removed (~15 mL) and passed through a small column of sodium sulphate. The sample was extracted a second time and the dried extracts were bulked (typically  $8.5 \pm 0.5$  mL was collected). Octadecane was added to the bulked extracts as an internal standard and the solution was concentrated to 1-2 mL under a stream of argon or nitrogen. A further portion of octadecane was added and the extract was concentrated to 200  $\mu$ L. Final extracts were analysed by GC-FID and the authors reported a detection limit of ~ 20 ng/L with a precision of 10-15 %. They pointed out that in their situation the detection limit was adequate and the procedure was simple, convenient and allowed them to analyse large numbers of samples without the need for specialised equipment.

Vegetable oil was used to extract trace amounts of geosmin from water (Dupuy *et al.*, 1986). The procedure used four grams of high purity vegetable oil which was added to pond water (2 L) in an Erlenmeyer flask and magnetically stirred for 30 min. After phase separation the oil layer was removed and centrifuged. An aliquot of the clear oil (300 mg) was transferred onto glass wool which was in the glass liner of an external GC injection port. Volatile compounds were swept onto a cold capillary column ( $-30^{\circ}\text{C}$ ) by carrier gas as the injection port was heated ( $140^{\circ}\text{C}$ ). After desorption ( $\sim 20$  min) volatile components of the mixture were separated by temperature programming. A method for the extraction of geosmin from off-flavoured catfish was also described. However, instead of using vegetable oil to extract geosmin the oil of the fish was used. Quantification although not detailed appears to have been by standard addition. Detection limits were in the order of parts per billion.

A microwave cooking technique was used by Martin *et al.* (1987) to extract volatile odour compounds (especially MIB) from pond raised catfish. The sample to be extracted (catfish muscle ( $\sim 40$  g) cut into  $\sim 1$  cm squares) was placed (sometimes with hexane) into a round bottom flask (100 mL capacity) and fitted with side arm adaptors to allow for continuous purging of the extracting vessel with nitrogen (80 mL/min). While nitrogen was flowing over the sample in the microwave (+ or - hexane) the fish was cooked in 15 sec. bursts at 40 % power (580 W) for 3.5 min. Volatiles generated by the cooking step were carried by the nitrogen stream and trapped in a flask, external to the microwave, which was kept at  $-80^{\circ}\text{C}$ . The hexane condensate, or the hexane extract of the distillate, was concentrated to  $\sim 100$ -200  $\mu\text{L}$  using a Kuderna Danish concentrator and analysed by GC-FID or GC-MS. The internal standard used was 1-

chlorododecane. The authors pointed out that the microwave technique had several advantages over vacuum distillation such as reduced analysis time, greater reproducibility and sensitivities in the nanogram per gram range.

Janda *et al.* (1988) reported an interesting observation made in their laboratory during the CLSA of volatiles in drinking water. Bromoalkanes, which were used as internal standards, generated chloroalkanes during the stripping of water samples containing chlorine. They pointed out that although conversion of bromoalkanes to chloroalkanes was unlikely to occur in dilute aqueous solution in the presence of chlorine it was possible on the carbon filter. They suggested that both the bromoalkanes and chlorine were transferred to the activated carbon surface of the filter where they were now present in relatively high concentrations and could react. Evidence was presented showing the formation of chloroalkanes with the same carbon number as the bromoalkanes. The phenomenon was not observed when chlorine was removed with sodium thiosulphate before stripping.

A bromine-based colorimetric technique for the detection of geosmin was described by Hensarling and Waage (1990). The technique takes advantage of a bromine-based colour reaction stated to be specific for compounds with ditertiary double bonds (the Tortelli-Jaffe reaction). Because geosmin dehydrates to argosmin and argosmin contains one of those ditertiary double bonds the authors explored the possibility of developing a colour reaction for geosmin. Colour which absorbed at 650 nm (molar absorptivity of  $800 \text{ L/ mol}^{-1} \text{ cm}^{-1}$ ) was formed with geosmin however, the timing and sequence of addition of reactants was critical for colour development to be achieved. Interestingly

when the reaction was tried with argosmin (produced by acidifying geosmin) and MIB no colour formation was observed. The lack of reaction with argosmin was suggested to be due to residual acid and further attempts with argosmin were not pursued as a positive result with geosmin had already been achieved. MIB on the other hand did not react because the formation of ditertiary double bonds on dehydration was not possible. The detection limit of the technique was very poor and the authors pointed out that the technique was not meant as a method for the direct determination of geosmin but suggested that it might prove useful combined with solid phase or liquid concentration techniques or as a post column HPLC derivatisation method.

An enzyme-linked immunosorbent assay (ELISA) for the determination of MIB was described by Chung *et al.* (1990). The technique relies on the production of an antibody which can recognise a specific part of the MIB molecule. The authors obtained their antibody from antisera raised against a conjugate of camphor linked to bovine serum albumin (BSA). Competition between the compound of interest (MIB) and a camphor-ovalbumin conjugate for the antibody adhering to the bottom of a microwell determines the amount of colour developed once a chromogen (Ig-G-horseradish peroxidase conjugate) was added. Colour developed when the camphor-ovalbumin conjugate bound to the antibody reacted with the chromogen. Maximum colour meant that no MIB was present whereas no colour meant that MIB was present in large concentrations. The intensity of colour was therefore inversely proportional to MIB concentration. Because the technique relies on shape recognition other compounds with partial MIB structural features interfered.

Compounds also evaluated were camphor, geosmin, 2-IP-3-MP and seven other MIB structurally related compounds. The percent cross-reactivity were as follows: MIB (100 %); camphor (100 %); isoborneol (100 %); borneol (100 %); camphorquinone (68 %); norcamphor (0 %); *endo*-norborneol (0 %); norbornane (0 %); geosmin (0 %); 2-IP-3-MP (0 %) and 2-methyl-2-bornene, an MIB dehydration product, (100 %). The authors pointed out that the methyl groups in camphor and MIB molecules were essential for antibody recognition. Although the sensitivity of the technique (1 µg/mL) was well above the odour threshold of MIB the authors pointed out that because it reacted specifically with MIB or MIB-like compounds and did not react with other odour compounds such as geosmin or methoxypyrazines it was a useful screening tool for the rapid, simple and routine detection of MIB, in some circumstances. They also suggested that the technique might be made more sensitive with different antibodies.

An attempt to raise monoclonal antibodies to geosmin analogues or geosmin derivatives as a first step to developing an ELISA kit with specificity to natural geosmin was outlined by Middlebrooks *et al.* (1987). Of thirty three clones tested, three had the desired characteristics however, details with respect to detection limits and possible cross-reactions were not given.

## 1.8 Limitations of Existing Internal Standards

To obtain the most accurate and precise results for a given analytical procedure an internal standard should be used that has physical and chemical properties identical to those of the target compound. Isotopically labelled analogues of the target compound are ideally suited for this purpose and their advantages in the analysis of important food

aroma compounds was highlighted by Whitfield and Shaw in 1987. However, if these are not available, the chosen internal standard should have properties as similar as possible to the target compound for at least the most critical part of the procedure. Because volatile odour compounds (particularly MIB and geosmin) are present in water at extremely low levels (ng/L) the most critical part of any analytical procedure designed for their detection is the extraction and concentration step. Concentrations of 100,000 fold are usually required before the compounds can be detected instrumentally. The accuracy and precision of the entire analytical technique therefore hinges on the ability of the internal standard to mirror the behaviour of the target compound, under all circumstances, during extraction and concentration. A number of internal standards for the quantitative determination of MIB and geosmin have been tried and are listed below:

Compound	Procedure	Reference
hexamethylbenzene	CLSA	Grob, 1973
camphor	SE	Wood and Snoeyink, 1977
	SDE	Swanson and Hernandez, 1984
1-undecanol	SDE	Swanson and Hernandez, 1984
<i>n</i> -butylbenzoate	SE	Yasuhara and Fuwa, 1979
2-undecanone	SE	Johnsen and Kuan, 1987
1-dodecanol		
1-naphthalene		
2-undecanone	SE	Dionigi <i>et al.</i> , 1990,91,92
octadecane	SE	Brownlee <i>et al.</i> , 1988
bromoalkanes	CLSA	Janda <i>et al.</i> , 1988
borneol	OLSA	Naes <i>et al.</i> , 1985
	CLSA	Wu and Jüttner, 1988
standard addition	P&T	Yagi <i>et al.</i> , 1983
	VOE	Dupuy <i>et al.</i> , 1986
1-chloroalkanes	CLSA	Grob and Grob, 1974



(added individually  
or as a homologous  
series)

CLSA	Grob <i>et al.</i> , 1975
CLSA	Grob and Zürcher, 1976
CLSA	Krasner <i>et al.</i> , 1981, 1983
CLSA	Hwang <i>et al.</i> , 1984
OLSA	Boéren <i>et al.</i> , 1982
OLSA	Sävenhed <i>et al.</i> , 1983
MCGP	Martin <i>et al.</i> , 1987
OLSA	Naes <i>et al.</i> , 1985

CLSA = closed-loop stripping analysis; OLSA = open-loop stripping analysis; SE = solvent extraction;  
MCGP = microwave cooking gas purging VOE = vegetable oil extraction; P&T = purge and trap  
SDE = simultaneous distillation and extraction

As can be seen from the table above 1-chloroalkanes are the most widely used internal standards particularly for open- and closed-loop stripping techniques. However, these compounds have a number of limitations, the most serious of which relate to the extraction and concentration required prior to analysis. Grob and Zürcher (1976) recognised this and emphasised that both the stripping equipment and procedure must be designed to minimise or eliminate errors and uncertainty. Most problems are caused for the following reasons:

1. Chloroalkanes are stripped from water at a different rate compared with MIB and geosmin. As a result, great care must be taken to control the following CLSA experimental parameters:

- \* stripping time
- \* water temperature
- \* air flow rate
- \* flow resistance of charcoal filter
- \* air leaks

To obtain accurate results the samples are usually required to be analysed twice, once with chloroalkanes only and a second time spiked with

geosmin or MIB in addition to chloroalkanes. Because stripping times are long (about 1.5 h), the need to run spikes to improve accuracy and precision is very time consuming.

2. Chloroalkanes cannot be added at the time of collecting the sample to compensate for loss of MIB or geosmin due to physical, chemical or biological processes (during sample transport and storage) because chloroalkanes are lost even more rapidly by the same processes. As a result, geosmin or MIB concentration measured in the laboratory are the concentrations remaining in the water at the time of analysis, not the concentration present at the time of sampling. To suppress biological degradation it is necessary to add mercuric chloride to the water sample.

The factors which influenced the determination of volatile organics were summarised by Grob and Zürcher (1976) as: the efficiency of stripping from the water sample; the efficiency of adsorption on the filter; the efficiency of extraction from the filter; the fraction of the total extract transferred to the GC and the sensitivity of the detector for the individual compound.

## **1.9 Potential Advantages of Labelled Internal Standards**

The inherent problems associated with an internal standard that does not have the same physical, chemical or biological properties as the target compound would potentially be overcome by using deuterated analogues of the compounds themselves. The isotopically

labelled compounds would be virtually identical with the natural compound in respect of:

- \* stripping behaviour to the natural compounds
- \* adsorptivity on the activated carbon filter
- \* extraction efficiency from the filter
- \* solubility in extracting solvent
- \* chromatographic behaviour
- \* mass spectral and FID response
- \* stability
- \* biodegradability (for the same enantiomer and assuming that breakage of C-H/C-D bonds are not involved in the rate determining step)

and would not be sensitive to changes in stripping parameters such as:

- \* stripping time
- \* sparging rate
- \* air leaks
- \* size and shape of stripping bottle
- \* temperature of
  - water bath
  - transfer tube before filter
  - carbon filter
- \* amount or type of salt added or whether added at all

The labelled internal standards could be added at the time of collecting the sample and thus compensate for any loss of analyte due to biodegradation, volatilisation or adsorption during sample transport and storage. Accurate results would be expected from a single analysis therefore running spikes would be unnecessary. Stripping times could be varied for every sample, depending on the initial concentration as judged by the odour of the water, without needing to recalibrate or recalculate response factors. Analytical runs would not have to be aborted if an air leak occurred. The deuterated standards would also provide greater precision and accuracy for the determination of MIB and geosmin in other matrices (eg foods and beverages) regardless of the extraction, concentration or analytical procedure used.

## 2 MATERIALS AND METHODS

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### 2.1 Chemicals

1-Chloro-3-pentanone, cyclohexene oxide, methylmagnesium iodide- $d_3$ , 1-chlorooctane, 1-chlorodecane, tridecane, pentadecane, m-chloroperbenzoic acid (MCPBA), lithium aluminium hydride (LAH), lithium aluminium deuteride (LAD) and all forms of camphor {(+), (-) and ( $\pm$ )} (Aldrich Chemical Company, Milwaukee, Wisconsin); 1-chlorododecane, carbon disulphide (E.Merck, Darmstadt); 6-methylhept-5-en-2-one, geranylacetone,  $\beta$ -ionone and  $\beta$ -cyclocitral were generously donated by Prof. F. Jüttner. Racemic geosmin was from Wako Pure Chemical Industries (Osaka, Japan).

### 2.2 Instrumentation

#### 2.2.1 IR and NMR

Infrared spectra were recorded with a Perkin-Elmer 783 spectrophotometer.  $^1H$  and  $^{13}C$  spectroscopy was performed on a Joel FX90Q NMR spectrometer with TMS as internal standard.

#### 2.2.2 GC-MSD

GC, Varian 3300; carrier gas He (0.75 mL/min); column, Hewlett-Packard methyl silicone, 25 m, 0.20 mm i.d., 0.30  $\mu m$  film. Samples were injected using the "hot needle" or "air sandwich" fast injection technique while the GC oven was held at 25°C (1.5 min.) with the splitter off (1 min.). The splitter was turned on and the column

heated at 10°C /min to 80°C, then 5°C /min to 250°C and held at 250°C (5 min). Splitter gas flow rate was 50 mL/min and both injector and interface were at 260°C. For concentrations of less than 1 ng/L, three consecutive injections of 2 µL of extract were made per analysis. Cumulative transfer to the column was achieved without band broadening by holding the column at 25°C until all three injections were made. After 5 min, for each of the first two injections, the splitter was opened for 20 sec and then closed again prior to the next injection. For the third injection the splitter remained open after 5 min and the oven was temperature programmed as described above.

**Table 8** Ions monitored and those used for quantification using GC-MSD (in multiple ion detection mode).

Compound	Ions (m/z) Monitored	
	A	B
1-chlorodecane	43 (91) 93	
MIB	(95) 107 135	(168)
geosmin	111 (112) 125	(112) (182)
MIB-d <sub>3</sub>		(171)
geosmin-d <sub>3</sub>		(115) (185)
6-methyl-hept-5-en-2-one	43 (108) 111 126	43 (108) 111 126
β-ionone	135 (177) 192	135 (177) 192
β-cyclocitral	109 123 (137) 152	109 123 (137) 152
geranylacetone	(43) 125 136 151	(43) 125 136 151

A = chlorodecane as internal standard

B = MIB-d<sub>3</sub> and geosmin-d<sub>3</sub> as internal standards

( ) = ions used for quantification

The GC eluant reported to a Hewlett-Packard 5970 mass selective detector, using electron impact ionisation (70 eV at a source pressure of  $2.8 \times 10^{-5}$  Torr) and operated in the SIM mode. The ions monitored and those used for quantification are shown in Table 8.

When comparing relative areas of peaks from labelled and unlabelled compounds, computed correlations were applied where necessary for the contribution of isotope satellites in the unlabelled compound to the labelled peak being monitored, and vice versa. For example, when using the base peaks 112/115 for quantification of geosmin the following equations were required to be solved simultaneously to obtain corrected ion area values for the two masses:

$$\text{area } 112_{\text{obs}} = \text{area } 112_{\text{corr}} + (\% \text{ area } 112_{\text{l}} \times \text{area } 115_{\text{corr}})$$

$$\text{area } 115_{\text{obs}} = \text{area } 115_{\text{corr}} + (\% \text{ area } 115_{\text{ul}} \times \text{area } 112_{\text{corr}})$$

$_{\text{obs}}$  = observed,  $_{\text{corr}}$  = corrected

$\% \text{ area } 112_{\text{l}} =$  peak area at mass 112, in labelled geosmin, expressed as a percentage of its peak area at mass 115

$\% \text{ area } 115_{\text{ul}} =$  peak area at mass 115, in unlabelled geosmin, expressed as a percentage of its peak area at mass 112.

e.g.

If

$$\text{area } 112_{\text{obs}} = 251736, \% \text{ area } 112_{\text{l}} = 2.40$$

$$\text{area } 115_{\text{obs}} = 210708, \% \text{ area } 115_{\text{ul}} = 0.185$$

Then

$$251736 = \text{area } 112_{\text{corr}} + (2.4/100 \times \text{area } 115_{\text{corr}}) \quad (\text{i})$$

$$210708 = \text{area } 115_{\text{corr}} + (0.185/100 \times \text{area } 112_{\text{corr}}) \quad (\text{ii})$$

Multiply (i) by 1 and (ii) by (2.4/100)

$$251736 = \text{area } 112_{\text{corr}} + (2.4/100 \times \text{area } 115_{\text{corr}}) \quad (\text{iii})$$

$$5057 = \text{area } 115_{\text{corr}}(2.4/100) + 0.0000444 \text{ area } 112_{\text{corr}} \quad (\text{iv})$$

Subtract (iv) from (iii)

$$246679 = (1-0.0000444) \text{ area } 112_{\text{corr}}$$

Rearrange

$$\text{area } 112_{\text{corr}} = 246679/0.9999556$$

Result

$$\text{area } 112_{\text{corr}} = 246689.96$$

Round Off

$$\text{area } 112_{\text{corr}} = 246690$$

Substitute area  $112_{\text{corr}}$  into (i) or (ii)

$$251736 = 246690 + (2.4/100) \text{ area } 115_{\text{corr}}$$

Rearrange

$$(251736 - 246690) \times (100/2.4) = \text{area } 115_{\text{corr}}$$

Result

$$\text{area } 115_{\text{corr}} = 210250$$

Equimolar solutions of the authentic MIB and geosmin were added to equimolar solutions of the corresponding labelled compounds to establish the purity of the latter.

### 2.2.3 GC-Enantioselective-MSD

GC, Varian 3300; injector temperature 230°C; interface temperature 270°C; column, SGE 50QC2/CYDEX-B, 0.22 mm ID, 0.25 µm film; temperature program, 30°C 1.5 min. then to 130 at 30°C/min hold 50 min then to 230°C at 30°C/min hold for 5 min; splitter off 1.5 min; split flow 50 mL/min; carrier gas, helium, linear velocity 24 cm/min. Ions monitored were 43, 91, 93 (1-chlorodecane), 112, 182 (geosmin) and 115, 185 (geosmin-d<sub>3</sub>). No mathematical correction for contribution of satellite peaks was necessary because labelled and unlabelled geosmin were baseline resolved.

### 2.2.4 GC-FID

GC, Varian 3400; detector, FID ( $4 \times 10^{-11}$ ); column, Alltech ECONO-CAP SE 30, 0.25 mm ID, 0.25 µm film; temperature program, 25°C for 1.5 min then to 250°C at 10°C/min hold for 5 min; splitter off 1.5 min; split flow 50 mL/min; carrier gas helium, linear velocity 24 cm/s; injector, 260°C; detector, 260°C. Data was captured by either the series 3000 in-board data handling system (IBDH) or a Varian series 600 chromatography data system (DS 601).

### 2.2.5 GC-Enantioselective-FID

GC, Varian 3400; detector, FID ( $2 \times 10^{-12}$ ); column, SGE 50QC2/CYDEX-B, 0.22 mm ID, 0.25 µm film; temperature program, 30°C for 1.5 min then to 127°C at 30°C/min. hold for 38 min then to 230 at 30°C/min hold for 5 min; splitter off 1.5 min; split flow



50 mL/min; carrier gas hydrogen, linear velocity 38 cm/s; injector temperature 230°C; detector temperature 260°C. Data was captured by either the series 3000 in-board data handling system (IBDH) or a Varian series 600 chromatography data system (DS 601).

## 2.3 CLSA

### 2.3.1 Equipment

CLSA equipment was from Brechbuehler AG, Switzerland, modified to take a tall form bottle (see Fig 14 (a), (b) and (c)). WISP-96 limited volume (100  $\mu$ L) micro vial inserts (#73024) and stainless steel springs (#73026) (Alltech); 2 mL clear glass screw cap vial (EC1200), Teflon faced septa (EC1250) and open top cap (1220) (Edwards Instrument Company).

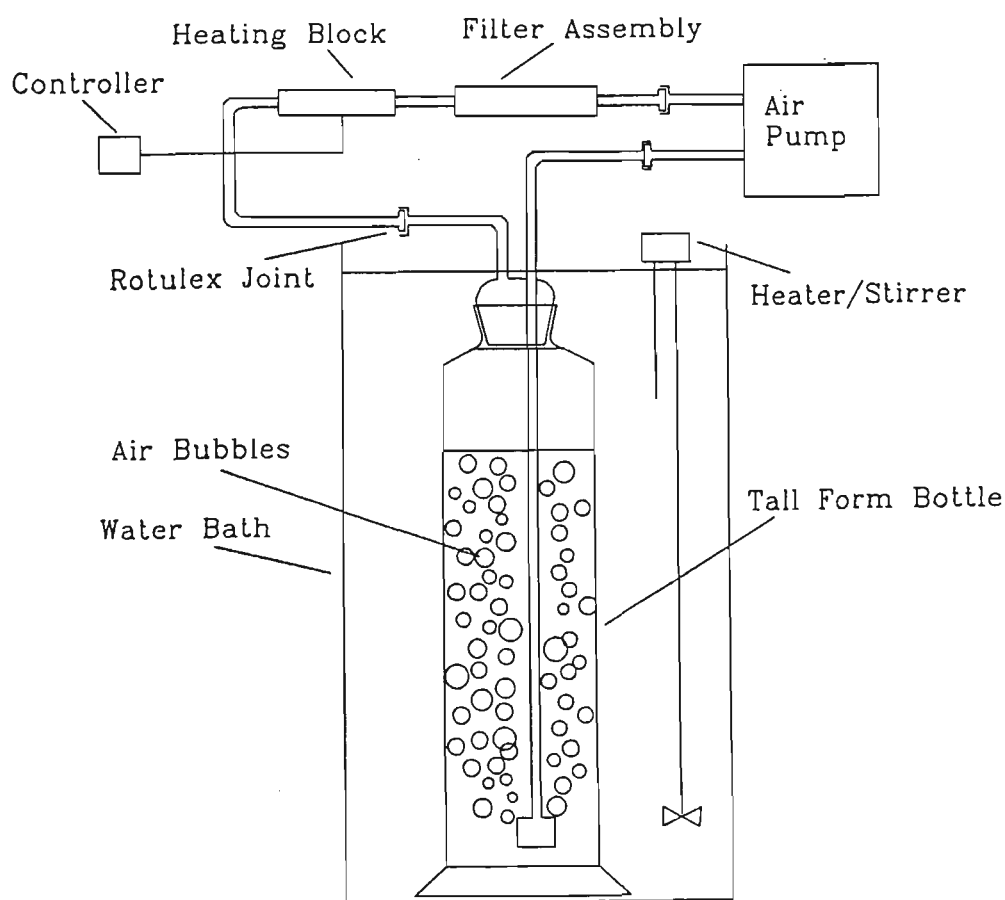


Figure 14 (A) Closed Loop Stripping Apparatus

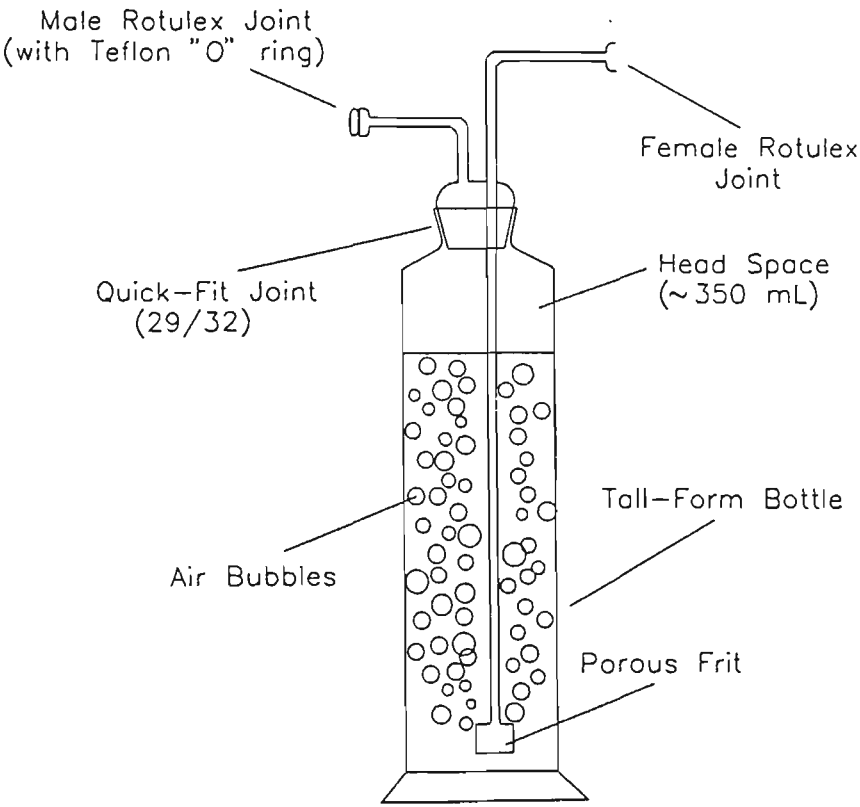


Figure 14 (B) Tall-Form Bottle

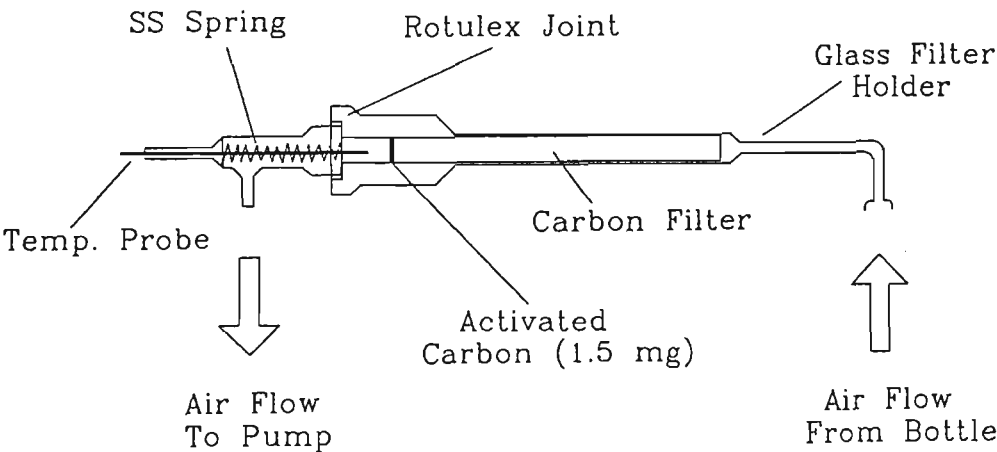


Figure 14 (C) Filter Holder Assembly

### 2.3.2 Stripping Procedure

A water sample (~ 800 mL or made up to ~ 800 mL with milli-Q water) was added to the tall-form bottle and 90 g of solid sodium chloride (previously heated to 700°C for 12 h) was added and dissolved (< 30 s) with magnetic stirring. The stirrer bar was removed and the solution made up to 900 mL with milli-Q water. The solution was held at 25°C and stripped for 1.5 h at an air flow of 2 L/min. Volatile organic compounds were adsorbed by a granular activated carbon (GAC) filter held at 40°C (block temperature 50°C). At the completion of the stripping cycle, the filter was eluted sequentially with 10, 10 and 5  $\mu\text{L}$  of  $\text{CS}_2$ . This was achieved by attaching the filter to a micro vial using a Teflon sleeve (Fig. 15). Organic compounds adsorbed to the surface of the carbon were eluted by moving the solvent from above the carbon bed to below it. The solvent was moved by pulling the micro vial and the filter apart (solvent moved below carbon bed Fig. 15 (i)) and pushing them back together (solvent moved above carbon bed Fig. 15 (ii)). This movement of the solvent was repeated 50 times for each addition of fresh solvent.

The extract was transferred into the micro vial by pushing the micro vial and the filter together to ensure a butt fit. The solvent, now above the carbon bed was moved below it by placing the end of the micro vial into a beaker containing ice and water (the small amount of  $\text{CS}_2$  vapour condenses causing a partial vacuum which was enough to pull the solvent below the carbon bed Fig. 15 (iii)). Once the solvent had moved below the carbon bed it was transferred into the micro vial by holding the micro vial with the fingers so that the filter was in the palm of the hand. A flick of the hand, as one would to reset a clinical thermometer, was sufficient to transfer the extract to the micro vial.

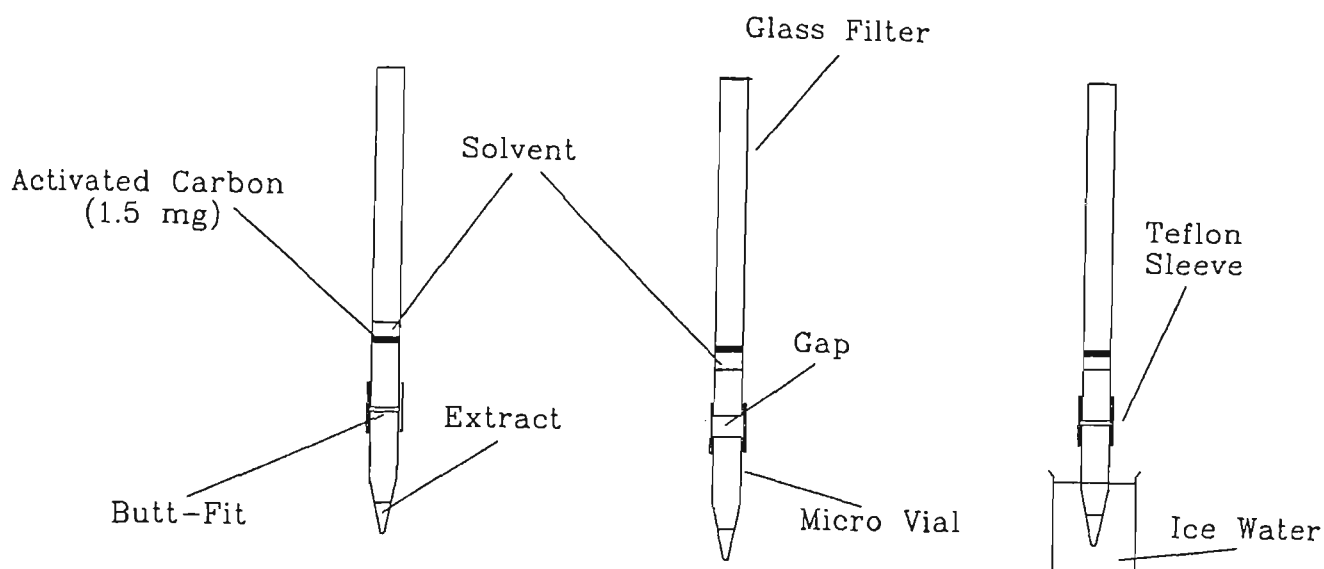


Figure 15 Filter Extraction Assembly

The combined extract was stored in a micro vial (100  $\mu$ L) placed in a 2 mL screw cap vial fitted with an open top cap and a Teflon septum. A stainless steel spring was used to push the micro vial up against the septum (see Fig 16). Filters were washed with 1 mL portions of acetone, water (milli-Q), acetone,  $\text{CS}_2$ , acetone (3x), methylene chloride (3x) and dried under vacuum.

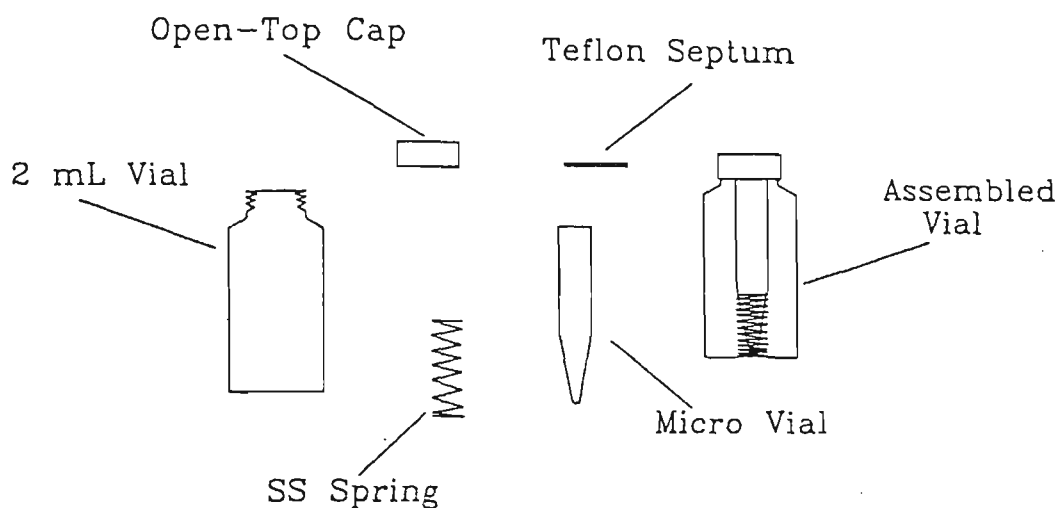


Figure 16 Vial Assembly

## 2.4 Synthesis

### 2.4.1 Geosmin- $d_3$

#### 2.4.1.1 2- $[^2H_3]$ methylcyclohexanol

Copper (I) iodide (1.91 g; 0.01 mol) was added to a 1 L flask fitted with a Friedrich condenser, two pressure equalising funnels and a magnetic stirrer. The flask was purged with dry argon. Dry THF (100 mL) was added and the stirred mixture cooled to  $-30 \pm 5^\circ\text{C}$ , using an ethanol/dry ice bath.  $[^2H_3]$ -Methylmagnesium iodide in ether (0.1 M; 100 mL) was added over 40 min and the mixture maintained at  $-30^\circ\text{C}$  for a further 10 min. Cyclohexene oxide (6.57 g; 0.067 mol) in THF (100 mL) was added over 10 min at  $-30^\circ\text{C}$ . The solution was allowed to warm to between  $-5$  and  $0^\circ\text{C}$  and was maintained in that range for 2 h. The reaction mixture was poured into saturated ammonium chloride (800 mL) and the organic layer separated. The aqueous layer was extracted with ether (3 X 50 mL) and the combined organic extract washed with sodium

thiosulphate (10%; 2 X 50 mL) then brine (3 X 50 mL). The solution was then dried ( $\text{Na}_2\text{SO}_4$ ) and the ether removed on a rotary evaporator. The oil was purified by distillation to give 2- $[\text{}^2\text{H}_3]$ methylcyclohexanol (9.0 g, b.p.  $73^\circ\text{C}/6\text{mm}$ )  $\nu_{\text{max}}$  3340, 2225, 2205, 2127, 2070  $\text{cm}^{-1}$ . Mass spectrum: (M+)  $m/z$  117.124  $\text{C}_7\text{H}_{11}\text{D}_3\text{O}$  requires 117.1232.

#### 2.4.1.2 2- $[\text{}^2\text{H}_3]$ methylcyclohexanone

2- $[\text{}^2\text{H}_3]$ methylcyclohexanol (11.4 g) was dissolved in ether (40 mL) in a 250 mL flask in a water bath at  $15\text{--}2^\circ\text{C}$ . A solution of sodium chromate (10.9 g) and sulphuric acid (7.3 mL) in water (30 mL) was diluted with water to 50 mL and added in small portions over 20-25 min to the stirred solution using a Pasteur pipette. When the addition of oxidant was complete, the mixture was allowed to react for a further 10 min. The phases were separated and the water phase extracted ether (2 X 10 mL). The combined ether extracts were shaken with saturated sodium bisulphite (50 mL) for 30 min, then washed with 10 mL each of water, 10% sodium bicarbonate, water, brine and then dried ( $\text{Na}_2\text{SO}_4$ ). The ether was removed by distillation and the residue distilled through a short Vigreux column at atmospheric pressure to give 2-methylcyclohexanone- $\text{d}_3$  (9.6 g, b.p.  $82\text{--}85^\circ\text{C}/17\text{mm}$ ).  $\nu_{\text{max}}$  (neat) 2220, 2150, 2120 2070 1710  $\text{cm}^{-1}$ . Mass spectrum: (M+)  $m/z$  115.106  $\text{C}_7\text{H}_9\text{D}_3\text{O}$  requires 115.1076.

#### 2.4.1.3 1,10 $\beta$ - $[\text{}^2\text{H}_3]$ dimethyldecal-(9)-en-2-one 4

A mixture of 2-methylcyclohexanone- $\text{d}_3$  (11.2 g, 0.1 mol), 5-chloro-3-pentanone (18.4 g, 0.15 mol), *p*-toluenesulphonic acid monohydrate (600 mg) and benzene was refluxed for 20 h using a Dean Stark apparatus to trap the water produced. The mixture was

cooled and diluted with ether (40 mL). The organic was washed with saturated sodium bicarbonate (20 mL) and water (40 mL) and the layers separated. The water and bicarbonate layers were combined and extracted with ether (2 X 20 mL). The ether extracts were combined with the original organic layer and dried ( $\text{Na}_2\text{SO}_4$ ). The ether and benzene were removed by distillation and the residual oil distilled under vacuum. The first fraction collected boiled at 85-98°C (bath temperature 150-160°C) and the second at 98-115°C (bath temperature 160-170°C). The GC trace showed fraction one contained 92 % of 4 and fraction two contained 87 % of 4. The two fractions were combined to give 11.5 g of 4 (59%).  $\nu$  max (neat liquid) 2220, 2060, 1710, 1665, 1610  $\text{cm}^{-1}$ ;  $^1\text{H}$  NMR ( $\text{CDCl}_3$ )  $\delta$  1.76 (s, 3, C-1  $\text{CH}_3$ );  $^{13}\text{C}$  NMR ( $\text{CDCl}_3$ )  $\delta$  10.55, 21.27 26.63 27.46 33.61 35.75 (C-10), 37.41, 41.80, 120.00 (C-1 or C-9), 128.05 (C-1 or C-9), 162.57 (C-2). Mass spectrum: (M+)  $m/z$  181.156  $\text{C}_{12}\text{H}_{15}\text{D}_3\text{O}$  requires 181.1544.

#### 2.4.1.4 $1\beta,10\beta$ -[ $^2\text{H}_3$ ]dimethyl-1,9-epoxydecal-2-one 5

The enone 4 (5.0 g) was dissolved in dichloromethane (20 mL) and cooled in ice. A suspension of *m*-chloroperbenzoic acid (8.0 g) in dichloromethane (80.0 mL) was added over 10 min and the mixture kept 18 h at 4°C. The mixture was shaken with sufficient 10%  $\text{NaHSO}_3$  to give a negative starch-iodide test, then washed with 5%  $\text{NaHCO}_3$ , 1 M  $\text{NaOH}$ , water and brine. The solution was dried ( $\text{MgSO}_4$ ) and the solvent removed by distillation. The residual oil was purified by distillation under vacuum to give 5 as a colourless oil, b.p. 110-117°C (4.4 g).  $\nu$  max (neat liquid) 220, 2160, 2120, 2065, 1755, 1708, 1665  $\text{cm}^{-1}$ ;  $^1\text{H}$  NMR ( $\text{CDCl}_3$ )  $\delta$  1.38 (s, 3, C-1  $\text{CH}_3$ )  $^{13}\text{C}$  NMR ( $\text{CDCl}_3$ )  $\delta$  11.04, 20.86, 23.90, 26.23, 32.01, 38.03, 65.10 (C-1 or C-9), 71.60 (C-1 or C-9), 207.40 (C-2). Mass spectrum: (M+)  $m/z$  197.149  $\text{C}_{12}\text{H}_{15}\text{D}_3\text{O}_2$  requires 197.1493.

#### 2.4.1.5 $1\alpha,10\beta$ ,-[ $^2\text{H}_3$ ]dimethyl-2,9 $\alpha$ -dihydroxydecalin 6

The epoxide 5 (1.2 g) was dissolved in dry dimethoxyethane (5 mL) and treated with LAH (10 mL; 0.5 M in dimethoxyethane). The mixture was refluxed for 2 h, then excess LAH was destroyed with ethyl acetate and the mixture diluted with ether and washed with 1 M HCl, water and brine. The solution was dried ( $\text{MgSO}_4$ ) and further dried by azeotropic distillation with benzene. The diol 6 was obtained as a colourless oil (1.16 g).  $\nu$  max (Nujol) 3400, 2240, 2220, 2175, 2070  $\text{cm}^{-1}$ ;  $^1\text{H}$  NMR ( $\text{CDCl}_3$ )  $\delta$  0.88 (d, 3,  $J=6.3$  Hz, C-1  $\text{CH}_3$ , 2.3 (OH), 3.29, 3.44 (C-2 H). Mass spectrum: (M+),  $m/z$  201.181  $\text{C}_{12}\text{H}_{19}\text{D}_3\text{O}_2$  requires 201.1806.

#### 2.4.1.6 $1\alpha,10\beta$ ,-[ $^2\text{H}_3$ ]dimethyl-9 $\alpha$ -hydroxy-2-*p*-toluenesulphonate 7

The diol 6 (1.16 g) was dissolved in chloroform (10 mL; freed of ethanol by filtration through alumina) and the solution treated with pyridine (1.0 g) and *p*-toluenesulphonyl chloride (1.44 g). The mixture was kept 14 h at  $4^\circ\text{C}$ , then diluted with ether (50 mL) and extracted with 2M HCl, 5%  $\text{NaHCO}_3$  then brine. The solution was dried ( $\text{MgSO}_4$ ) and the ether removed by distillation. The residual oil crystallised on standing at room temperature and the crude 7 was recrystallised from ether/hexane to give needles (1.3 g, m.p.  $110\text{--}111^\circ\text{C}$ ).  $\nu$  max (Nujol) 3620, 3060, 2240, 2220, 2175, 1597,  $\text{cm}^{-1}$ ;  $^1\text{H}$  NMR ( $\text{CDCl}_3$ )  $\delta$  0.72 (d, 3  $J=6.4$  Hz, C-1  $\text{CH}_3$ ), 2.44 (s, 3, Ar- $\text{CH}_3$ ), 4.60 (m, 1, C-2 H), 7.32 (d, 2,  $J=8.0$  Hz, Ar-H), 7.80 (d, 2,  $J=8.0$  Hz, Ar-H);  $^{13}\text{C}$  NMR ( $\text{CDCl}_3$ )  $\delta$  9.85, 19.30 (septet, C-10  $\text{CD}_3$ ), 19.96, 20.43, 27.90, 30.20, 33.14, 34.62, 36.37, 39.99, 75.82 (C-9), 86.02 (C-2), 127.70, 129.59, 134.55, 144.26. Mass spectrum: (M+)  $m/z$  355.191.  $\text{C}_{19}\text{H}_{25}\text{D}_3\text{O}_4\text{S}$  requires 355.1894.



#### 2.4.1.7 ( $\pm$ )-*trans*-1,10 $\beta$ -[ $^2\text{H}_3$ ]methyl-9 $\alpha$ -decalol (geosmin- $\text{d}_3$ ) 8

The tosylate (0.96 g) was dissolved in sodium dried THF and added to a solution of lithium aluminium hydride in 1,2-dimethoxyethane (0.5 M, 20 mL). The solution was heated at 80°C under reflux for 3h, then cooled and treated dropwise with ethyl acetate to destroy unreacted LAH. Water (5 mL) was added and the organic layer diluted with ether (20 mL). Dilute hydrochloric acid (2 M) was added to dissolve the white precipitate of aluminium hydroxide and the mixture shaken. The ether layer was then washed with sodium hydroxide (2 M), water and brine and the ether layer dried ( $\text{MgSO}_4$ ). The ether was removed by distillation on a rotary evaporator and the crude 8 was purified by chromatography on silica gel (40 g); elution with ether/hexane (2:98; 200 mL) gave geosmin- $\text{d}_3$  8 (0.15g).  $\nu_{\text{max}}$  (neat) 3620, 3510, 2240, 2200, 2170, 2080,  $\text{cm}^{-1}$ ;  $^1\text{H}$  NMR ( $\text{CDCl}_3$ )  $\delta$  0.77 (d, 3,  $J=6$  Hz, C-1  $\text{CH}_3$ );  $^{13}\text{C}$  NMR ( $\text{CDCl}_3$ )  $\delta$  14.79, 20.74, 20.93, 21.32, 29.85, 30.44, 34.24, 34.92, 35.56, 36.97, 74.27. Mass spectrum:  $m/z$  185 (0.4), 167 (0.6), 149 (1.5), 138 (0.8), 129 (8.0), 128 (7.3), 115 (100), 114 (19.7); ( $\text{M}^+$ ) 185.185  $\text{C}_{12}\text{H}_{19}\text{D}_3\text{O}$  requires 185.1857.

The synthesis of geosmin was identical with that described above for geosmin- $\text{d}_3$ , starting from step 2.4.1.3, except that 2-methylcyclohexanone- $\text{d}_3$  was replaced by 2-methylcyclohexanone.

#### 2.4.2 Methyloborneol- $\text{d}_3$ (MIB- $\text{d}_3$ )

##### 2.4.2.1 (-)-2-*exo*-hydroxy-2-[ $^2\text{H}_3$ ]methylbornane

Magnesium turnings (1.7 g) and dry ether (40 mL) were added to a 1 L flask fitted with a Friedrich condenser, two pressure equalising funnels and a magnetic stirrer.  $\text{CD}_3\text{I}$  (10.0

g, (4.6 mL)) was placed into one of the pressure equalising funnels and the reaction vessel was continuously flushed with argon. One crystal of  $I_2$  was added to the Mg turnings in ether prior to addition of  $CD_3I$ .  $CD_3I$  was added dropwise (over 30 min) and at a rate which ensured that the exothermic reaction sustained a gently reflux. When the addition of  $CD_3I$  was completed, (+)-camphor (8.2 g) in dry ether (11 mL) was added to the other pressure equalising funnel. The (+)-camphor in ether was added to the Grignard reagent in the reaction vessel dropwise (over 30 min) while stirring. With the addition of camphor, refluxing commenced and was continued for 2 h after addition of camphor was completed. After refluxing, the solution cooled to room temperature. The reaction mixture was poured onto crushed ice (55 g), allowed to warm to room temperature and the pH was adjusted to  $\sim 6.0$  using glacial acetic acid. The solution was transferred to a separating funnel and extracted with ether (3 X 50 mL). The ether layers were bulked, dried ( $Na_2SO_4$ ), filtered (Whatman 1) and the solvent was removed by rotary evaporation. The residue was dissolved in absolute ethanol (30 mL) and transferred to a three necked 500 mL flask fitted with a Friedrich condenser. To this solution hydroxylamine hydrochloride (5.5 g in 13.8 mL water) and NaOH (8.8 g in 13.8 mL water) was added. The reaction vessel was flushed with argon, heated using a paraffin bath to  $103^\circ C$  and refluxed (8 h). The mixture was cooled and allowed to stand overnight at room temperature then transferred to a 250 mL separating funnel. Milli-Q water (35 mL) was added and the solution shaken ( $\sim 1$  min) to form a cloudy/white emulsion. The emulsion was extracted with hexane (4 X 15 mL) and the hexane layers were combined and transferred back into the 250 mL separating funnel. The hexane was washed with 2N NaOH (70 mL) and left overnight. The aqueous layer was removed and the hexane layer was further washed with 2N NaOH (16 X 70 mL),

water (3 X 50 mL) and dried ( $\text{Na}_2\text{SO}_4$ ). The dry hexane was filtered and the solvent removed by rotary evaporation (water bath  $50^\circ\text{C}$ ). The white crystalline residue (2.6 g; 36 %) of (-)-2-methylisoborneol- $\text{d}_3$  had m.p.  $170^\circ\text{C}$  (sealed tube).  $\nu_{\text{max}}$  (Nujol) 3470, 2220, 2125, 2070  $\text{cm}^{-1}$ ;  $^1\text{H}$  NMR ( $\text{CDCl}_3$ )  $\delta$  0.84 (s, 3), 0.86 (s, 3), 1.10 (s, 3);  $^{13}\text{C}$  NMR  $\delta$  9.86, 21.12, 21.40, 26.82, 31.29, 45.43, 47.23, 48.89, 51.86, 79.5. Mass spectrum:  $m/z$  (M+) 171.170  $\text{C}_{11}\text{H}_{17}\text{D}_3\text{O}$  requires 171.170.

The synthesis of (-)-2-*exo*-hydroxy-2-methylbornane (methylisoborneol or MIB) was identical with that described above for (-)-2-*exo*-Hydroxy-2- $[\text{}^2\text{H}_3]$ methylbornane (methylisoborneol- $\text{d}_3$  or MIB- $\text{d}_3$ ) except that  $\text{CH}_3\text{I}$  was substituted for  $\text{CD}_3\text{I}$  to form the Grignard reagent. Different enantiomers of MIB were obtained by using different enantiomers of camphor as the starting material (i.e. (+)-camphor was used to synthesise (-)-MIB; (-)-camphor was used to synthesise (+)-MIB and ( $\pm$ )-camphor was used to synthesise ( $\pm$ )-MIB). The synthesis is based on the published method of Wood and Snoeyink (1977).

#### 2.4.3 Thioketal of 1,10 $\beta$ -dimethyldecal-1(9)-en-2-one

Dimethyldecalenone (1 g), synthesised in the same way as its labelled counterpart (2.4.1.3, **4**), was dissolved in glacial acetic acid (20 mL) and treated with ethanedithiol (5 mL) and borontrifluoride etherate (1 mL). The solution was kept at  $20^\circ\text{C}$  for 1 h then diluted with brine and extracted into ether. The ether was washed with 4 N NaOH, water and dried ( $\text{Na}_2\text{SO}_4$ ). The ether was removed by distillation and the residual oil crystallised twice from ether/methanol to give the thioketal as white needles, m.p.  $80\text{--}81^\circ\text{C}$ .  $^1\text{H}$  NMR ( $\text{CDCl}_3$ )  $\delta$  1.07 (3H, s, C-10  $\text{CH}_3$ ); 1.86 (3H, s, C-1  $\text{CH}_3$ ); 3.20 (1H, m);

3.35 (3H, m) (CH<sub>2</sub>-s). <sup>13</sup>C NMR (CDCl<sub>3</sub>) δ 16.15, 21.95, 24.15, 26.88, 27.46, 34.92, 39.31, 39.61, 39.85, 40.82, 42.43, 72.66 (C-2), 124.93 (C-9), 141.99 (C-1). Found: C, 65.8; H, 9.0; S, 25.2 %. Calculated for C<sub>14</sub>H<sub>22</sub>S<sub>2</sub>: C, 66.1; H, 8.7; S, 25.2 %.

#### 2.4.3.1 desulphurisation of thioketal

The thioketal was desulphurised with Raney nickel to produce a mixture of three argosmins.

#### 2.4.4 (±)-*trans*-1β-[<sup>2</sup>H],2α-[<sup>2</sup>H],10β-[<sup>2</sup>H<sub>3</sub>]methyl-9α-decalol (Geosmin-d<sub>5</sub>)

Lithium aluminium deuteride (1 g) was added to a solution of crude epoxide (2.4.1.5, **5**) (0.40 g), in sodium dried tetrahydrofuran (20 mL). The mixture was refluxed for 3 h, then worked up and the crude diol (0.19 g) was tosylated, reduced and purified as described above (2.4.1.6 and 2.4.1.7) to give geosmin-d<sub>5</sub> (0.07 g). Mass spectrum: (M<sup>+</sup>) m/z 187 (0.5 %), m/z 115 (100 %).

##### 2.4.4.1 Dehydration of Geosmin-d<sub>5</sub> with Sulphuric Acid

A few milligrams of geosmin-d<sub>5</sub> were treated with 18 M sulphuric acid (1 drop) and ether (0.5 mL) in a reactivial and stored overnight. Water (1 mL) was added and the mixture shaken with ether (1 mL). The ether layer was removed with a Pasteur pipette and dried over Na<sub>2</sub>SO<sub>4</sub> and examined by GC-MSD. The experiment was repeated using authentic geosmin purchased from Wako Chemicals.

## 2.5 Standards

### 2.5.1 Preparation of Stock Standards

Stock standards of each compound (see Table 9) were prepared by weighing the appropriate compound into a volumetric flask (10 mL) and making it up to volume with AR acetone. Each solution was analysed (GC-FID) to ascertain and correct for its GC purity. The concentrations of labelled MIB and labelled geosmin were further cross calibrated (GC-MSD) against the authentic compounds (Wako Chemicals) by analysing equimolar mixtures of the labelled and unlabelled solutions.

**Table 9** Concentration of stock standards. (\* = adjusted concentration after cross calibration against authentic compounds).

Compound		amount (mg) to 10 mL	GC-FID Purity (%)	Conc. (ng/ $\mu$ L)
MIB		20.0	100	2000
MIB-d <sub>3</sub>		19.8	96	1860*
geosmin		20.0	98	1960
geosmin-d <sub>3</sub>		21.7	94	2040*
chloroalkane mixed standard	1-Cl-C <sub>8</sub>	27.5	99	2694
	1-Cl-C <sub>10</sub>	19.5	95	1853
	1-Cl-C <sub>12</sub>	26.9	95	2555

Stock standards of tridecane and pentadecane were prepared by weighing an amount of each compound into separate amber screw cap vials and adding a known weight of AR acetone. The volume of acetone added was calculated from its specific gravity (at 20°C) and the concentration of each compound was determined (see Table 10).

**Table 10** Stock alkane standards.

Compound	amount (mg)	Acetone (mg)	Acetone (mL)	Conc. (ng/ $\mu$ L)
tridecane	9.60	3.15788	3.95	2430
pentadecane	12.86	2.85854	3.62	3550

Stock standards of the carbonyl compounds  $\beta$ -ionone,  $\beta$ -cyclocitral, 6-methylhept-5-en-2-one and geranylacetone were prepared by weighing an amount of each compound into separate amber screw cap vials (4 mL capacity) and adding a known weight of carbon disulphide. The volume of carbon disulphide added was calculated from its specific gravity (at 20<sup>0</sup>C) and the concentration of each compound was determined (see Table 11).

**Table 11** Concentration of Carbonyl Stock Standards

Compound	Amount (mg)	Weight CS <sub>2</sub> (mg)	Volume CS <sub>2</sub> (mL)	Conc. (ng/ $\mu$ L)	GC Purity (%)	Conc. (ng/ $\mu$ L)
$\beta$ -ionone	11.1	1.0563	0.8362	13270	98.4	13058
$\beta$ -cyclocitral	2.4	0.7045	0.5577	4303	66.3	2853
6-methylhept-5-en-2-one	4.0	0.6313	0.500	8000	92.6	7408
geranylacetone	4.5	0.5650	0.5650	7965	93.2	7423

## 2.5.2 Preparation of Working Standards

Working standards of geosmin-d<sub>3</sub> (geos\*WS), MIB-d<sub>3</sub> (MIB\*WS), geosmin (geosWS), MIB (MIBWS) and chloroalkane mix (ClmixWS) were prepared by transferring (Gilson 0-100 µL Varipette) the appropriate stock standard (100 µL) to a volumetric flask (10 mL) and making it up to volume with AR acetone. An alkane mix working standard (AlkmixWS) was prepared by transferring (Gilson 0-100 µL Varipette) stock solutions (100 µL) of tridecane and pentadecane to a volumetric flask (10 mL) and making it up to volume with AR acetone. A mixed carbonyl working standard (TuebmixWS) of β-ionone, β-cyclocitral, 6-methylhept-5-en-2-one and geranylacetone was prepared by transferring (Gilson 0-100 µL Varipette) the appropriate stock solutions (50 µL) to a volumetric flask (10 mL) and making it up to volume with AR acetone (see Table 12).

**Table 12** Concentration of working standards.

Compound		Stock Standard (ng/µL)	Working Standard (ng/µL)
	MIB	2000	20.00
	MIB-d <sub>3</sub>	1860	18.60
	geosmin	1960	19.60
	geosmin-d <sub>3</sub>	2040	20.40
chloroalkane mixed standard	1-Cl-C <sub>8</sub>	2694	26.94
	1-Cl-C <sub>10</sub>	1853	18.53
	1-Cl-C <sub>12</sub>	2555	25.55
alkane mixed standard	C <sub>13</sub>	2430	24.3
	C <sub>15</sub>	3550	35.5
Carbonyl mixed standard	β-ionone	13058	18.3
	β-cyclocitral	2926	10.9
	6-methylhept-5-en-2-one	7408	16.3
	geranylacetone	7423	16.7

## 2.5.3 Preparation of Calibration Mixes

### 2.5.3.1 deuterated calibration mix

A concentrated calibration mix (Hcal\*) of the deuterated standards was prepared by transferring (Gilson 0-100  $\mu$ L Varipette) stock standards of MIB, MIB-d<sub>3</sub>, geosmin and geosmin-d<sub>3</sub> (100  $\mu$ L of each) to an amber screw cap vial (4 mL capacity) and adding AR acetone (1.6 mL). The high calibration standard (Hcal\*) was serially diluted (10 fold) with acetone to give medium (Mcal\*), low (Lcal\*) and trace (Tcal\*) deuterated calibration mixes (see Table 13).

**Table 13** Concentration of standards used for calibration, precision and accuracy trials employing labelled compounds as internal standards.

Compound	Concentration (ng/ $\mu$ L)				
	Stock Standard	Hcal*	Mcal*	Lcal*	Tcal*
MIB	2000	100	10	1.0	0.20
MIB-d <sub>3</sub>	1860	93	9.3	0.93	0.186
geosmin	1960	98	9.8	0.98	0.196
geosmin-d <sub>3</sub>	2040	102	10.2	1.02	0.204

### 2.5.3.2 unlabelled calibration mix

A high calibration mix containing chloroalkanes (HcalCl) was prepared by transferring (Gilson 0-100  $\mu$ L Varipette) stock standards of MIB, geosmin and chloroalkane mixed standard (100  $\mu$ L of each) to an amber screw cap vial (4 mL capacity) and adding AR acetone (1.7 mL). The high calibration standard (HcalCl) was serially diluted (10 fold) with acetone to give medium (McalCl), low (LcalCl) and trace (TcalCl) chloroalkane calibration standards (see Table 14).



**Table 14**

Concentration of calibration standards used for calibration, precision and accuracy trials employing chloroalkanes as internal standards.

Compound		Concentration (ng/ $\mu$ L)				
		Stock	HcalCl	McalCl	LcalCl	TcalCl
MIB		2000	100	10	1.00	0.10
geosmin		1960	98	9.8	0.98	0.098
chloroalkane mixed standard	1-Cl-C <sub>8</sub>	2694	134.7	13.5	1.35	0.135
	1-Cl-C <sub>10</sub>	1853	92.7	9.3	0.93	0.093
	1-Cl-C <sub>12</sub>	2555	127.8	12.8	1.28	0.128

### 2.5.3.3 solvent stability calibration mix

Stock solutions (~100  $\mu$ L) of MIB, geosmin, tridecane, pentadecane and chloroalkane mix were transferred (Gilson 0-100  $\mu$ L Varipette) to an amber screw cap vial (4 mL capacity). The solutions were mixed thoroughly (shaking by hand ~ 1 min) and concentrations calculated from the volume added per component relative to the total volume (see Table 15, column 4). A portion of this mix (50  $\mu$ L) was transferred (Gilson 0-100  $\mu$ L Varipette) to six amber screw cap vials (4 mL capacity) each containing (1950  $\mu$ L) of one of the following solvents; acetone, methanol, ethanol, hexane, carbon disulphide or dichloromethane. Final concentrations for each component in each solvent are also shown in Table 15, column 5.

**Table 15**                      Concentration of compounds in each solvents used in solvent stability trial.

Compound		Stock (ng/μL)	Volume Taken (μL)	Conc. in Mix (ng/μL)	Conc. in each Solvent (ng/μL)
MIB		2000	100	459.8	11.5
geosmin		1960	100	450.6	11.3
tridecane		2430	80	446.9	11.2
pentadecane		3550	55	448.9	11.2
chloroalkane mix	1-Cl-C <sub>8</sub>	2694	100	619.3	15.5
	1-Cl-C <sub>10</sub>	1853		426.0	10.7
	1-Cl-C <sub>12</sub>	2555		587.3	14.7
Total Volume			435		

**2.5.3.4                      salting out calibration mix**

A salting out calibration mix (SOM) was prepared by transferring (Gilson 0-100 μL Varipette) stock solutions (100 μL) of MIB, MIB-d<sub>3</sub>, geosmin, geosmin-d<sub>3</sub> and chloroalkane mix to a volumetric flask (10 mL) and making it up to volume with AR acetone. A spiking salting out mix (SSOM) was prepared by transferring (Gilson 0-100 μL Varipette) salting out mix (SOM, 100 μL) to an amber screw cap vial and adding AR acetone (400 μL) (see Table 16).

**Table 16** Concentration of each component of the salting out mix (SOM) and the spiking salting out mix (SSOM).

Compound		Stock Standard (ng/ $\mu$ L)	Working Salting Out Mix (ng/ $\mu$ L)	Spiking Salting Out Mix (ng/ $\mu$ L)
MIB		2000	20.0	4.00
MIB-d <sub>3</sub>		1860	18.6	3.72
geosmin		1960	19.6	3.92
geosmin-d <sub>3</sub>		2040	20.4	4.08
chloroalkane mixed standard	1-ClC <sub>8</sub>	2694	26.9	5.39
	1-Cl-C <sub>10</sub>	1853	18.5	3.70
	1-Cl-C <sub>12</sub>	2555	25.6	5.12

A salting out mix alkane internal standard (SOM AIS) was prepared by transferring (Gilson 0-100  $\mu$ L Varipette) a portion of the n-alkane mixed working standard (AlkmixWS, 100  $\mu$ L) to a screw cap amber vial (4 mL capacity) and adding AR acetone (400  $\mu$ L) see Table 17.

**Table 17** Concentration of salting out mix alkane standards.

Compound	Concentration (ng/ $\mu$ L)	
	(AlkmixWS)	(SOM AIS)
tridecane	24.3	4.86
pentadecane	35.5	7.10

## 2.6 Quantification

Quantification was carried out by stripping the sample (CLSA) [2.3] followed either by gas chromatography with flame ionisation detection (GC-FID) [2.2.4] or gas

chromatography with mass spectrometric detection in multiple ion detection mode (GC-MS-MID) [2.2.2].

### 2.6.1 Using Unlabelled Internal Standards

A system (CLSA-GC-MSD or CLSA-GC-FID) response factor ( $RF_{sys}$ ) was calculated by spiking Milli-Q water (900 mL) with known amounts of an internal standard (1-chlorodecane) and the compounds of interest. The solution was stripped, extracted and analysed as described earlier using the standard CLSA procedure. Peak areas were determined for each compound by integration of either the total FID signal or, when mass spectrometry was used, the signal of the quantifying ion.  $RF_{sys}$  were calculated for each compound as follows:

$$RF_{sys} \text{ Compound} = \frac{\text{area compound} \times \text{amount 1-Cl-C}_{10}}{\text{area 1-Cl-C}_{10} \times \text{amount compound}}$$

1-Cl-C<sub>10</sub> = internal standard 1-chlorodecane  
amount = amount (ng) of compound added

Quantification of compounds with established  $RF_{sys}$  was achieved by stripping and analysing the sample (CLSA-GC-MSD or CLSA-GC-FID), determining peak areas of unknown analyte and internal standard and calculating the unknown concentration as follows:

$$[\text{compound}] \text{ (ng/L)} = \frac{\text{area compound} \times \text{amount 1-Cl-C}_{10} \times 1000}{\text{area 1-Cl-C}_{10} \times RF_{sys} \text{ compound} \times Vol_{sple}}$$

$Vol_{sple}$  = volume of sample added (mL)

### 2.6.2 Using Labelled Internal Standards

When using a labelled internal standard to quantify its unlabelled analogue it was not necessary to calculate a system response factor because the two compounds have identical physical and chemical characteristics. Concentrations of unlabelled compound could be determined readily by a direct comparison of their respective molecular ions ( $m/z$  168/171 for MIB/MIB- $d_3$  and  $m/z$  182/185 for geosmin/geosmin- $d_3$ ). In the case of geosmin- $d_3$ , because the base peak was also labelled, greater sensitivity could be achieved by using the base peaks for quantification ( $m/z$  112/115). However, it was necessary to correct for contributions of isotope satellites in the unlabelled compound to the labelled peak being monitored, and vice versa (see 2.2.2).

### 2.6.3 ( $\pm$ )-Geosmin- $d_3$ and Enantioselective GC-MSD

Quantification using a chiral column and mass spectrometric detection, in multiple ion detection mode, was identical with that described above (2.6.2) except that a mathematical correction for the effect of satellite peaks on the  $m/z$  112/115 ratio was not necessary as (-)-geosmin and (-)-geosmin- $d_3$  are resolved.

### 2.6.4 ( $\pm$ )-Geosmin and Enantioselective GC-FID or GC-MSD

When using ( $\pm$ )-geosmin to quantify natural (-)-geosmin, (+)-geosmin was used as an internal standard. Total (-)-geosmin concentrations were determined and the contribution of (-)-geosmin, from ( $\pm$ )-geosmin, was subtracted from the total to give the concentration of natural (-)-geosmin.

### 2.6.5 ( $\pm$ )-Geosmin- $d_3$ and Enantioselective GC-FID

Quantification of natural (-)-geosmin using chiral column GC-FID and labelled racemic geosmin utilised (+)-geosmin- $d_3$  as the internal standard. Concentrations of natural (-)-geosmin were determined in an identical way to those described above when using (+)-geosmin as an internal standard.

## 2.7 Storage of MIB and Geosmin in Organic Solvents

A stock solution of MIB (460 ng/ $\mu$ L), geosmin (450.6 ng/ $\mu$ L), 1-chlorooctane (619.3 ng/ $\mu$ L), 1-chlorodecane (426 ng/ $\mu$ L), 1-chlorododecane (587.3 ng/ $\mu$ L), tridecane (446.9 ng/ $\mu$ L) and n-pentadecane (448.9 ng/ $\mu$ L) was prepared in acetone. An aliquot (50  $\mu$ L) of this solution was added to each of six 4 mL amber screw cap vials containing 2 mL of acetone, dichloromethane, ethanol, methanol, hexane and carbon disulphide respectively. Each solution was mixed thoroughly and analysed immediately. The solutions were split into 2 sets of duplicate aliquots (1 mL) and stored. The 1st set was stored at  $-15^{\circ}$  C in the dark and the 2nd set at room temperature ( $22^{\circ}$ C) under normal light conditions. All solutions were analysed at various time intervals (GC-FID) and the area of each compound was normalised relative to the area of tridecane.

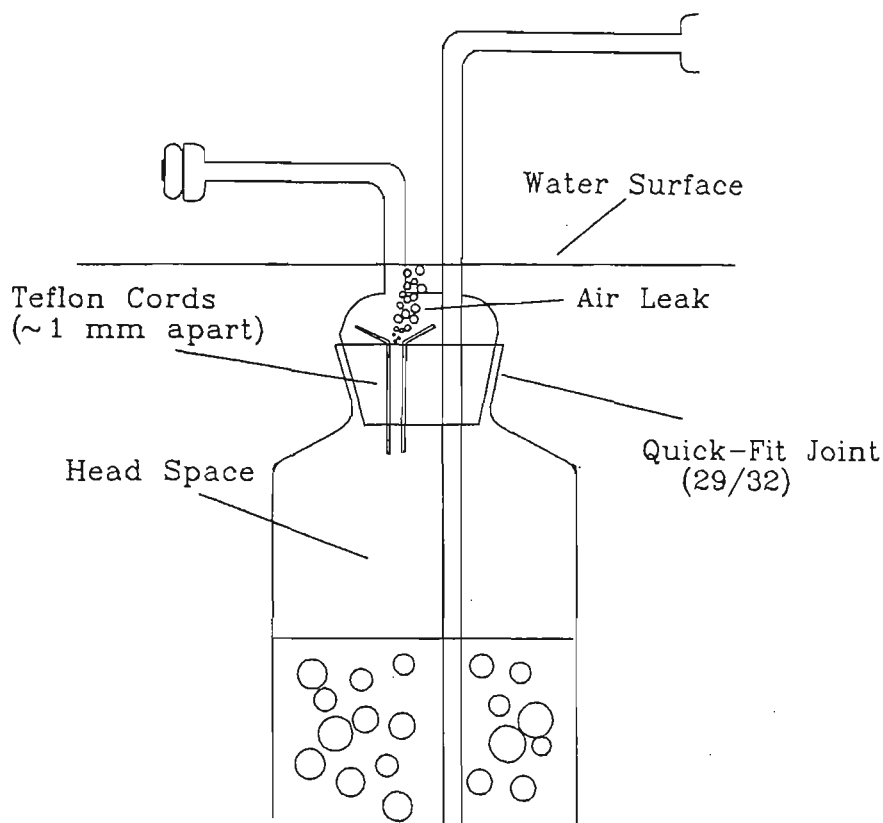
## 2.8 Chemical Degradation of MIB

MIB (180 mg) was added to  $\text{CH}_2\text{Cl}_2$  (1 mL) in a 4 mL amber screw cap vial. An aliquot (1 drop) of this solution was transferred to another vial containing  $\text{CH}_2\text{Cl}_2$  (2 mL) and analysed by GC-FID. To the initial solution 1N HCl(aq) (1 mL) was added and the vial placed in a wrist action shaker (48 h). After 27 h a further 0.9 mL of 10 M HCl was added to increase the acid concentration to 5 M. The degradation was

monitored as follows: at various time intervals aliquots (1 drop) were added to  $\text{CH}_2\text{Cl}_2$  (2 mL) and 5%  $\text{NaHCO}_3(\text{aq})$  (2 mL) in a 4 mL amber screw cap vial. The mixture was vortexed (1 min) and allowed to settle until the phases separated. The aqueous phase was removed and  $\text{Na}_2\text{SO}_4$  was added to dry the organic layer. This layer was analysed using GC-FID.

## 2.9 Air Leak Trial

To a tall-form stripping bottle containing Milli-Q water (900 mL) and NaCl (90 g) was added MIB (100 ng), MIB- $\text{d}_3$  (93 ng), geosmin (102 ng) and geosmin- $\text{d}_3$  (98 ng). Prior to stripping this solution an air leak was deliberately introduced into the system. This was achieved by placing two pieces of Teflon tape (previously twisted into two cords each 5 cm long) approximately 1 mm apart and between the neck of the stripping bottle and the flange of the sparger (see Fig. 16). To ensure that an air leak could be observed the stripping bottle was placed into the water bath so that the neck of the bottle was under the water surface. A standard stripping cycle (90 min.) was commenced and allowed to continue (i.e. judged to be successful, for this trial) when bubbles were seen emanating from the neck of the bottle within the first 30 min. This procedure was repeated until 5 "successful" air leak strips were completed. Although the flow of the air leak could not be measured its severity was changed by varying the separation and thickness of the two Teflon cords for every strip.



**Figure 16** Neck of stripping bottle showing the position of the two Teflon cords used to create an air leak.

## 2.10 Precision and Accuracy

### 2.10.1 Multiple Injections

To a sample of Milli-Q water (900 mL) containing NaCl (90 g) was added MIB (100 ng), geosmin (98 ng) and 1-chlorodecane (83.4 ng). The solution was stripped using CLSA and the extract was analysed ten times (GC-MS in multiple ion detection mode). Response factors for 1-chlorodecane versus MIB and geosmin were calculated from the first two injections and these were used to determine the concentrations of MIB and geosmin for the rest of the injections. To a second sample of Milli-Q water (900 mL) containing NaCl (90 g) geosmin (98 ng), geosmin- $d_3$  (84.6 ng), MIB (100 ng) and



MIB-d<sub>3</sub> (83.4 ng) was added. The solution was stripped using CLSA and the extract was analysed eight times (GC-MS in multiple ion detection mode). Concentrations of MIB and geosmin were calculated from a direct comparison between the labelled and unlabelled analogues.

### **2.10.2 Calibration Curve and Detection Limit**

To a sample of Milli-Q water (900 mL) containing NaCl (90 g) was added 10 µL of the trace deuterated calibration mix (Tcal\*). The solution was stripped using CLSA and the extract was analysed in duplicate (GC-MS in multiple ion detection mode). This procedure was repeated until five strips of Tcal\* were completed. A similar five strips were also performed on Milli-Q water (900 mL, NaCl 90 g) spiked with 10 µL of Lcal\*, Mcal\*, Hcal\*, TcalCl, LcalCl, McalCl and HcalCl.

### **2.10.3 Enantioselective-GC**

To Murrumbidgee River water (1 L) was added (-)-geosmin (1960 ng) and HgCl<sub>2</sub> (40 mg). Portions of this solution were stripped in lots of five with addition of either (±)-geosmin or (±)-geosmin-d<sub>3</sub> as internal standards added at the time of stripping. Each strip was analysed in duplicate using both enantioselective GC/FID and enantioselective GC/SIM.

## **2.11 Biodegradation Trials**

### **2.11.1 Additional Odour Compounds vs Geosmin-d<sub>3</sub> and 1-Chlorodecane**

Murrumbidgee River water (5 L) was collected and chloroalkanes and labelled geosmin were added to an aliquot (900 mL) as internal standards. This was then stripped and

analysed as a blank. The remaining river water (4 L) was spiked with the following: 6-methylhept-5-en-2-one (3560 ng/L); geranylacetone (3570 ng/L);  $\beta$ -ionone (6280 ng/L) and  $\beta$ -cyclocitral (315 ng/L). The spiked water was then split into two equal portions A and B (2x2 L Schott bottles). To portion B geosmin- $d_3$  was added (1000 ng/L) and both bottles were stored at room temperature (22°C) and under normal light conditions. Sub-samples were taken at ~12 h time intervals over the next 130 h and stripped in duplicate.

Prior to stripping sub-samples from bottle A, chloroalkanes (90 ng/L, 1-ClC<sub>10</sub>) and geosmin- $d_3$  (100 ng/L) were added whereas prior to stripping sub-samples from bottle B only chloroalkanes (90 ng/L, 1-ClC<sub>10</sub>) were added. Response factors for each of the four odour compounds and geosmin- $d_3$  relative to 1-ClC<sub>10</sub> as well as each of the four odour compounds and 1-ClC<sub>10</sub> relative to geosmin- $d_3$  were determined from the duplicate strips of sub-samples from each bottle at time = 0. These response factors were used to follow the biodegradation of the four odour compounds added using either 1-ClC<sub>10</sub> or geosmin- $d_3$  as internal standards.

### 2.11.2 Natural (-)-Geosmin vs Synthetic ( $\pm$ )-Geosmin- $d_3$

Natural (-)-geosmin produced in culture by *Anabaena circinalis*; isolated in 1985 by Dr. R.L. Oliver from the Hay Weir pool on the Murrumbidgee River, New South Wales, (strain designation 852E) was used to spike Murrumbidgee River water. An aliquot of the *A. circinalis* culture (200 mL) was probe sonicated (6x30 sec.) and centrifuged (6000 rpm) for 15 min to clarify. The clarified supernatant was then used to spike raw water.

River water (5 L) was collected and chloroalkanes and labelled geosmin were added to an aliquot (900 mL) as internal standards. This was then stripped and analysed as a blank. To a portion of the remaining river water (2 L) all of the clarified supernatant of *A. circinalis* was added. This solution was mixed and made up to volume (4 L) with raw water to give a final concentration for (-)-geosmin of 1290 ng/L.

The spiked raw water (4 L) was split into two portions A and B (2x2 L Schott bottles) and portion B was spiked with geosmin-d<sub>3</sub> to give a final concentration of 1000 ng/L. Both 2 L portions were stored at room temperature (22°C) and under normal light conditions. A sub-sample (50 ml) was taken from each bottle, preserved with HgCl<sub>2</sub> (40 mg/L) and stored under the same conditions next to the 2x2 L portions. Further sub-samples were taken at ~ 12 h time intervals over the next 90 h preserved with HgCl<sub>2</sub> and stored in a cold room (4°C) until ready for stripping. Each sub-sample was stripped in duplicate and each strip analysed in duplicate by GC-MSD.

Prior to stripping sub-samples from bottle A, chloroalkanes (90 ng/L, 1-ClC<sub>10</sub>) and geosmin-d<sub>3</sub> (100 ng/L) were added as internal standards whereas only chloroalkanes (90 ng/L, 1-ClC<sub>10</sub>) were added prior to stripping sub-samples from bottle B. Response factors for 1-ClC<sub>10</sub> relative to geosmin-d<sub>3</sub> were calculated at each time interval from the duplicate strips of sub-samples of bottle A. These response factors were used to determine the concentration of natural (-)-geosmin and synthetic (±)-geosmin-d<sub>3</sub> of the sub-samples from bottle B.

### 2.11.3 Using Enantioselective GC

To Murrumbidgee R. water (2 L) was added (-)-geosmin (829 ng/L) and either (±)-geosmin (980 ng/L) or (±)-geosmin-d<sub>3</sub> (1000 ng/L) at the time of collection. The solution was stored for up to 192 h at room temperature (22°C) and under diffuse daylight. For each storage time interval, two subsamples were taken. Each subsample was stripped in duplicate and each extract was analysed twice by enantioselective GC/SIM and a mean concentration calculated.

## 3 RESULTS AND DISCUSSION

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### 3.1 General Strategy For Synthesis Of Labelled Compounds

Several options for the incorporation of an isotopic label were available albeit restricted by the types of atoms present in the target molecule. In the cases of MIB and geosmin, because oxygen, carbon and hydrogen were the only atoms present, the most obvious candidates for labelling were  $^{18}\text{O}$ ,  $^{13}\text{C}$ ,  $^{14}\text{C}$ ,  $^2\text{H}$  or  $^3\text{H}$ .  $^{18}\text{O}$ ,  $^{13}\text{C}$ , were ruled out for cost reasons while  $^{14}\text{C}$ ,  $^3\text{H}$  were rejected for a combination of cost and safety reasons (both being radioactive). Deuterium remained as the most obvious choice and was used in all subsequent work.

Because mass spectrometry was to be used for detection incorporation of at least three deuterium atoms was desirable to ensure the mass of the resultant molecular ion was three daltons (or more) higher than the parent compound. In this way the mass of labelled compound would be high enough to be away from the natural  $^{13}\text{C}$  and  $^{14}\text{C}$  isotope region of the unlabelled compound. Therefore, if molecular ions were used for quantification, no mathematical correction would be required for isotope contribution from the unlabelled compound to the labelled compound and vice versa.

The chosen mechanism of label incorporation must be such that deuterium is not labile in the intermediates to the synthesis of the final compound (i.e. must not be exchangeable by keto-enol tautomerism or solvent-hydrogen solute-deuterium exchange during synthesis or storage). Ideally the label should reside in the part of the molecule

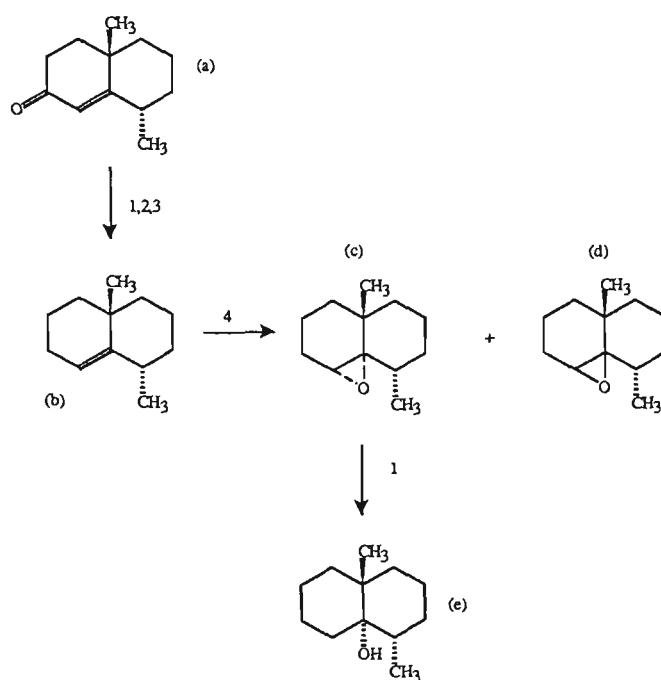
that produces the base peak. In this way maximum analytical sensitivity would be achieved when using the labelled compound as an internal standard followed by gas chromatography and mass spectrometry (GC-MS).

For cost and efficiency reasons the label should be introduced as late as possible in the synthetic sequence because it is inevitable that portions of the labelled precursor is lost during successive steps of the synthesis. However, the synthesis should be as flexible as possible to allow for modification should incorporation of the label be required in a different part of the molecule.

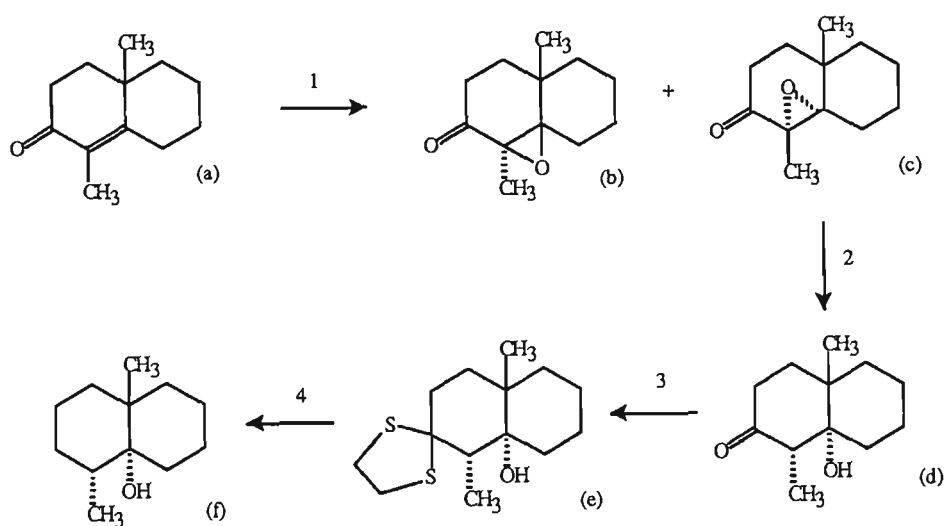
As can be seen below some of these objectives proved to be mutually exclusive.

### 3.2 Synthesis of ( $\pm$ )-Geosmin- $d_3$ and ( $\pm$ )-Geosmin- $d_5$

A literature search showed that two syntheses for ( $\pm$ )-geosmin were available. The first was described in 1968 by Marshall and Hochstetler (Fig. 17) and the second in 1976 by Ayer *et al.*, (Fig. 18). Because the Ayer synthesis appeared to be more direct, less complicated and offered greater flexibility for the introduction of a deuterium label it was the more desirable of the two. Although the introduction of less than three deuterium atoms was not ideal, for the reasons given above (section 4.1), it was an attractive option because this could be achieved in the final step of the synthesis. The thioketal (Fig. 18, e) was desulphurised with Raney nickel prepared by dissolving Raney nickel Al-Ni alloy in a solution of NaOD/D<sub>2</sub>O. However, analysis (GC-MS) of the crude product showed the presence of geosmin but not the expected geosmin- $d_2$ . Evidently exchange of H for D had occurred during storage of the catalyst under ethanol.



**Figure 17** Synthesis of (±)-geosmin (e) (Marshall and Hochstetler, 1968). 1.  $\text{LiAlH}_4$ ; 2.  $\text{Ac}_2\text{O}$ ; 3.  $\text{Li}$ ,  $\text{EtNH}_2$ ; 4.  $m\text{-ClC}_6\text{H}_4\text{CO}_3\text{H}$



**Figure 18** Synthesis of (±)-geosmin (f) (Ayer et al., 1976). 1.  $\text{H}_2\text{O}_2/\text{NaOH}$ ; 2.  $\text{Li}/\text{NH}_3$ ; 3.  $\text{HSCH}_2\text{CH}_2\text{SH}/\text{BF}_3$ ; 4. Raney nickel

To avoid loss of deuterium through exchange mechanisms during the synthesis it was necessary to introduce the label such that it would remain inert, once introduced and during subsequent reactions. One way this could be achieved was through the introduction of a  $\text{CD}_3$  group and attaching it to  $4^\circ$  carbon e.g. labelling a precursor to 1,10-dimethyldecal-(9)-en-2-one (Fig. 18, a) very early in the synthesis. The possibility of obtaining 2-methylcyclohexanone- $\text{d}_3$  in one step from commercially available materials via the enamine synthesis (Stork *et al.*, 1963) or in two steps by reaction of cyclohexene oxide with  $\text{CD}_3\text{MgI}$  followed by oxidation to the ketone (Fig. 19) was evaluated. Because a literature search showed that the Stork enamine synthesis gave poor yields (44 %) and that the product was contaminated with 5-10 % of 2,6-dimethylcyclohexanone, which would be very difficult to remove, the cyclohexene oxide synthesis was chosen. To conserve labelled Grignard reagent the success of the procedure was ascertained using unlabelled Grignard reagent first.

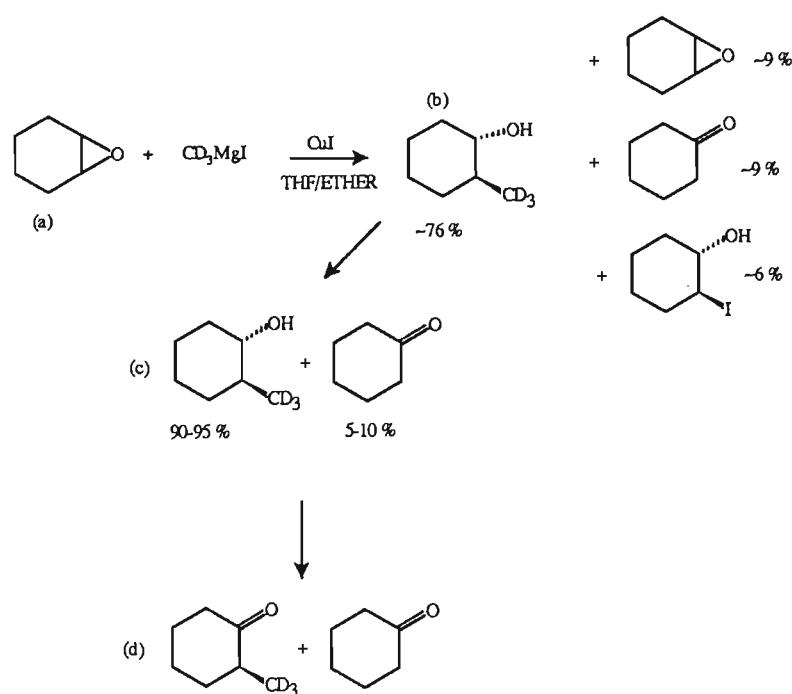


Figure 19

Synthesis of deuterated methylcyclohexanone (d)

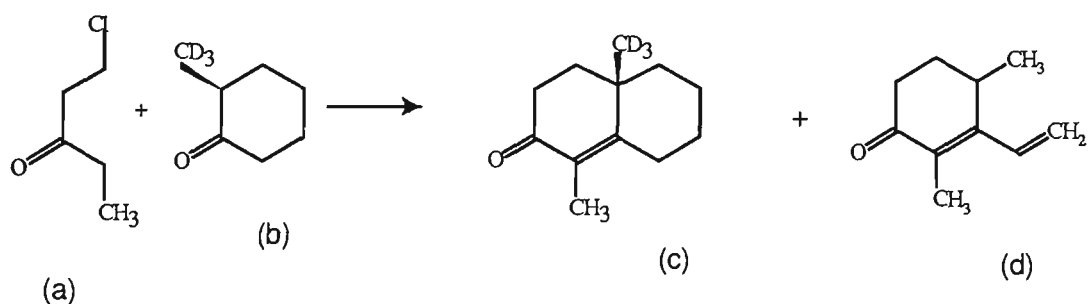


However, the alkylation of cyclohexene oxide was not successful giving poor yields (< 5 %) which were very much lower than would be expected for the alkylation of a ketone. The yield was improved dramatically (> 75 %) by using copper (I) iodide as a catalyst (Huynh *et al.*, 1979) with the main by-products being cyclohexanone and 2-iodocyclohexanol. The latter was easily removed by distillation but cyclohexanone (which had almost the same boiling point as 2-methylcyclohexanol) was not eliminated. Rather than finding alternate purification procedures it was decided to oxidise the mixture with chromic acid and to purify the resultant 2-methylcyclohexanone instead. A two phase oxidation procedure was selected (Brown *et al.*, 1971) which extracted the ketone into ether as fast as it was being formed from the alcohol thus reducing the possibility of further oxidation. The optimum stoichiometry for chromic acid oxidation of an alcohol has been shown to be 1.0 mole alcohol : 0.4 mole dichromate : 1.33 mol sulphuric acid (Hussey and Baker, 1960) with the reaction temperature held below 30°C.

Once the mixture was oxidised, two techniques for the separation of 2-methylcyclohexanone from cyclohexanone were tried. The first utilised column chromatography on alumina but recoveries were low (60 %). Although mass balance was restored by washing the column with methanol the recovered material showed mass spectral fragmentation patterns which suggested that an aldol reaction had been catalysed by the alumina. The second technique involved shaking the ketones with saturated  $\text{NaHSO}_3(\text{aq})$  for 30 min and removing cyclohexanone as the crystalline bisulphite addition compound. Small scale experiments, using decane as an internal standard, showed that the more sterically hindered 2-methylcyclohexanone did not react with

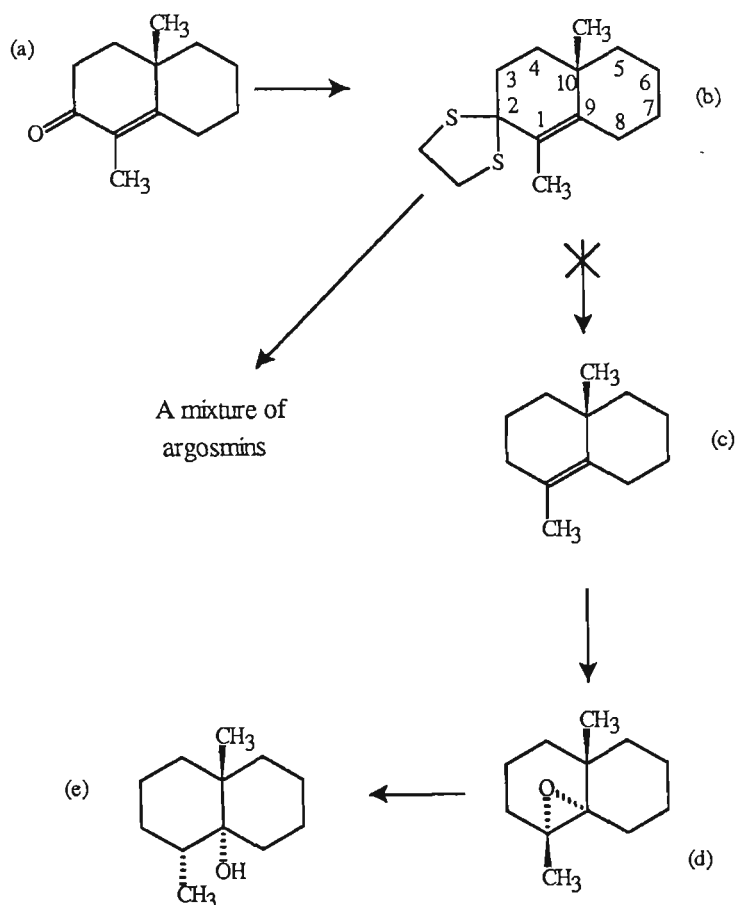
$\text{NaHSO}_3(\text{aq})$  and was recovered quantitatively. Once the method was optimised 2-methylcyclohexanone- $\text{d}_3$  was synthesised in an identical manner using deuterium labelled Grignard reagent.

2-Methylcyclohexanone- $\text{d}_3$  and 1-chloropentan-3-one were condensed, to form the conjugated ketone (Fig. 20, c) in ~60 % yield, by an *in situ* acid-catalysed Michael addition followed by Robinson annelation (Zoretic *et al.*, 1975). Water was removed with a Dean-Stark apparatus (Flaugh *et al.*, 1980) and the crude product was purified by short path length vacuum distillation. To avoid loss of product, due to formation of non-volatile gum in the still pot, it was necessary to vacuum distil the crude mixture as quickly as possible. This was achieved by pre-heating the oil bath to  $130^\circ\text{C}$ , then lifting it up to the distillation flask (which was under vacuum) using a lab jack. The oil bath was then rapidly heated to  $\sim 160^\circ\text{C}$  (hot plate) and the distillate was collected in the range  $98\text{--}115^\circ\text{C}$  (GC purity ~ 90 %). The major impurity (Fig. 20, d) had a lower retention time and did not contain a labelled methyl group.



**Figure 20**      Synthesis of 1,10β-[ $^2\text{H}_3$ ]dimethyldecal-(9)-en-2-one (c)

The molecular weight (150) suggested that it was the compound (Fig. 20, e) formed from 1-chloro-3-pentanone by dehydrohalogenation to ethyl vinyl ketone followed by Michael addition and Robinson annelation. Enantioselective GC (see section 4.5) gave two peaks, consistent with the presence of a single chiral centre.



**Figure 21** Attempted synthesis of geosmin via thioketal formation

Having optimised the synthesis of 1,10-dimethyldecal-(9)-en-2-one, the starting material of both geosmin syntheses (i.e. Marshall and Hochstetler, 1968 and Ayer *et al.*, 1976), it was decided to try different approaches for the synthesis of geosmin which may require fewer steps and or provide better yields. The first attempt converted the ketone

readily to a crystalline thioketal by dissolving it in glacial acetic acid and treating it with ethanedithiol and borontrifluoride etherate (Fig. 21, b). If the single alkene (Fig. 21, c) could have been obtained the plan was then to epoxidise this with MCPBA followed by reduction to geosmin with LAH (Fig. 21, d). However, when the thioketal was desulphurised (Raney nickel) a mixture of argosmins was obtained similar to those obtained by the acid catalysed dehydration of geosmin (see 3.4.5.2) and this approach was not pursued further.

A new synthesis of geosmin appeared in the literature (Gosselin *et al.*, 1989)(Fig. 22) while this work was in progress. The new Gosselin synthesis used the same starting material as the Ayer synthesis (Fig. 18, a) however they oxidised with MCPBA instead of  $\text{H}_2\text{O}_2$ -NaOH. Unlike the 60:40 mixture of  $\alpha$  and  $\beta$  epoxy ketones as produced by  $\text{H}_2\text{O}_2$  oxidation they obtained 94 % of the  $\alpha$  isomer. The epoxy ketone (Fig. 22, b) was reduced with sodium borohydride to give the epoxy alcohols (Fig 22, c) which were not separated but immediately tosylated (Kabalka *et al.*, 1986) and the epoxy tosylates were reduced with LAH to give hydrogenolysis of the tosylate group and opening of the epoxide to form geosmin (Fig 22, e).

Unfortunately we were not able to obtain a crystalline tosylate and were therefore unable to purify this compound. Although geosmin was obtained, its purity was very low and attempts to further purify the crude product by sublimation to remove excess tosyl chloride failed due to the instability of the tosylate which decomposed at  $> 100^\circ\text{C}$ .

A different synthetic pathway was explored (Fig 23). The unsaturated ketone (Fig 23, a) was epoxidised, in the same manner as described by Gosselin *et al.*, (1989), to

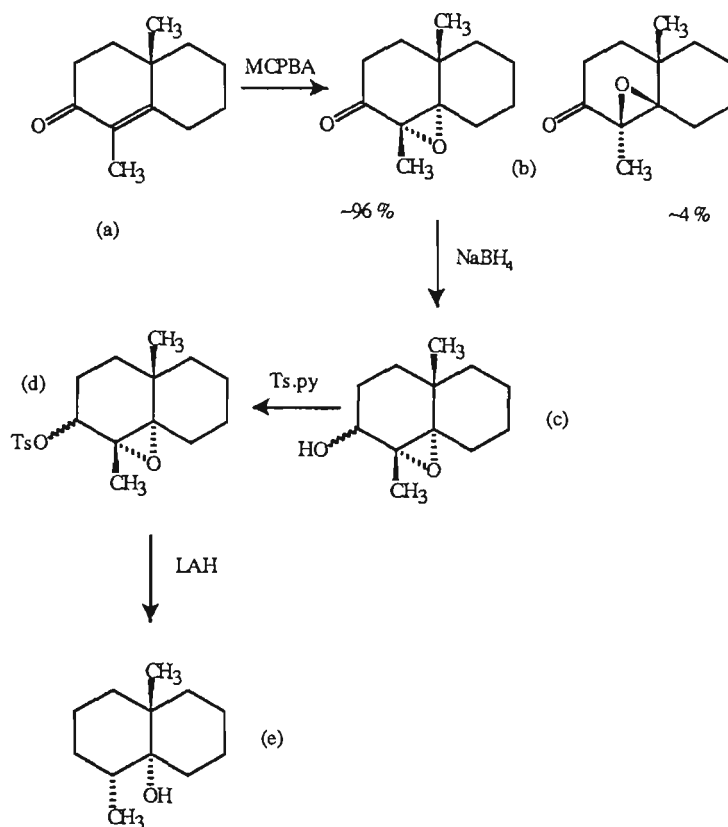
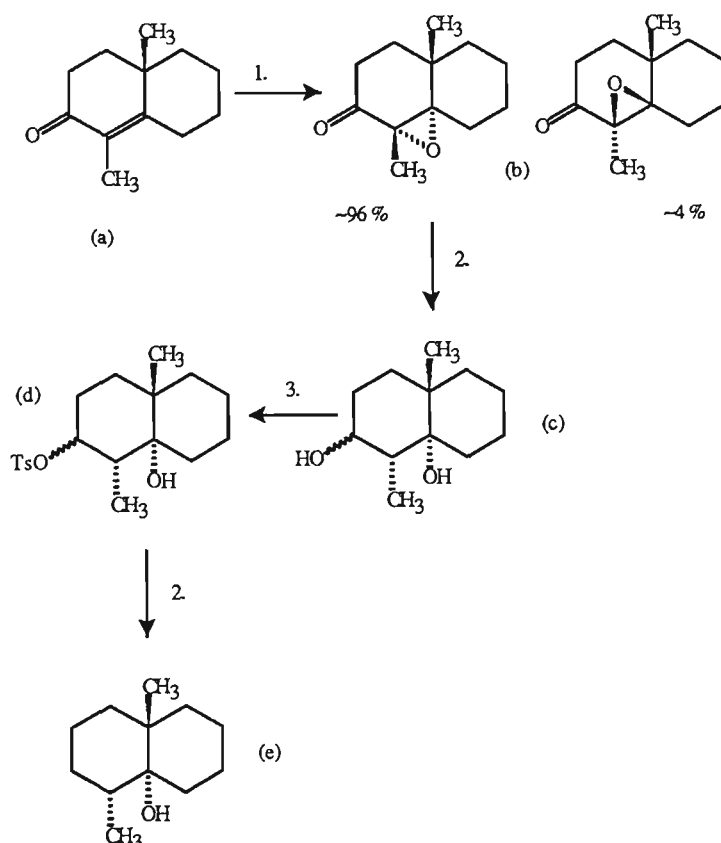


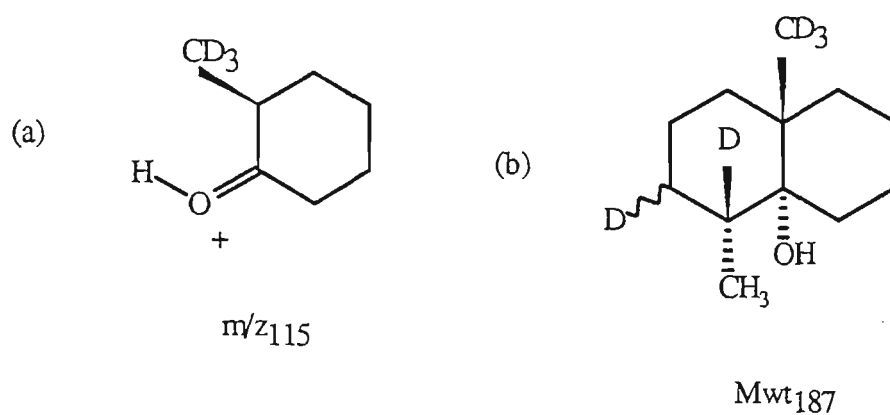
Figure 22 geosmin (e) synthesis of Gosselin *et al.*, (1989)

maintain the high yield of  $\alpha$ -epoxy ketone and reduced with LAH to the diol mixture (Fig. 23, c) rather than with sodium borohydride to produce the epimeric alcohols (Fig. 22, c). The secondary hydroxyl group was then selectively tosylated and reduced with LAH to give ( $\pm$ )-geosmin-d<sub>3</sub> (Fig. 23, e). Gosselin *et al.*, (1989) were able to separate the two epimers (Fig. 22, d) by chromatography on silica gel and identified each by reduction to the diol with LAH. In the present case, the mixture of epimeric tosylate alcohols crystallised spontaneously and were more stable than the tosylate epoxide. After a single crystallisation the compound was reduced with LAH by inverse addition (tosylate added to LAH) to give ( $\pm$ )-geosmin-d<sub>3</sub> in high yield. This was further purified on silica gel as described by Gosselin *et al.*, (1989).

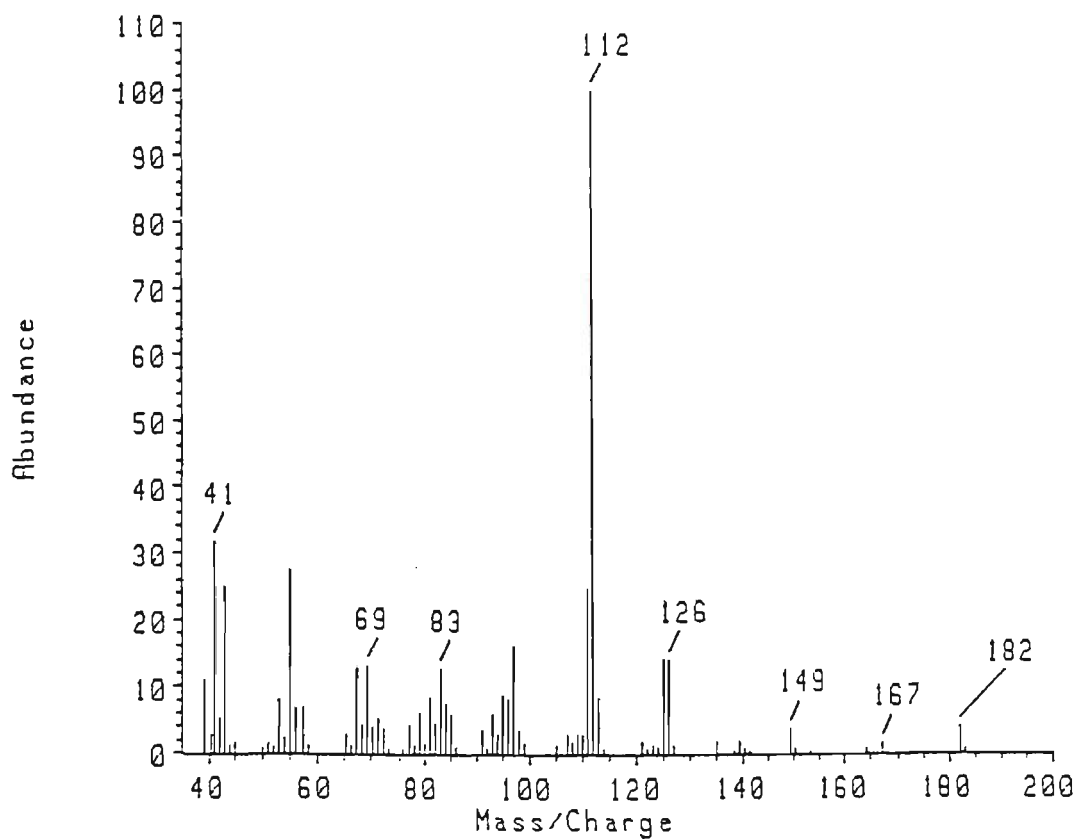
The mass spectrum of ( $\pm$ )-geosmin- $d_3$  (Fig. 26) shows complete incorporation of the label with the base peak (115) and molecular ions (185) both three daltons higher than in the unlabelled analogue (Fig. 25). The mass of the base peak shows total retention of the label and is consistent with the fragment (Fig. 24, a). This was confirmed by a similar base peak being obtained from ( $\pm$ )-geosmin- $d_5$  (Fig. 24, b) indicating that the left hand ring fragments and is not part of the base peak. The pentadeuterated geosmin was synthesised from a labelled enone (equivalent to Fig 23 (a) except that  $CD_3$  replaces  $CH_3$  at the ring junction) and by substituting LAD for LAH in the reduction of the correspondingly labelled keto epoxides (Fig. 23, b). It had a molecular ion at  $m/z$  187 and a base peak at  $m/z$  115.



**Figure 23** Alternative synthesis of geosmin (e) via tosylation of diols 1. MCPBA, 2. LAH, 3. Ts.py



**Figure 24** Structure of geosmin- $d_3$  and proposed structure of fragment comprising the base peak at  $m/z$  115



**Figure 25** Mass spectrum of (±)-geosmin

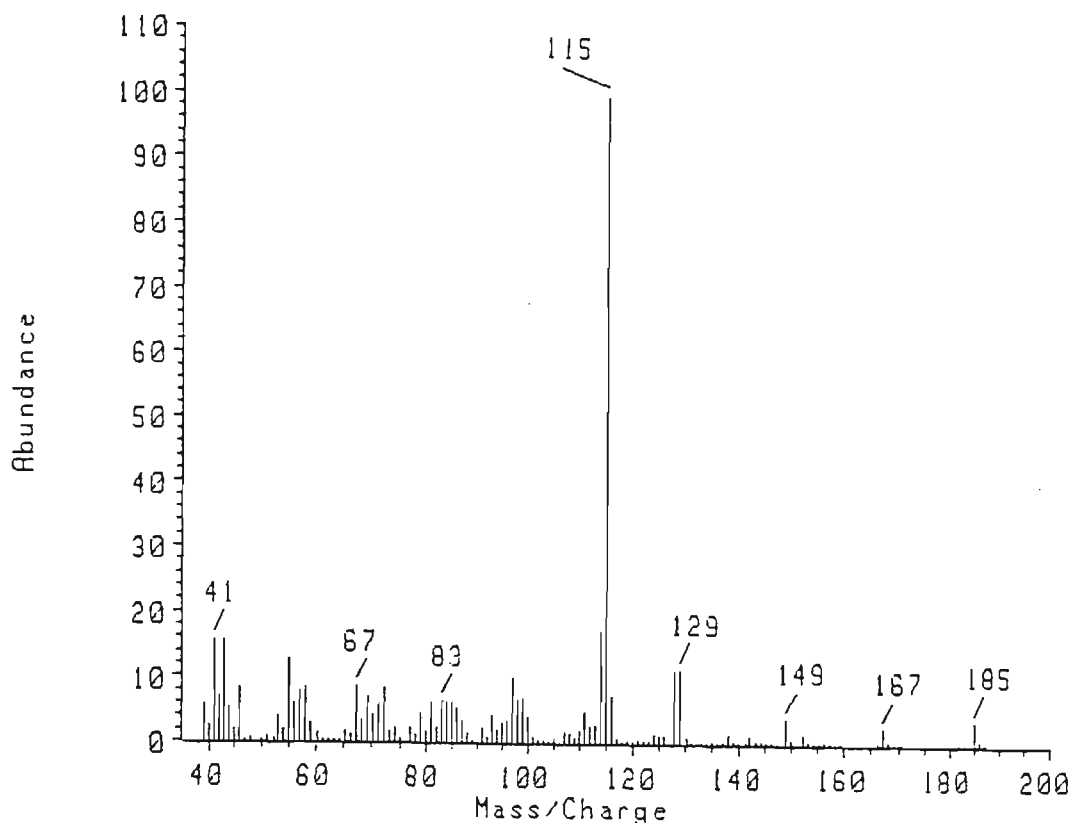


Figure 26 Mass spectrum of (±)-geosmin-d<sub>3</sub>

### 3.3 Synthesis of (-)-MIB-d<sub>3</sub>

Labelled MIB could be synthesised in one step from (+)-camphor by the method of Tyler *et al.*, 1978. However, the competitive formation of an enolate salt during the synthesis, produced when camphor reacted with CD<sub>3</sub>MgI, reduced the overall yield of the method. The enolate converted back to camphor when the reaction mixture was worked up with HCl<sub>(aq)</sub>, but was difficult to remove and contaminated the product. The problem was overcome by Wood and Snoeyink (1977) who selectively converted the unwanted camphor to an oxime by reacting the crude product with hydroxylamine hydrochloride at reflux. The oxime was removed by taking advantage of its acidic nature and washing the reaction mixture repeatedly with NaOH<sub>(aq)</sub> leaving essentially pure MIB behind.



Labelled MIB was synthesised as described by Wood and Snoeyink (1977) and the mass spectrum of the purified product showed a molecular ion at  $m/z$  171 (~7 % of the base peak), but the base peak ( $m/z$  95) was not labelled (Fig. 27 and 28). This was to be expected given the mass spectral fragmentation pattern of MIB proposed by Medsker *et al.*, (1969) (Fig. 29).

Incorporation of a deuterium label into the fragment which produced the base peak was attempted by synthesising camphor from camphene. The C-10 carbon was to be labelled with one deuterium atom by reaction of camphene with  $CD_3COOD/D_2SO_4$ , however, scrambling of the label occurred due to rearrangements of an intermediate carbocation and this approach was not pursued. To label camphor in the correct position such that a labelled MIB base peak was obtained  $CD_3$  labels would need to be introduced into  $\beta,\beta$ -dimethyl glutaric acid during the early stages of the ten step total synthesis of camphor (Komppa, 1909). Because detection limits of MIB using MIB- $d_3$  were already below the odour threshold of the compound (see later) the extra analytical sensitivity which would be obtained did not justify such a lengthy synthesis.

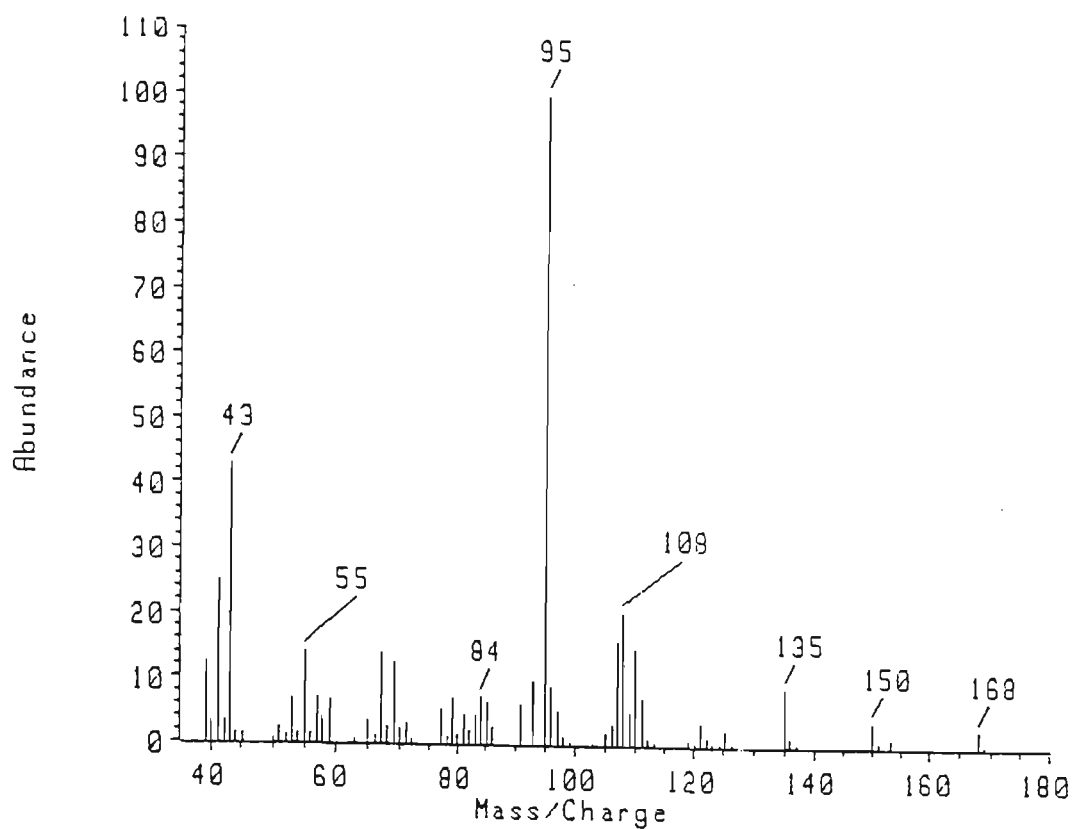


Figure 27 Mass spectrum of (-)-MIB

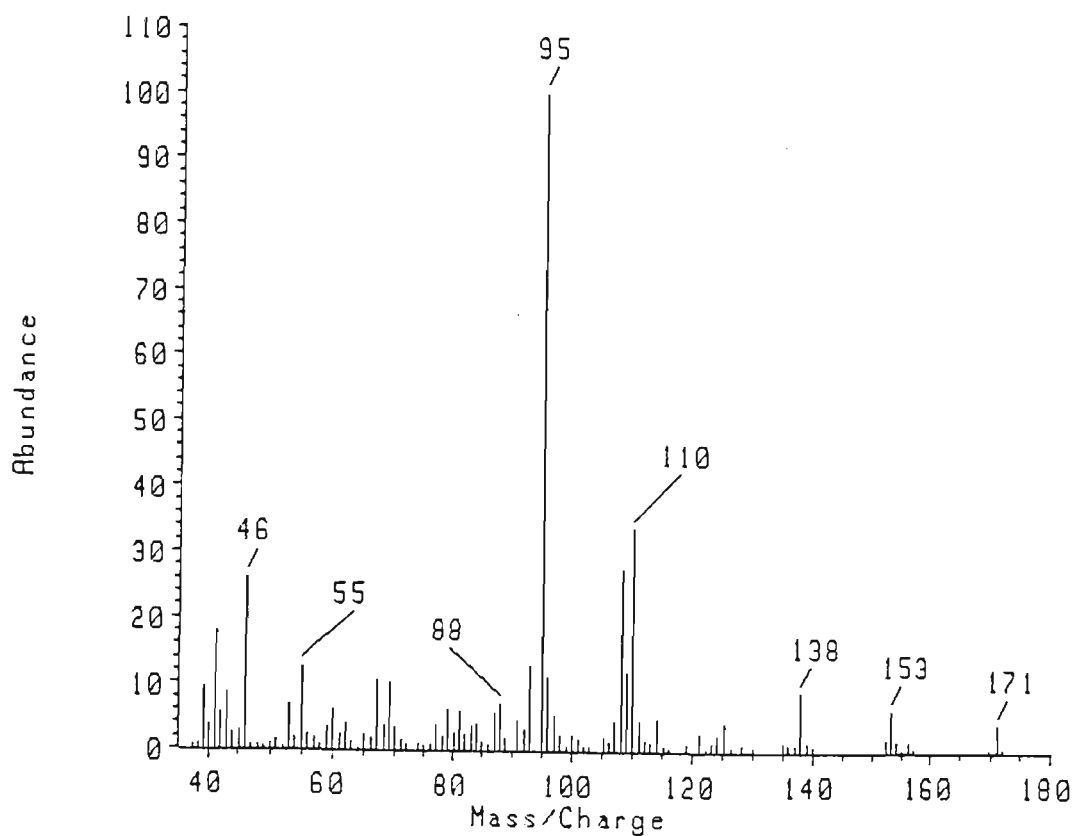
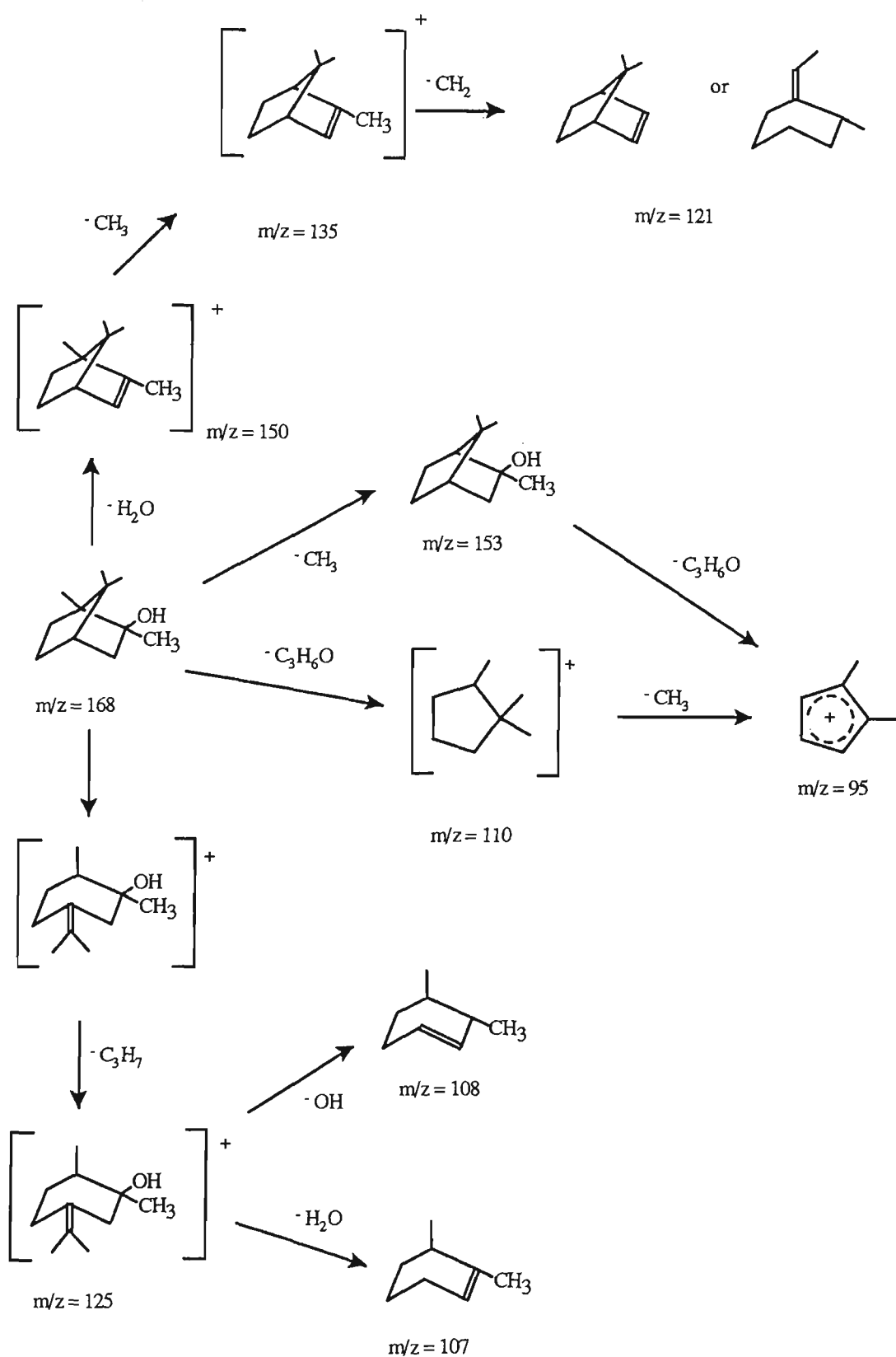


Figure 28 Mass spectrum of (-)-MIB-d<sub>3</sub>



**Figure 29** Proposed fragmentation pattern for MIB (Medsker *et al.* (1969), parent compound  $m/z$  168, base peak  $m/z$  95

### 3.4 Application of Labelled Compounds as Internal Standards

#### 3.4.1 Comparison of Labelled Internal Standards vs 1-chlorodecane

The deuterated internal standards were compared to the traditionally used 1-chlorodecane for the determination of MIB and geosmin at various concentrations. The CLSA results using 1-chlorodecane were calculated using the procedure of Mallevalle and Suffet (1987) and described in the Materials and Methods section (2.6.1). Response factors (RF's) for MIB and geosmin were calculated from the CLSA of one water sample containing known concentrations of MIB, geosmin and 1-chlorodecane calibration standard. These response factors were then used to calculate the MIB or geosmin concentrations of unknown samples (u) for a further four CLSA runs, using the formula:

$$M/g_u \text{ (ng/L)} = \frac{\text{area } M/g_{(u)} \times \text{amount (IS)}_{(u)}}{\text{RF (M/g)} \times \text{area (IS)}_u}$$

where:

M/g = MIB or geosmin

area M/g = peak area of the ion used for quantification (m/z 95, MIB; m/z 112, geosmin)

area (IS)<sub>u</sub> = peak area of internal standard (m/z 91, 1-chlorodecane)

u = unlabelled

In the case of the labelled internal standard, the response factors of MIB and geosmin were the same as their labelled analogues (i.e. RF = 1).

**Table 18** Precision and accuracy of measurement of MIB by GC/MS (MID) using MIB-d<sub>3</sub> or 1-chlorodecane as internal standard

(a) MIB-d<sub>3</sub> internal standard: m/z 168/171

Concn present (ng/L)	Concn of int. std. (ng/L)	Concentration found (ng/L)					Mean	CV %
1000	930	999	1065	1051	1040	1034	1038	2.1
100	93.0	100.5	100.2	100.3	100.2	99.9	100.2	0.2
10.0	9.30	10.42	10.5	10.40	10.79	10.97	10.6	2.3
1.00	0.930	1.1	1.18	1.08	1.10	1.09	1.11	3.5*
0.200	0.186	ND	ND	ND	ND	ND		

(b) 1-chlorodecane internal standard: m/z 95 (MIB)/91 (1-ClC<sub>10</sub>)

Concn present (ng/L)	Concn of int. std. (ng/L)	RF	Concentration Found (ng/L)				Mean	CV %
1000	930	1.39	968	991	949	928	959	2.4
100	93.0	1.56	89.3	101.0	84.9	82.6	89.4	8.8
10.0	9.30	1.48	11.88	11.96	12.05	12.51	12.1	2.0
1.00	0.93	1.91	0.97	1.07	1.00	0.93	0.99	5.2
0.10	0.186	3.07	0.19	0.21	0.35	0.32	0.27	25.5

\* S/N = 5:1; ND = not detected; RF = response factor

Table 18 shows the comparative behaviour of MIB-d<sub>3</sub> and 1-chlorodecane internal standards for different concentrations of MIB (and internal standard). Labelled internal standard gave better precision than 1-chlorodecane in all cases except for the 0.2 ng/L concentration, where the molecular ion intensity was too low to give an acceptable signal. However, using the multiple injection technique, good results were obtained at the 1 ng/L concentration, which was below the odour threshold of MIB.

**Table 19** Precision and accuracy of measurement of geosmin by GC/MS (MID) using geosmin-d<sub>3</sub> or 1-chlorodecane as internal standard

(a) geosmin-d<sub>3</sub> internal standard: m/z 112/115. Numbers in parentheses were determined using m/z 182/185

Concn present (ng/L)	Concn of int. std. (ng/L)	Concentration found (ng/L)					Mean	CV %
980	1018	982 (978)	988 (969)	989 (985)	986 (975)	988 (949)	987 (971)	0.3 (0.6)
98.0	101.8	95.7 (95.7)	97.0 (99.2)	96.0 (97.2)	96.3 (98.0)	95.6 (96.6)	96.1 (97.3)	0.6 (1.4)
9.80	10.18	10.15 (10.47)	10.14 (9.93)	10.09 (10.59)	10.05 (10.68)	9.89 (9.69)	10.1 (10.3)	1.1 (4.2)
0.98	1.018	0.975 (1.06)	0.986 (1.10)	0.981 (1.04)	0.965 (1.02)	0.957 (1.01)	0.973 (1.03)	1.2 (3.3)
0.196	0.204	0.219 (0.248)	0.192 (0.265)	0.232 (0.304)	0.193 (0.250)	0.216 (0.229)	0.210 (0.259)	8.3 (10.8)

(b) 1-chlorodecane internal standard: m/z 112 (geosmin)/91 (1-ClC<sub>10</sub>)

Concn present (ng/L)	Concn of int. std. (ng/L)	RF	Concentration Found (ng/L)				Mean	CV %
980	930	1.81	911	957	949	880	924	3.2
98.0	93.0	2.09	92.6	98.8	91.1	94.6	94.3	3.0
9.80	9.30	1.87	11.1	11.7	11.3	11.2	11.3	2.5
0.980	0.93	2.12	1.05	0.98	1.03	1.04	1.03	2.8
0.196	0.186	2.63	0.19	0.25	0.21	0.27	0.23	13.7

RF = reponse factor

Table 19 shows the comparative behaviour of geosmin-d<sub>3</sub> and 1-chlorodecane as internal standards for different initial concentrations of geosmin (and for internal standard). The required response factors were determined as for MIB (or shown above). Using the respective base peaks (*m/z* 112/115), the geosmin-d<sub>3</sub> internal standard gave better precision and accuracy than the 1-chlorodecane over the whole concentration range (0.2-

1000 ng/L). A (visual) signal to noise ratio (S/N) was readily obtained, even at concentrations of 0.2 ng/L. Using the molecular ions, S/N was 2:1 at 0.2 ng/L and 8:1 at 1 ng/L. For all concentrations the precision using the base peak was better than using the molecular ion.

### 3.4.2 Storage of MIB and Geosmin

Table 20 shows the effect of storage on the concentration of MIB, geosmin and 1-chlorodecane which had been added to a surface water samples to give concentrations of 1000, 980, 927 ng/L respectively. The water sample was then divided into two portions, the first of which was dosed with MIB-d<sub>3</sub> and geosmin-d<sub>3</sub> (1020 ng/L). Each of these portions was then divided into a set of sub-samples (of varying volume depending on the intended storage time). Each sub-sample was stored in a completely filled, sealed container and the separate containers analysed after varying times up to three weeks. This procedure was followed to avoid the loss of volatile solute into the head space which may occur when sequential aliquots are withdrawn from a single container (Cline and Severin, 1989). A set of containers one from each portion, was stored at both 20 and 5°C. All containers were stored in the dark.

Each sub-sample was made up to 900 mL in the stripping bottle, to which 90 g of NaCl had been added. For each time/temperature storage condition, three sub-samples with and three sub-samples without initial addition of labelled internal standard were analysed. Apparent concentrations of MIB and geosmin were determined using the corresponding labelled internal standard (added initially) while the concentration of MIB, geosmin and 1-chlorodecane remaining at the end of each storage interval were

determined by adding a known mass of MIB-d<sub>3</sub> and geosmin-d<sub>3</sub> to the water immediately before stripping. For storage times < 1 week, the sub-samples were treated with mercuric chloride at the selected sampling time to suppress biological degradation during the course of replicate analyses.

For samples stored at 20<sup>0</sup>C the loss of geosmin was very rapid while the MIB concentration declined more slowly; in three days the losses were 94 and 15 % respectively. The loss of 1-chlorodecane was even more rapid; after one day it was no longer detectable. The initial loss of 1-chlorodecane was probably due to adsorption; the water was very turbid (70 NTU). For samples stored at 5<sup>0</sup>C, the rate of loss of MIB, geosmin and 1-chlorodecane was much lower (see Table 20). When mercuric chloride (40 mg/L) was added at the outset, loss of MIB and geosmin was suppressed. The presence of sunlight did not alter this result. This suggest that MIB and geosmin are lost mainly by biological pathways rather than photochemical means. The greater loss of geosmin due to biological degradation had been noted previously (Hwang, 1982).



**Table 20** Effect of storage on the concentration of MIB, geosmin and 1-chlorodecane<sup>#</sup> in surface water determined using MIB-d<sub>3</sub> and geosmin-d<sub>3</sub> as internal standards.

Temp.	Time (days)	Apparent concn <sup>†</sup> (ng/L)		Concn. found* (ng/L)		
		MIB	geosmin	MIB	geosmin	1-chlorodecane
5°C	0.1	997	1000	949	949	165
	2	1006	974	951	875	93
	4	1006	980	912	797	23
	8	1022	965	889	656	4
	15	988	949	851	475	ND
	22	993	956	808	252	ND
20°C	0.1	997	1000	949	949	165
	1	985	975	943	603	ND
	3	1006	958	853	509	ND
	7	989	974	823	36	ND
	14	992	979	783	29	ND
	21	967	945	714	1	ND

<sup>#</sup> Initial concentration (ng/L): MIB 1000, geosmin 980, 1-chlorodecane 927

<sup>†</sup> Determined by adding MIB-d<sub>3</sub> and geosmin-d<sub>3</sub> at the time of dosing the water with MIB, geosmin and 1-chlorodecane

\* Determined by MIB-d<sub>3</sub> and geosmin-d<sub>3</sub> at the time of stripping

The most important result from the storage trial was that the samples dosed with MIB-d<sub>3</sub> and geosmin-d<sub>3</sub> (on day 0) still indicated the correct initial concentration of MIB and geosmin, even when < 10 % of the original concentration (in the case of geosmin) remained in solution. By contrast, the chloroalkane was lost rapidly and, at 20°C, all chloroalkane had disappeared after one day. This is a major advantage of the labelled internal standards: for our surface water, assuming an initial concentration of about 50

ng/L of MIB and geosmin, the labelled internal standards may be added at the time of sampling and provided the sample is analysed within 2 weeks (for samples stored at room temperature) or 3-4 weeks (for samples stored under refrigeration) accurate results for the initial concentration can still be obtained. This obviates the need for toxic preservatives such as mercuric chloride and eliminates the health and waste disposal problems associated with its use.

### **3.4.3 Variability due to Stripping Bottle or Salt Concentration**

The ability of the internal standards (1-chloroalkane, MIB-d<sub>3</sub> and geosmin-d<sub>3</sub>) to compensate for changes in size and shape of stripping bottle as well as salt addition was evaluated. To minimise sources of error due to spiking each sample with analyte prior to analysis it was decided to prepare a stock solution of MIB and geosmin (~10,000 ng/L each) in Milli-Q water. The stock solution was stored at 4°C and portions (10 mL) were used for all subsequent analyses. For each set of stripping conditions four strips were performed, two using 1-chlorodecane as the internal standard and two using MIB-d<sub>3</sub> and geosmin-d<sub>3</sub> as internal standards. The stripping conditions evaluated were as follows:

- A) short-form bottle, no salt, stripping time 2 h
- b) tall-form bottle, no salt, stripping time 1.5 h
- c) tall-form bottle, plus salt, stripping time 1.5 h
- d) tall-form bottle, plus salt, stripping time 1.5 h, small air leak

Because the amount of analyte was the same for all strips, any observed variation in the area ratios of the compound of interest to the internal standard were due to changes in stripping efficiency of one or both compounds caused by the change in stripping conditions. Although an error due to the syringe technique, when adding the internal standard, was also possible it was anticipated to be small compared with the those due to the change in stripping conditions. Each sample was stripped twice and analysed in duplicate.

Table 21 shows the variation between duplicate injections for all area ratios is comparatively small (see section 3.4.8). The difference between the area ratios of duplicate strips, on the other hand, is also low for those calculated using labelled internal standards (see Table 21, columns 5,6,7) whereas those calculated using 1-chlorodecane are quite high (see Table 21, columns 3,4). However, the most significant differences were found to be between separate treatments (i.e. short-form bottle vs tall-form bottle) but only for the area ratios calculated using the unlabelled internal standard. To obtain meaningful data using 1-chlorodecane as an internal standard it would be necessary to calibrate the CSLA system for each treatment regime and to use the calculated response factors for that particular treatment exclusively.

In contrast the variation in area ratios between duplicate injections, strips and treatments using labelled internal standards was small and independent of CLSA parameters. These results reinforce the ability of the labelled internal standards to compensate for even the most drastic changes in CLSA parameters and confirms that accurate and precise results may be obtained under conditions which would force the termination of the analytical

procedure if chloroalkanes were used as internal standards.

**Table 21** Comparison of 1-chlorodecane, MIBd<sub>3</sub> and geosmin-d<sub>3</sub> as internal standards under varying stripping conditions

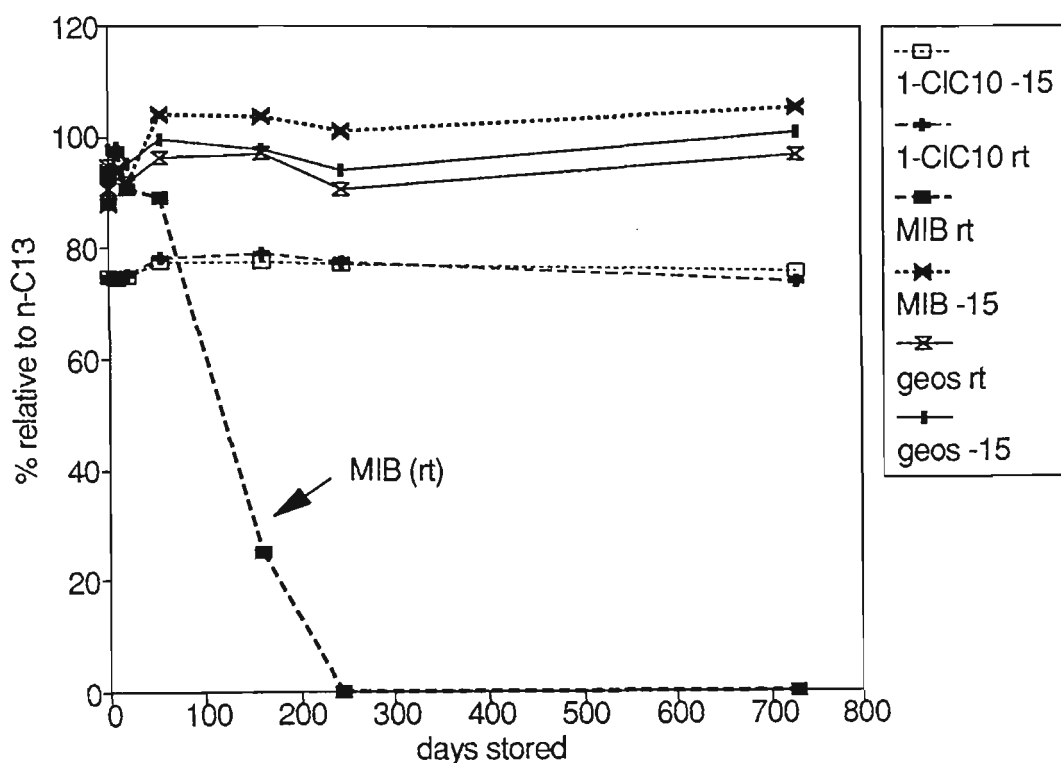
		Area ratios MIB and geosmin:internal standard				
		1-chlorodecane int. std.		labelled int. stds.		
Treatment	Strip	m/z 95 (MIB)/ 91 (1-ClC <sub>10</sub> ) inj.1, inj.2	m/z 112 (geos)/ 91 1-ClC <sub>10</sub> ) inj.1, inj.2	m/z 168 (MIB)/ 171 (MIB-d <sub>3</sub> ) inj.1, inj.2	m/z 112 (geos)/ 115 (geos-d <sub>3</sub> ) inj.1, inj.2	m/z 182 (geos)/ 185 (geos-d <sub>3</sub> ) inj.1, inj.2
short-form, no salt	1	0.337, 0.325	0.552, 0.554	1.53, 1.56	1.61, 1.63	1.61, 1.71
	2	0.253, 0.260	0.422, 0.425	1.50, 1.60	1.64, 1.63	1.58, 1.58
tall-form, no salt	1	0.147, 0.155	0.243, 0.248	1.50, 1.54	1.66, 1.64	1.73, 1.66
	2	0.229, 0.225	0.338, 0.333	1.54, 1.64	1.75, 1.74	1.68, 1.72
tall-form, plus salt	1	0.933, 1.02	1.44, 1.54	1.57, 1.57	1.80, 1.79	1.79, 1.79
	2	1.32, 1.35	2.02, 2.04	1.39, 1.42	1.61, 1.62	1.63, 1.66
tall-form, plus salt, plus air leak	1	1.31, 1.28	1.91, 1.91	1.53, 1.58	1.72, 1.71	1.76, 1.70
	2	1.13, 1.21	1.54, 1.65	1.58, 1.57	1.82, 1.82	1.87, 1.89
Mean		0.72	1.07	1.53	1.70	1.71
% CV		70.4	68.0	4.1	4.5	5.8

### 3.4.4 Stability in common laboratory solvents

The chemical stability of MIB, geosmin, 1-chlorooctane, 1-chlorodecane and 1-chlorododecane in six commonly used laboratory solvents was evaluated using C<sub>13</sub> and C<sub>15</sub> as internal standards (see materials and method 2.7). The area of each compound was normalised to the area of C<sub>13</sub>. A decrease in the ratio to that obtained for each compound at day 0 would indicate a breakdown of the compound. If the internal standard degrades then the ratio of all compounds relative to C<sub>13</sub> would increase.

A stock solution containing each of the compounds mentioned above was prepared in acetone ( $\sim 500$  ng/ $\mu$ L per component). An aliquot (50  $\mu$ L) of this stock solution was added to six amber screw cap vials each containing 2000  $\mu$ L of one of the following solvents: hexane, dichloromethane, acetone, methanol, ethanol and carbon disulphide. Acetone was used as the solvent for the stock solution as it was miscible with all other solvents. By adding 50  $\mu$ L of the stock solution to a particular solvent a forty fold dilution was achieved and this was assumed to be sufficient to make any effect due to the carrier solvent negligible. Once the stock solution had been added to each solvent the individual solvent solutions were mixed thoroughly and analysed immediately to give the initial concentration of each component for each solvent at day zero. The solutions were then split into two sets of duplicate aliquots (1 mL) and stored. The first set of each solvent was stored at  $-15^{\circ}\text{C}$  and in the dark whereas the second set was simply left on the laboratory bench at room temperature ( $22^{\circ}\text{C}$ ) and under diffuse light conditions. The solutions were then analysed at various time intervals and the area of each component was normalised to the area of tridecane. Total storage time was 729 days ( $\sim 2.0$  years).

All compounds were found to be stable when stored at  $-15^{\circ}\text{C}$  in the dark and all except MIB were stable when stored at room temperature ( $22^{\circ}\text{C}$ ) under normal light conditions. In dichloromethane (DCM), MIB degrades (see Fig. 30).



**Figure 30** Stability of 1-chlorodecane, MIB and geosmin stored in DCM at room temperature (rt) and -15°C

Figure 30 clearly shows that MIB degrades totally when stored at room temperature in dichloromethane for more than 245 days. However, the concentrations of geosmin and MIB stored at -15°C and geosmin stored at room temperature, in the same solvent, did not decrease. The fact that geosmin did not degrade when stored at room temperature in dichloromethane, an environment in which MIB degrades, reflects the greater susceptibility of MIB to acid catalysed dehydration and reinforces findings (see 3.4.5 and 3.4.6) that geosmin is much more stable under acidic conditions but is biodegraded, in river water at near-neutral pH, more rapidly than MIB. This difference in biodegradability is not unique to our particular waters; similar differences in the

biodegradation of MIB and geosmin were observed in California for two separate drinking water source waters (Hwang, 1982).

Erratic results ( $\pm 10\%$ ) were obtained for the GC analyses of all components stored in methanol and ethanol, up to day twenty. The problem was traced to active surfaces in the injection port coupled with the unsuitability of methanol and in particular ethanol to the Grob splitless injection technique under the selected GC temperature conditions. Because of the polar nature of both methanol and ethanol and their higher boiling points not enough time or high enough temperatures were set to allow for complete venting of the injection port prior to temperature programming of the column. This resulted in peak splitting with a smaller peak eluting just before the main peak. The integration system did not detect these peaks in all instances and consequently erratic results were obtained. To establish whether or not the early eluting peak of a doublet was in fact a breakdown product of that compound the methanol and ethanol solution were analysed using GC-MSD after day twenty. In both cases (i.e. methanol and ethanol) no breakdown products were observed. The fact that compounds were not degrading in these two solvents was shown by the results of the next storage interval after day twenty (i.e. day 55) to the last storage interval (i.e. day 729). After the injection port liner was cleaned and the split ratio increased (to vent the injection port more quickly) the erratic results observed earlier were eliminated.

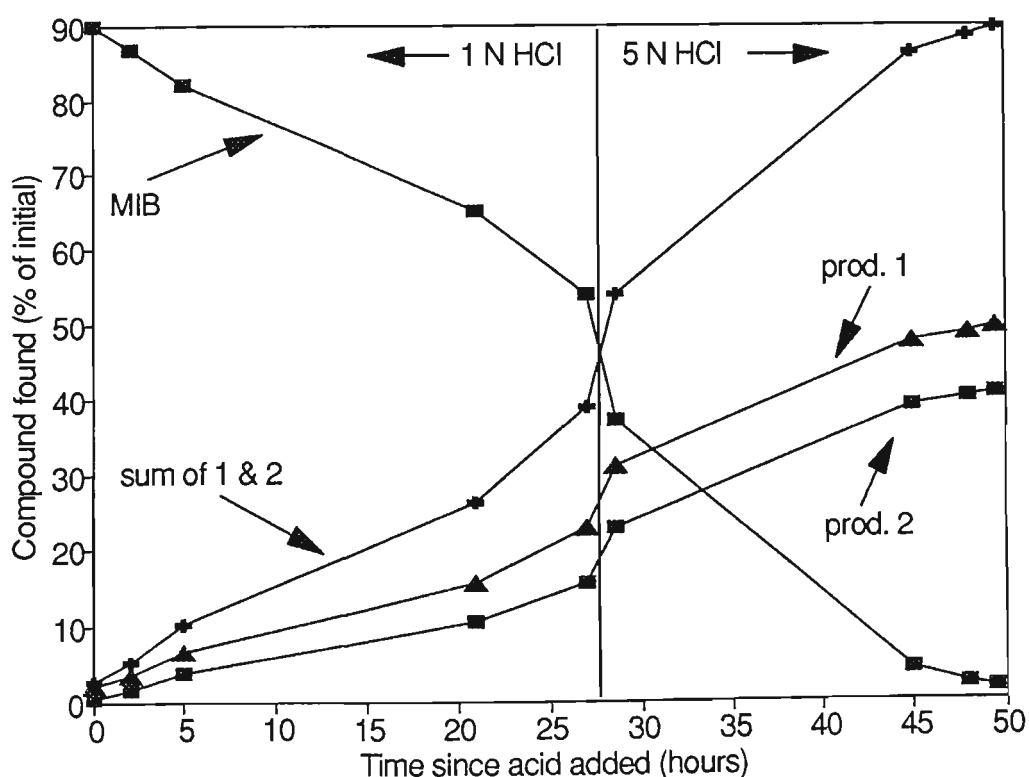
The results obtained during this storage trial indicate that MIB, geosmin, 1-chlorooctane, 1-chlorodecane and 1-chlorododecane were stable, for as long as two years, at  $-15^{\circ}\text{C}$  and all (except MIB) are stable at room temperature (in amber screw cap vials) for the same

period. If dichloromethane were used as a solvent for the storage of MIB care must be taken to keep the solution cold ( $-15^{\circ}\text{C}$ ) and in the dark otherwise total degradation of the compound to 2-methyl-2-bornene and 2-methylenebornane will occur. A more detailed description of the likely degradation process is given in the following section.

### 3.4.5 Reaction of MIB and Geosmin with Acid

#### 3.4.5.1 chemical breakdown of MIB

The observed breakdown of MIB in dichloromethane (DCM) (3.4.4) is evidently due to acid catalysed dehydration in the presence of HCl formed by the photolytic breakdown of DCM. A similar breakdown of MIB could be observed by shaking MIB in DCM with 1N HCl (see materials and method 2.8). The disappearance of MIB and the appearance of two earlier eluting breakdown products was also identical with those observed for the original solution (see Fig. 31 and 32).

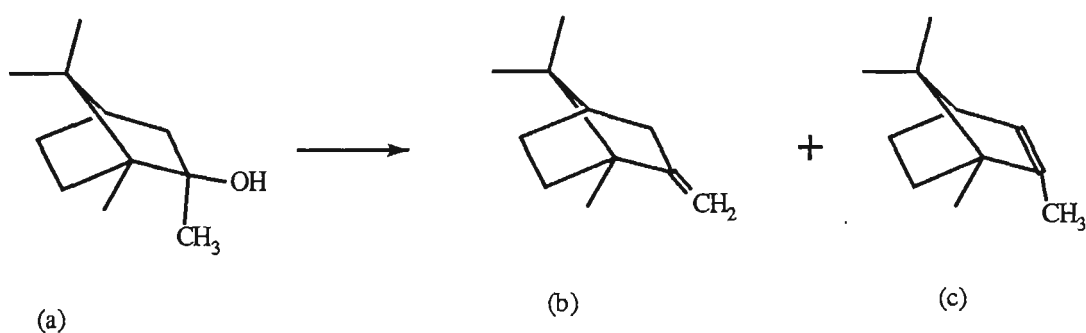


**Figure 31**

Acid catalysed decomposition of MIB. Product 1 = 2-methylenebornane, product 2 = 2-methyl-2-bornene



Mass spectra of the two breakdown products (Fig. 32 and 33) showed a molecular ion at  $m/z$  150 (168 for MIB) which is consistent with the loss of water and were identical with published mass spectra of 2-methylenebornane and 2-methyl-2-bornene (Martin *et al.*, 1988). Prior to the formation of the two breakdown products the chromatographic peak for MIB was observed to split into two almost co-eluting peaks. The left hand side of the doublet had the characteristic MIB mass spectrum however the right hand side (RHS) differed. Although the molecular ion was still  $m/z$  168 for the RHS peak it was lower in intensity ( $\sim 1\%$ ) and the base peak had changed to  $m/z$  107 from the usual  $m/z$  95. At first it was thought that the RHS peak could be 2-methylborneol: MIB could protonate and dehydrate to a carbocation which could then either lose  $H^+$  to give the mixture of alkenes or re-hydrate and lose  $H^+$  to form the epimeric alcohol. However, the mass spectrum is not the same as that published by Tyler *et al.*, (1978). Possibly it is a metastable intermediate resulting from an acid-catalysed skeletal rearrangement or a different alcohol formed from dehydration and rehydration of MIB.



**Figure 32** Methylisoborneol (a) and its dehydration products 2-methylenebornane (b) and 2-methyl-2-bornene (c)

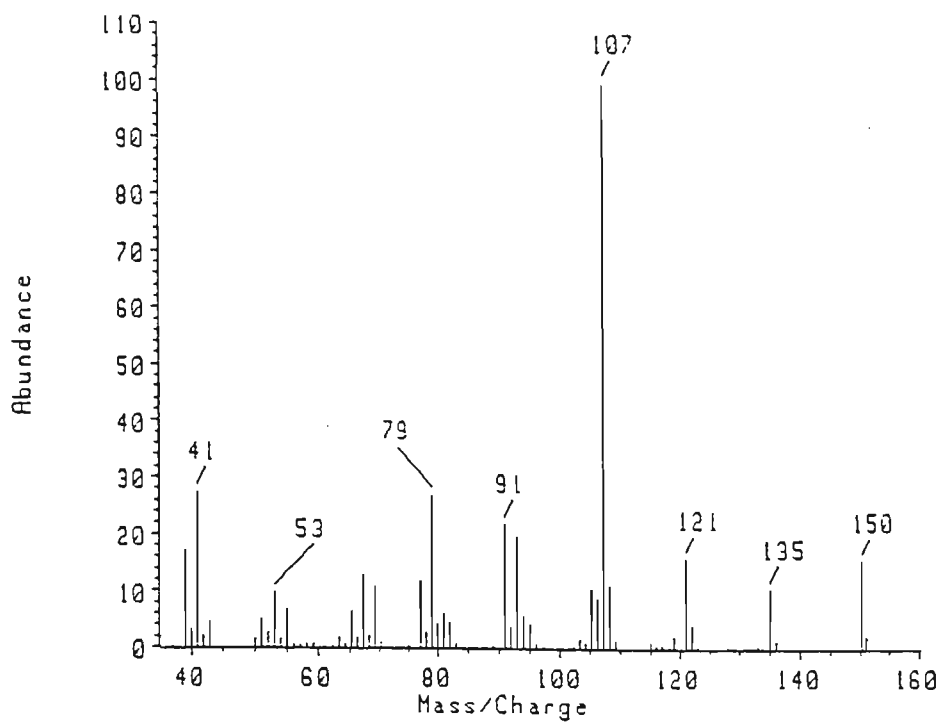


Figure 33 Mass spectrum of 1st eluting MIB dehydration product 2-methylenebornane

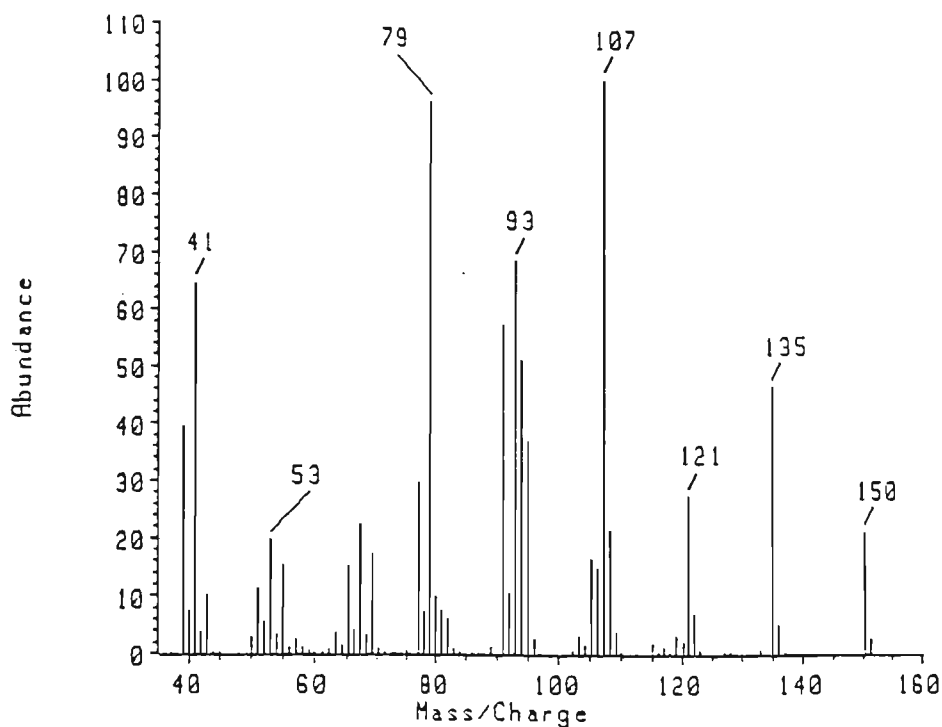
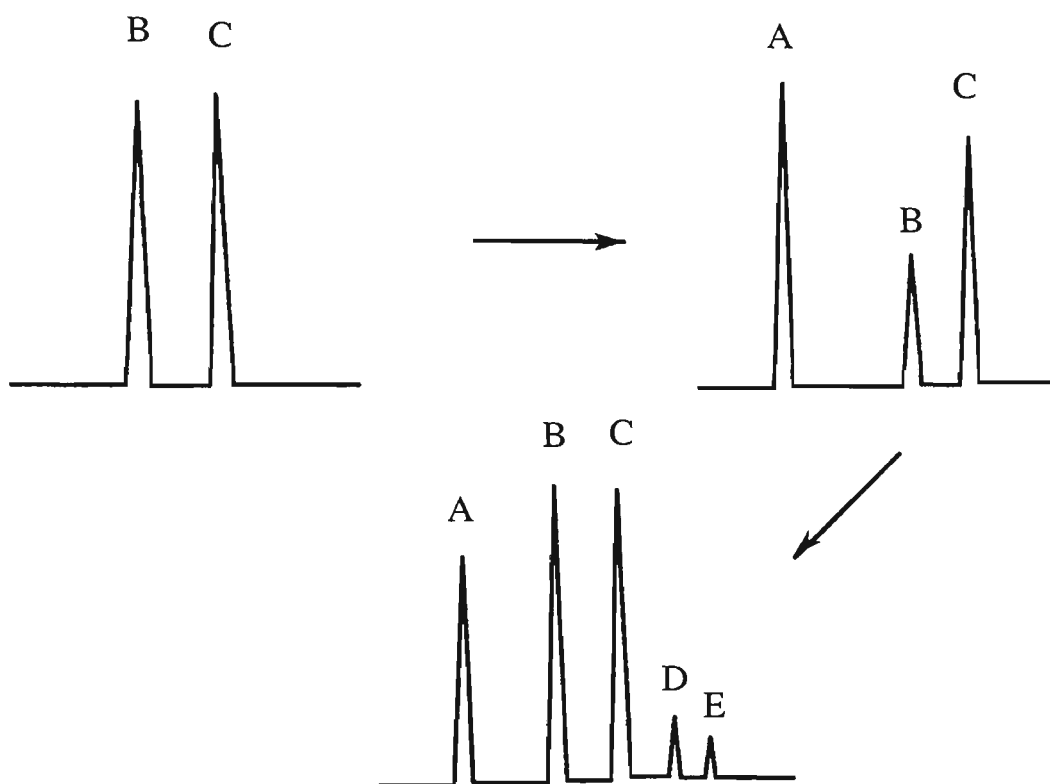


Figure 34 Mass spectrum of 2nd eluting MIB dehydration product 2-methyl-2-bornene

### 3.4.5.2 chemical breakdown of geosmin

Gerber and Lechevalier (1965) were the first to establish that geosmin could be transformed by acid catalysed dehydration to another compound that was odourless. They named the new compound argosmin (from the Greek, "argos" = inactive and "osme" = odour). Initially they believed that only one argosmin was formed by this process, however, in later work, by other researchers and Gerber herself, and with the benefit of higher resolution gas chromatography they found that quite a number of argosmins were actually formed. As many as nine argosmin have been reported (Medsker *et al.*, 1968).

When geosmin is dehydrated with aqueous HCl, the number and relative proportions of the argosmins formed varied with the severity of acid treatment. With short reaction times (about 2h, leaving a small amount of unreacted geosmin) there were two major product peaks in the gas chromatogram of the mixture. With longer reaction times a third major peak was evident and with longer reaction times again, two further small peaks were evident (Fig. 35). Under extremely severe acid conditions and long contact times (conc. HCl, 3 days) five argosmins as well as five peaks at higher retention times were formed. The peaks at higher retention times had mass spectra consistent with those expected for chloroargosmins i.e. compounds formed by the dehydration of geosmin followed by addition of HCl (Fig. 41). The three major argosmin GC peaks were labelled argosmin A, B and C respectively (Gerber, 1968) in order of elution from a nonpolar GC column. Similar results have previously been found (Rosen *et al.*, 1968; Medsker *et al.*, 1968; Gerber, 1968).



**Figure 35** Reconstructed gas chromatograms of argosmins formed with increasing severity of acid treatment of geosmin

Gerber (1968) established that argosmin C was 1,10-dimethyl-(9)-octalin from NMR data and DNP derivatisation of its degradation product as well as by comparison with an authentic sample of the compound sent to her by Dr. Marshall (Marshall and Hochstetler, 1966; Fig. 37, c). In an effort to identify the other two argosmins, GC-MS data were acquired for the three major argosmins, their chloro analogues and labelled argosmins derived from acid catalysed dehydration of geosmin- $d_3$  and geosmin- $d_5$  (Fig. 36).

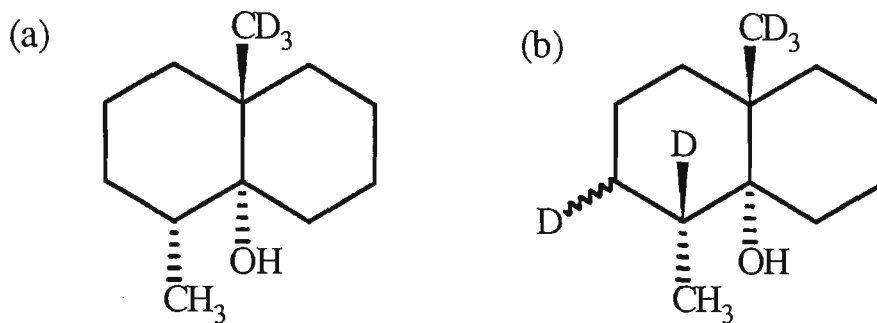


Figure 36 Chemical structure of geosmin- $d_3$  (a) and geosmin- $d_5$  (b)

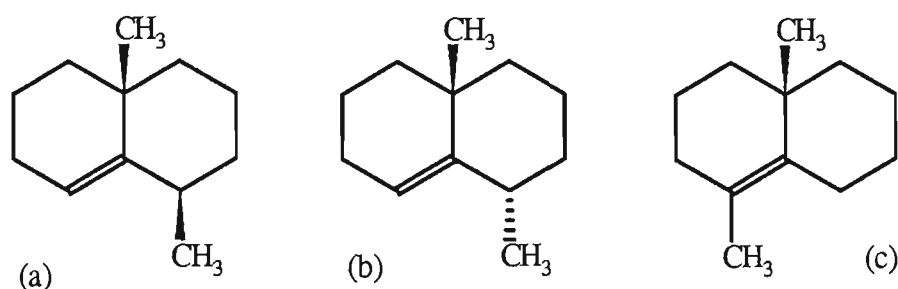
#### 3.4.5.2.1 interpretation of mass spectra of argosmins

The molecular ion of unlabelled argosmin was at  $m/z$  164, whereas in all three labelled argosmins it was at  $m/z$  167 i.e. the two extra deuterium atoms introduced at C-1 and C-2 in geosmin- $d_5$  (Fig. 36, b) prepared from dimethyldecalenone- $d_3$  have been lost during the dehydration of the geosmin- $d_5$  to argosmins; only the labelled methyl group attached to C-10 had survived. The unlabelled argosmins all give a prominent peak at  $m/z$  149 (M-15). This could be due to loss of either or both methyl groups (but only one from any one molecular ion). The labelled argosmin gave both the  $m/z$  149 (M-18) ion and a weaker  $m/z$  152 (M-15) ion, which suggests that both losses occur in parallel, with the loss of the C-10 methyl being favoured.

All three show an M-29 peak at  $m/z$  135 or  $m/z$  138 for the unlabelled and labelled argosmins respectively (loss of  $C_2H_4$ ) and further fragments corresponding to loss of  $C_3H_6$  and  $C_4H_8$ . Although the base peaks for all three argosmins were at  $m/z$  149 argosmin C could be distinguished easily from the other two because of the high abundance of the  $m/z$  93 ion (abundance  $\sim 80\%$  of base peak).

The low  $m/z$  ions were similar for all three argosmins at  $m/z$ : 41, 55, 67, 79, 93 but with some variation in the relative intensities of the  $m/z$  79 and  $m/z$  81 peaks. Further differences were seen at higher  $m/z$  values: in argosmin A, there is a peak (abundance ~50 % of base peak) at  $m/z$  109 ( $m/z$  112 labelled). In argosmin B, there are peaks at  $m/z$  108 ( $m/z$  111 in labelled) and  $m/z$  121 ( $m/z$  124 in labelled) whereas in argosmin C, there are peaks at  $m/z$  107 ( $m/z$  107,  $m/z$  110 in labelled) and  $m/z$  121 ( $m/z$  121 in labelled).

The identity of racemic argosmin B was confirmed to be *trans*-8,10-dimethyl-1(9)-octalin by comparison (GC-MS) with an authentic specimen of the compound provided by Dr. Revial (Revial, 1989) (see Fig. 37, b). Given that argosmin C was previously identified (Gerber, 1968) as the tetrasubstituted alkene (Fig. 37, C) it is probable that the remaining argosmin (argosmin A) is the diastereoisomer of argosmin B (Fig. 37, a).



**Figure 37** Chemical structure of argosmin B, (b) and C, (c) and proposed structure of argosmin A, (a)

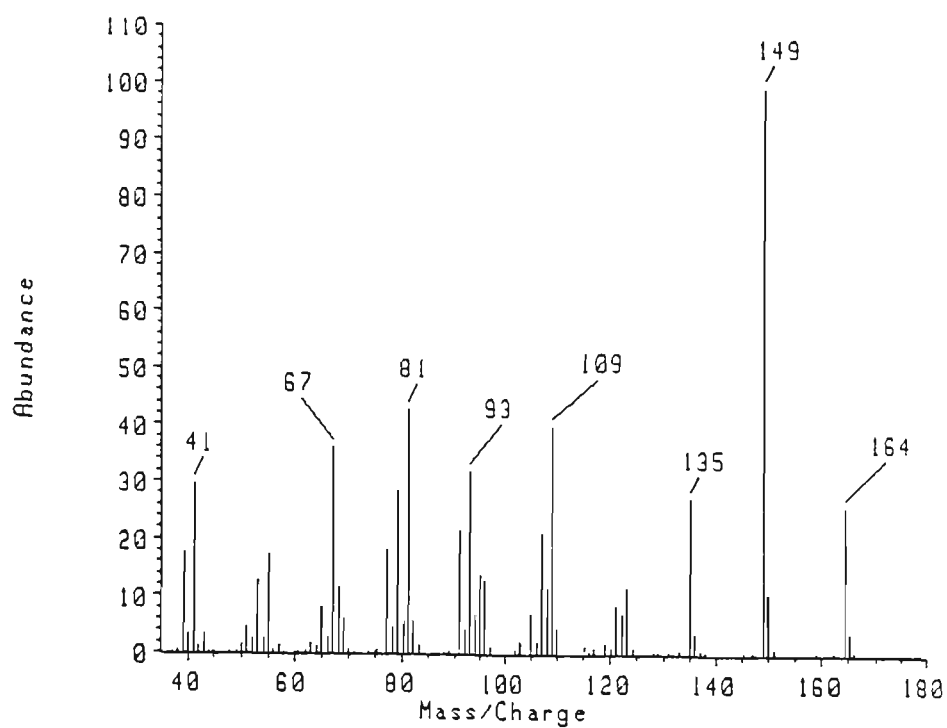


Figure 38 Mass spectrum of argosmin A

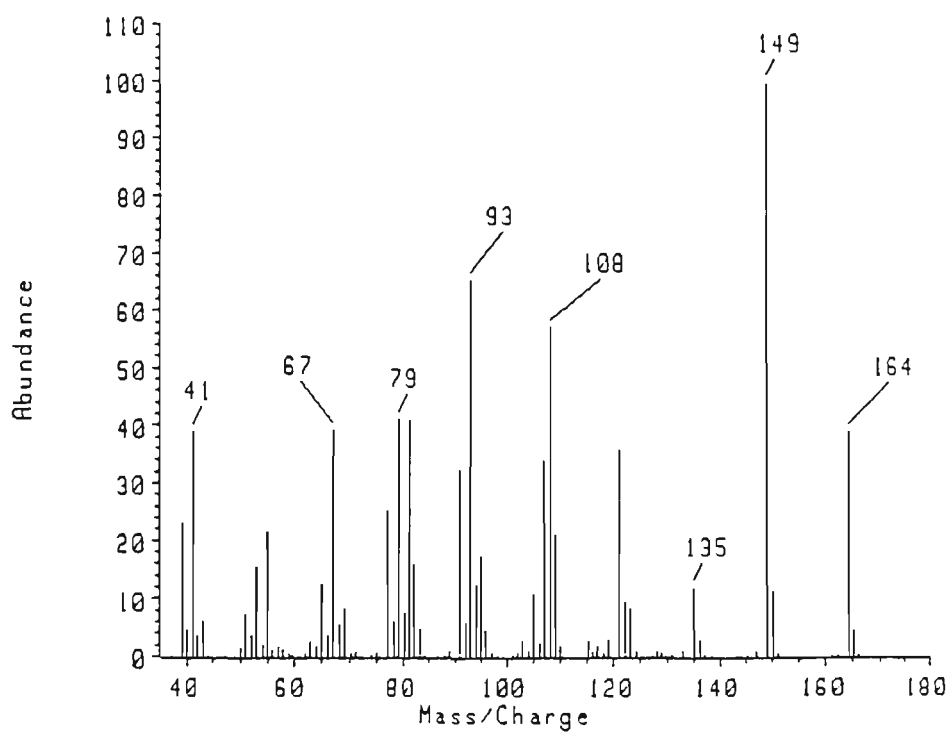


Figure 39 Mass spectrum of argosmin B

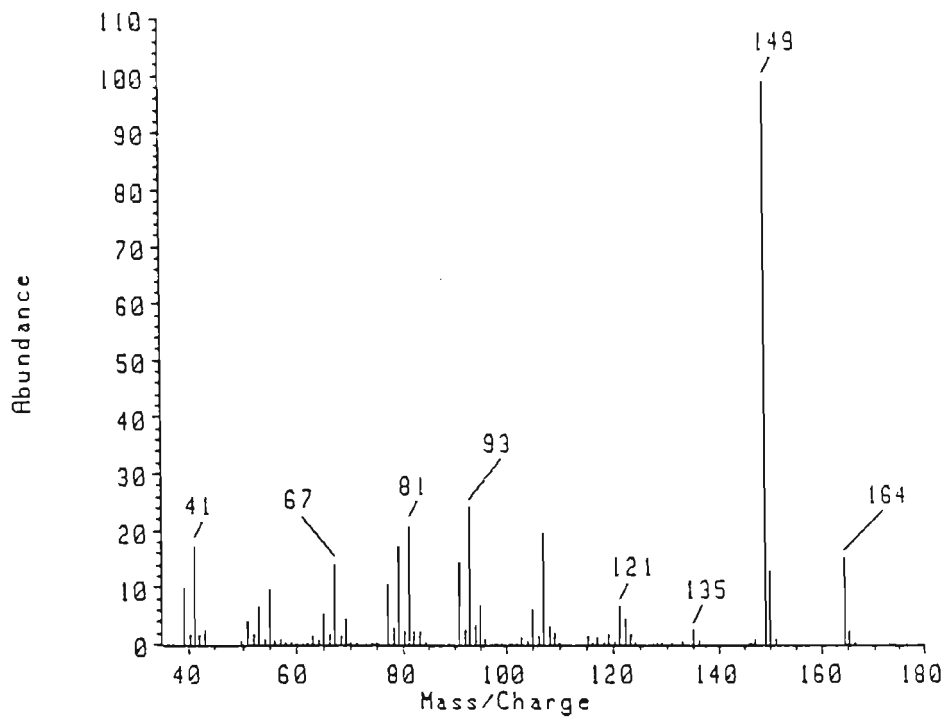


Figure 40 Mass spectrum of argosmin C

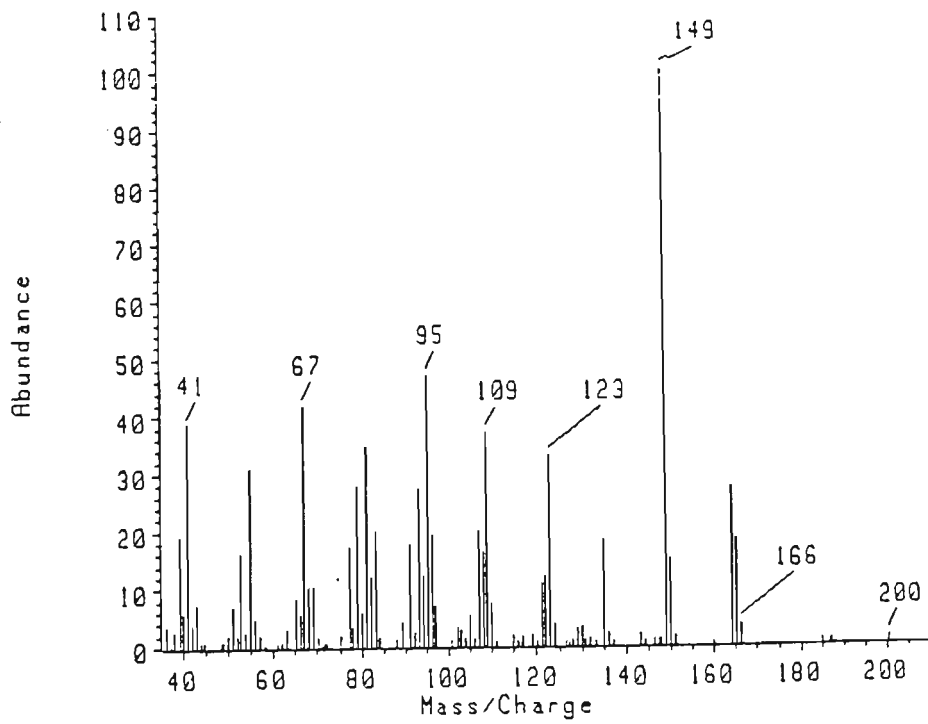


Figure 41 Mass spectrum of chloroargosmin C



Evidence that would support the designation of argosmin A as *cis*-8,10-dimethyl-1(9)-octalin (Fig. 37, a) was obtained by analysing the three argosmins by enantioselective GC. Using either the J&W or the SGE  $\beta$ -cyclodextrin columns baseline resolution of argosmin B and C was achieved but argosmin A remained unresolved. In principle all the argosmins should be optically active as the asymmetric carbon at C<sub>10</sub> would remain intact after the acid catalysed dehydration of geosmin. The inability of the  $\beta$ -cyclodextrin column to resolve the enantiomers of argosmin A (or any of the other possible argosmins) is more likely due to subtle differences in shape between the molecules rather than a loss of optical activity. Because  $\beta$ -cyclodextrin columns separated compounds largely by shape recognition (see section 3.5) there must be enough of a difference in the structural configuration between the object and mirror images of a particular compound for one to be retained more securely. The variation in shape between the (+) and (-) *cis* enantiomers of argosmin A may be insufficient to cause differential retention of one or the other enantiomers in the chiral cavity of the  $\beta$ -cyclodextrin molecule.

Inspection of models shows the methyl group on C<sub>8</sub> in argosmin B and C<sub>1</sub> in argosmin C is equatorial (and at right angles to the C<sub>10</sub> methyl) whereas in argosmin A it is axial (and parallel to the C<sub>10</sub> methyl) and the whole molecule is more compact and closer to spherical in shape. Thus, it may be significant that the two argosmins which do resolve have an exposed methyl group whereas the argosmin which is unresolved does not.

In summary, the three unlabelled argosmins show very similar mass spectra, with a base peak at  $m/z$  149 and the main differences being:

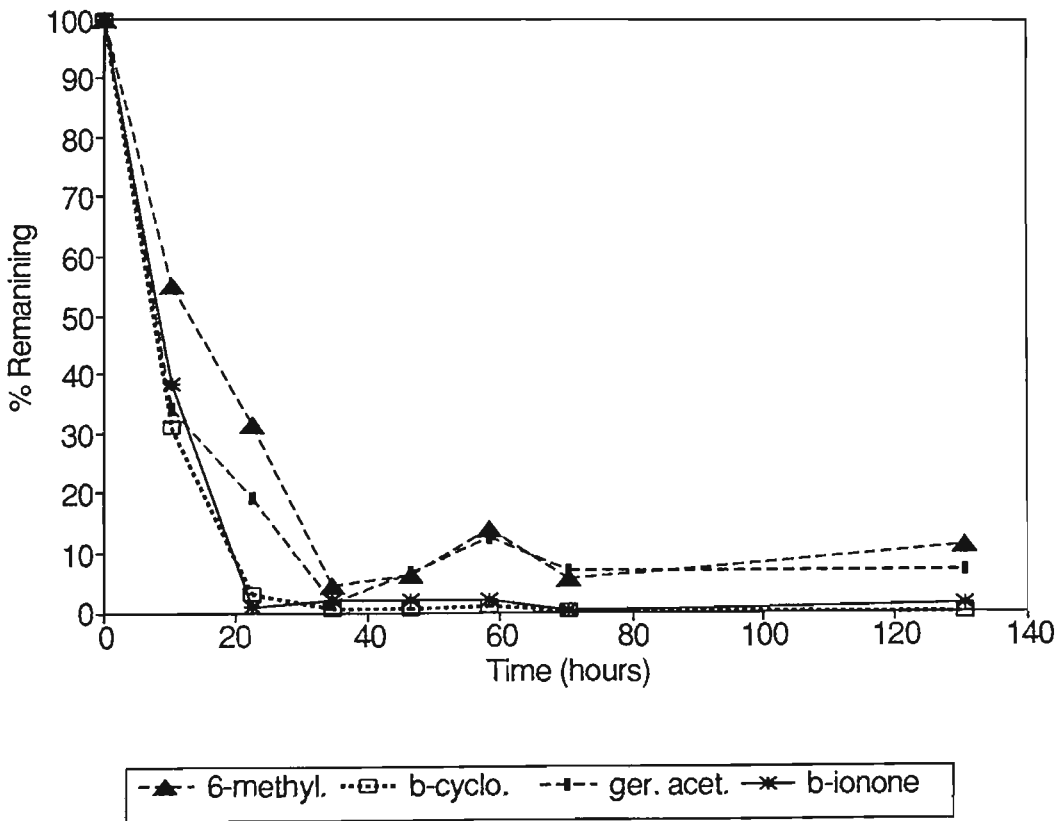
- i) the abundance of the ion at  $m/z$  93 compared with the abundance of the base peak at  $m/z$  149, argosmin A (~50%), argosmin B (~80%) and argosmin C (~20%). However, when a MAT 44 mass spectrometer was used the base peak of argosmin B was at  $m/z$  93
- ii) the relative intensities of the  $m/z$  135 peak (argosmin A > argosmin B > argosmin C)
- iii) the relative intensities of the cluster of peaks at  $m/z$  105-109. The most intense peak is  $m/z$  109, 108, 107 for argosmin A, B, C, respectively

### **3.4.6 Biodegradation Trials**

#### **3.4.6.1 determination of additional odour compounds using geosmin- $d_3$ and 1-chlorodecane**

The suitability of the labelled standards for the determination of other odour algal metabolites was also investigated. Murrumbidgee River water spiked with four carbonyl odour compounds ( $\beta$ -cyclocitral,  $\beta$ -ionone, 6-methylhept-5-en-2-one, geranylacetone) was analysed with addition of chloroalkanes, MIB- $d_3$  and geosmin- $d_3$  prior to stripping (see materials and methods).

The labelled compounds were as effective as chloroalkanes when added at the time of stripping giving comparable results for each of the odour compounds (see Fig. 42, 43 and 44). However, as for chloroalkanes, the biodegradation of all compounds (i.e., target compounds and internal standards) differ sufficiently to preclude addition of the labelled standards at the time of sampling. The rapid loss of the four carbonyl compounds in Murrumbidgee river water, also observed for lake water in Germany (Jüttner, 1984), suggests that these compounds may be less troublesome pollutants than MIB and geosmin because they biodegrade more rapidly (see Fig. 45).



**Figure 42** Biodegradation of carbonyl odour compounds using 1-chlorodecane as internal standard.  
 (6-methyl. = 6-methylhept-5-en-2-one; b-cyclo. =  $\beta$ -cyclocitral;  
 ger.acet. = geranylacetone; b-ionone =  $\beta$ -ionone)

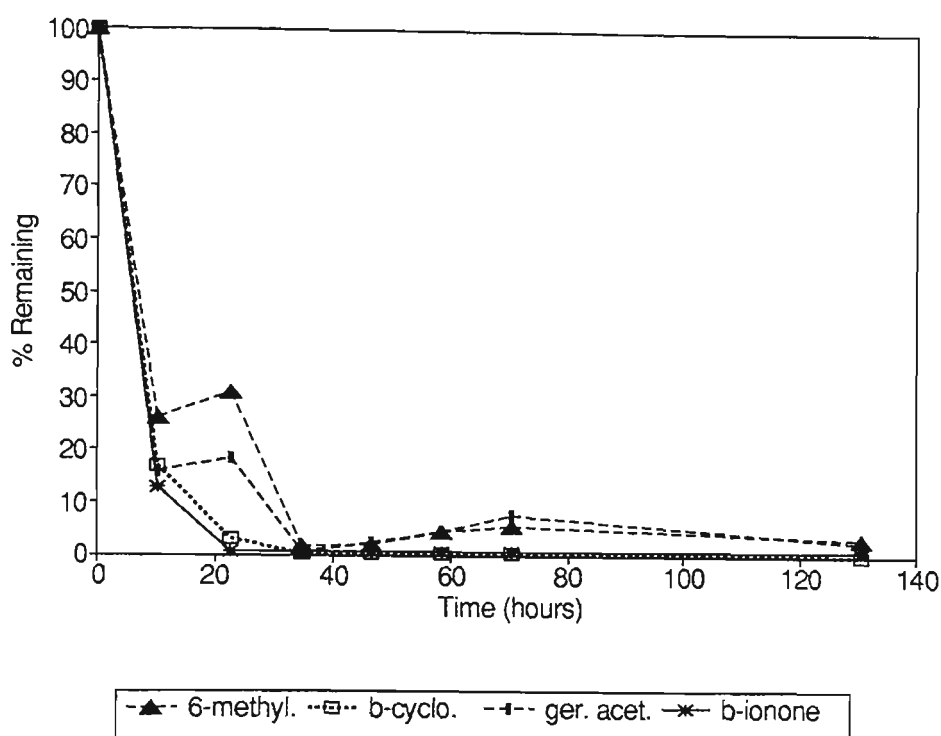


Figure 43

Biodegradation of carbonyl odour compounds using geosmin- $d_3$  as internal standard.  
 (6-methyl. = 6-methylhept-5-en-2-one; b-cyclo. =  $\beta$ -cyclocitral;  
 ger.acet. = geranylacetone; b-ionone =  $\beta$ -ionone)

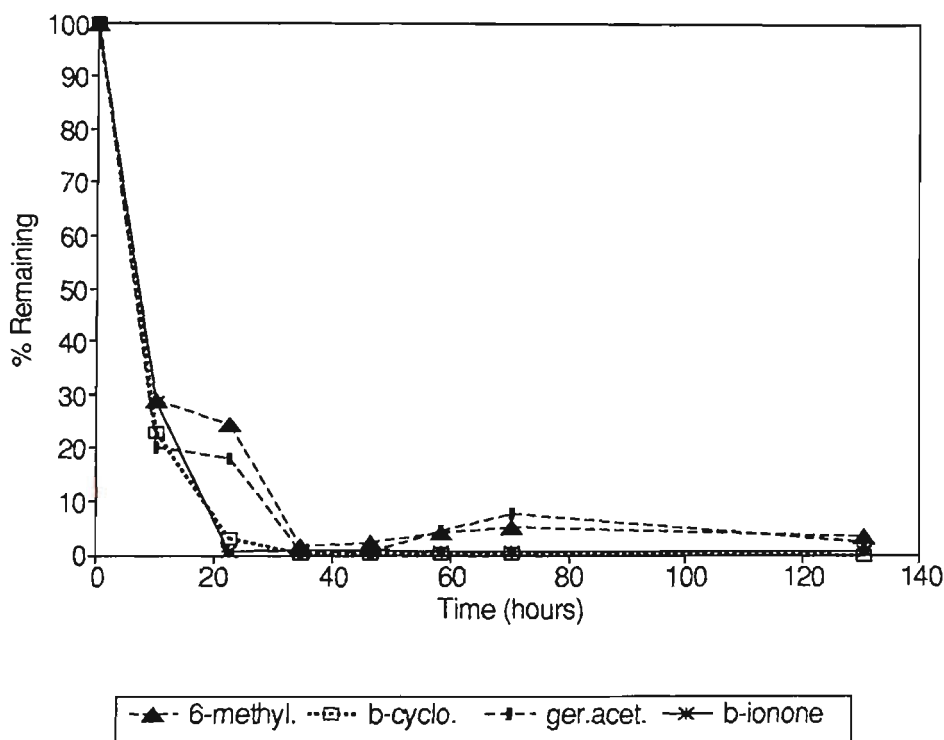
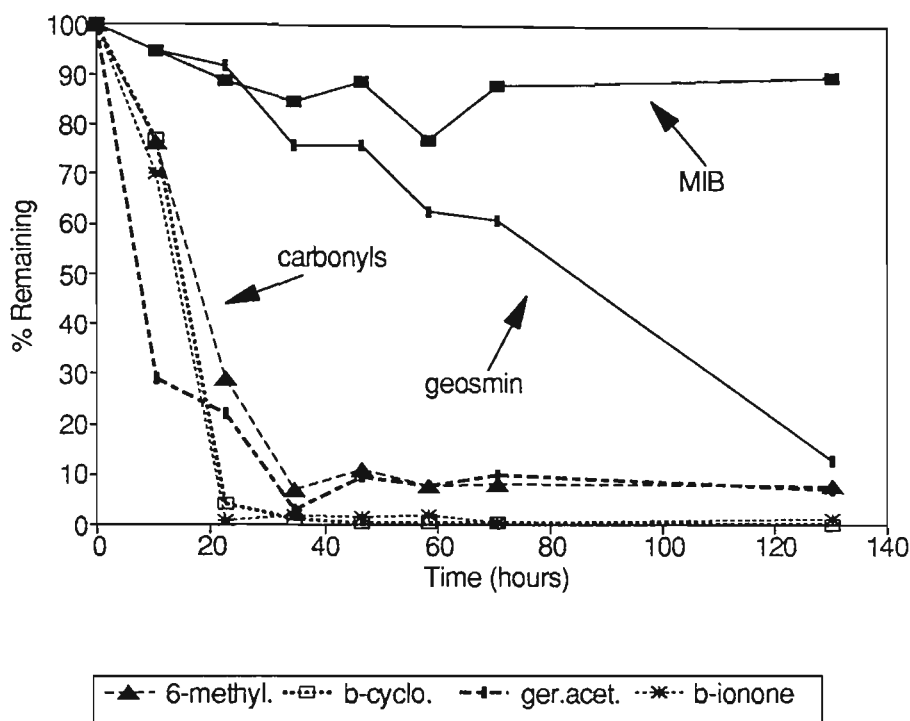


Figure 44

Biodegradation of carbonyl odour compounds using MIB- $d_3$  as internal standard.  
 (6-methyl. = 6-methylhept-5-en-2-one; b-cyclo. =  $\beta$ -cyclocitral;  
 ger.acet. = geranylacetone; b-ionone =  $\beta$ -ionone)



**Figure 45** Biodegradation of carbonyl odour compounds as well as MIB and geosmin (20°C).  
 (6-methyl. = 6-methylhept-5-en-2-one; b-cyclo. =  $\beta$ -cyclocitral;  
 ger.acet. = geranylacetone; b-ionone =  $\beta$ -ionone)

### 3.4.6.2 natural geosmin/MIB vs synthetic geosmin-d<sub>3</sub>/MIB-d<sub>3</sub>

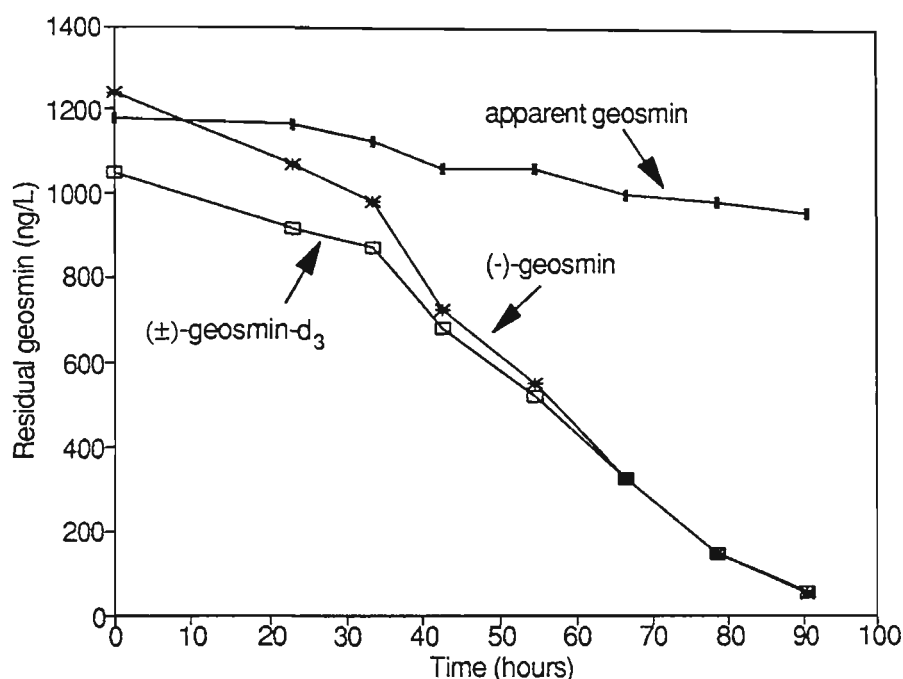
To be suitable as an internal standard that can be added in the field at the time of sampling, the rates of biological loss of the target compound and the internal standard must be identical. In the previous section Table 20 showed that MIB-d<sub>3</sub> could be added as an internal standard at the time of sampling. Because the synthetic compound was prepared from a chiral precursor ((+)-camphor), it was optically pure and had the same configuration (-) as MIB from natural sources (Wood and Snoeyink, 1977). Both materials were therefore biodegraded at the same rate. The synthetic geosmin-d<sub>3</sub> was racemic and, when added to a water sample spiked with synthetic geosmin, both

materials were biodegraded at the same rate. However, one cannot assume that the rates of biodegradation of (+) and (-)-geosmin are the same, and so an experimental study was made of the comparative rates of biodegradation of (-)-geosmin and ( $\pm$ )-geosmin- $d_3$ .

Geosmin from natural sources is (-) (Gerber and Lechevalier, 1965). An aqueous solution of (-)-geosmin from a culture of *Anabaena circinalis* (Bowmer *et al.*, 1992) was used to spike a Murrumbidgee River water sample, together with ( $\pm$ )-geosmin- $d_3$ . 1-chlorodecane was added as an internal standard at the time of analysis. The ratio of  $m/z$  185/182 (molecular ion geosmin- $d_3$ / molecular ion geosmin) increased slowly with storage time (by ca. 10% after 40 h). By measuring the residual concentration of (-)-geosmin and ( $\pm$ )-geosmin- $d_3$ , the residual concentration of (+)-geosmin- $d_3$  and (-)-geosmin- $d_3$  at each storage time could be calculated.

Fig. 46 shows that the (+) enantiomer is biodegraded at a slightly lower rate than the (-) enantiomer. The initial concentrations were: (-)-geosmin 1238 ng/L and ( $\pm$ )-geosmin- $d_3$  1049 ng/L (i.e. 525 ng/L of (+)-geosmin- $d_3$  and 525 ng/L (-)-geosmin- $d_3$ ). After 43 h the concentration of (-)-geosmin had fallen 41 %, so the concentration of (-)-geosmin- $d_3$  must also have fallen 41 %. Thus, the concentration of (-)-geosmin- $d_3$  after 43 h was calculated to be 309 ng/L (i.e.  $525 \times 0.59 = 309$ ). The measured concentration of ( $\pm$ )-geosmin- $d_3$  after 43 h was 683 ng/L which meant that the (+)-geosmin- $d_3$  concentration at 43 h was 374 ng/L (i.e.  $683 - 309 = 374$ ). This had the effect of increasing the ratio of  $m/z$  185/182 (and hence lowering the apparent initial concentration of (-)-geosmin in the water) with increasing storage times. However, such errors did not become serious until the geosmin concentration had been greatly reduced by biodegradation during

storage and hence did not preclude the addition of the racemic labelled standard at the time of sampling.



**Figure 46** Geosmin biodegradation in Murrumbidgee River water stored at room temperature under normal light conditions

Even if the concentration of natural geosmin was allowed to degrade to 5 % of the initial value an apparent concentration of 80 % of the initial value could still be obtained. If however the degradation was kept below 10 %, by storing the sample in the cold and in the dark, an apparent concentration greater than 90 % of the initial value was found (see Fig. 46). This source of error could potentially be avoided by using pure (-)-geosmin-d<sub>3</sub> as an internal standard (obtained by stereoselective synthesis or by formation and separation of diastereoisomers at a suitable point in the geosmin-d<sub>3</sub> synthesis). Alternatively, a chiral GC column could be used to resolve the (+) and (-) enantiomers of geosmin-d<sub>3</sub> (or even of unlabelled (±)-geosmin) (see section 3.5).

### 3.4.7 Air Leak Trial

Air leaks during a CLSA procedure can occur at any time and usually require the analytical run to be terminated because it is impossible ascertain the relative extent to which the compound of interest and the internal standard have been affected. When using unlabelled internal standards (e.g. 1-chlorodecane) the accuracy of the determination relies on the ability to reproduce the exact analytical conditions used when first calibrating the system for subsequent analyses of unknowns. Clearly if an air leak occurs, particularly early on in the stripping procedure, the previously calculated response factors do not apply and the run is usually terminated. However, when using labelled analogues of the compounds themselves as internal standards the analytical procedure does not have to be aborted as both analyte and internal standard are lost by leakage to the same extent.

To test this an air leak was deliberately introduced during each of five CLSA procedures by placing pieces of teflon tape, which were twisted into a cord, into the neck of the stripping bottle. Although the magnitude of the leak was not measured its severity was altered for each strip by varying the separation and thickness of the teflon cords (see section 2.10). As can be seen (Table 22) the reproducibility of the analytical procedure did not suffer, when determining MIB or geosmin, as a result of air leaks in the CLSA apparatus. This is very important, because it is often the case (and usually with the most important samples) that only sufficient sample is available for a single determination and if an air leak occurred during the stripping procedure the sample could not be re-analysed. However, using labelled internal standards meaningful data could still be obtained even if an air leak occurred as long as enough compound and



labelled internal standard was transferred to the carbon filter for detection.

**Table 22**                      Reproducibility of 5 CLSA determinations with introduced air leaks. Expected concentration (ng/L); MIB = 100, geosmin = 102

Air leak sample	Concentration Found		
	MIB	geosmin	
	m/z 168/171	m/z 182/185	m/z 112/115
1	105.8	98.6	101.7
2	100.9	102.1	100.1
3	101.3	95.8	98.1
4	101.8	104.6	101.1
5	105.6	98.6	96.7
Mean	103.8	100.0	99.5
% CV	2.3	3.4	2.1

**3.4.8                      Reproducibility of Injection Technique**

The variability of the injection technique (manual, air-sandwich hot-needle-fast) was evaluated by repeated injections (~1 µL) of the same sample. In order to avoid bias resulting from the injection of standards added to clean solvent (thereby obtaining an artificially low background) samples that had been previously stripped and extracted with CS<sub>2</sub> were used. The area ratios of MIB and geosmin vs the internal standard for each injection were recorded and a mean and % CV calculated for each. When labelled internal standards were used the ratio of the of the molecular ions of the unlabelled vs

the labelled analogue were used (i.e.  $m/z$  168/171 for MIB/MIB- $d_3$ ;  $m/z$  182/185 for geosmin/geosmin- $d_3$ ). For geosmin, the base peak ratio ( $m/z$  112/115) was used in addition. When chlorodecane was used as the internal standard, the ratios of the base peaks of both odour compounds (MIB  $m/z$  95, geosmin  $m/z$  112) versus the base peak of the internal standard (1-chlorodecane  $m/z$  91) were used.

**Table 23** Reproducibility of injection technique.

Inj. No.	Area Ratio: MIB and geosmin vs internal standard				
	$m/z$ 168/171	$m/z$ 112/115	$m/z$ 182/185	$m/z$ 95/91	$m/z$ 112/91
1	1.338	1.096	1.111	0.839	1.175
2	1.301	1.100	1.095	0.849	1.192
3	1.334	1.097	1.092	0.820	1.150
4	1.312	1.095	1.096	0.818	1.116
5	1.322	1.088	1.085	0.826	1.174
6	1.286	1.082	1.086	0.821	1.144
7	1.281	1.078	1.086	0.879	1.214
Mean	1.311	1.091	1.093	0.836	1.174
% CV	1.71	0.76	0.84	2.64	2.04

Table 23 shows the % coefficient of variance calculated for ratios using labelled internal standards were better, for both MIB and geosmin, than those obtained using 1-chlorodecane as the internal standard. However, the % variance for all area ratios were low and confirmed the fact that the major problem area in the CLSA procedure lies in the extraction and concentration steps before analysis rather than in the analytical

technique once the sample had been extracted.

### 3.4.9 Solvent Concentration Trial

The possibility of lowering the detection limit of the CLSA technique, without sacrificing precision or accuracy, by concentrating the final extract under a stream of nitrogen was investigated. Carbon disulphide (85  $\mu\text{L}$ ) was spiked with MIB (149.5 ng/ $\mu\text{L}$ ), geosmin (83.7 ng/ $\mu\text{L}$ ), MIB- $\text{d}_3$  (112.7 ng/ $\mu\text{L}$ ), geosmin- $\text{d}_3$  (119.6 ng/ $\mu\text{L}$ ) and 1-chlorodecane (102.0 ng/ $\mu\text{L}$ ). The solution was analysed five times and the area ratios of the following ions were recorded:  $m/z$  168/171 (MIB vs MIB- $\text{d}_3$ );  $m/z$  112/115 and  $m/z$  182/185 (geosmin vs geosmin- $\text{d}_3$ );  $m/z$  95/91 (MIB vs 1-chlorodecane) and  $m/z$  112/91 (geosmin vs 1-chlorodecane). Table 24(a) shows the initial area ratios of MIB and geosmin to the labelled internal standards as well as to 1-chlorodecane.

Although the volume of the initial extract was concentrated  $\sim 5$ -6 fold (85 to  $\sim 15$   $\mu\text{L}$ ) the area of each component only increased approximately 2.5-3 fold. This was most likely due to the fact that some of the volatile components were lost as the solvent was being evaporated. However, because the losses of labelled and unlabelled compounds were proportional at all levels no reduction in precision was observed. On the contrary the precision actually increased because the amount of each compound "on column" was now greater, making the peaks per component larger, and therefore the integration of their areas more precise (Table 24, b). This was particularly important for the molecular ions of MIB and geosmin as their abundance is less than 10 % of the base peaks ( $m/z$  95, MIB; and  $m/z$  112, geosmin) making integration at low levels more critical.

**Table 24**      Area ratios of five consecutive injections: (a) = before solvent concentration and (b) = after solvent concentration

(a)

Area Ratio vs Internal Standard					
Inj.	MIB			geosmin	
	m/z 168/171	m/z 95/91	m/z 182/185	m/z 112/115	m/z 112/91
1	1.364	1.701	0.709	0.781	0.679
2	1.343	1.789	0.689	0.734	0.704
3	1.344	1.743	0.704	0.732	0.684
4	1.317	1.675	0.716	0.740	0.682
5	1.281	1.770	0.684	0.741	0.679
Mean	1.330	1.736	0.700	0.746	0.686
% CV	2.4	2.7	1.9	2.7	1.5

(b)

Area Ratio vs Internal Standard					
Inj.	MIB			geosmin	
	m/z 168/171	m/z 95/91	m/z 182/185	m/z 112/115	m/z 112/91
1	1.323	1.724	0.695	0.781	0.707
2	1.323	1.679	0.695	0.734	0.724
3	1.322	1.712	0.686	0.730	0.698
4	1.346	1.731	0.678	0.732	0.717
5	1.371	1.808	0.692	0.746	0.750
Mean	1.337	1.731	0.689	0.730	0.719
% CV	1.6	2.7	1.1	0.9	2.8

The results for the labelled internal standards were expected but because of the large differences in volatility between the odour compounds and 1-chlorodecane, when being stripped from water during the CLSA procedure, it was anticipated that the chloroalkane would be lost at a greater rate than both MIB and geosmin. However, Table 24 (b)

shows that the difference due to evaporation was minimal. The similarity between the loss of 1-chlorodecane and MIB and geosmin during sample evaporation is most likely due to the extreme solubility of all compounds in the matrix ( $\text{CS}_2$  in this case as opposed to water in the CLSA procedure) and their relatively close boiling points ( $210^\circ\text{C}$  MIB,  $270^\circ\text{C}$  geosmin and  $223^\circ\text{C}$  1-chlorodecane).

To ascertain whether or not the difference in evaporation rates between the odour compounds and 1-chlorodecane would be greater if the sample was concentrated more than 5-6 fold, dichloromethane (100 mL) was spiked at the same concentrations as the  $\text{CS}_2$  in the first part of this trial and concentrated ~2000 fold (100 mL to ~50  $\mu\text{L}$ ) under a stream of nitrogen. The results obtained for duplicate injections of the sample concentrated 2000 fold were as follows:

	m/z 168/171	m/z 95/91	m/z 182/185	m/z 112/115	m/z 112/91
area ratio	1.304	1.759	0.728	0.765	0.725
Diff. (%) from Mean in Table 24 (a)	- 2.0	+ 1.3	+ 4.0	+ 2.5	+ 5.7

These area ratios were within 5 % of those obtained in Table 24(a) (with the exception of m/z 112/91) and suggest that solvent concentration was not a major contributor to inaccuracies of the technique once the sample had been extracted. The largest error was caused by the differences in physical properties between the compounds of interest and the internal standard during the CLSA extraction procedure.

### 3.5 Enantioselective GC for the Analysis of Geosmin and MIB

#### 3.5.1 Chiral Stationary Phases (CSP)

The earliest CSP's were based on amino acid derivatives (Frank *et al.*, 1977) or chiral metal complexes (Schurig and Nowotny, 1988) and these often showed enantioselectivity for only a single class of functional groups because they relied on chemical interaction between the analyte and the CSP, giving reversible diastereoisomer formation. A much more versatile CSP became available with the use of cyclodextrins, which operate primarily on shape recognition. In principle,  $\beta$ -cyclodextrin could resolve any racemic compound that can fit in the cavity, irrespective of the functional groups present.

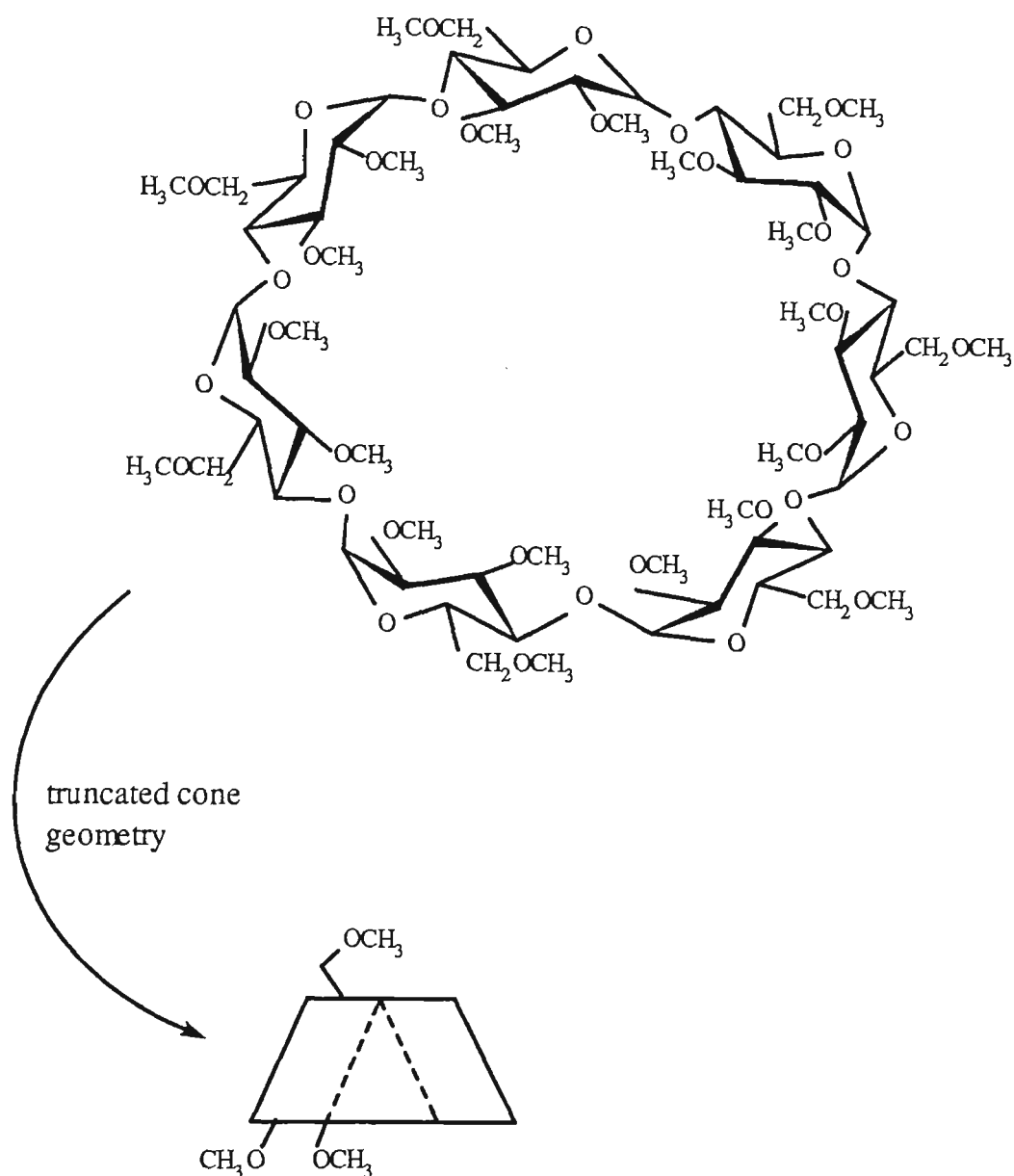
The cyclodextrins are macrocyclic compounds containing varying numbers of glucopyranose units giving an overall shape of a truncated cone. The  $\alpha$ ,  $\beta$  and  $\gamma$ -cyclodextrins contain 6, 7 and 8 glucopyranose units respectively. The structural arrangement for  $\beta$ -cyclodextrin is shown in Fig. 47. The interior of the cavity is relatively hydrophobic, being composed of methylene and 1,4-glucoside linkages which provide a high electron density for interaction with the analyte. The exterior faces are hydrophilic. The larger opening is surrounded by fixed 2<sup>o</sup> hydroxyls (with 2-hydroxyls rotated clockwise and 3-hydroxyls anticlockwise). The 1<sup>o</sup> hydroxyls are at the smaller end of the cone and can rotate.

Because of differences in chemical reactivity (the OH acidity decreases from the 6-position to 2- and 3- positions) some or all of the hydroxyl groups can be chemically modified by acetylation, methylation etc. in a stepwise fashion. This modifies the size of the internal cavity and/or the extent of H-bonding between the cyclodextrin and the

functional group on the asymmetric carbon of the analyte.

Modified cyclodextrins have been used as stationary phases in both HPLC and GC for the resolution of racemates and for many other separations e.g. structural isomers such as *o*, *m*, *p*-substituted benzenes, positional isomers of alkenes, PAH's by ring number etc.

For GC the first cyclodextrin for the resolution of optical isomers was by Koscielski *et al.*, 1983, who resolved  $\alpha$ -pinene on a packed column with a solution of  $\alpha$ -cyclodextrin in formamide. In 1987 Juvancz *et al.* coated a glass capillary column with molten permethylated  $\beta$ -cyclodextrin while Schurig and Nowotny (1988) dissolved the permethylated  $\beta$ -cyclodextrin in OV-1701 silicone. Schurig *et al.* (1990) and Fischer *et al.* (1990) immobilised a permethylated  $\beta$ -cyclodextrin by chemical binding to a polydimethylsiloxane to achieve inertness and good thermal stability. The melting point of cyclodextrins can be lowered by introducing longer chain, hydrophobic groups via esterification and thereby eliminating the need for solvent e.g. dipentylacetyl cyclodextrins (König *et al.*, 1988), dipentyl butyryl  $\gamma$ -cyclodextrin (König *et al.*, 1989), tripentyl  $\alpha$ - and  $\beta$ -cyclodextrin, dipentylated methyl-, butyryl- and acetyl-  $\alpha$ ,  $\beta$  and  $\gamma$ -cyclodextrins (Wenz *et al.*, 1990). Keim *et al.* (1991) found permethylated  $\beta$ -cyclodextrin in polysiloxane to have the widest applicability, showing excellent chiral selectivity to compounds as diverse as hydrocarbons, bicyclic acetals, cyclic ethers, cyclic carbonates, lactones, esters, ketones, aldehydes, alcohols, halocarbons and some N-compounds.



**Figure 47** Heptakis(2,3,6-tri-*O*-methyl)β-cyclodextrin Schurig *et al.*, (1990) and overall geometric shape König *et al.*, (1990)

Mosandl *et al.*, (1990 a,b) reported the use of β-cyclodextrin chiral phases for the separation of a large number of stereoisomeric flavour compounds such as 2-alkylated carboxylic acids and their corresponding 2-alkylated alkanols, ethyl 2-methylbutanoate,



1,2 (1,3)-ketones and 3-hydroxy carboxylic esters as well as four furanoid linalool oxides and chiral 3[2H]-furanones. Direct chiral evaluation of compounds from complex flavour matrices was achieved by using multidimensional gas chromatography (MDGC) employing "heart-cutting" techniques from a suitable nonchiral precolumn (e.g. carbowax 20M) on to a permethylated  $\beta$ -cyclodextrin enantioselective column.

Mono and sesquiterpenes were successfully separated using  $\beta$ -cyclodextrin capillary columns by Takeoka *et al.*, (1990). They reported for the first time the separation of  $\alpha$ -copaene (a fruit fly attractant) and  $\delta$ -elemene which although it contained two asymmetric carbons was optically inactive because it existed as a racemic mixture.

Lindström *et al.*, 1990 reported an interesting observation while attempting to obtain a reliable technique for the enantioselective separation of monoterpenes. In their hands the separation of the two enantiomeric pairs of  $\alpha$ - and  $\beta$ -pinene at 35<sup>o</sup> with dry helium (according to the method of Koscielski *et al.*, (1983)) was not reproducible. Retention times fluctuated and peak shape deteriorated with time while the selectivity factor  $\alpha$  was rapidly reduced from 2.2 to 1.5 (for  $\alpha$ -pinene). They attributed this behaviour to the dehydration of the stationary phase matrix due to the dry carrier gas. The situation was rectified by wetting the carrier gas stream with water. This produced stable enantioselectivity and improved the life of the column. They noted that the performance of the  $\alpha$ -cyclodextrin columns might be improved if the organic liquid of the stationary phase could be changed from formamide possibly to triethanolamine.

### 3.5.2 Performance Indicators

There are two parameters ( $\alpha$  and  $R$ ) commonly used to quantify the performance of a CSP:

- i) the separation factor  $\alpha$ , where  $\alpha$  = retention time of more retained peak/retention time of less retained peak. However, this is not very satisfactory as peak separation can be insufficient even at large  $\alpha$  values. Moreover, for a given separation,  $\alpha$  depends on the absolute retention time i.e.  $\alpha$  decreases as  $\Delta t$  increases.
- ii) a better measure of efficiency is the peak resolution factor  $R$ , where  $R$  = distance between peak maxima/sum of half widths of peaks.

### 3.5.3 Structure Resolvability Correlations

Only limited progress has been achieved so far in the development of structure-resolvability correlations and in being able to predict a suitable chiral phase for a given racemic compound. Enantioselective inclusion in the cavity of  $\beta$ -cyclodextrin is very sensitive to slight variations in structure of analyte e.g. in the separation of chiral long-chain acetates, molecules with the chiral carbon near the centre of the chain were more difficult to separate than those where the acetyl group was near the end of the chain (Keim *et al.*, 1991). With acyl-substituted cyclodextrins, the position of the acyl group is critical. (3-*O*-acetyl-2,6-di-*O*-methyl-) $\beta$ -cyclodextrin gave good separation of polar racemates but if the 6-OH was acetylated, almost total loss of enantioselectivity results (König *et al.*, 1990). Keim *et al.*, (1991) showed that mandelic acid methyl and ethyl esters were well-resolved by the small permethyl- $\alpha$ -cyclodextrin cavity, whereas tartaric

acid diisopropyl ester was better separated by the large cavity permethyl- $\beta$ -cyclodextrin. Thus "host-guest complexation between cyclodextrin derivative and the chiral volatile cannot be the only mechanism of enantiomer discrimination". Takeoka *et al.* (1990) also pointed out that enantioselectivity is affected by subtle changes in constituent structure. Thus they were able to achieve separation of ethyl 2-methylbutanoate but propyl 2-methylbutanoate was not separated and 2-methylbutyl acetate only slightly. At this time it is clear that there are many exceptions to any rules and the selection of a chiral phase is still very much a matter of trial and error.

The scope of enantiomer separation on diluted permethylated  $\beta$ -cyclodextrin (phase I) was extended by Nowotny *et al.*, (1989) by using a novel 2,6-dimethyl-3-trifluoroacetyl  $\beta$ -cyclodextrin (phase II). The trifluoroacetyl group, which can form hydrogen bonds via the fluorine atoms and exert dipole-dipole interaction via the carbonyl group, was introduced to provide additional selectivity at the polar entrance to the cyclodextrin cavity. The two phases were used in a complementary fashion for the analysis of over 30 underivatised volatile (predominantly cyclic) chiral compounds. They observed that although some compounds were resolved by both phases, albeit to a different extent, other compounds were either not resolved on phase I but baseline resolved on phase II or vice versa. Surprisingly, no common trends, such as apolar compounds being separated preferentially on phase I and polar compounds on phase II, were observed. The authors attributed this to the fact that the trifluoroacetyl group not only affects polarity but also changes the shape of the entrance to the cyclodextrin cavity and that these factors would influence enantiomer separation in an unpredictable way. They suggested that in some instances the different contributions to chiral recognition could

oppose each other with a resulting loss of chiral separation.

### 3.5.4 Laboratory Trials with Geosmin and MIB

The recent availability of enantioselective capillary columns based on permethylated cyclodextrin and the ability to obtain baseline-resolved peaks of the two enantiomers of a natural product raised the possibility of quantification by standard addition of either the racemate or (better) the opposite enantiomer to the natural product. In the case of MIB and geosmin because both compounds exist in nature as the (-) enantiomers the potential use of the corresponding (+) enantiomers for quantification was an attractive option as GC-FID only would be required as opposed to the more expensive GC-MSD system required when using the labelled compounds as internal standards.

(+)-MIB could be synthesised from (-)-camphor and (±)-MIB from (±)-camphor using the same techniques outlined earlier for the synthesis of (-)-MIB or (-)-MIB- $d_3$  from (+)-camphor. Optically pure (+)-geosmin could be synthesised by the method of Revial (1989) who reacted the chiral amine (*R*)-(+)- $\alpha$ -methylbenzylamine with (±)-2-methylcyclohexanone to obtain an imine. The imine was stereoselectively condensed with ethylvinylketone using sodium methoxide as a catalyst to form chiral 1,10-dimethyldecal-(9)-en-2-one. The chiral ketone was converted to (+)-geosmin by the method of Gosselin *et al.*, (1989).

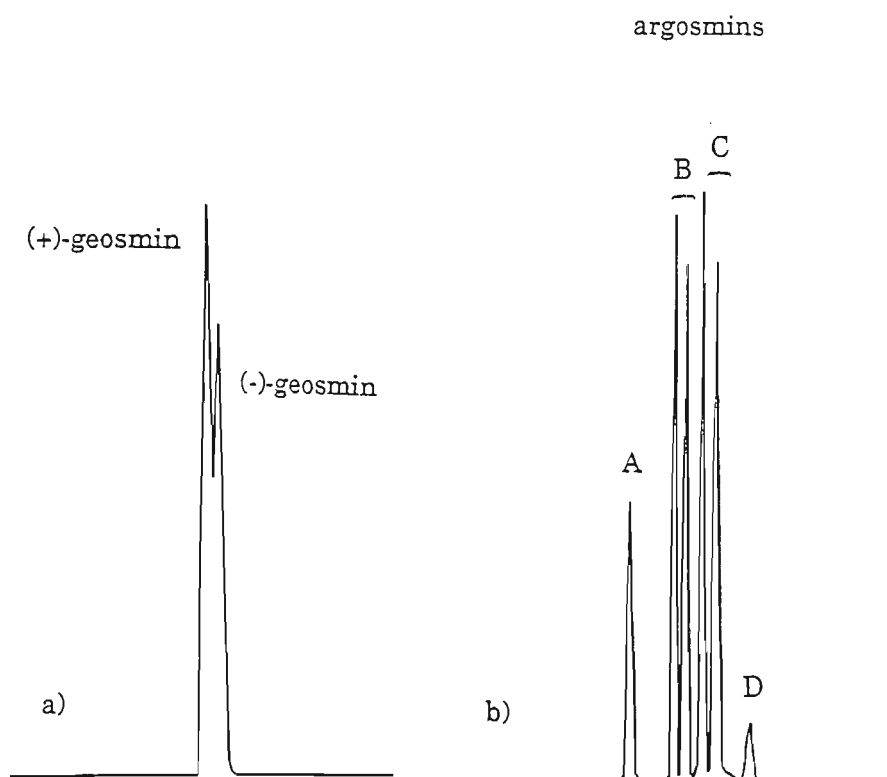
To establish the feasibility of using the (+) enantiomers of MIB or geosmin added either in pure form or as part of a racemic mixture it was necessary to achieve baseline

resolution of the two enantiomers on a suitable analytical column. The first column tried, without success, was a permethylated  $\beta$ -cyclodextrin based column (Cyclodex-B, J&W). ( $\pm$ )-Geosmin was poorly resolved (Fig. 48, a) whereas ( $\pm$ )-MIB was not resolved at all. However, when geosmin was acidified to produce a series of argosmins (dehydration products of geosmin) baseline resolution of two of the argosmins was observed (Fig. 48, b).

Interestingly, only two of the four argosmins formed (argosmin B and C) were separated whereas the remaining two argosmins were not resolved at all. It is difficult to believe that the two argosmins which were not resolved had lost their optical activity as the asymmetric carbon at the ring junction at C<sub>10</sub> would remain intact after the dehydration process. It is more likely due to subtle differences in the shape of the argosmin A compared with argosmin B. The fact that subtle differences in molecular shape can have a large bearing on the ability of the cyclodextrin column to resolve enantiomers is evidenced by the poor separation of the geosmin enantiomers and the baseline resolved argosmin enantiomers which were run under identical GC conditions.

A chiral column being developed by SGE, Australia, and not commercially available at the time of our earlier experiments, was donated to us by SGE for the purpose of evaluating its ability to separate the MIB and geosmin enantiomers. Because this column was also a permethylated  $\beta$ -cyclodextrin column (like the J&W column) it was anticipated that the performance with respect to separation of the MIB and geosmin enantiomers would be similar. However, although ( $\pm$ )-MIB was not resolved, baseline resolution of ( $\pm$ )-geosmin was achieved on the 50 m column, albeit at retention times

in excess of fifty min (see Fig 49). The retention times were reduced and the performance indicators  $\alpha$  and R increased when ( $\pm$ )-geosmin was analysed by GC-FID using hydrogen as the carrier gas (Table 25). The order in which the individual enantiomers eluted from the column was established by co-injection of the optically pure natural (-)-geosmin enantiomer with the synthetic racemic mixture.

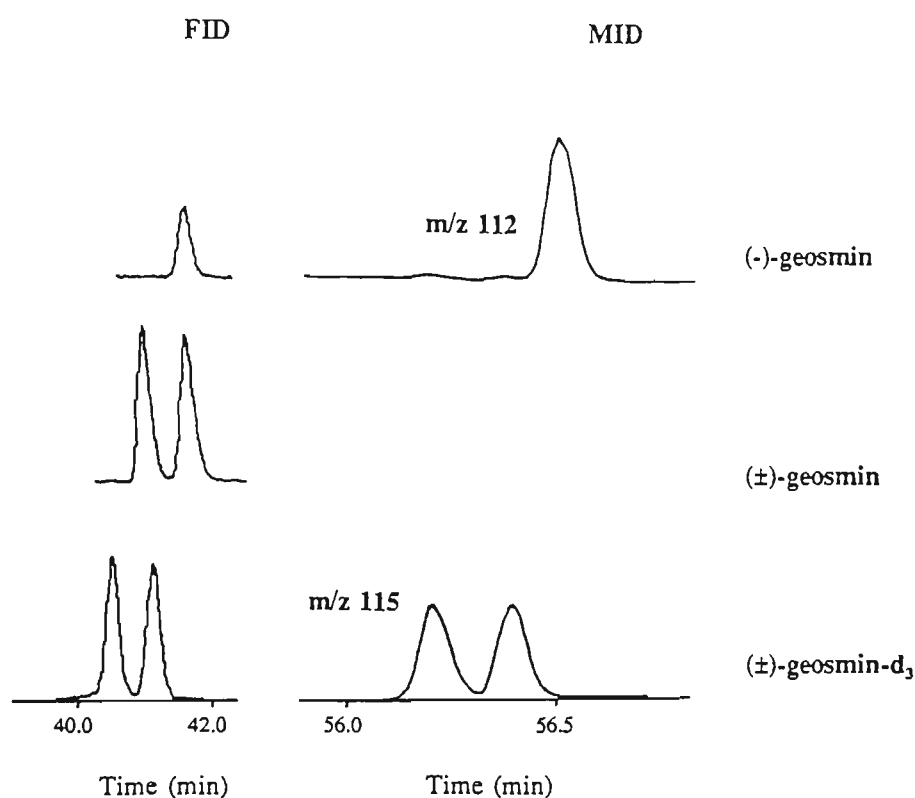


**Figure 48** Enantiomeric separation of (a) ( $\pm$ )-geosmin ( $R_t$  = ~41 min) and (b) ( $\pm$ )-argosmins ( $R_t$  = ~20 min) analysed on the J&W column Cyclodex-B. Argosmin  $\alpha$  = 1.01,  $R$  = 2.0

**Table 25** Performance indicators  $\alpha$  and R calculate for the separation of (-) and (+) geosmin using hydrogen and helium carrier gas

Compound	Carrier gas	Retention time (min)		$\alpha$	R
		(+)	(-)		
( $\pm$ )-geosmin	helium	56.212	56.404	1.004	1.25
( $\pm$ )-geosmin	hydrogen	40.869	41.434	1.014	1.38

As was expected ( $\pm$ )-geosmin- $d_3$  gave identical  $\alpha$  and R values when analysed under the same conditions as the unlabelled racemate. Surprisingly however, separation between both labelled enantiomers and (-)-geosmin was also achieved. The order of elution using the SGE column was (+)-geosmin- $d_3$ , {(-)-geosmin- $d_3$  and (+)-geosmin} and (-)-geosmin (Fig. 49).



**Figure 49** Enantioselective GC of (-)-geosmin, and (±)-geosmin- $d_3$  using FID and MID detection

### 3.5.5 Analytical Application for Geosmin in Water

The ability to obtain baseline resolved peaks of the two enantiomers of geosmin as well as geosmin- $d_3$  was used to quantify (-)-geosmin (the enantiomer from natural sources) in surface water using commercially available (±)-geosmin or (±)-geosmin- $d_3$  as internal standards, added either at the time of stripping or the time of sampling of the water.

Quantification was performed by CLSA followed by gas chromatography with flame ionisation detection (GC-FID) or GC-MS with multiple ion detection (GC-MID) (see Materials and Methods). Table 26 shows the results obtained for the determination of (-)-geosmin in water, using standard addition of (±)-geosmin (both labelled and unlabelled).

**Table 26** Determination of (-)-geosmin in Murrumbidgee river water by enantioselective GC using (±)-geosmin or (±)-geosmin-d<sub>3</sub> as internal standards

Internal standard	Concentration present (ng/L)	Concentration detected (ng/L)		
		GC-FID	GC-MID <sup>#</sup>	
			A <sup>!</sup>	B <sup>*</sup>
(±)-geosmin	16.3	15.9 (4.6)	15.4 (8.4)	14.0 (2.1)
(±)-geosmin-d <sub>3</sub>	16.3	19.7 (1.2)	15.5 (1.7)	16.5 (1.4)
(±)-geosmin	126	125 (9.2)	127 (3.6)	121 (3.4)
(±)-geosmin-d <sub>3</sub>	126	152 (1.7)	118 (2.6)	123 (2.3)

# numbers in parenthesis = % coefficient of variance (n=5)

! obtained using molecular ions m/z 182 and m/z 185

\* obtained using base peaks m/z 112 and m/z 115

Concentrations of geosmin added to river water were determined by CLSA followed by (a) enantioselective GC with FID detection or (b) enantioselective GC with MID detection. The analytical data obtained can be compared with those obtained using deuterated internal standards and a non-chiral column (see section 3.4.6.2). In both (a) and (b) above the internal standard was added at the time of stripping. Somewhat better results would be expected for the GC-FID technique if pure (+)-geosmin (rather than (±)-geosmin) were used as internal standard. (+)-geosmin can be synthesised from a



chiral imine as described earlier (Fig. 9).

When detection is by FID alone, there is a greater possibility that the analyte peak can be inflated by overlap with other components of the sample. This was the case with a sample of Murrumbidgee river water (previously shown to be free of geosmin) and dosed with (-)-geosmin derived from a culture of *Anabaena circinalis* (Bowmer *et al.*, 1992) (Table 26). Significant interference was found from another volatile organic component of the water, derived from natural sources. The more contaminated the surface water (from domestic and industrial sources) the greater the risk of accidental coelution.

In earlier work indirect means were used to determine the relative rates of biodegradation of (-) and (+) geosmin in stored water samples (3.4.6). Use of a chiral column enables these to be determined directly from the relative peak areas of the two enantiomers. The ratio does not, of course, indicate the absolute concentrations remaining after selected storage times, but the latter can be determined by adding either 1-chlorodecane or, better, a deuterium labelled standard at the time of stripping followed by GC-MID. Experiments using enantioselective GC to follow the biodegradation of (-)-geosmin from *Anabaena circinalis* (Bowmer *et al.*, 1992) and (+)-geosmin from (±)-geosmin confirmed our earlier findings (section 3.4.6.2) that the two enantiomers degrade at different rates. The (-) enantiomer is biodegraded more rapidly than the (+) enantiomer and consequently using (+)-geosmin as an internal standard to calculate the apparent initial concentration of (-)-geosmin is only valid at time 0 (i.e. before biodegradation has commenced). Once biodegradation has commenced, using (+)-

geosmin as an internal standard results in an underestimation of the apparent initial concentration. This underestimation becomes more pronounced the longer the degradation is allowed to proceed.

Better results were obtained when a deuterium-labelled internal standard was used with FID detection. Table 27 (column 3) shows the results from enantioselective GC-FID with ( $\pm$ )-geosmin- $d_3$  being added at the time of sampling. Peaks elute in the order (+)-geosmin- $d_3$ , {(-)-geosmin- $d_3$  and (+)-geosmin}, (-)-geosmin (Fig. 49). Since (+)-geosmin is not produced in nature, one can readily determine the ratio (-)-geosmin/(-)-geosmin- $d_3$ . As both labelled and unlabelled (-)-geosmin degrade at the same rate, and are chromatographically resolved, it is possible to follow the biodegradation of natural (-)-geosmin using (-)-geosmin- $d_3$  as an internal standard. Determination by FID has not been possible with achiral columns, because the deuterated and unlabelled compounds are only slightly resolved, with the deuterated compound giving a slight shoulder to the leading edge of the peak of the unlabelled compound.

In Table 27 (column 3), where detection is by FID alone, the apparent initial concentration of (-)-geosmin, measured after short storage times, are inflated by co-eluting peak of another organic compound. This compound was evidently biodegraded very rapidly, because after four days storage, the apparent initial concentration was 97 % of the concentration added originally. On further storage the apparent initial concentration rose again (Table 27, column 3), presumably owing to a co-eluting microbial metabolite. Problems of co-eluting metabolites are minimised by using MID instead of FID detection (Table 27, columns 4 and 5). Here, very accurate

determination of initial concentration was possible (using  $m/z$  ratios 112/115 and 182/185), even when as little as 2 % of the initial 829 ng/L remained after prolonged storage (192 h).

**Table 27** Use of enantioselective GC with FID and MID detection to follow the biodegradation of (-)-geosmin in stored water samples.

Storage time (h)	Storage temperature (°C)	Apparent initial concentration of (-)- geosmin (ng/L) <sup>a)</sup>		
		GC-FID	GC-MID	
			A <sup>b)</sup>	B <sup>c)</sup>
0	22	987	829	843
24	22	1036	825	800
72	22	910	835	845
96	22	811	849	825
120	22	934	850	867
144	30	994	856	859
192	30	870	870	ND

a) all concentrations were determined by adding ( $\pm$ )-geosmin- $d_3$  at the beginning of the storage period and measuring the peak area ratio (-)-geosmin/(-)-geosmin- $d_3$  after each time interval

b) determined using base peaks  $m/z$  112/115

c) determined using molecular ions  $m/z$  182/185

ND not detected

Over the whole storage period, the mean apparent recovery of geosmin was  $102 \pm 2$  %. Thus, it is now possible to determine accurately the concentration of (-)-geosmin which was present in the water at the time of sampling, without the need to either analyse immediately or to preserve the water by storage at 4°C or by the addition of mercuric chloride.

In principle, it should be possible to determine natural MIB (the (-) enantiomer) by enantioselective GC using (+)-MIB as the internal standard. However, in our laboratory only partial separation of the two MIB enantiomers has been achieved.

Enantioselective GC enables the use, for the first time, of (+)-geosmin (applied via commercially available (±)-geosmin) as an internal standard for the determination of (-)-geosmin, with detection either by FID or MID. The latter reduces the risk of errors created by peak overlap. These risks are reduced even further by using a deuterium-labelled geosmin as the internal standard. The latter has the further advantage that it may be added at the time of sampling rather than the time of analysis, when the (-)-labelled standard compensates perfectly for loss of natural (-)-geosmin by biodegradation, volatilisation etc. during sample storage and transport. When a labelled internal standard is used, the chiral column offers the further advantage that the complete separation of (-)-geosmin and (-)-geosmin- $d_3$  means there is no need to correct mathematically for the effect of satellite peaks on the  $m/z$  112/115 ratio. The main disadvantage of enantioselective GC is the longer retention times arising from the use of a 50 m column. However, recently similar resolution of (±)-geosmin was achieved using a shorter version of the same column and reduced temperatures (see 3.5.6).

The order of precision and accuracy for the various techniques is: enantioselective GC-MID (labelled (±) standard) > GC-MID (labelled (±) standard) > enantioselective GC-FID (unlabelled (±) standard) > enantioselective GC-MID (labelled (±) standard). Enantioselective GC should, where the racemate is easily synthesised or is commercially available, be readily applicable to the determination of other chiral volatile odour

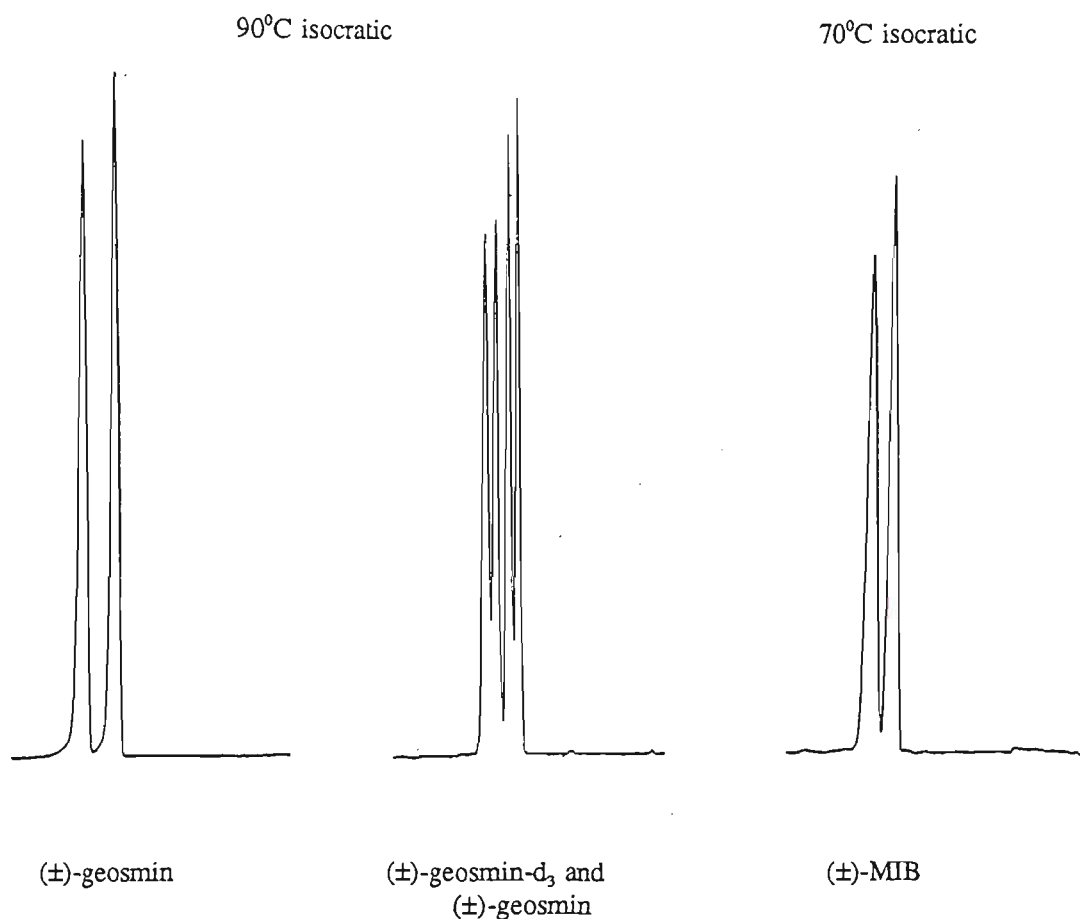
compounds found in water.

### 3.5.6 Recent Improvements in Enantioselective GC of MIB and Geosmin

In 1991 Keim *et al.* (Aachen, Germany) reported a comparison of five different cyclodextrins with 250 racemic compounds. Given their baseline resolution of camphor (which was not achieved in our laboratory), and the structural similarity of camphor and MIB, a decision was made to send the following samples to Aachen: ( $\pm$ )-camphor, ( $\pm$ )-MIB, (-)-MIB- $d_3$ , ( $\pm$ )-geosmin and ( $\pm$ )-geosmin- $d_3$ . The main aim was to achieve baseline resolution of MIB and, it was hoped, baseline resolution of both MIB and geosmin in a single chromatographic run. Dr. Meltzow at Aachen tried five different cyclodextrins but only trimethyl- $\beta$ -cyclodextrin was successful with MIB. Camphor was not resolved, but ( $\pm$ )-geosmin and ( $\pm$ )-geosmin- $d_3$ , respectively, were both baseline resolved. However, a mixture of the labelled and unlabelled geosmin racemates gave partially overlapping peaks, as was the case with the SGE column. (-)-MIB could not be resolved from (-)-MIB- $d_3$ . The separations achieved in their laboratory for ( $\pm$ )-MIB, ( $\pm$ )-geosmin and a mixture of ( $\pm$ )-geosmin and ( $\pm$ )-geosmin- $d_3$  are shown in (Fig. 50).

Both the J&W and the SGE columns use cyclodex-B, which is a permethylated  $\beta$ -cyclodextrin like that used in the successful resolution of MIB in Aachen. The different performance is the more surprising given they used only a 25 m column whereas the SGE column was 50 m in length. The key difference in formulation of the stationary phase may be that the Aachen group's column has the cyclodextrin dispersed in polysiloxane. Keim *et al.*, (1991) have noted that this gives better performance (resolution, reproducibility and thermal stability) than neat permethylated cyclodextrin.

The formulation of the J & W and SGE columns is proprietary, so this possible explanation for the difference in performance could not be explored further.



**Figure 50** Chromatograms showing resolution of (±)-MIB ( $R_t = \sim 34$  min), (±)-geosmin and a mixture of (±)-geosmin and (±)-geosmin-d<sub>3</sub> ( $R_t = \sim 32$  min) analysed on the same  $\beta$ -cyclodextrin column (courtesy of Dr. Meltzow, Aachen)

Recently, Lindström (1991) improved enantiomer separation with permethylated  $\beta$ -cyclodextrin columns dramatically using very short columns. Separations as good or better than those obtained on 50 m columns were obtained on columns as short as 2 m. The authors noted that for enantiomer separation the longest columns do not necessarily provide the best separation and that the separation factor  $\alpha$  was very dependent on

temperature. Thus using shorter columns, at reduced temperature, efficient separation of larger more polar compounds was achieved. Because extended retention times was one of the drawbacks when using the SGE 50 m column for the separation of ( $\pm$ )-geosmin it was decided to try a shorter version of this column, at reduced temperatures, in an effort to reduce the overall retention time of the enantiomeric separation.

Approximately 3 m was broken off the 50 m SGE column and connected, as normal, to a GC-FID system using hydrogen as the carrier gas. Several oven temperature and carrier gas flow regimes were tried until baseline resolution of ( $\pm$ )-geosmin was achieved. The optimum parameters for baseline resolution were; carrier gas back pressure  $\sim$  5 psi with the column held at 70°C for 15 min.

The retention times for complete separation of both enantiomers was reduced from  $\sim$  40 min to  $\sim$  14 min with comparable performance indicators ( $\alpha$  = 1.050 and  $R$  = 1.14). Unfortunately, separation between ( $\pm$ )-geosmin- $d_3$  and (-)-geosmin, as was achieved using the 50 m SGE column, was not possible. In principle, the short column could be used for the quantification of (-)-geosmin using either ( $\pm$ )-geosmin or ( $\pm$ )-geosmin- $d_3$  as internal standards although, greater accuracy would be expected if optically pure (+)-geosmin was used with GC-FID or ( $\pm$ )-geosmin- $d_3$  with GC-MID. It is likely that a technique similar to that described by Monsadl *et al.*, (1990 a,b) involving "heart cutting" of the geosmin peak from a suitable non-chiral pre-column would be required when analysing extremely complex matrices.

## 4 CONCLUSION

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The present study achieved the synthesis of deuterium labelled analogues of MIB and geosmin and has evaluated them extensively as internal standards in the CLSA/GC/MS determination of MIB and geosmin in surface waters. Compared with the conventional internal standards (1-chloroalkanes) the labelled compounds offer many advantages. They offer greater precision and accuracy at, or below, the threshold odour concentration of both MIB and geosmin and compensate for variations in sparging rate and other parameters of the CLSA procedure. Much time is saved by not having to determine response factors regularly and to analyse unknowns with and without spikes where an accurate determination of concentration is required.

The stability of MIB, geosmin, 1-chlorooctane, 1-chlorodecane and 1-chlorododecane was tested in six commonly used laboratory solvents. All compounds may be stored as dilute solutions in acetone, methanol, ethanol, hexane and carbon disulphide at either room temperature (in amber vials) or at - 15°C for periods of up to two years without decomposition. In dichloromethane (for the same storage period i.e. two years) all compounds were stable stored at - 15°C and all except MIB were stable stored at room temperature. MIB was found to decompose in dichloromethane stored at room temperature due to acid catalysed dehydration in the presence of HCl formed by the photolytic breakdown of dichloromethane.

Standard solutions of MIB-d<sub>3</sub> and/or geosmin-d<sub>3</sub> in acetone can be added to environmental samples at the time of stripping to determine the concentration of other



volatile compounds present in the water. The labelled standards when used in this way provide results comparable with those obtained using chloroalkanes as internal standards. They can be added at the time of sampling and will compensate for loss of MIB and geosmin due to physical, chemical or biological means during sample transport and storage. Because MIB- $d_3$  is optically pure and the same enantiomer as the natural compound (i.e. (-)) its biodegradation is identical to that of the natural compound and so will compensate perfectly for any losses due to biological processes. On the other hand geosmin- $d_3$  is racemic and because a slight biological discrimination against the (+) enantiomer is evident in our waters an underestimation of initial concentration of natural (-)-geosmin results when using ( $\pm$ )-geosmin- $d_3$  as the internal standard added at the time of sampling. However, the underestimation, which is not significant unless the sample is allowed to biodegrade substantially, may be overcome using enantioselective gas chromatography.

Enantioselective GC enables the use, for the first time, of (+)-geosmin (applied via commercial ( $\pm$ )-geosmin) as the internal standard for the determination of (-)-geosmin, with detection by either FID or MID. The latter reduces the risk of errors created by peak overlap. These risks are reduced even further by using deuterium-labelled geosmin as the internal standard. The latter has the further advantage that it may be added at the time of sampling rather than at the time of analysis, when the (-)-labelled standard compensates perfectly for losses of natural (-)-geosmin by biodegradation, volatilisation etc. during sample storage and transport. When a labelled standard is used the chiral column offers an added advantage since the complete separation of (-)-geosmin and (-)-geosmin- $d_3$  means that there is no need to correct mathematically for the effect of

satellite peaks on the  $m/z$  112/115 ratio. The main disadvantage of enantioselective GC (i.e. long retention times) may be overcome by using short  $\beta$ -cyclodextrin columns at reduced temperatures.

The order of precision and accuracy for the various techniques is : GC-MID (labelled ( $\pm$ )-standard) > GC-MID (labelled ( $\pm$ )-standard) > enantioselective GC-FID (unlabelled ( $\pm$ )-standard) > enantioselective GC-FID (labelled ( $\pm$ )-standard). Where the racemate is easily synthesised or is commercially available enantioselective GC should be readily applicable to the determination of other chiral volatile odour compounds found in water.

Although MIB and geosmin impart an earthy/musty taste and odour to drinking water, have extremely low odour thresholds and are resistant to normal water treatment processes (e.g. chlorination) they are useful as sensitive indicators for predicting the onset of algal blooms. Because the labelled internal standards, with their inherent high precision and accuracy, may be used to analytically detect both compounds at very low concentrations ( $\sim 1$  ng/L) the presence of potential bloom-forming organisms may be established well before they are discernible by standard visual techniques (Bowmer *et al.*, 1992). Management strategies can therefore be implemented while the concentration of either or both compounds is still low enough to make an early treatment option viable (McGuire *et al.*, 1983). In this way a potential bloom may be treated before it becomes unmanageable and any effect due to release of cell metabolites, which may include volatile odour compounds as well as algal toxins, is minimised.

The labelled compounds should also make excellent internal standards for the determination of MIB and geosmin in other matrices in which they are found (e.g. foods and beverages). Their identical physical and chemical behaviour during procedures employed to isolate and concentrate them means that precision and accuracy of the determination is maximised.

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