The Development of Methods for the Selective Capture and Characterisation of Fragrances and Flavours from Nature.

by

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CONTENTS

List of Tables

List of Figures

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Declaration

I hereby declare that this thesis is my own work and that, to the best of my knowledge and belief, it contains no materials previously published or written by another person, nor materials which to a substantial extent has been accepted for the award of any other degree or diploma of a university or other institute of higher learning, except where due acknowledgement is made in the text.

Julia Jackson

Summary

The chemical senses of taste and smell are the two least well understood of our senses. Recent advances in our understanding of the genetic and molecular mechanisms have led to increasing interest in olfaction and gustation. Amongst the practical applications of fragrances and flavours are commercial consumer products. The primary source for inspiration for these is nature and the work discussed in this thesis addresses methods for isolating selected aroma and taste molecules from natural sources, for use as new ingredients in food and fragrance applications.

The methods are designed to deal with the challenges of isolating and identifying species present at very low concentrations (as low as parts per trillion), of unstable nature and with the desire to target specific functional groups. Carbonyl and thiol compounds have been selected for trapping due to their interesting fragrance and flavour characteristics respectively.

A portable reactive trap has been designed, built, and tested to isolate aldehyde (or ketone) compounds from the headspace of living plants. The trap uses the derivatising reagent, *O*-(2,3,4,5,6-pentafluorobenzyl)hydroxylamine hydrochloride coated on to a solid sorbent, Tenax TA™. Reagents with immobilised reactive groups capable of selective reacting with thiol compounds have been investigated and the proof of principle has been illustrated for three different methods.

The nature identical status of the cooling compound, L-monomenthyl succinate, has been demonstrated by using highly sensitive and selective analytical techniques to identify this compound in the berries and leaves of plant *Lycium barbarum*. A preparative liquid chromatography system was used to simplify plant extracts which were subsequently analysed using nano liquid chromatography-electrospray ionisation-tandem mass spectrometry (nLC-ESI-MS/MS). This system could be applied to the analysis of other natural source extracts.

Abbreviations

A	
APCI	Atmospheric pressure chemical ionisation
ATP	Adenosine 5'-triphosphate
ATR	Attenuated total reflectance
B	
BIPM	N-[4-(2-Benzimidazolyl) phenyl]-maleimide
B_v	Breakthrough volume
C	
cAMP	3'-5'-cyclic adenosine monophosphate
CI	Chemical ionisation
D	
Da	Daltons
DACM	N-(7-Dimethylamino-4-methyl-2-oxo-3-chromenyl)maleimide
DBMP	2,6-Ditertbutyl-4-methylpyridine
DBPM	N-[4-(6-Dimethylamino-2-benzofuranyl)phenyl]maleimide
DBU	1,8-Diazabicyclo[5.4.0]undec-7-ene
DCM	Dichloromethane
DNA	Deoxyribonucleic acid
DNPH	2,4-Dinitrophenylhydrazine
DNSH	Dansylhydrazine
DTD	Direct thermal desorption
DTNB	5,5'-Dithiobis(2-nitrobenzoic acid)
DTT	Dithiothreitol
E	
ECD	Electron capture detection
EI	Electron impact or electron ionisation
EPA	Environmental Protection Agency
ESI	Electrospray ionisation
EWG	Electron-withdrawing group
F	
FEMA	Flavour and Extract Manufacturers' Association

FID	Flame ionisation detector
FPA	Focal plane array
FPD	Flame photometric detector
FTICR	Fourier transform ion cyclotron resonance
FTIR	Fourier transform infrared
G	
GC	Gas chromatography
GC-FTIR	Gas chromatography-Fourier transform infrared
GC-MS	Gas chromatography-mass spectrometry
GC-O	Gas chromatography-olfactory
GCxGC	Two-dimensional gas chromatography
GPC	Gel-permeation chromatography
GPCR	G-protein coupled receptor
GRAS	Generally recognised as safe
H	
HPLC	High performance liquid chromatography
HSSE	Headspace sorptive extraction
I	
ICAT	Isotope-coded affinity tags
ID	Internal diameter
IEC	Ion exchange chromatography
IOFI	International Organisation of the Flavour Industry
IR	Infrared
L	
LC	Liquid chromatography
LC-MS	Liquid chromatography-mass spectrometry
LC-NMR	Liquid chromatography-nuclear magnetic resonance
M	
m/z	Mass to charge ratio
MAE	Microwave-assisted extraction
MALDI	Matrix-assisted laser desorption/ionisation
mCPBA	*m*-Chloroperoxybenzoic acid
MIMS	Membrane inlet mass spectrometer

MS	Mass spectrometry
MS/MS	Tandem mass spectrometry
N	
NAM	N-(9-Acridinyl)maleimide
NEM	N-ethyl maleimide
NI	Nature identical
NIOSH	National Institute of Occupational Safety and Health
NIR	Near infrared
NMR	Nuclear magnetic resonance
NP-HPLC	Normal phase-high performance liquid chromatography
NPM	N-(1-pyrenyl)maleimide
O	
OD	Outer diameter
P	
PAGE	Polyacrylamide gel electrophoresis
PDMS	Polydimethylsiloxane
PET	Polyterephthalic acid
PFBBr	Pentafluorobenzyl bromide
PFBHA	*O*-(2,3,4,5,6-pentafluorobenzyl)hydroxylamine hydrochloride
PFBO	Pentafluorobenzyloxime
PFPH	Pentafluorophenylhydrazine
POM	Particulate organic matter
ppb	Parts per billion
ppm	Parts per million
ppt	Parts per trillion
PTFE	Polytetrafluoroethylene
PTGC	Programmed temperature gas chromatography
R	
RF	Radio-frequency
RP-HPLC	Reverse phase-high performance liquid chromatography
S	
SAR	Structure activity relationship
SBSE	Stir-bar sorptive extraction

SEC	Size-exclusion chromatography
SFC	Supercritical fluid chromatography
SIM	Selected ion monitoring
SIMS	Secondary ion mass spectrometry
SPME	Solid-phase microextraction
SVOC	Semi-volatile organic compound
T	
t-BDMS	*t*-butyldimethylsilyl
TD	Thermal desorption
THF	Tetrahydrofuran
TIC	Total ion count
TLC	Thin-layer chromatography
TMS	Trimethylsilyl
TNB	2-Nitro-5-sulfhydrylbenzoic acid
TOF	Time-of-flight
Tris	*Tris*(hydroxymethyl)aminomethane
U	
UV/Vis	Ultraviolet/visible
V	
VOC	Volatile organic compound
VVOC	Very volatile organic compound
W	
WGMA	Working Group on Methods of Analysis

1

Introduction

1.1 Synopsis

The chemical senses of taste (gustation) and smell (olfaction) are the two least well understood of our senses. Recent advances in our understanding of the genetic and molecular mechanisms have led to ever expanding research into these topics. Flavour and fragrances have been used by humans for millennia and in this century flavour and fragrance compounds form the basis of multi-million pound industries (Leffingwell & Associates, 2006). Amongst the practical applications of fragrances and flavours are commercial consumer products. The flavour and perfume industries both use nature as a guide and source of inspiration with the aim of replicating a flavour or fragrance to use in new products. In order to do this, the chemical compounds responsible for flavour and fragrance need to be isolated, characterised and synthesised. As isolation, separation, analysis and synthetic techniques have developed over the last fifty years, new challenges have arisen regarding the identification of new flavour and fragrance molecules. These challenges include identifying species present at very low concentrations, of low stability and of wide chemical diversity. This thesis is primarily concerned with development of methods to isolate and characterise these species.

To help understand the importance of the chemosenses of taste and smell, this introduction provides an overview of the current state of knowledge on the function of these senses. This introduction also sets out some of the history and drivers of the modern flavour and fragrance industries. Key stages and challenges to be considered during the process of discovering new ingredients from nature are discussed, followed by an overview of the tools that provide solutions to isolation and characterisation problems. The characteristics of three families of fragrance and

flavour compounds: aldehydes and ketones, sulfur compounds and menthyl esters, which are of particular focus in this thesis, are reviewed. Finally, in the light of the information presented, the aims of this thesis are discussed.

1.2 Chemosensation

Taste and smell are the most ancient senses for all organisms. They are the central mechanism by which most organisms sample their environment whether finding food or a mate, evading predators or avoiding ingesting toxic food. Smell has proved the most perplexing sense to decipher, with the major advances in understanding of the mechanisms of recognition of smell and taste made only recently. In the early 1990's, Richard Buck and Linda Axel (Buck and Axel, 1991) discovered odorant receptors and the organisation of the olfactory system, for which they received the Nobel prize in Physiology or Medicine in 2004. Since that time, significant progress has been made in explaining how animals detect and discriminate between thousands of different odour compounds. Similar advances have been made in understanding taste: the receptors responsible for bitter taste were first identified in 2000 (Adler *et al.*, 2000; Chandrashekar *et al.*, 2000; Matsunami *et al.*, 2000), with another recent development being the discovery of the receptor responsible for sweet sensation in 2005 (Zhao *et al.*, 2005).

Both chemical senses are also intimately linked with two neural systems: emotion and memory (Rouby, 2002). This means that the perception of smell and taste consists not only of the sensation of the odorant or tastants themselves but of the experiences and emotions associated with these sensations. Smells in particular can evoke strong emotional reactions. This has led to increasing speculation and interest in the potential commercial and therapeutic applications of exploiting odorous molecules.

1.2.1 Definition of flavour and fragrance

Fragrance or aroma is due to the volatile compounds emitted by the odour-producing source, be it blooming rose, or delicious roast dinner. To provide sensory properties, the odorant must have some water solubility, a sufficiently high vapour pressure, low polarity, some ability to dissolve in fat (lipophilicity), and surface activity.

Flavour is most often associated with taste, and therefore the tongue, but in fact, the nose is also the main organ for flavour perception, because flavours can be made of up to 80% volatile odour compounds, released during eating. Flavour also contains another sensory aspect, trigeminal, which is an irritant sensation felt in the general pain, tactile and temperature receptors in the mouth, nose and eyes. It is this trigeminal sensation that makes chillies feel hot, spices pungent and menthol cool. The three aspects of flavour are shown in Figure 1.1.

Figure 1.1 Flavour utilises three of our senses: smell, taste and trigeminal (Image courtesy of Quest International).

Odorous molecules run the gamut of chemical diversity, encompassing functional groups including aldehydes, esters, ketones, alcohols, alkenes, carboxylic acids, amines, imines, thiols, halides, nitriles, sulfides and ethers. The question of how the nose manages to detect this wide range of airborne materials and distinguish between them is considered next.

1.2.2 Olfaction

The nose is a remarkable organ capable of detecting about ten thousand different odours, even in infinitesimal quantities, which the brain can then recognise and remember. From an evolutionary perspective, it is speculated that this ability is required because of the diversity of the molecules that may be encountered in an organism's lifetime. The evolutionary importance of smell is also reflected in the

fact that a high percentage (up to 4%) of the genomes of higher eukaryotes is devoted to encoding the proteins of smell (Firestein, 2001).

In humans, odorants are detected by ciliated olfactory sensory neurons coated in mucus. They are located in the olfactory epithelium, found in the upper reaches of the nasal cavity (see Figure 1.2).

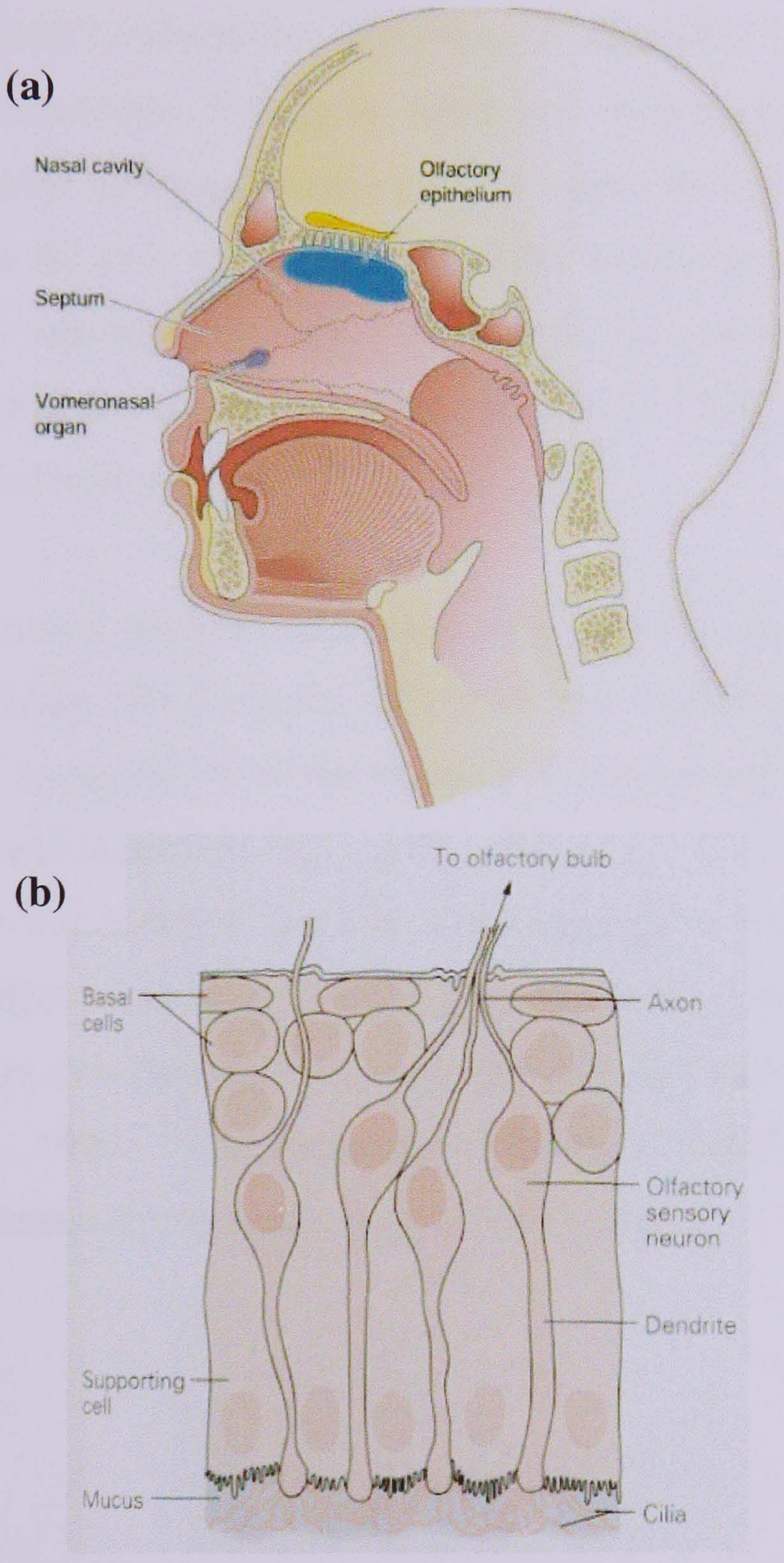
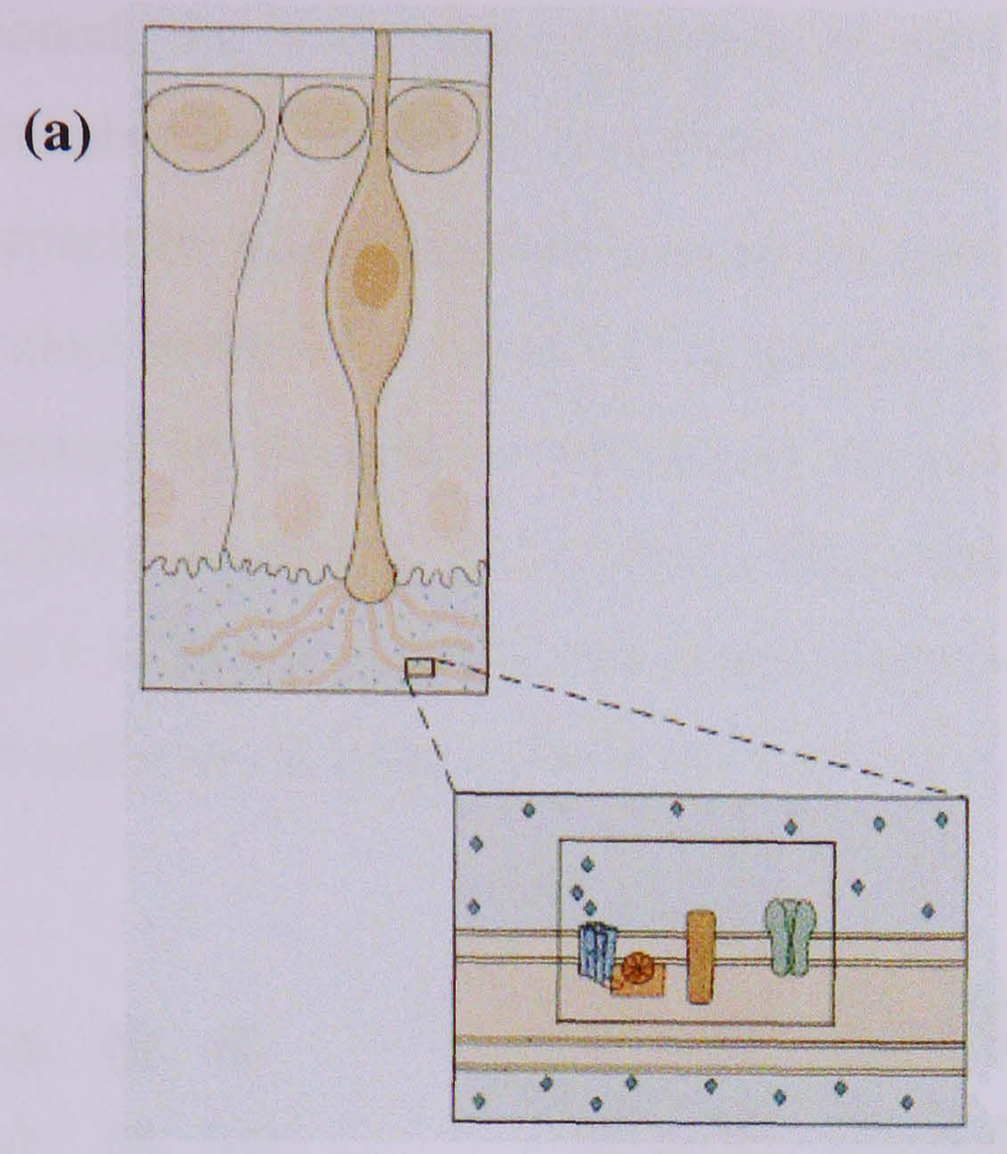


Figure 1.2 The olfactory organs: (a) side view of the human head showing the olfactory epithelium, the location of the cells responsible for smell. (b) Four olfactory sensory neurons are shown with their cilia projecting into the mucus (Mann, 2003).

When an animal or person sniffs an odorant, the odorant molecules are dissolved in mucus that coats the cilia of the sensory neurons. The lipids in the mucus solubilise

odour molecules and hence help transport them to the receptors. These receptors were discovered to be of the G-protein coupled receptor (GPCR) type, '7–pass' transmembrane proteins also important for neurotransmission, photoreception and other cellular process (Buck and Axel, 1991). Once the odorant has bound, it activates a G-protein coupled to the receptor on its cytoplasmic side, which in turn activates adenylyl cyclase, an enzyme embedded in the plasma membrane of the cilia. Adenylyl cyclase catalyses the conversion of adenosine 5'-triphosphate (ATP) to the secondary messenger, 3'-5'-cyclic adenosine monophosphate (cAMP). The release of cAMP opens up ligand-gated cation channels which facilitate the diffusion of sodium ions into the cell, reducing the potential across the plasma membrane. If this depolarisation reaches threshold, it generates an action potential which is conducted back along the olfactory nerve to the brain. This sensory transduction pathway is illustrated in Figure 1.3.

Each olfactory sensory neurone expresses only one of around 1000 olfactory receptor genes (Zhang and Firestein, 2002). These olfactory system genes and pseudogenes make up nearly 2% of the human genome, second only to the receptors of the immune system (Glusman *et al.*, 2001). The axons from all cells expressing a particular receptor are bundled together and converge onto only one or a few glomeruli in the olfactory bulb, which increases the sensitivity of the signal sent to the brain. The brain evaluates the olfactory signals reaching it as a particular odour (Mombaerts *et al.*, 1996). The brain also receives a signal through the limbic system, which generates an emotional response to the smell.



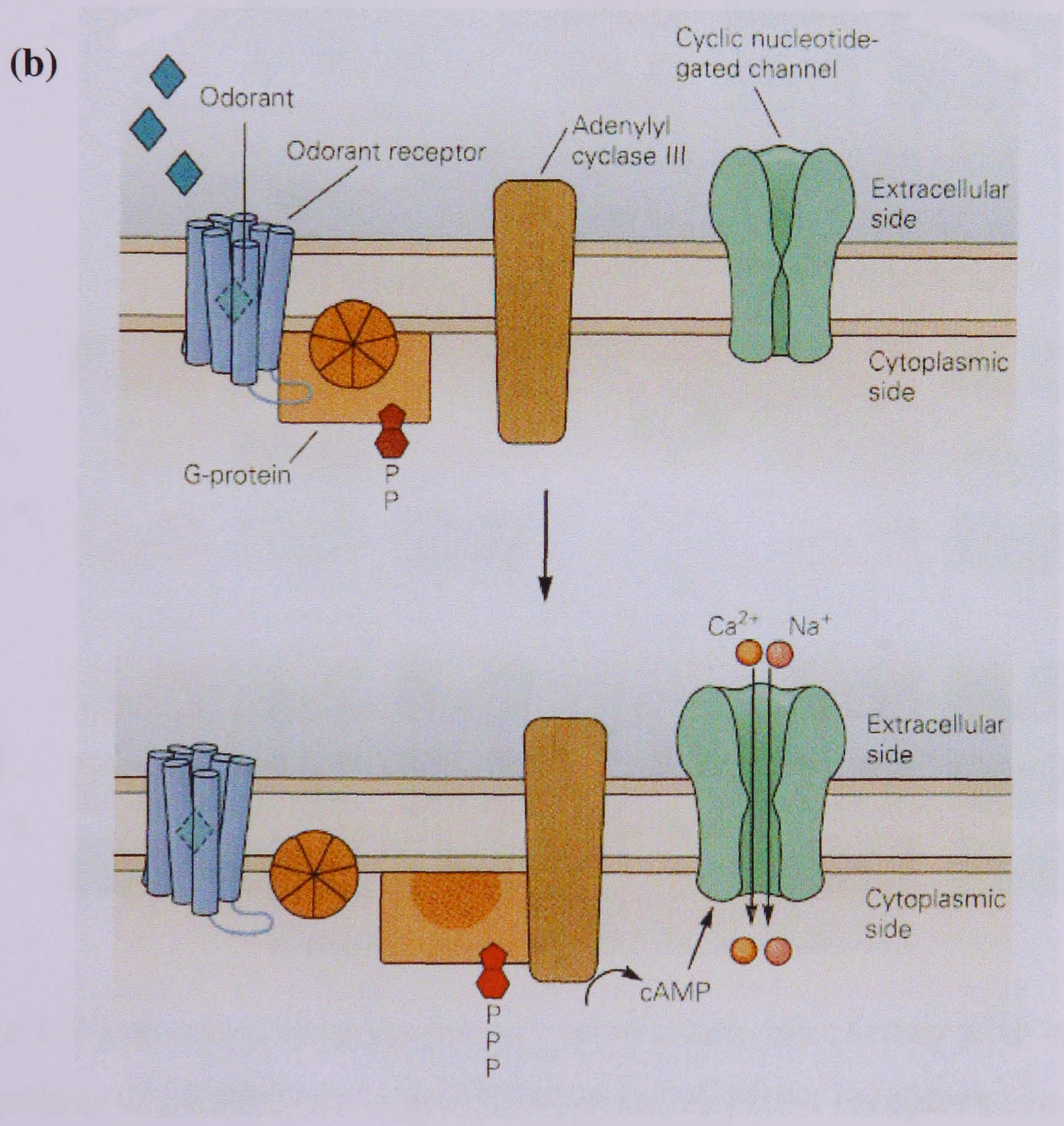


Figure 1.3 (a) An inset showing an enlargement of a portion of the membrane of a cilium showing the location of the receptor and the G-protein system. (b) The process of sensory transduction: the G-protein system in the resting condition (above) and the active condition (below). In the active condition, an ion channel opens admitting cations (Mann, 2003).

As already mentioned, the nose can detect tens of thousands of odours with a relatively small number of olfactory receptors. This is achieved by using a combinatorial approach to recognition and processing odours (Malnic *et al.*, 1999). There is no individual receptor dedicated to a specific odour, but rather different odorants are recognised by different combinations of receptors to create a specific odour response within the neurons of the brain. This pattern recognition process is illustrated in Figure 1.4. This is based on the current experimental evidence, which is likely to be refined and revised in the light of the very active research on this topic.

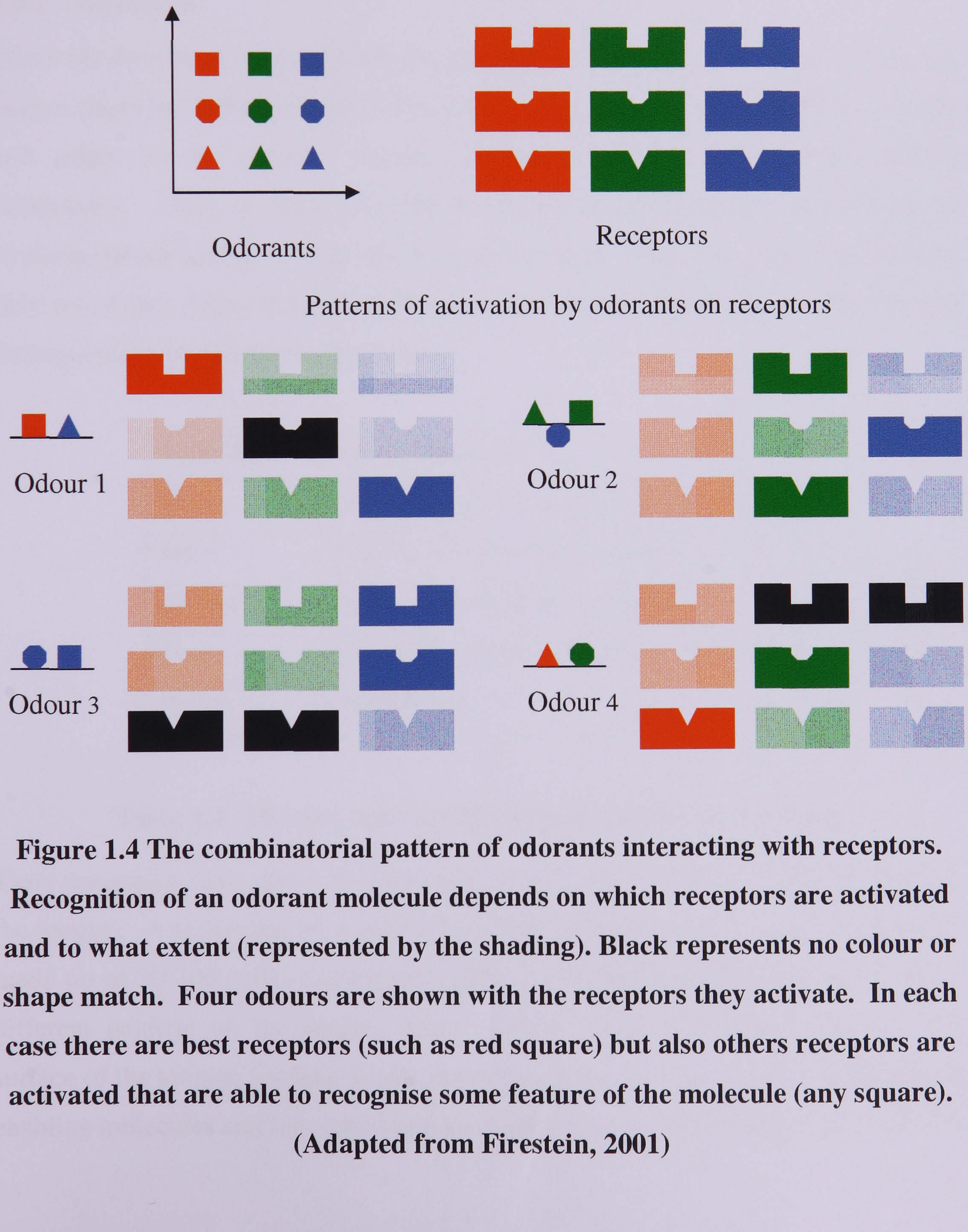


Figure 1.4 The combinatorial pattern of odorants interacting with receptors. Recognition of an odorant molecule depends on which receptors are activated and to what extent (represented by the shading). Black represents no colour or shape match. Four odours are shown with the receptors they activate. In each case there are best receptors (such as red square) but also others receptors are activated that are able to recognise some feature of the molecule (any square). (Adapted from Firestein, 2001)

Different chemical structures activate different combinations of receptors, even when the change in structure is slight. For example, octanol smells like oranges, but the related octanoic acid smells like sweat. Malnic and co-workers (1999) also discovered that a large concentration of an odorant binds to a wider variety of receptors than smaller amounts of the same chemical. This explains why a chemical's odour can vary with concentration. As an illustration, indole smells of sweet violets at trace level concentrations but putrid at higher concentration.

1.2.3 Gustation

There are five basic tastes that the tongue responds to: sour, salt, bitter, sweet and umami (literally 'delicious taste'). The latter is relatively recent to the Western world and refers to the savoury flavour of monosodium L-glutamate and similar compounds. Each of these provides a specific function in allowing animals to evaluate the nutritional content of a food as shown in Table 1.1. Although there are only five tastes, these provide an effective and reliable system for recognising and distinguishing key dietary components.

Taste bud	**Nutritional role**
Sweet	Identification of energy-rich nutrients
Salty	Control of electrolyte balance
Umami	Identification of amino-acids
Sour	Warning against ingesting poisonous substances
Bitter	

Table 1.1 The five taste sensations and their nutritional role

Taste perception takes place through taste receptor cells located in the taste buds on the tongue. A schematic of a taste bud is shown in Figure 1.5. Each taste bud is made up of 50-100 cells (Lindemann, 2001). The taste buds are distributed across different papillae on the tongue. Taste receptor cells project microvillae on the surface of the tongue, forming a taste pore that opens out to the surface of the tongue enabling molecules and ions taken into the mouth to reach the receptor cells inside.

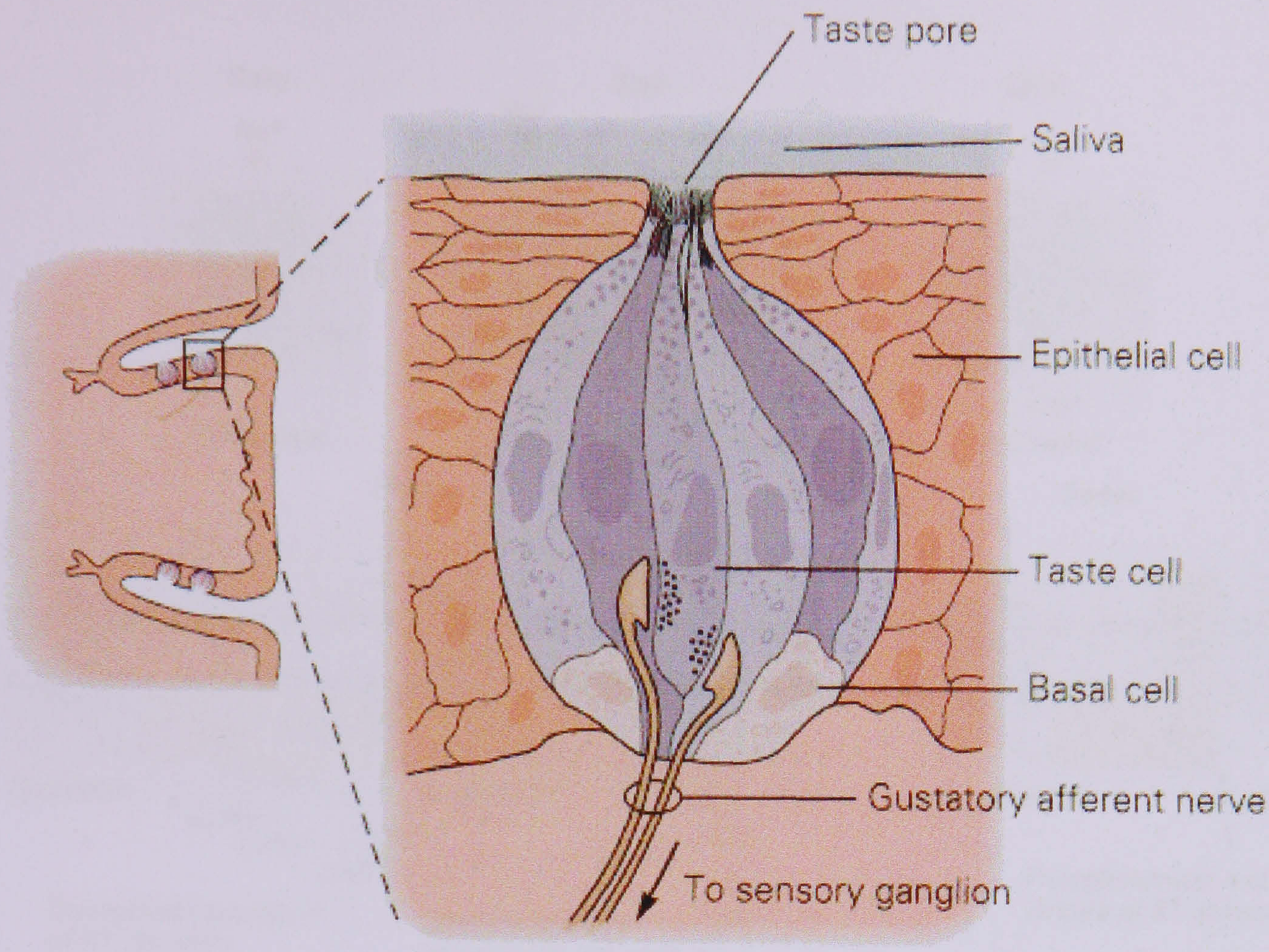


Figure 1.5 A schematic of a taste bud on the surface of the tongue. Microvilli in the taste pore interact with the taste compounds, producing an electrical charge, triggering release of a neurotransmitter. This activates nerves resulting in an impulse to the brain (Mann, 2003).

Mechanisms for detection of taste compounds are illustrated in Figure 1.6. Salty and sour tastants are primarily ionic compounds and these act directly though ion channels on the cell membrane of taste receptors. The ion flow triggers neuron activation and transmission of the signal to the brain. Salty substances often contain sodium ions, whereas sour substances often contain acidic hydrogen ions. Some bitter substances are also ionic and can activate potassium ion channels.

A more complicated process occurs for the non-ionic compounds responsible for bitter, sweet and umami sensations. As with olfactory receptors, the G-protein coupled receptors are involved (Adler *et al.*, 2000; Matsunami *et al.*, 2000).

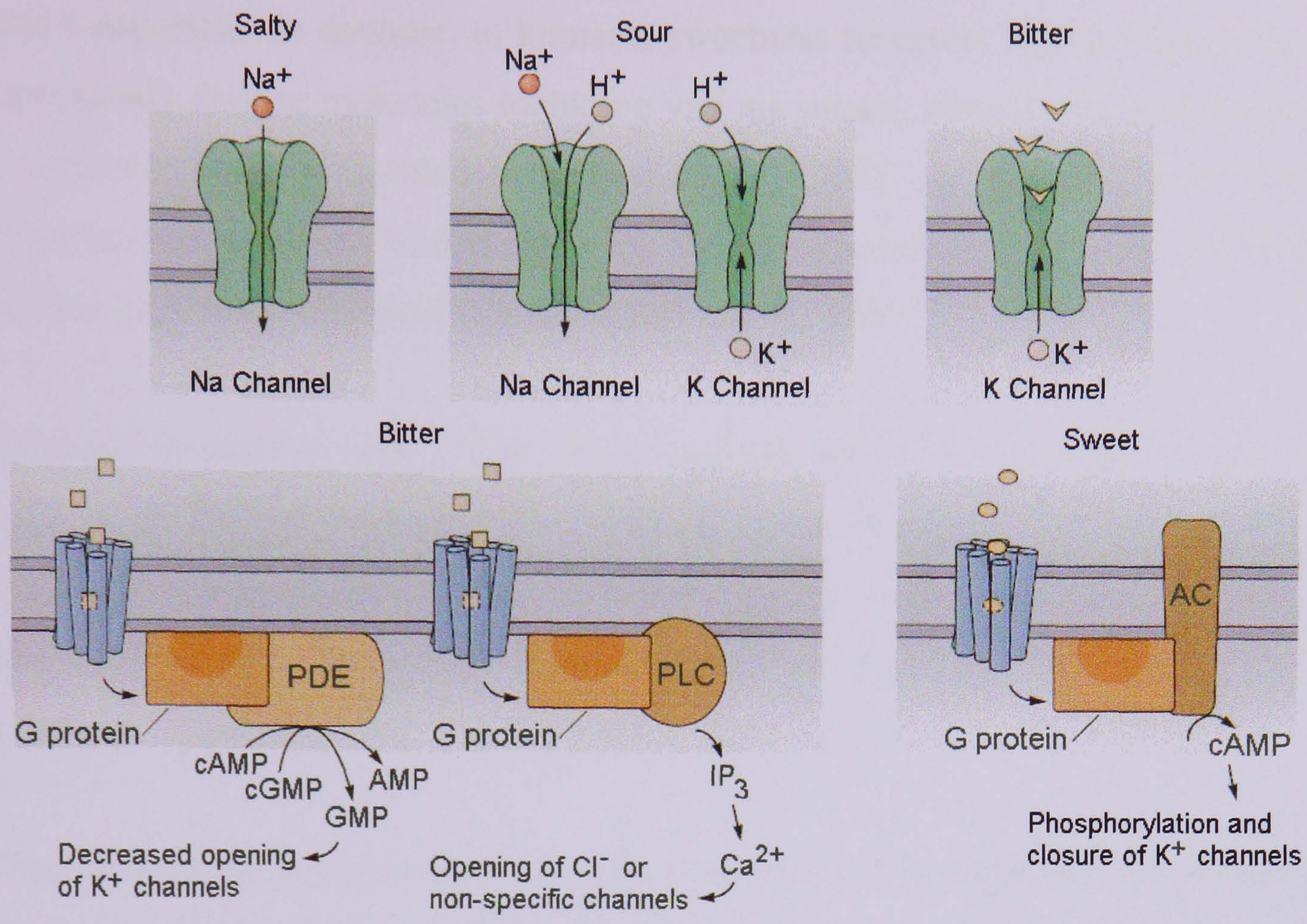


Figure 1.6 The mechanisms of detection of taste substances. Ionic substances such as those which can generate a salty, sour or bitter sensation can act directly on sodium or potassium ion channels. For non-ionic substances (bitter and sweet) interaction of the tastants is with a GPCR receptor followed by subsequent activation of potassium or chloride ion channels (Mann, 2003).

The membrane proteins that serve as receptors for the transduction of taste have for a long time remained elusive, but screening the mass of genome sequence data that has recently become available has provided a new means to identify key receptors for bitter and sweet taste (Lindemann, 2001). For bitter tastes, the G-protein receptors are known as the T_2R family of proteins (Chandrashekar *et al.*, 2000; Mueller *et al.*, 2005). The extra-cellular regions show much variation which corresponds well with the ability to detect the chemically diverse species which taste bitter, representing the wide range of toxic substances that an animal may encounter.

In mammals, sweet and umami tastants are detected by T_1R receptors, a family of three GPCRs. These receptors form heterodimers in which different T_1R's come

together. For example, the T_1R_1-T_1R_3 dimer binds only monosodium L-glutamate and L-aspartate. In contrast, in humans, sweetness receptors bind a wide variety of structurally diverse molecules including various sugars, sweet proteins and artificial sweeteners such as saccharin (Nelson *et al.*, 2001). A detailed review of the experiments conducted to determine the receptors responsible for each of the tastes has recently been published (Chandrashekar *et al.*, 2006).

Contrary to popular belief, there are no specific areas on the tongue for detecting each taste. Instead, receptors for particular tastes are spread all over the tongue. Although a single taste cell may have representatives of several types of receptor, one type may be more active than the others on that cell. No single taste cell contains receptors for both bitter and sweet tastants.

One fascinating and active area of taste research is to understand how information flows from the tongue to the sensory evaluation centres in the brain and hence controls our behaviour. Such research should reveal how it is that taste perception varies according to context. For example, the sourness of lemon juice is masked by the addition of sugar, but retains its acidity. It could also reveal how our perception of taste is affected by other factors such as how a substance smells, looks, or feels in the mouth, as well as how this relates to feelings such as prior experience of the taste or whether a person is hungry or satiated. This research will require molecular genetic and novel physiological approaches.

1.2.4 Trigeminal sensation

A discussion of flavour would not be complete without mentioning trigeminal sensations. The trigeminal nerve runs into the olfactory epithelium and has a set of endings in the mouth, eyes and nasal cavity which are responsible for tactile, pressure, pain and temperature sensations in these areas. A number of chemical trigeminal stimulants produce effects described as hot, cold, tingling or irritating. Common chemicals which stimulate a trigeminal sensation include menthol (mint), capsaicin (hot chili powder) and diallyl sulfide (onion). It has been suggested that about 70% of all odours stimulate the trigeminal nerve, but in general the response is several times less sensitive than olfactory receptors (Ohloff, 1994). Menthyl esters,

(discussed in Section 1.9.3) are used in applications where a trigeminal sensation is desirable, such as mouthwash or beverages.

1.2.5 Applications of the latest research in olfaction and gustation.

Until recently, uses of fragrance and flavour compounds have been rather limited mainly to use in direct commercial products, such as fine fragrances and flavourings in food products. These are industries which depend strongly on the sensory properties of the products. As such, the work reported in this thesis has focussed on this type of application. Nevertheless, future potential applications of benefit to society are many and include those important for health, nutrition and quality of life. An example of one such important issue is pest control. Insects are responsible for damaging crops and carrying deadly diseases such as malaria. Alternative solutions to pesticides are highly desirable, due to the detrimental effects of these compounds on the environment and the increasing emergence of resistant strains. Odours are showing strong potential in acting as insect control measures, underpinned by an understanding of the odour receptor genes in the fruit fly Drospophila (Clyne *et al.*, 1999; Vosshall *et al.*, 1999). An up-to-date review of the role of molecular genetics of insect olfaction in controlling insects has been published (van der Goes van Naters and Carlson, 2006).

Another area of current research which has immediate application is chemosensory neuroscience, that is, understanding the direct connection between an odour, the brain and the effect it has on us physically and emotionally. One study in rats found that the scent of grapefruit oil and its active components stimulated an increase in blood pressure (Tanida *et al.*, 2005). Another study showed the aroma of hops initiated a relaxing effect in humans (Kaneda *et al.*, 2005). A recent review examines how chemosensory neuroscience could be used to understand the connection of the brain's flavour system and eating behaviours (Shepherd, 2006). The hope for such research is that it will lead to better nutrition, for example, by using odours to control appetite stimulation.

Other opportunities include tailoring fragrances to individuals' odour phenotype; testing for deficits in smell for diagnosis of diseases such as Alzheimer's disease; and bitter flavour blockers which could be used to make medicines more palatable.

A review has been published on the commercial opportunities in olfaction and taste (Gilbert and Firestein, 2002). Many of these applications will require the challenging task of an extensive screening program to match tastants and odorant molecules with their respective receptors.

1.3 Flavours and fragrances: a brief history of usage

1.3.1 Ancient history

Both fragrances and flavours have been in use by societies for millennia, playing a role in religious and pleasurable pursuits for ancient civilisations. Initially, fragrances and flavours were obtained from natural sources, such as flowers, aromatic plants and animals (musk scents). In the classical world of ancient Greece and Rome, the technique of distillation was developed. This is still used today to extract essential oils from aromatic plants to create perfumes and essences. Herbs and spices have always been used for flavouring foods. The trade of spices could even be said to be a major driving force to the discovery by the West of the Americas, in search of an alternative trade route of the Silk Road from the East via Constantinople.

1.3.2 The industrial age

The flavour and fragrance industry as it is today began to develop in the industrial age of the nineteenth century. Isolation of many aroma compounds from nature, especially essential oils, led to the identification of aroma active materials such as benzaldehyde (almond aroma). At the same time, advances in organic chemistry led to the development of synthetic fragrance compounds. For example, the important perfumery compound, cinnamic aldehyde was first synthesised in 1856. In this age of discovery, five Nobel Prize winners in the first half of the twentieth century were involved with aspects of aroma chemistry, indicating the importance of aromatics to chemistry at that time (Pybus, 1999).

1.3.3 The modern era

The real expansion in the flavour and fragrances industries took off in the latter half of the twentieth century thanks to both technical and sociological factors. From a technical point of view, advances in analytical techniques such as infrared (IR)

spectroscopy, nuclear magnetic resonance (NMR) spectroscopy and in particular, gas chromatography (GC) and mass spectrometry (MS) have been instrumental in allowing the key odorants of fragrant flowers and food stuffs to be identified at increasingly lower concentrations. The use of 'trapping' methods such as headspace extraction means that the trace level compounds can be identified. These often define the character of an odour. Isolation methods are discussed in Section 1.5. Comparable advances in synthetic techniques have also led to a vast increase in the number of materials available to the perfumer or flavourist at a reasonable cost for the finished formulation.

Alongside the scientific factors, social factors have also been important in the development of today's flavour and fragrance industry. Post World-War II, the standard of living in the developed world has led to more 'disposable income' to spend on luxury or convenience goods, so that a fine fragrance is no longer the preserve of the wealthy, and fragrances are now found in hundreds of consumer products such as bleach, washing powder and soap. These fragrances need to meet the challenges of harsh chemical environments such as the highly alkaline pH of bleach. Perfumery is now a multi-million pound industry and of considerable interest to commercial enterprises and also in therapeutic research. Social and geographic mobility has also led to people experiencing a much wider palette of flavours. This is an important driving force in developing new and improved flavour sensations.

1.3.4 Future trends

A key trend in both flavour and fragrance industries is the search for ever more 'exotic' ingredients (Figure 1.7). This quest has led to several expeditions to untapped sources of plant material such as the canopy of the tropical rainforest, reached by hot air balloon and sampled by non-destructive techniques (Boelens and Boelens, 2003; Gassenmeier *et al.*, 2001). Other novel developments include Quest's Aquaspace™ technology for capturing the aroma molecules in water using a system of pumping and filtering water through an odour trap. The technique gives perfumers a new olfactory experience from a previously inaccessible part of nature and opens up new creative possibilities.

Figure 1.7 Images from an expedition to Sri Lanka to discover new and exciting flavour and fragrance compounds (courtesy of Quest International)

Strong drivers of innovation in the flavour industry are improving nutrition and creating authentic flavours. With increasing concern about obesity and unhealthy diets, flavours and tastes which can give low-fat products the same sensation as a full-fat product are highly desirable. Replacing salt in products whilst still retaining its flavour enhancing qualities is also a prominent movement. Finally, with today's busy lifestyles, the trend for purchasing convenience foods shows no sign of halting, and consequently there is a desire to produce healthy, ready-made meals with a similar taste sensation to a home-cooked meal.

There are two primary reasons that finding flavour or fragrance compounds in nature is so important. These are external factors experienced by these industries, namely, consumer perception and regulatory restrictions. There is wide-spread consumer perception that natural is best. This is particularly pronounced in the flavour industry where artificial flavourings are seen as undesirable. In the European Union, and in some other countries world-wide, flavour chemicals are classified in the legislation into three categories (European Union, 1988). These are:

- natural; flavour chemicals obtained using a natural process or of natural extraction or distillation from material of vegetable or animal origin;
- nature identical (NI), flavour chemicals of synthetic origin but indistinguishable from a substance naturally present in material of vegetable or animal origin; and

- artificial; flavour chemicals of synthetic origin with no known natural occurrence.

Establishing NI status is therefore of key importance to the flavour industry because NI labelling status is greatly preferred over artificial labelling in the European Union. The identification of an NI component is demonstrated in Chapter 5.

Recently, even nature-identical has been seen as 'not natural enough', and the flavour industry is shifting to natural extracts. This is despite the fact that in many ways, synthetic flavouring products are preferable to natural extracts: they have longer shelf-lives, and more reliable in concentration and flavour. Production volumes of natural flavours are also far less predictable because of uncontrollable factors such as weather.

1.4 The discovery process

There are several paths for fragrance and flavour chemists to follow in the search for new ingredients. These include: analysis of natural products, optimisation of leads, and serendipity. The primary route considered in this thesis is identifying products from nature by extracting them in some way. This is discussed in more detail below. It is worth mentioning, however, alternative methods such as utilisation of structure activity relationships (SAR's) (Rossiter, 1996; Turin, 2004). The underlying principle behind SAR's is that the molecular structure of an organic compound determines the properties of the compound. Thus, a set of compounds which exhibit the same type of activity and which are detected by the same mechanism must have one or more structural features in common. If such a relationship is found, it can be used to predict the activity of compounds that have not yet been made, and as such is a very useful tool in the design of new, potentially active compounds. The same principle is used in the design of other molecules that interact with the body, such as drugs. Molecules which are likely to have aroma or flavour properties can be synthesised first, and their odour/taste profile assessed afterwards. This approach can be risky given the still developing understanding of the mechanisms of olfaction and gustation.

The steps for the discovery of new molecules from nature are illustrated in Figure 1.8. Extraction of the molecules of interest involves a wide variety of techniques which are explored in more detail in Section 1.5. The various analytical tools required to characterise the molecules of interest are described in Section 1.6. The ultimate verification of the assignment of a correct structure is by synthesis of a sample so that properties can be assessed. If the natural material contains structural features which make them difficult to synthesise or susceptible to degradation, synthetic analogues can be made which are close in odour properties but which overcome these issues.

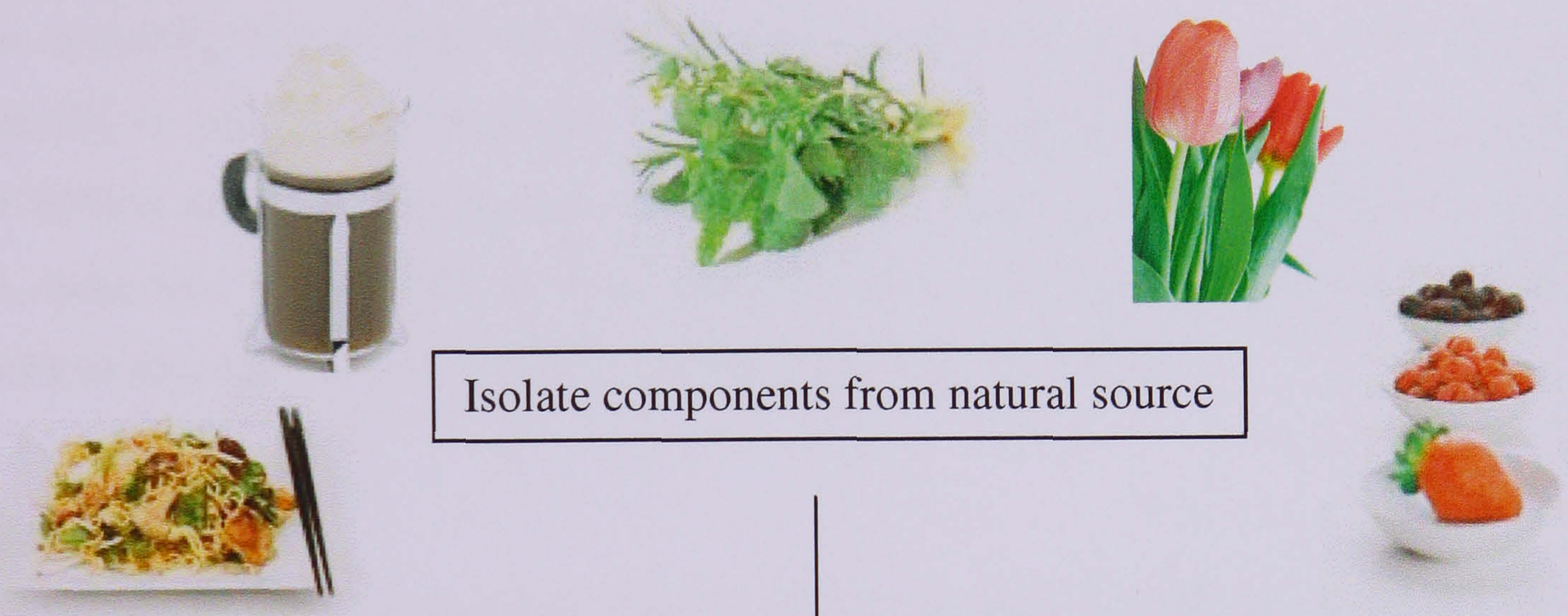


Figure 1.8 The steps involved in discovery new compounds from nature

1.4.1 Challenges in discovery from nature

It has already been mentioned that nature is the primary source of inspiration in both flavour and fragrances industries. There are some specific problems associated with identifying flavours and fragrances from natural sources, including:

- the complexity of the natural source,
- concentration issues,
- the varied chemical natures and physical properties,
- the full spectrum of chemical functionality that is found.

Each of these is discussed in more detail below.

Concentration

The aromatic components of interest are generally present of very low levels, typically in parts per million (ppm), parts per billion (ppb), or even as low as parts per trillion (ppt). Although some analytical techniques are capable of detecting at the same low limits (such as mass spectrometry), generally any isolation method needs to concentrate the sample by several orders of magnitude.

Matrix

The sample matrix containing the aroma chemicals may cause interference. In many cases, volatile compounds are intracellular and the sample must be liberated in some way. Frequently, there are non-volatile components in a sample such as lipids, proteins and carbohydrates, which usually cause emulsification and foaming during the isolation procedures.

Complexities of Aromas

The aroma composition of foods can be very complex. For example, coffee contains almost 800 identified components of almost every chemical class, as illustrated in Table 1.2. These compounds will also cover the full range of solubilties, volatilities, polarities and pH's.

Instability

Many components in an aroma are unstable, and may be oxidised by air, or degraded by heat or extremes of pH. The instability of many compounds generates artefacts

during the isolation of aroma compounds. Flavours also change with time and processing conditions. The reactivity of different functional groups may make the trapping and analysis of some classes of compounds particularly difficult: thiols, for example, are easily oxidised to disulfides. This can lead to the formation of artefacts and uncertainty about the nature of the flavour or fragrance.

Chemical class	**Number of compounds**
Hydrocarbons	74
Alcohols	20
Aldehydes	30
Ketones	73
Acids	25
Esters	31
Lactones	3
Phenols (and ethers)	48
Furans	127
Thiophenes	26
Pyrroles	71
Oxazoles	35
Thiazoles	27
Pyridines	19
Pyrazines	86
Amines and miscellaneous nitrogen compounds	32
Sulfur compounds	47
Miscellaneous	17
Total	*791*

Table 1.2 Classes of aroma compounds in coffee (Wasserman *et al.*, 1993)

Field trips

The drive for finding ever more exotic ingredients has led to more trips to isolated field locations which place restrictions on the methodologies that can be used.

Equipment needs to be light, portable and robust. Samples collected may also require protection from heat and light. If properly treated, samples can be stored for long periods or transported over long distances before analysis in the laboratory (Clery, 1999).

1.5 Isolation

For both flavours and fragrances, the isolation of the desired compounds is a multi-stage process. At each stage there is the possibility of losing the compound of interest. Collection of the sample influences the method used, as does the nature of the components. Strategies for isolating and identifying one type of compound may be unsuitable for another class. For example, polar compounds like alcohols often require some modification before they can be detected, whereas light hydrocarbons can be very volatile. It is unlikely, therefore, given these complexities that one single technique of isolation will capture the full aroma of the sample. This has been illustrated by Jennings and Filsoof who compared different sample preparation techniques (1977).

Most isolation methods use some form of distillation (this relies on differences in vapour pressures) or extraction (this relies on solubilities in different solvents) or a combination of both. Isolation of compounds of interest can be destructive or non-destructive to the sample. Destructive techniques, which extract the component of interest from the sample, are used in research but also for large-scale acquisition of fragrance or flavour ingredients. In general, non-destructive techniques are used for isolation of volatile components from living plants, fruits and flowers. Whichever method is chosen, the aim is to minimise artefacts of the isolation process which can lead to misleading results. Care must be taken so that heat labile compounds are not destroyed by harsh conditions, highly volatile compounds not lost in concentration by distillation, or low solubility compounds lost in extractions. It is also desirable that the isolation method be easy to employ, require minimal equipment, and produce reproducible, meaningful results. Excellent discussions of the topic of isolation of aroma compounds have been written (Bicchi *et al.*, 2006; Da Costa and Eri, 2004; Maarse and Belz, 1982; Sugisawa, 1971; Weurman, 1969).

1.5.1 Destructive isolation techniques

Flavour and fragrance components can be isolated from a natural source in three basic ways that are destructive to the material. These are expression, distillation and solvent extraction, the choice of which depends on the nature and the amount of material available and the fate of the extract. Expression refers simply to subjecting a sample to physical pressure. For example, squeezing a piece of orange peel produces a spray of orange oil expressed through the ruptured glands. This is a common route for producing citrus oils. Distillation, particularly steam distillation, is often used for extracting essential oils from flowers such as lavender or rose and from wood. Widespread use is made of organic solvents or other extraction fluids such as supercritical carbon dioxide to strip flavour and fragrance compounds from their natural source. Discussions on extraction techniques have been published for fragrances (Sell, 1999) and more generally on aqueous and solid flavour samples (Parliament, 2002).

1.5.1.1 Solvent extraction

Solvent extraction has been utilised for the work described in Chapter 5. During solvent extraction, the extract is obtained by mixing or agitating a liquid or solid sample with organic solvent. The components are separated from insoluble materials and collected in the solvent phase. The choice of solvent is based on the nature of the desired extractants, for example, polar or non-polar components. It should also have a low enough boiling point so that it can be easily removed without loss of volatiles. Typical organic solvents include dichloromethane, a pentane/diethyl ether mixture and ethanol.

There are several different extraction methods currently employing solvents. The most common and oldest solvent extraction technique is Soxhlet extraction. In this technique, the sample is placed in a porous thimble and continuously extracted with a suitable solvent. The extraction typically uses large volumes of solvent and involves long extraction times (several hours). In recent years, there have been developments in the various solvent extraction routines which are designed to reduce extraction times, minimise the use of solvent and allow for more automated and high throughput processing. These include ultra-sonic extraction, microwave-assisted extraction, accelerated solvent extraction and supercritical fluid extraction. A recent

comparison of the merits of various techniques for extracting plant material has been published (Romanik *et al.*, 2007).

Microwave-assisted extraction (MAE) has been utilised for the work described in Chapter 5 and compared with traditional Soxhlet extraction. In MAE, a sample in contact with a solvent is rapidly heated using microwave energy. The use of closed vessels allows elevated temperatures to be used. MAE is based on the principle that polar chemical compounds absorb microwave energy. The energy absorbed is proportional to the dielectric constant of the medium, resulting in rotation of dipoles in an electromagnetic field (usually 2.45 GHz). The efficiency of MAE depends on the properties of the solvent, sample material, the components being extracted, and the dielectric constant of the solvent. The higher the dielectric constant, the more energy is absorbed by the molecules and the faster the solvent reaches boiling point. Water has the highest dielectric constant. Acetonitrile, ethanol, acetone and methanol are also appropriate solvents.

The advantages of microwave-assisted extraction include the reduced amount of solvent required, the shorter extraction time, the ease of control of sample heating and the reduced size of extraction apparatus. It is not so suitable for the extraction of non-polar analytes from non-polar materials. In these cases, mixtures of solvents can be used such as n-hexane and acetone replacing solely n-hexane. MAE has been used in a wide range of applications, initially for extracting pollutants from soil sediments (Lopezavila *et al.*, 1994) and in several other environmental applications (Camel, 2000). MAE has also been used extensively in extractions from natural products. It has been shown to be as successful as conventional extraction techniques for recovering components (Kaufmann and Christen, 2002). A comprehensive review on the principles of microwave extraction and the optimisation of parameters affecting the extraction process has been published (Eskilsson and Bjorklund, 2000).

Microwaves can also be used in organic synthesis, with similar benefits to those observed with microwave-assisted extraction, that is, shorter reaction times, minimal use of solvents, potential for higher throughput and automation. This is touched on here because microwave synthesis was used in Chapter 5 to make a standard compound. Several comprehensive reviews of the role of microwaves in organic

synthesis have been published (Abramovitch, 1991; Lidstrom *et al.*, 2001; Nuchter *et al.*, 2004; Whittaker and Mingos, 1994).

1.5.2 Non-destructive techniques

For many purposes it is necessary or desirable to have the living plant intact while sampling, particularly for volatile components. This is especially true on field trips where the plants may be in a protected area, such as a nature reserve, or the volatiles may only be emitted at a specific time of the day, due to the biorhythms of the plant. For example, certain orchids release different volatiles in the day or night depending on the type of insect pollinator they attract (Huber *et al.*, 2005). A destructive extraction technique may also not be representative of the fragrance of fresh flowers, or there may be insufficient material available for normal extraction. The most common isolation technique used is headspace sampling.

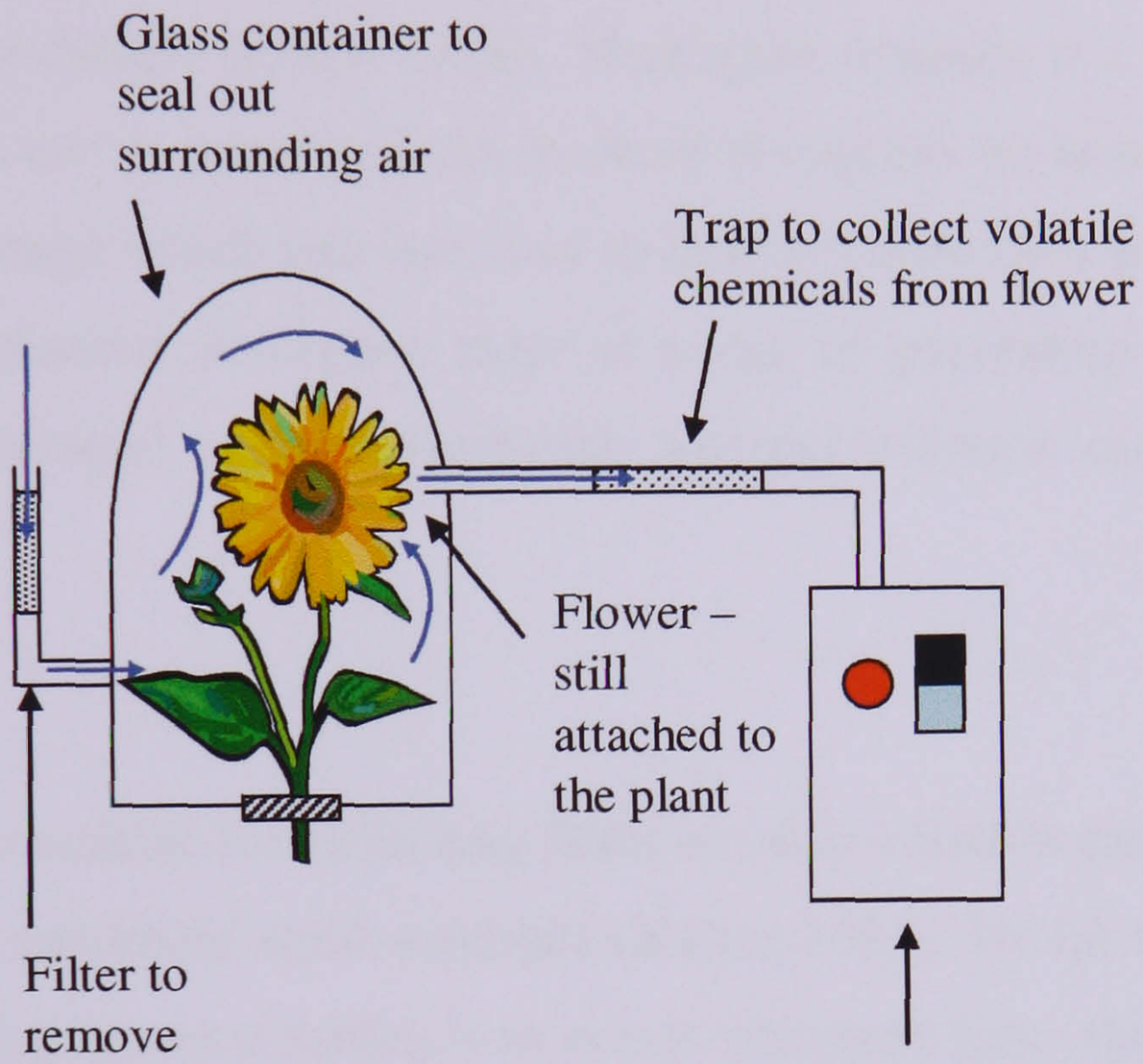
1.5.2.1 Headspace sampling

There are three main forms of headspace sampling: static, dynamic and 'purge-and-trap'. For each the basic principle is the same: volatile analytes from a solid or liquid material are investigated by sampling the atmosphere around the sample, leaving the actual sample behind. In static headspace, the sample is enclosed for a period of time to allow the volatiles to enter the headspace and reach equilibrium. A sample is collected by syringe for direct injection into the gas chromatograph for analysis (see Section 1.6.1.1). A disadvantage of static headspace sampling is that it requires the sample to be collected over a relatively long period. For a flower, this means an average of the flower's scent is sampled. This is undesirable if the biorhythm of the plants means that the composition of volatiles varies with time. Static headspace does have an advantage in that the sample is usually analysed without the presence of a solvent. This can be important if the solvent peak masks a peak from the sample volatiles, especially those of high volatility.

Dynamic headspace techniques continuously draw the headspace through a trap thereby concentrating the volatile compounds in the headspace. This means enough of the scent can be collected for analysis in a relatively short time during the period when the flowers scent emission is at a maximum. Therefore dynamic headspace sampling is the preferred technique when analysing flower scents. Note that the term

'dynamic headspace' is usually used when referring to the analysis of solid materials; the term 'purge-and-trap' generally refers to the analysis of liquid samples where a purge gas is bubbled through the sample.

A schematic of dynamic headspace sampling of a flower is shown in Figure 1.9 along with photographs of the equipment used in the field.



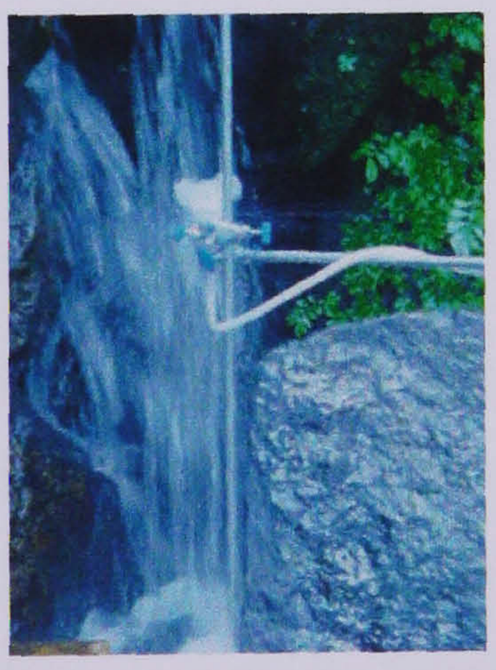


Figure 1.9 Schematic of the headspace experimental set-up and images of the headspace apparatus in use. The apparatus is being used in the field to capture the headspace from leaves found in the Sri Lankan rainforest and the molecules around water. (Photographs courtesy of Quest International).

The volatile vapours around the plant part are pumped through a trap, usually a porous polymer adsorbent material. The adsorbent provides a method of concentrating the samples as air and water pass through un-retained. Collection volumes can vary from 100 mL to several litres which in effect may result in quantitative removal of the volatile analytes from the sample. The trap can then be stored and desorbed later for analysis. Ideally, several traps are used with different adsorbents so that they can be analysed by more than one method. Different traps can be thermally desorbed or solvent desorbed, potentially generating more information on the sample (Clery, 1999). Headspace trapping is a relatively artefact free technique for use in odour analysis because it requires no heat to be applied. It is the desorption stage which can then lead to analyte breakdown and introduction of artefacts. With thermal desorption there is a risk of generating thermal artefacts either from the sample, or the adsorbents, whereas solvents can be a source of contamination.

Adsorbents

Several traps are available for collecting floral or other volatiles including cold traps, solvent traps and adsorbent solid materials (Kaiser 1991). By far the most common method for trapping flower volatiles is to use an adsorbent trap. One review of floral scents estimated that 76% of studies (90 out of 118) on analysing floral volatiles used headspace trapping with adsorbents (Knudsen *et al.*, 1993). The adsorbent material is finely divided to increase the surface area for retaining the analytes. When choosing an adsorbent material, it is necessary to evaluate the characteristics of the material for its ability to adsorb the analytes of interest and the ease with which they can be desorbed. Most sorbent materials are porous polymers. Tenax TA® (poly-2,6-diphenyl-p-phenylene oxide) is perhaps the most widely used, general purpose sorbent for dynamic headspace. It can trap a fairly wide range of organic volatiles, is especially good with aromatics, it may be heated to relatively high temperatures for desorption, and is long lasting. It is not very suitable for very volatile hydrocarbons (pentane and below) or for low molecular weight alcohols. Other sorbents are shown in Figure 1.10 with their applicability for trapping organic compounds.

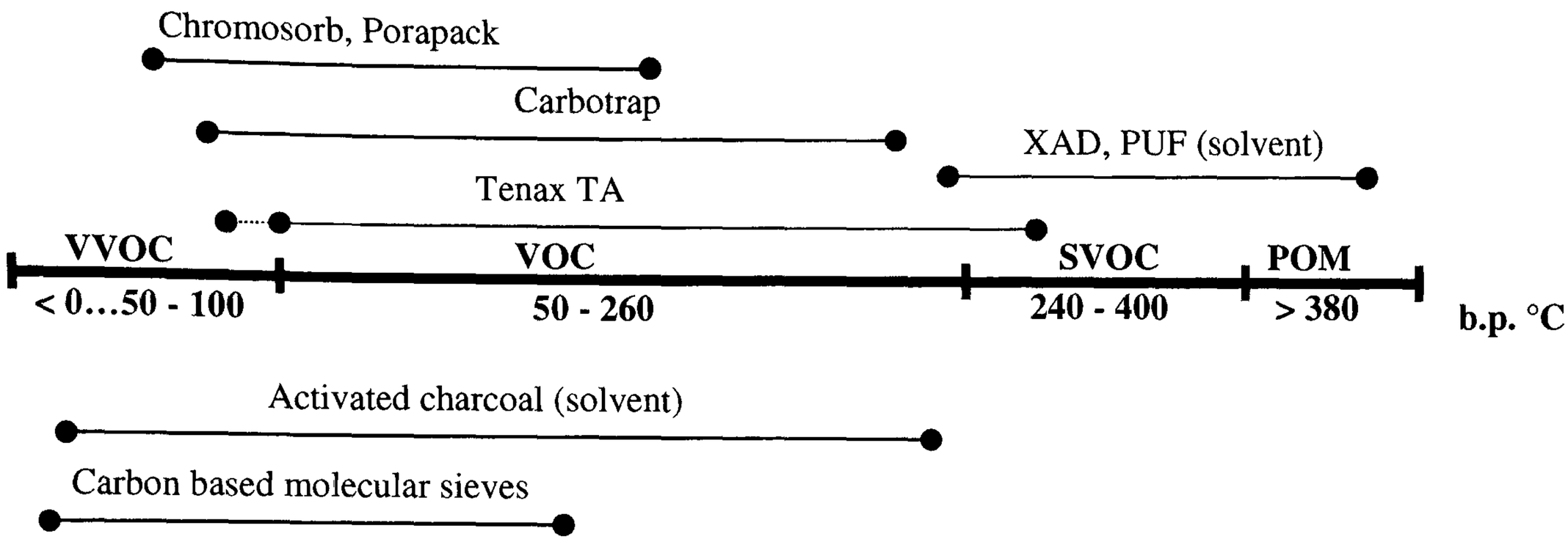


Figure 1.10 The ability of sorbents to trap volatile compounds according to the boiling point of the compound (VVOC = very volatile organic compound, VOC = volatile organic compound, SVOC = semivolatile organic compound, POM = particulate organic matter). (Woolfenden, 1997)

As a general rule, the more retentive the adsorbent material, or the lower the molecular weight of the compounds it is capable of retaining; the more heat is required to regenerate the trap. More detailed consideration of the factors involved in trapping are considered in Chapter 3, in which headspace adsorbent trapping was carried out.

1.5.2.2 Direct thermal desorption

In direct thermal desorption (DTD), samples are placed directly in a thermal desorption tube between glass wool plugs. The tube is placed in a thermal desorption unit connected to a GC. The volatiles are driven onto the GC column by controlled heating of the sample in a carrier gas flow. This technique is most suitable for solid samples, and has the considerable advantage in that this is a solvent-less, one-step process, thereby minimising the chances of contamination and simplifying sample preparation. Applications for the fragrance and food industry have been reviewed (Eri *et al.*, 2000; Grimm *et al.*, 2002; Hartman *et al.*, 1991). DTD is not suitable for field sampling due to the need for proximal equipment.

1.5.2.3 Solid-phase microextraction

Solid-phase microextraction (SPME) is a sorptive technique, introduced in 1990 by Arthur and Pawliszyn (Arthur and Pawliszyn, 1990) in which a fused silica fibre

coated with a stationary phase is either immersed in a sample or exposed to its headspace and the target analytes partition from the sample matrix to the fibre coating. After extracting for a set period of time, the fibre is transferred to the heated injection port of a gas chromatograph for analysis (see Section 1.7). SPME is an alternative to the more traditional sorbents described above, and its use is growing rapidly. The method has been applied widely in recent years to the determination of the volatile chemical components of plants, flowers, foods, environmental samples, pharmaceuticals and forensic samples. Six-hundred and eighty-two references on food analysis and botanical applications using SPME have been recorded as of November 2006 (Tugulea, 2006).

Initially, there were some disadvantages and limitations to the use of SPME, mainly around quantification: SPME is an equilibrium technique and accurate quantitation requires that the extraction conditions be carefully controlled. Quantitative work can be carried out by incorporation of an internal standard. The aroma profile of the collected volatiles is dependent upon the type, thickness and length of the fibre as well as on the sampling time and temperature. For this reason, for comparison studies, it is best if the same fibre is used on all the samples. The advantages of SPME is that it is quick, easy to use, solvent-less and especially appropriate for comparison of samples. Typical examples from the food and fragrance industries include: determining the volatiles from cheese (Chin *et al.*, 1996); identifying the volatile components of aromatic and medicinal plants (Bicchi *et al.*, 2000b) and verification of the source of hops (Kovacevic and Kac, 2001). Excellent reviews of use of this technique in food analysis have been completed (Harmon, 2002; Wardencki *et al.*, 2004). An overview of the development of the technique in the decade since its invention can be found in Lord and Pawliszyn (2000).

1.5.2.4 Headspace sorptive extraction and stir-bar sorptive extraction

Headspace sorptive extraction (HSSE) and stir-bar sorptive extraction (SBSE) are two recently introduced sorption techniques (Baltussen *et al.*, 1999; Bicchi *et al.*, 2000a; Bicchi *et al.*, 2002). As with SPME, the absorption medium is polydimethylsiloxane (PDMS) which is coated as a film onto a glass-jacketed magnetic stirrer bar. The stir bar has been commercialised under the name Twister™. In HSSE, the Twister is suspended above the headspace of a sample. For

SBSE, the Twister is immersed in a liquid sample. After a fixed sample time, the Twister is desorbed either with solvent, or more commonly by thermal desorption. These techniques are quick and easy to use, require little sample and can be solvent-less. A potential disadvantage is that the sample needs to be desorbed soon after sampling, because the volatiles are retained less strongly than with traditional adsorbent materials. There is also poor discrimination of polar compounds because of the use of the non-polar (PDMS) coating. SBSE has already been used in a number of liquid food related examples, such as extracting benzoic acid from lemon flavoured beverages (Tredoux *et al.*, 2000), quantification of wine components (Fang and Qian, 2006) and identification of volatiles from malt whiskies (Demyttenaere *et al.*, 2003). A useful review of sorption techniques such as SBSE and SPME has been published (Baltussen *et al.*, 2002).

1.6 Characterisation

Once compounds have been isolated from a sample by whichever method, they then require chemical identification. This frequently involves some form of separation, usually chromatographic. Analytical techniques such as mass spectrometry, nuclear magnetic resonance, infrared, ultra-violet and Raman spectroscopy can be used to characterise species. These techniques are discussed in more detail below.

1.6.1 Chromatography

Chromatography is the most powerful and applicable technique for the separation of complex mixtures. Chromatographic techniques are essential to both fragrance and flavour research because natural extracts obtained during the isolation stage can contain hundreds of compounds, only some of which will be of interest to the flavourist or perfumer. The principle of any chromatographic technique is partioning of analytes between a mobile phase and a stationary phase, which separates the analyte to be measured from other molecules in the mixture and allows it to be isolated. The time it takes for a particular compound to elute from the column (its retention time) is reproducible under a given set of conditions. This retention time can be used for identification of compounds by comparison with standards. Those compounds that are poorly absorbed onto the stationary phase have short retention times; strongly adsorbed compounds emerge at a later retention time.

The choice of chromatography technique depends on the nature of the analyte, such as its volatility and polarity. Aroma compounds, by their very nature, must be volatile enough to travel through the air to reach the nose, which makes gas chromatography (GC) the most appropriate separation technique, and the industry standard. Taste active components are often highly polar or of higher molecular weight (greater than approximately 300 Da) in which case high performance liquid chromatography (HPLC) would be the first choice analytical tool. Both of these techniques are considered in more detail below because they have been used extensively in the research described in this thesis.

An analytical instrument can be combined with a separation method for on-line analysis. Examples of such 'hyphenated techniques' include gas and liquid chromatography with mass spectrometry (GC-MS and LC-MS), GC with Fourier-transform infrared spectroscopy (GC-FTIR), and HPLC with diode-array UV/Vis absorption spectroscopy (HPLC-UV/Vis). Only GC-MS and LC-MS are considered here.

Other types of chromatography include supercritical fluid chromatography (SFC), ion exchange chromatography (IEC); size-exclusion chromatography (SEC) (also called gel-permeation chromatography (GPC)); and thin-layer chromatography (TLC), all of which are used in analytical chemistry depending on the nature of the analyte. Preparative chromatography is used to purify sufficient quantities of a substance in a non destructive manner for further use. This technique has been utilised for the work discussed in Chapter 5. Affinity and covalent (or chemospecific) chromatography are two related tools used for the separation of biochemical mixtures, particularly the isolation and purification of biopolymers. These are described in Section 1.6.1.3. Covalent chromatography has been utilised in the work described in Chapter 4.

1.6.1.1 Gas chromatography

GC is the method of choice for the separation of volatile organic compounds. A schematic of the basic instrument is shown in Figure 1.11. In GC (or more correctly, gas-liquid chromatography) the sample is vaporised and injected onto the head of the chromatographic column. The sample is then transported through the column by the

flow of an inert, gaseous mobile phase such as helium or hydrogen. The column contains a liquid stationary phase which is adsorbed onto the surface of an inert solid. The detector monitors the composition of the gas stream as it emerges from the column carrying separated components. Optimisation of separation of components is achieved by varying the injection system, the column and the temperature profile of the experiment.

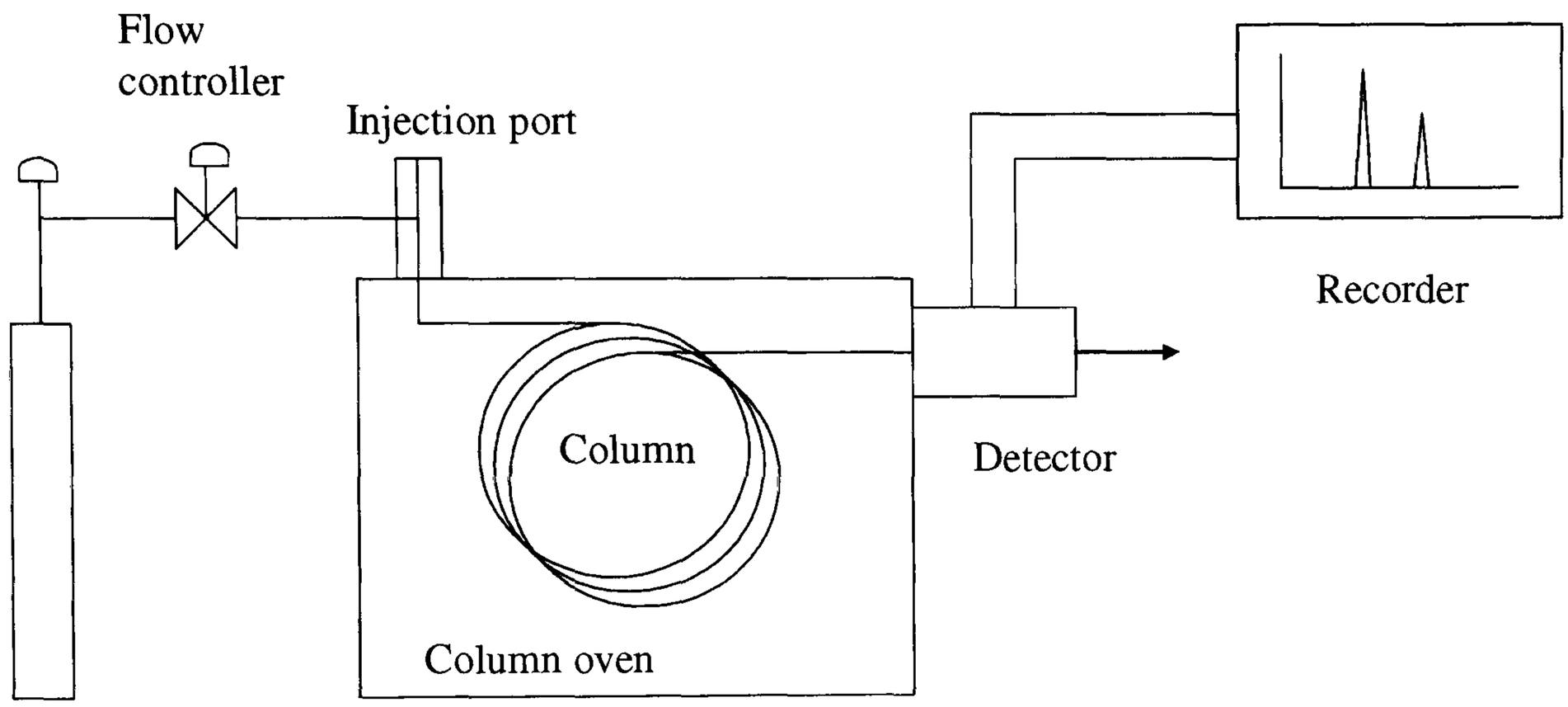


Figure 1.11 Schematic of a basic gas chromatograph

The sample is normally introduced onto the column through a port as a 'plug' of vapour, which gives sharper peaks and better resolution than with a slow injection. The sample (typically a few microlitres) is introduced into the port by micro-syringe. The temperature of the sample port is usually 50°C higher than the boiling point of the lowest volatility component of the sample, to allow for full vaporisation of the sample. To elute all analytes, a programmed temperature GC (PTGC) is often used. In this programme, the column temperature is raised continuously to elute the more retained peaks in a reasonable time during the run. This prevents the spreading of late-eluting compounds and is particularly useful for complex mixtures with a wide range of volatiles.

The typical operating range of GC is between room temperature and 300°C, and consequently is suitable for compounds with a similar range of boiling points to this.

Derivatisation can considerably increase this range by altering the volatility of compounds. The use of derivatisation, a technique used in the work of this thesis, is discussed in Section 1.7.

GC is capable of detecting compounds at concentrations of parts per billion (ppb), depending on the sensitivity of the detector used. The most common detector is a flame ionisation detector (FID) which is sensitive to a wide range of components, and detects over a wide range of concentrations. This works by mixing hydrogen and air with the column effluent and burning it at a small jet. Organic compounds burning in the flame produce ions and electrons which can conduct electricity through the flame. A large electrical potential is applied at the burner tip, and a collector electrode is located above the flame. The resulting current is measured.

Specialist detectors also exist for specific elements such as sulfur, nitrogen and phosphorous. Electron-capture detection (ECD) is very sensitive for halogenated compounds. The most common detector for the identification of unknowns is the mass spectrometer (see Section 1.6.2.1).

In GC, the sample size can be as low as picograms in analytical applications, whilst tens of grams can be handled in preparative work. Identification of analytes can be achieved by comparison of the retention time of an analyte through a column with the retention time of a standard run under the same conditions. Quantification of analytes is achieved by calibration against standards of known concentrations.

The column is critical in gas chromatography for influencing the quality of the separation. The modern GC column is capillary based in which the stationary phase is coated on the inner wall. Current columns are made with fused silica tube and coated with polyimide for strength. They are highly flexible, durable and chemically inert. Fused silica columns are applicable for the separation of almost all GC suitable-mixtures.

The choice of stationary phase and other column properties is dependent on the chemical nature of the analyte, the sample matrix and the solvent, and particularly on the nature of the molecular interaction between analyte and stationary phase. If there are non-polar interactions then separations are based on the analytic volatility, which

is measured simply as boiling temperature. When the analyte or stationary phase contains polar groups then this leads to dipole-dipole interactions between analyte and stationary phase and consequently increased retention and selectivity. A specialised example would be for analytes capable of hydrogen bonding: these can be separated by using a stationary phase containing ether functionality, typically polyethylene glycol. It is also possible to have chiral stationary phases to provide shape-selective separations.

The two most common stationary phases are: a) those based on a cross-linked polysiloxane backbone with pendant groups of varying functionality (e.g. methyl, phenyl, cyanopropyl, or trifluoropropyl) and b) polyethylene glycol phase ('wax' columns). Other variables in the choice of column are:

- Column internal diameter (ID) -typically 100 - 500 μm
- Film thickness - typically 0.25 - 1 μm
- Length - typically 5 m to greater than 100 m

The narrower the column, the greater the resolution but the lower the capacity, and the increasing trend is for shorter, narrower columns. Longer columns provide more resolving power, but increase analysis times and are more expensive. For an interesting review of the development of GC, see Bartle and Myers (2002). A comprehensive look at gas chromatographic techniques and applications has also recently been published (Handley and Adlard, 2001).

An important tool specifically for the flavour and fragrance industries is GC-Olfactory (GC-O), or more colloquially, GC-Sniff. In this technique, the effluent is split between the usual detector and a sniffing port. This allows the eluting peaks to be evaluated for their odour by a trained perfumer or flavourist and correlated with the response from the instrumental detector. This allows for the further investigation of only those compounds with an interesting olfactory response. The nose can be more sensitive than the instrumental detector and some of the key contributors to an odour are often present at trace amounts. An overview of the use of GC-Olfactory in food aroma analysis has been published (Blank, 2002).

Recent developments in GC of relevance to the flavour and fragrance industries include the use of fast GC and two-dimensional GC (GCxGC) for the separation of complex mixtures. The latter is considered the most significant development in gas chromatography in the last decade, introduced by John Phillips and co-workers (Liu and Phillips, 1991; Phillips and Beens, 1999). A typical GC×GC system consists of two chromatographic columns in series. The first-dimension column is (usually) a conventional capillary GC column, with a non-polar stationary phase column, so that it separates components largely based on their boiling points. The second-dimension column is considerably smaller (narrower diameter, shorter length) than the first-dimension column, so that separations in the second dimension are much faster. The result of a two-dimensional separation can be visualized as a very detailed two-dimensional chromatogram. This technique has been used in for several applications in the fragrance and food industries, for example to analyse the volatiles of coffee (Ryan *et al.*, 2004), or for the detection of suspected allergens found in fragrances (Dunn *et al.*, 2006). A recent study has combined two dimensional chromatography with GC-olfactory to analyse essential oils (Zellner *et al.*, 2007). Reviews of the use of comprehensive two-dimensional chromatography in food analysis have been published (Luigi Mondello *et al.*, 2002; Marriott and Kinghorn, 2001; Tranchida *et al.*, 2004).

1.6.1.2 High performance liquid chromatography

In contrast to GC, HPLC utilises a liquid mobile phase to separate the components of a mixture. These components are first dissolved in a solvent, and then forced to flow through a chromatographic column under a high pressure using pumps. In the column, the mixture is resolved into its components. As with other forms of chromatography, the resolution is dependent upon the extent of interaction between the solute components and the stationary phase. The stationary phase is the immobile packing material in the column. Capillary columns can also be used. A schematic of the components of an HPLC system are shown in Figure 1.12.

The interaction of the solute with mobile and stationary phases can be manipulated through different choices of both solvents and stationary phases. As a result, HPLC acquires a high degree of versatility not found in other chromatographic systems, giving it the ability to separate easily a wide variety of chemical mixtures. It is also

easily automated, and highly-sensitive. Detection limits extend to femtogram quantities when a mass spectrometer is interfaced to the LC system.

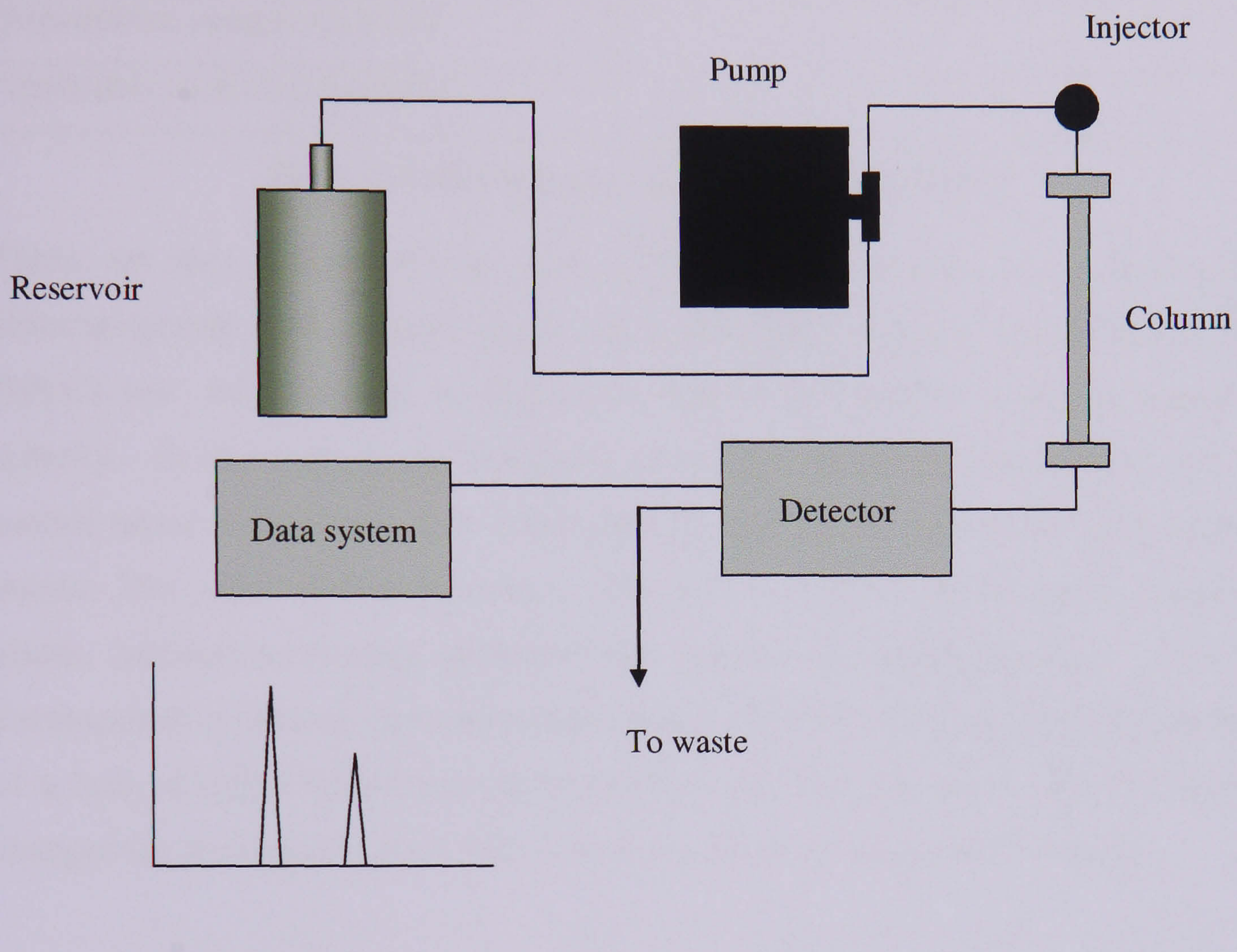


Figure 1.12 Schematic of the components of an HPLC system

As with GC, HPLC can also be used as a preparative technique for materials in milligram to kilogram quantities. A disadvantage of HPLC is that unlike GC, there is no universal detector such as an FID. This makes detection more problematic if compounds do not absorb UV rays or are not easily ionised for mass spectrometric analysis. Separation efficiency is also substantially less than with GC and the number of operating parameters make the operation of an LC instrument more difficult for the beginner. The advantages and limitations of HPLC are summarised in Table 1.3.

Advantages	**Limitations**
Rapid and precise quantitative analysis	No universal detector
Automated operation	Less separation efficiency than GC
High-sensitivity detection	More difficult for novice
Quantitative sample recovery	
Applicable to diverse samples	

Table 1.3 Advantages and limitations of HPLC.

There are two primary modes that HPLC can be operated in: normal phase chromatography and reverse phase chromatography. Normal phase-HPLC (NP-HPLC) was the first type of HPLC developed and separates analytes based on polarity. In this method the stationary phase is a polar stationary phase and the mobile phase is nonpolar. It is used when the analyte of interest is fairly polar in nature. The polar analyte associates with and is retained by the polar stationary phase. Adsorption strength increases with increase in analyte polarity. With the development of reverse phase chromatography, NP-HPLC fell out of favour because of a lack of reproducibility of retention times as water or protic organic solvents changed the hydration state of the silica or alumina chromatographic media.

Reversed phase-HPLC (RP-HPLC) is now the most common mode of operating HPLC. As the name suggests, the relative polarities of column and mobile phase are reversed: there is a non-polar stationary phase and a moderately polar mobile phase. The commonest stationary phase is silica which has been treated with RMe_2SiCl, where R is a straight chain alkyl group such as $C_{18}H_{37}$ or C_8H_{17}. The retention time is longer for molecules which are more non-polar in nature, allowing polar molecules to elute more readily. Retention time is increased by the addition of polar solvent to the mobile phase and decreased by the addition of more hydrophobic solvent. RP-HPLC operates on the principle of hydrophobic interactions which result from repulsive forces between a relatively polar solvent, the relatively non-polar analyte, and the non-polar stationary phase.

The retention of the analyte molecule is strongly dependent on the molecular structure. In general, an analyte with a longer alkyl chain length results in a longer retention time because it increases the molecule's hydrophobicity. Mobile phase

modifiers can be added to affect the mobile phase hydrophobicity, such as by the addition of inorganic salts which changes the properties of aqueous solutions. Another important factor is pH since this can change the hydrophobicity of the analyte. For this reason, most methods use a buffering agent such as sodium phosphate to control the pH. (Note that this is not suitable when MS is used as the detector, because of suppression of ionisation). An organic acid such as formic acid or most commonly trifluoroacetic acid is often added to the mobile phase. These serve multiple purposes: they control pH, neutralize the charge on any residual exposed silica on the stationary phase, and act as ion pairing agents to neutralize charge on the analyte, generally improving the chromatography. A useful introductory textbook describing the fundamentals, applications and developments of HPLC has been published (Wong, 2006).

HPLC is used extensively in many industries, particularly the pharmaceutical industry (Rao and Nagaraju, 2003) and for biological applications, especially in the booming field of proteomics, where two-dimensional liquid chromatography is now being used increasingly in preference to the more common two-dimensional gel electrophoresis method to simplify the analysis of highly complex mixtures of proteins (Peng *et al.*, 2003). The applicability of HPLC to flavours is mainly for non-volatile and polar compounds (Rouseff, 1985). HPLC has been employed to quantify thiosulfates contributing to the flavour of the allium family (garlic, chives, onion etc.) (Block *et al.*, 1992). It is also notable in the literature how important HPLC has proved to be for identifying components in various cheeses including peptides, organic acids and sugars (Addeo *et al.*, 1992; Bouzas *et al.*, 1991; Champion and Stanley, 1982; Engels and Visser, 1994).

Two HPLC systems have been used for the work described in Chapter 5 of this thesis: a preparative HPLC system and a capillary LC coupled with nano-MS. The preparative HPLC system is used to provide an initial separation of a complex extract from plant materials, and fractions are collected for a second separation on the high sensitivity, capillary-LC nano MS system. Both of these systems and the benefits they provide are described in more detail in that chapter.

1.6.1.3 Affinity and covalent chromatography

Affinity chromatography is a separation technique used mainly in biological applications. It is based on selective reversible interaction between an analyte and specific molecules, for example between antigen and antibody, enzyme and substrate, or receptor and ligand. Affinity chromatography can be used in a number of applications, including nucleic acid purification, protein purification from cell free extracts and antibody purification from blood serum. The technique is highly selective. It is the only technique that allows purification of a biomolecule on the basis of its biological function, or individual chemical structure. Covalent chromatography is different from other types of chromatography because, as the name suggests, it depends on a covalent bond forming between the analyte and the stationary phase. It is most commonly applied with thiol compounds, which are capable of forming disulfide bonds with the column material (a gel). The release of the bound species containing the thiol groups is achieved by addition of a reducing agent. There are several examples of how this technique has been used to purify peptides and proteins containing thiol groups (Egorov *et al.*, 1975; Norris and Brocklehurst, 1976; Ryden and Norder, 1981; Svenson *et al.*, 1977; Zvonar *et al.*, 1979). A thiol specific system has been used for the work described in Chapter 4. The general principle of affinity or covalent chromatography is shown in Figure 1.13.

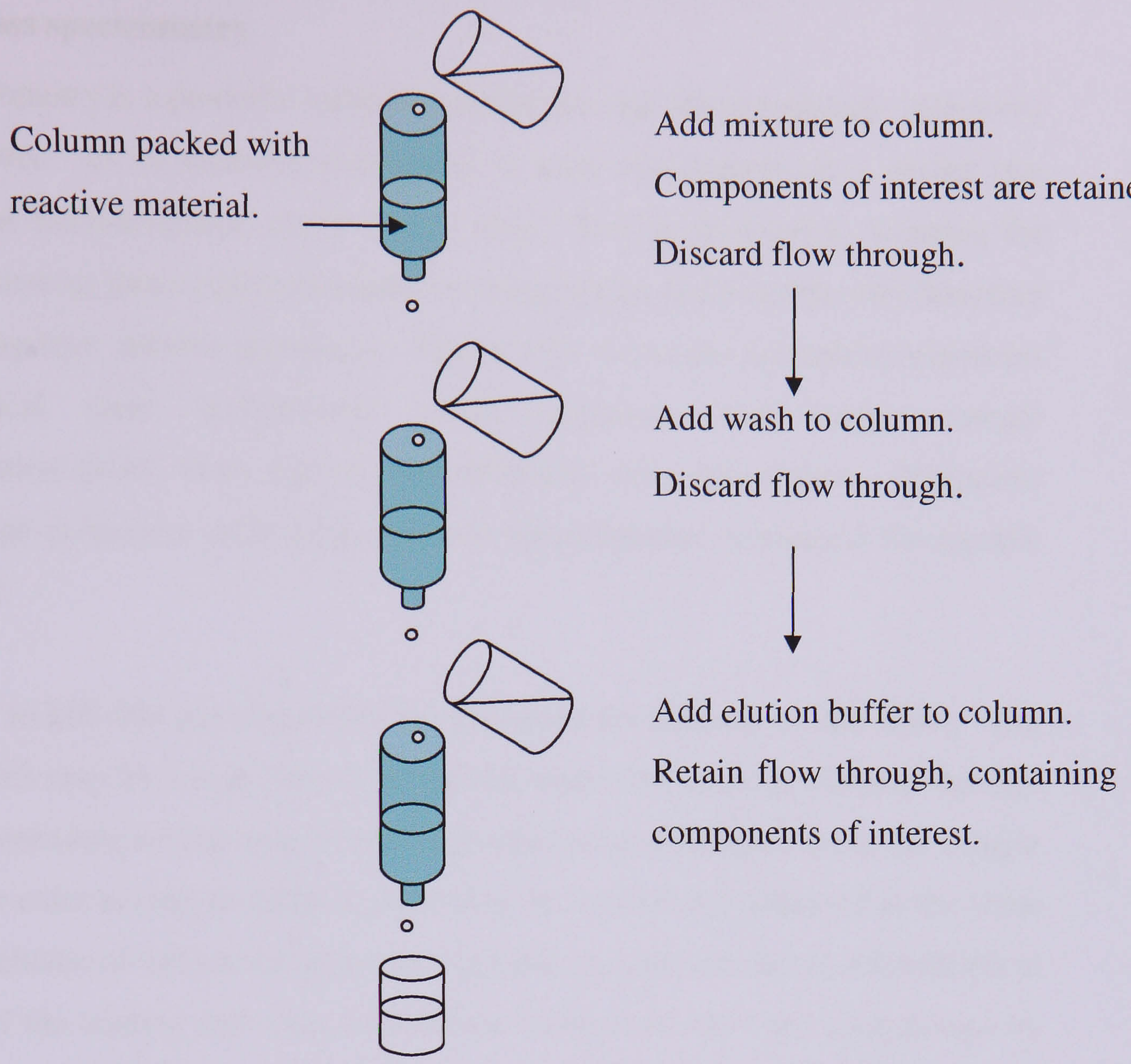


Figure 1.13 The principle of covalent chromatography: the column contains the reactive material; the mixture containing the components of interest is added to column and the components bind to the column. The non-bound species are washed away and the interesting components are released by an eluting buffer and collected.

1.6.2 Analytical techniques

Numerous analytical techniques are available for the identification of compounds each with their specific advantages and disadvantages. The basic principles, merits, limitations and applications to the flavour and fragrance industries of mass spectrometry, nuclear magnetic resonance, infrared, Raman and ultra-violet spectroscopies are considered in this section. These techniques often provide complementary information, and more than one may be required for definitive identification of a compound.

1.6.2.1 Mass spectrometry

Mass spectrometry is a powerful analytical technique that offers both high selectivity and sensitivity. In its simplest description, a mass spectrometer is a device that measures the mass-to-charge (*m/z*) ratio of ions. This is achieved by ionizing the sample, separating ions of differing mass-to-charge ratios and subsequently detecting and recording their relative abundance. Figure 1.14 shows the essential components of a typical mass spectrometer which comprises four stages: sample introduction/ionisation, mass analysis, ion detection and a data system. Variations on each stage produce a wide range of mass spectrometers, optimised for specific applications.

It is critical to MS that gas phase ions are generated for analysis to take place. The sample, which may be a solid, liquid, or vapour, enters the vacuum chamber through an inlet. Depending on the type of inlet and ionisation techniques used, the sample may already exist as ions in solution, or it may be ionised and volatised at the same time. The choice of ionisation technique depends strongly on the physicochemical properties of the analyte and what information is required about the analyte such as the molecular weight, structural information or both. This information is determined by the extent of fragmentation which is in turn influenced by the amount of internal energy transferred during ionisation. Both positive and negative ions can be generated.

Some ionisation techniques such as electron ionisation (or electron impact) (EI) are very energetic and generally cause extensive fragmentation of the ions. Other techniques are 'softer', such as chemical ionisation (CI) and generally produce molecular species. Both EI and CI are only suitable for gas-phase ionisation and therefore their use is limited to samples sufficiently volatile and thermally stable. These techniques are often used when the mass spectrometer is interfaced to a GC. Only EI was used in the GC-MS work described in this thesis. EI has the benefit of generating a mass spectrum that is often unique for each compound and therefore can be compared to reference libraries for positive identification. If the compound is not in a reference library, manual interpretation may be required, or comparison can be made to a standard.

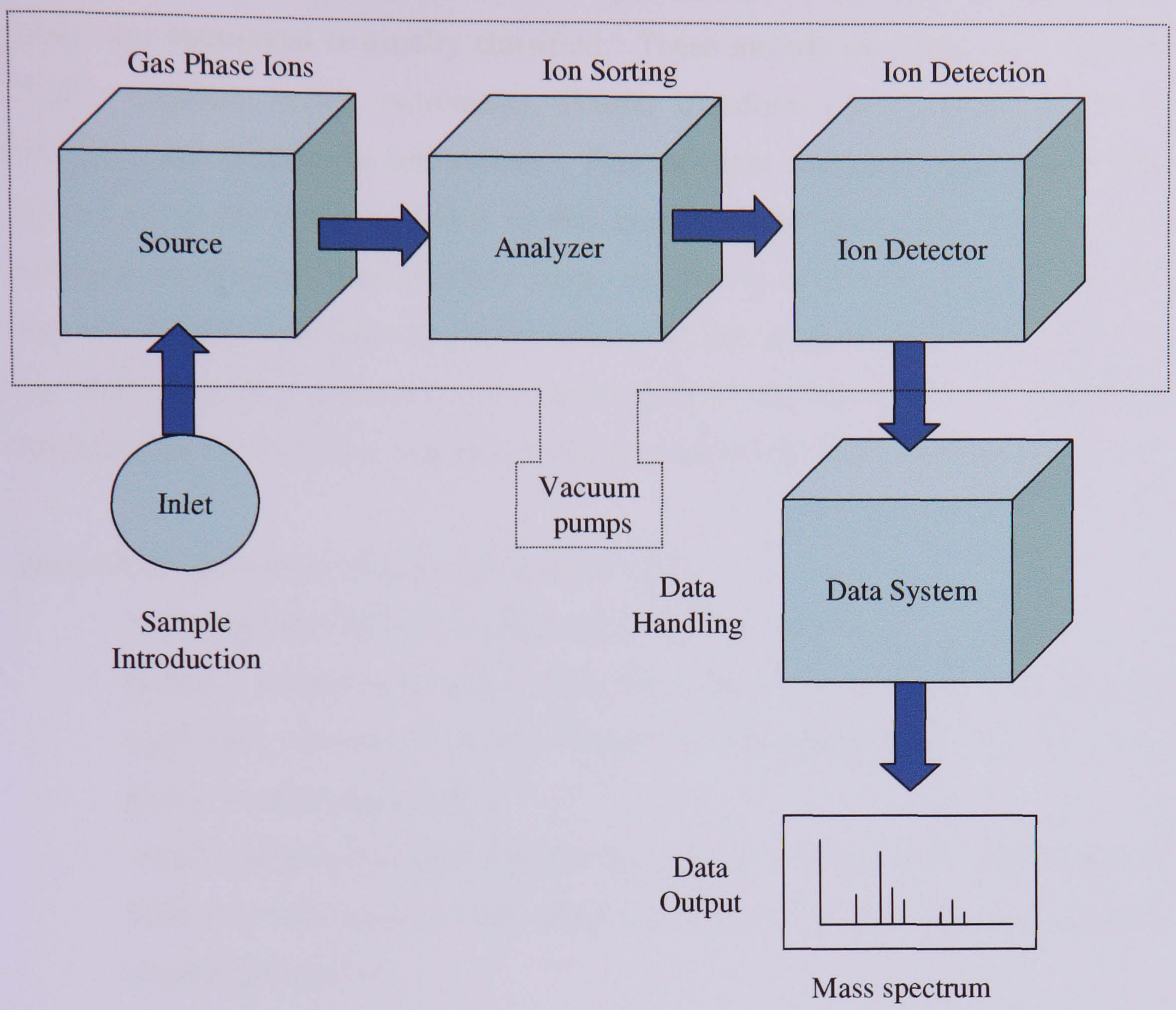


Figure 1.14 The components of a mass spectrometer. (Redrawn from (Chiu and Muddiman, 2001)).

For samples that are thermally labile or have insufficient vapour pressure, ions must be generated directly from either liquid or solid phase into the gas phase. Ionisation techniques which ionise from the analyte in solution include electrospray ionisation (ESI) and atmospheric-pressure chemical ionisation (APCI) both of which were utilised in the research described here. They are commonly used in conjunction with HPLC. ESI has the advantage that multiply charged ions are often generated, so extending the mass range that can be analysed. Other ionisation techniques include matrix-assisted laser desorption/ionisation (MALDI) which is designed to ionise and volatilise solid phase samples. Secondary ion mass spectrometry (SIMS) is another technique aimed at the solid phase to gas phase ion transition, primarily used for examining surfaces.

The second major variable of the mass spectrometer is the mass analyser, upon which the instrument is usually classified. These include ion traps, time-of-flight (TOF), magnetic sector instruments, Fourier transform ion cyclotron resonance (FTICR), and quadrupole instruments. Time-of-flight and quadrupole instruments were used in the work described in this thesis. Quadrupole mass analysers are scanning instruments that produce mass spectra by sequentially measuring the intensity of individual mass-to-charge ratios over the range selected. In contrast, in the TOF mass spectrometer all the ions present in the source are simultaneously extracted and subsequently separated by their varying flight times to the detector.

Some of the limitations of mass spectrometry are:

- it is sometimes difficult to distinguish between isomers;
- isobaric compounds (those with the same nominal mass but different molecular formulas) can be difficult to distinguish unless the resolving power is sufficiently high;
- some compounds may decompose or isomerise during the ionisation process leading to misleading results, especially if heat is used as part of the process to generate the ions;
- it is often not possible to predict the mass spectrum of a compound because the detailed mechanisms of ionisation processes are not fully understood;
- quantification can only be achieved by the addition of a suitable internal standard.

Good reference texts on modern mass spectrometry have been published (de Hoffmann and Stroobant, 2002; Downard, 2004; Gross, 2004).

It is now increasingly common for mass spectrometers to be used in tandem (MS/MS) or to even greater degrees in $[MS]^n$ to generate more structural information. In the basic MS/MS experiment, a precursor ion is selected by the first mass analyser. This ion is passed into a collision cell containing a low pressure of inert gas. A small fraction of the translational energy of the precursor ion is converted into internal energy on collision with the inert gas. Fragmentation occurs and product ions are formed from the chosen precursor ion. These product ions are collected by the second mass analyser. The main advantage of this experiment is the increased specificity and greater sensitivity as the ions from the interfering

components are reduced. The sensitivity is such that detection levels are 1 femtomole or lower which makes MS ideal for trace analysis work.

The mass of ions or molecules can be determined to different levels of accuracy. The nominal mass may be quoted which is calculated using the mass of the most abundant isotope of each element rounded to the nearest integer value. Accurate mass measurement is the process of determining the mass of an ion to a high level of accuracy (i.e. to one or more decimal places). This is a very useful tool for increasing the certainty of structural identification, since in favourable cases, the elemental composition of the ion can be determined. Generally, accurate mass measurements require the use of higher resolution instruments such as TOF or FTICR analysers. The high resolution is required when there is interference from contaminant ions of similar mass to the sample ion. Higher resolution can also lead to a more reliable calibration which is crucial for accurate mass measurement. Isotopic patterns can also be used to aid determination of the elemental composition of an ion. These are sets of peaks related to ions with the same chemical formula but containing different isotopes. The intensity ratios in the isotope patterns are due to the natural abundance of the isotope and can be used to determine the number of atoms of each type in the ion. For example, the two isotopes of chlorine, ^{35}Cl and ^{37}Cl, are naturally abundant in the ratio 3:1.

For complex fragrance and flavour extracts, MS techniques are used in conjunction with GC and LC. The extra structural information generated from accurate mass analysis and tandem mass spectrometry experiments, along with retention times from the preceding chromatographic separation may be the only method of unambiguously identifying the structure of an analyte. For example, the guidelines for establishing nature-identical status of a compound, give much higher weighting to this kind of evidence (European Union, 2002). A summary of studies which have utilised high resolution GC-MS for structural determination in flavour and fragrance analysis has been published (Holland and Gardner, 2002). For reference texts on hyphenated techniques see McMaster (2005; 1998).

A particular mass spectrometry interface used in the work described in Chapter 3, is the MS Nose™. This technique is designed for the analysis of volatile compounds in

real time. The system is suitable for a range of applications, and can be used to analyse volatile compounds from a wide variety of sources including human breath, perfumes, headspace samplers etc. (Taylor *et al.*, 2000). This instrument is described further in that chapter.

1.6.2.2 Nuclear magnetic resonance spectroscopy

Nuclear magnetic resonance spectroscopy is one of the most highly-utilised and powerful analytical techniques for structural determination. It is based on a quantum mechanical property of certain nuclei, called spin (I), which makes these nuclei behave in a similar fashion to bar magnets. In the presence of a strong magnetic field, the nuclear magnets can orient themselves in $2I + 1$ ways. Those nuclei with an odd mass number have nuclear spins of $^1/_2$, or $^3/_2$, or $^5/_2$ and so on. Nuclei with spin $^1/_2$ can only take up two orientations when a magnetic field is applied: a low energy orientation when aligned with the applied field, and a high energy orientation opposed to the applied field. The levels are populated to different extents. When the nuclei within the magnetic field are excited with radio frequency (RF) electromagnetic waves, some of the nuclei are promoted from the low energy state to the high energy state. The nuclei are observed as they 'relax' from this excited state, and it is this response that is measured in the NMR experiment.

All modern NMR spectrometers make use of a Fourier transform (FT) to transform the signal mathematically into the resultant NMR spectrum. In this technique a range of frequencies is probed at once which decreases the time required for a scan and gives increased sensitivity.

The most commonly measured nuclei are ^{1}H (the most sensitive isotope at natural abundance) and ^{13}C which both have spins of $^1/_2$. Nuclei from isotopes of many other elements can also be observed such as ^{31}P, ^{15}N or ^{19}F. The NMR spectrum gives information about the chemical environment of the nuclei and is interpreted to give information on molecular structure such as the relative position of different nuclei within the molecule. NMR spectra can be predicted and experiments are highly reproducible because NMR is based on a quantum mechanical property. Spectra of unknowns can be compared to databases of spectra to aid structure elucidation.

The main limitation of NMR is that it is inherently insensitive, relying as it does on detecting a small fraction of excited nuclei, and on the abundance of those nuclei with spin. For example, the natural abundance of ^{13}C is 1.1% which makes ^{13}C NMR much less sensitive than ^{1}H NMR. NMR is, therefore, significantly less sensitive than other analytical techniques such as mass spectrometry. The majority of NMR experiments are also conducted in solution, and thus rely on the solubility of the compound to be studied. This can be the limiting factor for experimentation with large biological polymers, for example. NMR measurements of samples in the solid state have also been developed but are less widely utilised since the resolution of the spectra is very poor so that less detailed information can be obtained. NMR can also be used in conjunction with chromatographic techniques such as in LC-NMR to simplify the analysis of mixtures.

In the fields of fragrance and flavour research, NMR has limited applicability to the identification of trace species because of the lack of sensitivity. It is primarily used in these industries for determination of structure when chemists synthesise novel compounds. In the work described in this thesis, NMR is used for structural determination only. One-dimensional experiments using ^{1}H and ^{13}C NMR were conducted. The latter are correlation experiments that allow additional information about how nuclei are connected to each other to be determined. For further information on the principles and applications of NMR spectroscopy and interpretation of NMR spectra, see a general textbook (Friebolin, 2005; Hore, 1995; Keeler, 2005; Levitt, 2001; Williams and Fleming, 1995).

1.6.2.3 Infrared and Raman spectroscopy

Infrared spectroscopy is a reliable and well-established technique utilising the electromagnetic waves from the infrared region of the spectrum, typically of wavelengths in the range of 2.5-16 μm. Vibrations of the atoms of a molecule are excited when IR energy is absorbed. The vibrations are characteristic of the type of bonds between atoms so that specific functional groups always absorb infrared radiation of similar wavelengths. The absorption pattern can be interpreted to determine functional groups and acts as 'fingerprint' recognition of a compound which can be compared with libraries of spectra.

IR has the advantage of being easy to use and requiring little sample preparation so that it is one of the simplest, most rapid techniques for establishing classes of compounds. IR spectra can be obtained from solids, liquid or gases, and can also be used for monitoring reactions in situ. It is, however, not very sensitive and often does not provide full structural information.

Most modern instruments are Fourier transform infrared (FTIR) spectrometers which have many advantages over older designs, including greater sensitivity and higher resolution. FTIR spectrometers can be operated in two common modes: transmission and reflectance. Transmission spectroscopy is used frequently and involves passing infrared radiation completely through a sample and measuring the extent of absorption. Some sample preparation may be required as the physical nature of the sample needs to be considered, such as sample thickness. There are also several reflectance techniques in use the most common being attenuated total reflectance (ATR) spectroscopy. A comprehensive text on all aspects of infrared spectroscopy can be found in the five volume reference by Chalmers and Griffiths (2001).

Raman spectroscopy is the measurement of the wavelength and intensity of inelastically scattered light from molecules. The Raman scattered light occurs at wavelengths that are shifted from the incident light by the energies of molecular vibrations. The mechanism of Raman scattering is different from that of infrared absorption, so that the information revealed is complementary to that derived from IR spectroscopy. For example, carbon-carbon double bonds absorb poorly in infrared but give a strong signal in a Raman spectrum.

The most common light source in Raman spectroscopy is an argon ion laser. Spontaneous Raman scattering is typically very weak, and as a result the main difficulty of Raman spectroscopy is separating the weak inelastically scattered light from the intense Rayleigh scattered laser light. A recent text on the use of Raman spectroscopy has been published (Smith and Dent, 2005).

As with IR spectroscopy, Raman spectroscopy is attractive because of the lack of sample preparation required. Both techniques can also be used on a whole range of

physical states: for example, solids, liquids or gases, in hot or cold states, in bulk, as microscopic particles, or as surface layers. Raman spectroscopy has an advantage over FTIR spectroscopy in that samples containing water can be examined because water provides very little interference in Raman spectroscopy. Raman spectroscopy can also be used to analyse samples inside glass containers and is therefore attractive for remote monitoring of reactions. Fluorescence of samples can, however, be a major hindrance in the generation of data. Typical applications are in structure determination and multi-component qualitative analysis.

In the context of flavour and fragrance work, IR and Raman are generally insufficiently sensitive for trace level analysis but can be used for structural identification of synthesised compounds. In the work described in this thesis, IR and Raman spectroscopies are used in the conventional manner for structure verification but both are also used in conjunction with microscopy to generate chemical images, a more specialised and novel application.

In chemical imaging or mapping techniques, a spectroscopy technique is combined with spatial resolution to generate maps of the distribution of chemical species in a sample. An example of a Raman image is shown in Figure 1.15. In the simplest mapping techniques, an image is built up by acquiring a spectrum from the sample and moving across the sample to acquire another spectrum in a sequential manner. An image is constructed based on some feature of the spectrum, such as the intensity of a particular band. This method of constructing an image is necessarily slow. Multi-channel detectors have been available in conjunction with Raman spectroscopy for some time. Only recently have arrays of IR detectors become available which allow for spectra from different regions of a sample to be acquired simultaneously (known as 'Global' IR imaging). This considerably speeds up acquisition times.

The main advantage of these imaging techniques is the richness of the information contained in the image compared to that obtained by the use of more traditional imaging techniques. The time to acquire data and the relatively lower spatial resolution (approximately 5 μm for IR and 1 μm for Raman) mean that these

techniques are not universally applicable. Improving this spatial resolution is an active area of research; see for example, Chan and Kazaria (2003). Applications of IR and Raman imaging have been reviewed (Bugay, 2001; Kidder *et al.*, 2002; Krafft and Sergo, 2006; Pudney *et al.*, 2002). Widespread use is found in the fields of polymers, pharmaceuticals, and particularly biomaterials.

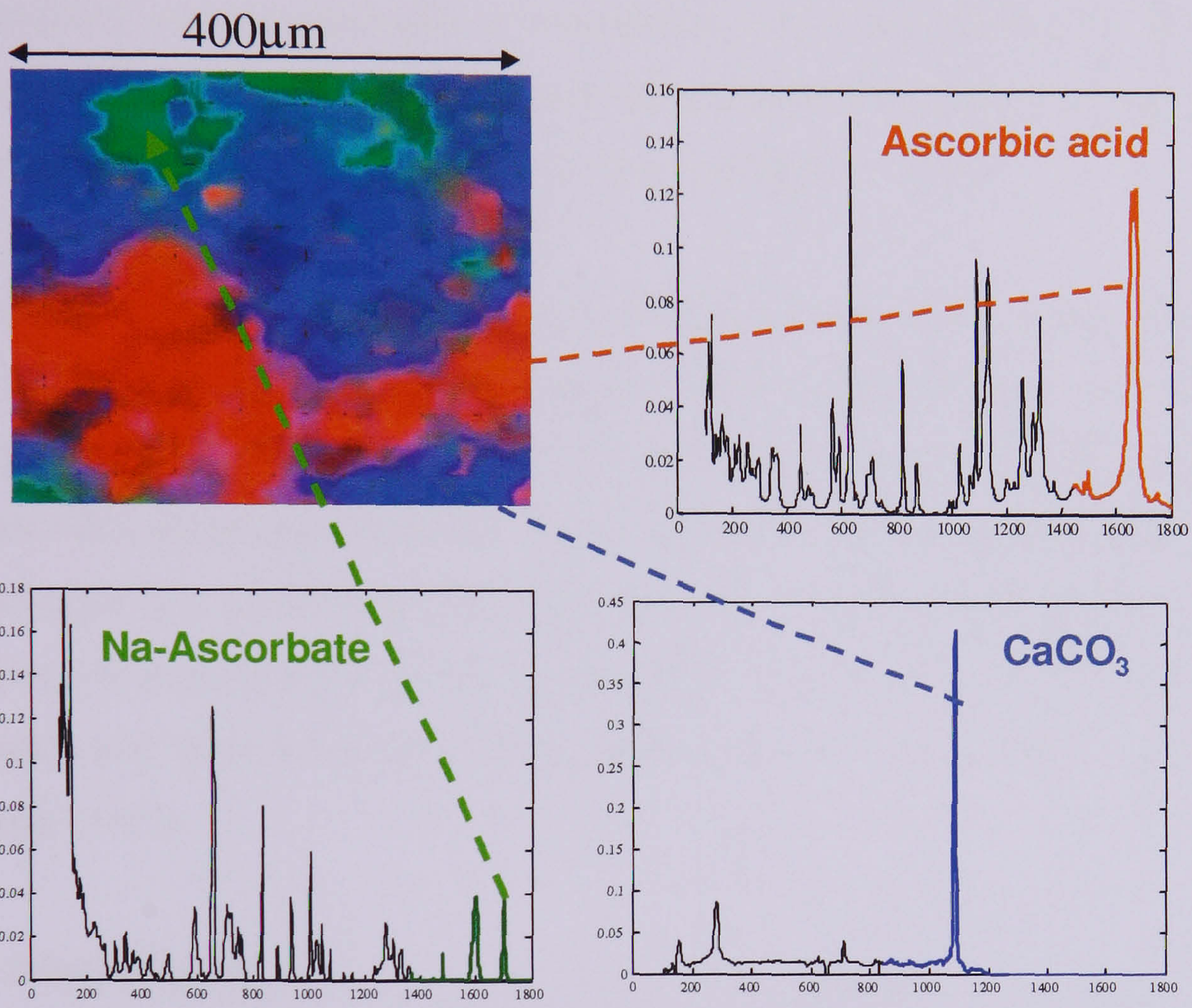


Figure 1.15 Raman image reconstructed from intensities of bands identified as belonging to ascorbic acid, sodium ascorbate and calcium carbonate. This shows that the component distribution is inhomogeneous on the µm scale. (Everall, 2002).

1.6.2.4 Ultraviolet-visible spectroscopy

Ultraviolet-visible spectroscopy (UV/Vis) uses light in the visible and adjacent near ultraviolet (UV) and near infrared (NIR) ranges. In this region of the electromagnetic spectrum, molecules undergo electronic transitions. In organic chemistry, UV/Vis spectroscopy is used primarily for the identification and quantification of compounds containing conjugated systems, where there are electronic transitions involving π orbitals and lone pairs.

UV/Vis spectroscopy is commonly used to determine the concentration of a solution: the absorbance (A) of a solution is measured by the spectrometer which is directly proportional to the solution's concentration. This is the Beer-Lambert law, expressed as $A = \varepsilon cl$, where ε is the molar absorbtivity with units of $L.mol^{-1}.cm^{-1}$ (a constant), c is the concentration of the compound in solution, expressed in $mol.L^{-1}$ and l is the path length of the sample - that is, the path length of the cuvette in which the sample is contained, usually expressed in centimeters. A is absorbance and this itself is defined as $\log_{10} (P_0 / P)$ where P_0 is the radiant power of the beam entering a sample, and P is the power of the beam after passing through the sample.

The use of UV/Vis spectrometry for flavour and fragrance applications is fairly limited, primarily due to complex nature of most compositions. It is, however, routinely used as a detector for HPLC when this is used for separating flavour extracts. This is the context in which it is used for the work described in this thesis. It is also used for monitoring reactions involving conjugated systems used in the covalent chromatography system described in Chapter 4. An overview of the principles and applications of UV/Vis spectroscopy can be found in Williams and Fleming (1995).

1.7 Derivatisation

Derivatisation is the process of chemically modifying a compound to produce a compound with new desirable properties. It is most commonly used in conjunction with chromatographic analysis. Derivatisation is a special type of microscale synthetic chemistry and can often be the key to unlocking and simplifying complex separation problems. The work described in Chapter 3 used derivatisation to do just that.

The many reasons for using derivatisation are:

- to increase the volatility and decrease the polarity of compounds;
- to increasing the thermal stability of samples;
- to increase detector response by incorporating functional groups into the derivative which produce a higher detector signal, for example, CF_3 groups for electron-capture detectors;

- to form fragmentation-directing derivatives for MS analysis;
- to improve separation and peak shape (reduce tailing) in GC and HPLC; and
- to improve extraction efficiency (e.g. acylation of phenolic amines before extraction from aqueous media).

Derivatisation is most often applied to compounds containing polar groups such as carboxylic acids, amines, alcohols and thiols, replacing the active hydrogen nuclei with thermally stable, non polar groups in order to protect the chromatographic columns from undesirable interactions between column and analyte leading to possible irreversible adsorption. The derivatisation procedure applied depends on the functional group to be derivatised, the other functional groups in the molecule and the purpose of the derivatisation. The goal in analytical derivatisation is 100% yield of a single pure product, which is achieved on a micro-scale by the use of high purity reactive reagent, often in large excess, so allowing for quantitative and rapid reactions.

Some of the disadvantages which have been observed with derivatisation include difficulties removing the excess derivatising agent and interference in the analysis. This is particularly disadvantageous when the purity of the compounds is being measured. The derivatisation conditions might also cause unintended chemical changes in the compound such as rearrangements or structural alterations. The derivatisation step also has the disadvantage of increasing the total analysis time.

Derivatisation has been in use for many years and therefore there is a huge range of well characterised, commercially available reagents available mainly based on the following transformation chemistry: acylation, alkylation and silyation (Supelco, 1997). Acylation converts the groups -NH, -OH, -SH into amides, esters and thioesters respectively using a carboxylic acid or a carboxylic acid derivative. Acylating reagents are used particularly with highly polar multi-functional compounds, such as carbohydrates and amino acids. They are also used to introduce per-halogenated groups for enhanced ECD detection.

Alkylation replaces the proton with an alkyl group and is usually the first choice for derivatisation of acids. The most popular method of alkylation is esterification,

(reaction of an acid with an alcohol in the presence of a catalyst to form an ester) due to the availability of reagents and ease of use.

In silylation, an active hydrogen is replaced by an alkylsilyl group such as trimethylsilyl (TMS) or *t*-butyldimethylsilyl (*t*-BDMS). Silylation is the most widely used derivatization procedure for sample analysis by GC. The popularity of silylation reagents is enhanced by their ease of use and formation of derivatives. Although silylating derivatives are prone to hydrolysis, the use of the t-BDMS groups enables the formation of derivatives 10,000 times more stable to hydrolysis than the TMS ethers. Silylation is particularly common when a mass spectrometer is used as the detector because the introduction of a silyl group can also enhance mass spectrometric properties of derivatives, by producing either more favourable diagnostic fragmentation patterns for use in structure investigations, or characteristic ions of use in trace analyses (employing selected ion monitoring and related techniques). Excellent texts on the selection of suitable analyte-specific derivatising reagents have been published (Blau and Halkett, 1993; Knapp, 1979). Derivatising reagents for aldehydes and ketones are described in more detail in Chapter 3, and those for thiols in Chapter 4, where reagents have been utilised with these functional groups.

1.8 Solid-phase reagents

Supported reagents have been in use in synthetic chemistry for 50 years but have only seen wide-spread usage in the last two decades. They are generally used in organic synthesis with recent interest particular driven by high-throughput parallel synthesis. Parallel synthesis allows for the rapid production of large numbers of compounds (combinatorial chemistry), and is therefore particularly important in industries such as pharmaceuticals where there is a critical need to develop new, patentable drugs (Zaragoza Dörwald, 2000). The principle of conventional solid-phase synthesis is that reagents are immobilised on an insoluble support, normally a polymer. The reaction is performed by shaking the support with a mixture of solvents and reagents for a given time, filtering the mixture and washing the support with suitable solvents. The product is released by cleavage from the resin, usually yielding a high purity product. These solid-phase reagents, therefore, offer the possibility of facile and simple reaction protocols with particularly simple isolation

steps. Solid-phase synthesis is now used routinely for the synthesis of peptides, deoxyribonucleic acid (DNA) and any molecule that need to be synthesised in a certain sequence.

Alternatively, an increasingly popular way of using solid-supported reagents is to carry out reactions in solution with excess reagent removed by solid-supported scavengers. Reagents and catalysts can also be used on solid-supports. This method combines the advantages of solution-phase and solid-phase reactions. The two processes are illustrated in Figure 1.16. For a review of reactions using solid-phase chemistry in this way see Ley *et al.* (2000).

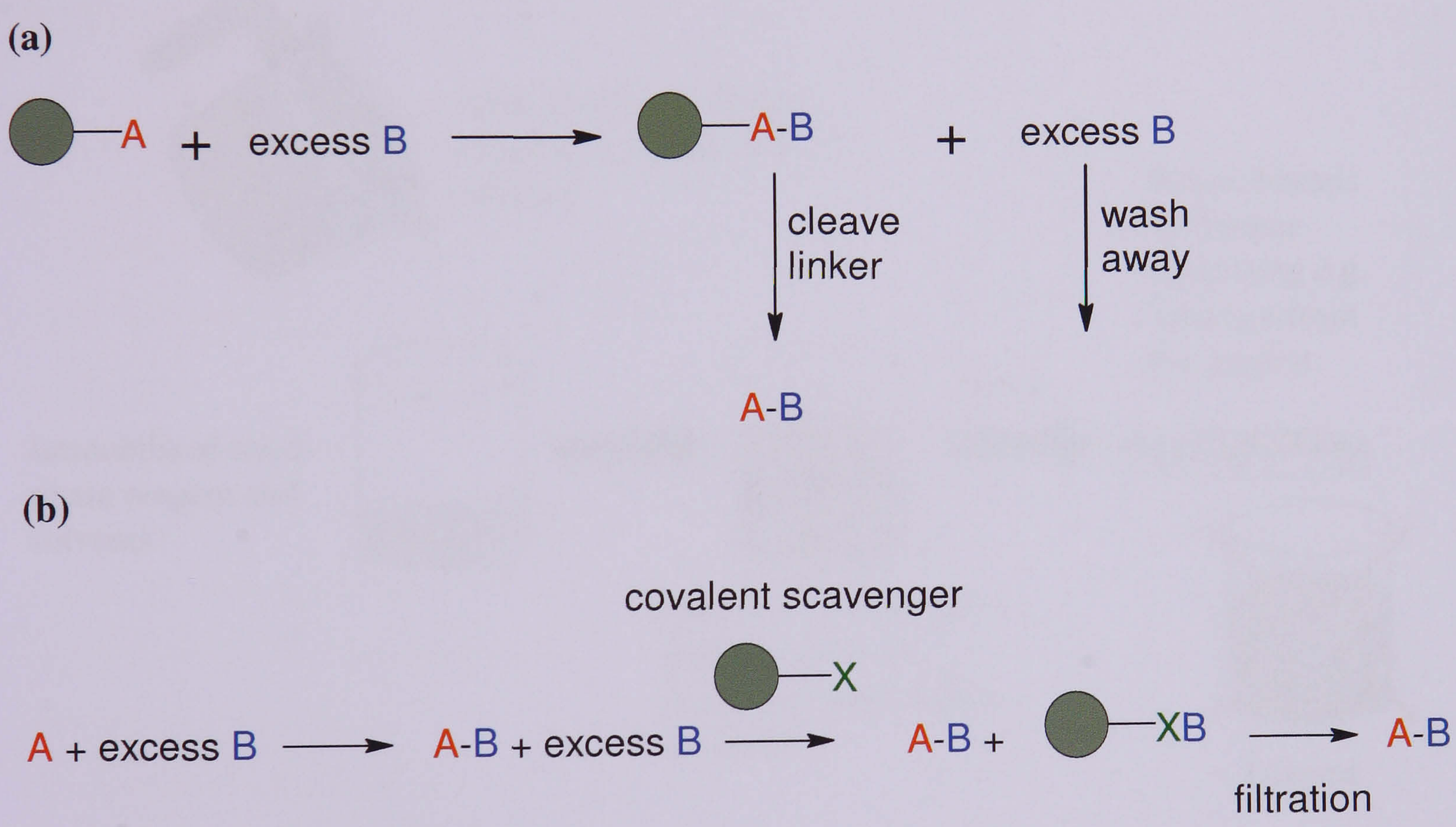


Figure 1.16 (a) Conventional solid-supported chemistry as used in combinatorial chemistry programmes and (b) reactions in solution with solid-supported scavengers.

In the work described in this thesis, a polymer supported reagent was used to immobilise compounds of interest by chemically reacting with them, thereby isolating them from the matrix. Once reacted, all other extraneous reagents, matrix, etc. can be washed away. The reacted bead can be used in further reactions such as

releasing the trapped species from the bead. This provides for the possibility of regenerating the molecule of interest, clean chemistry steps and enrichment of target compounds. The principle of the process is illustrated in Figure 1.17, as an example of how target compounds could be isolated from a complex food matrix such as coffee. An extensive review of the literature suggested that there were no published studies on using these polymer-supported reagents to isolate flavour and fragrance compounds. The use of these beads for isolating a particular class of compounds, namely thiols, is described in Chapter 4. This brief overview explains some of the principles of use of these reagents and their advantages and limitations.

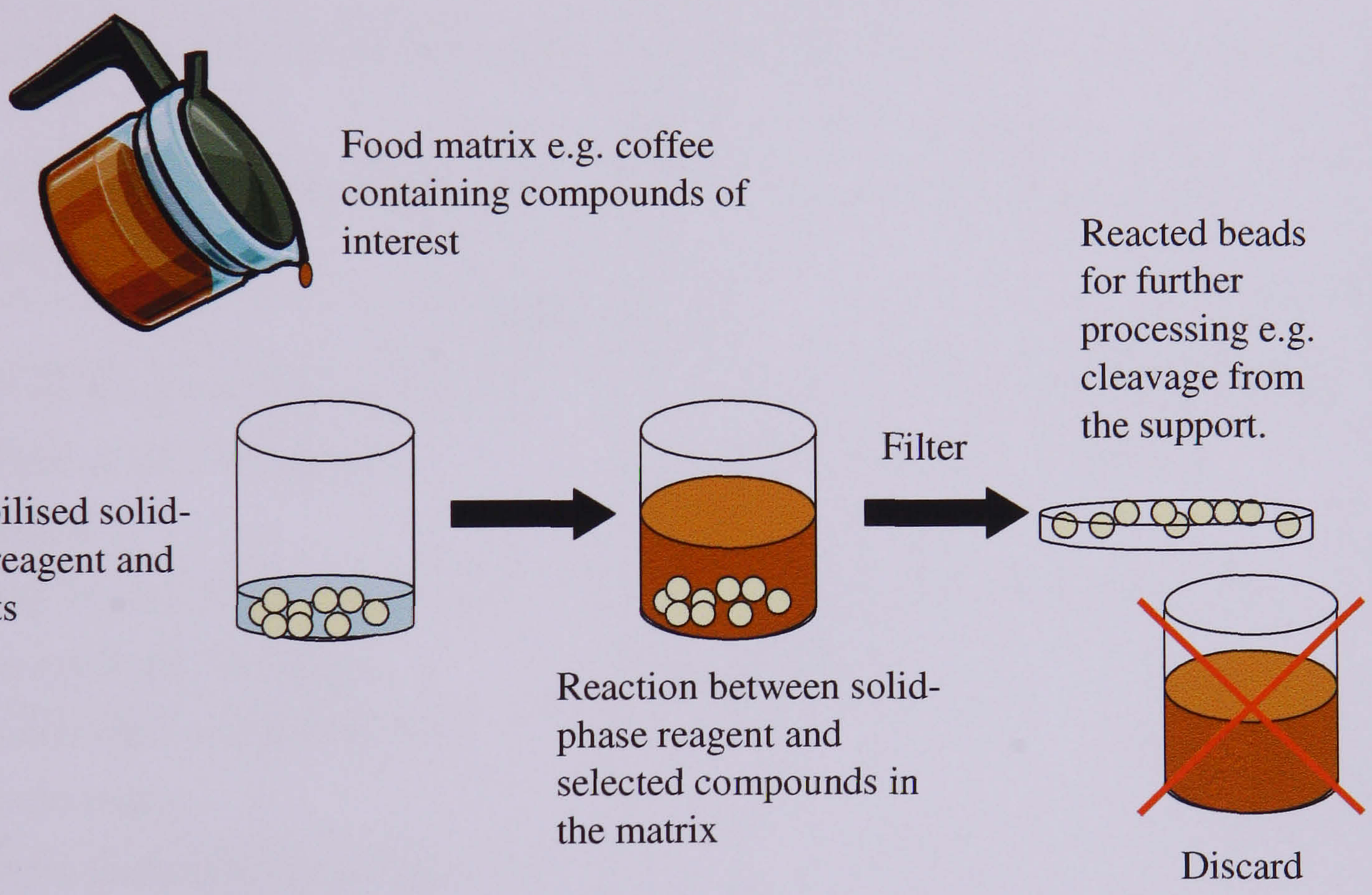


Figure 1.17 An illustration of how solid-supported reagents could be used to extract selected compounds from a complex food matrix.

There are several advantages to using solid-phase reagents as opposed to solution-phase, particularly the simplicity of work-up and purification steps, and the ease of automation of reactions. A major draw-back of solid-phase reactions is that standard solution-phase methods for analysis in chemistry are not applicable. This can be overcome by cleaving some of the reaction intermediate or product from the support and characterising it by the usual solution-phase methods. Several techniques also exist for analysing the beads with analytes still attached, including combustion

analysis, colorimetric assays for specific functional groups, IR, MALDI-TOF MS and TOF-SIMS. These are reviewed in the text by Zaragoza Dörwald (2000). A comparison of the advantages and disadvantages of solid-phase and solution-phase synthesis is made in Table 1.4.

On Solid-Phase	**Solution-Phase**
Advantages	
Reactants can be used to excess without separation problems later, and thus the reaction can be driven to completion.	All organic reactions can be used in principle.
Simple purification of the product by washing the support.	No adaptation of known reaction conditions required.
Automation of reactions easily achievable.	No additional reaction steps for linkage to and cleavage from support.
Split synthesis possible ("one bead, one compound").	Unlimited amounts of product can be produced.
Disadvantages	
Not well developed which leads to an expenditure of time on method development.	Reactants cannot be used in excess unless additional work is invested during purification.
Additional reaction steps for linkage to and cleavage from the support.	Automation of isolation and purification steps is difficult.
Support and linker availability limit possible chemistry.	
Methods for analytical monitoring of the reactions are not well developed.	

Table 1.4 A comparison of the merits and limitations of organic synthesis with solid-phase reagents and in solution.

Solid-phase reagents are usually polymer based, with a linker group used to anchor an active functional group which undergoes the reaction (Figure 1.18).

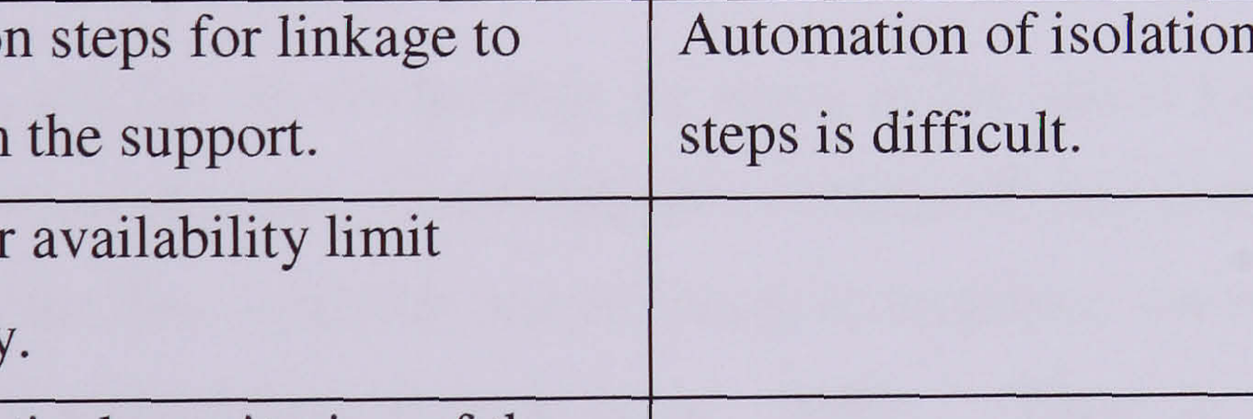


Figure 1.18 Components of a solid-phase reagent.

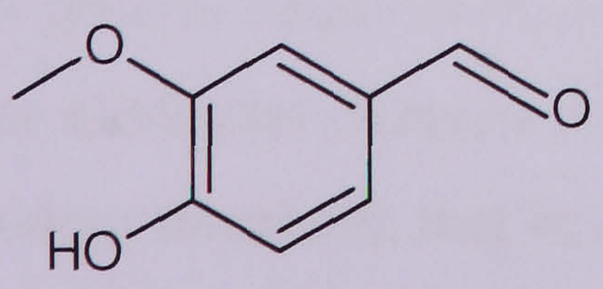
The solid supports should be mechanically stabile and chemically inert. They also need to be chemically functionalised so that the linker can be attached. The most common supports are copolymers of styrene with divinylbenznene. Other possibilities are polyacrlyamides, poly(ethylene glycols) and silica. The polymers must be able to swell (like a gel) when placed in solution which increases the surface area to reactants. Many commercially available reagents are available with a choice of resin, linker and functional group depending on the usage. Several comprehensive reviews have been published of reactions carried out on the solid-phase (Balkenhohl *et al.*, 1996; Booth *et al.*, 1998; Brown *et al.*, 1998; Drewry *et al.*, 1999; Hermkens *et al.*, 1996; Ley *et al.*, 2000). Specific reactions involving thiols are discussed in Chapter 4.

1.9 Specific families of fragrance and flavours compounds

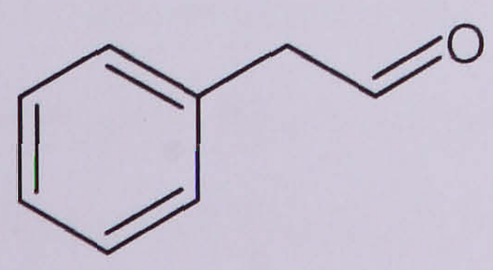
The full spectrum of chemical functional groups is odour and taste active and therefore this investigation of novel compounds was required to be selective. Three classes of odour and flavour active compounds of primary interest to this thesis are discussed below.

1.9.1 Aldehydes and ketones

Volatile aldehyde and ketone compounds are some of the oldest known natural and synthetic fragrance compounds. The aldehydes vanillin (**1**) and phenyl acetaldehyde (**2**) were amongst the first synthetic nature identical fragrance chemicals, first made in 1876 and 1883 respectively (Frater *et al.*, 1998). There is no single odour descriptor for this class of compound. Several, aldehydes and ketones, however, are character-impact compounds, that is, they are the main contributors to the character of the flavour or fragrance. Examples include benzaldehyde (**3**) which is the characteristic smell of almonds, 4-phenyl-2-butanone (**4**) the major contributor to raspberry aroma, 2-*trans*-6-*cis*-nonadienal the major contributor to cucumber (**5**) and (+)-nootakatone (**6**) which is one of two high-impact compounds that make up the smell of grapefruit (the other being a thiol).



(1)



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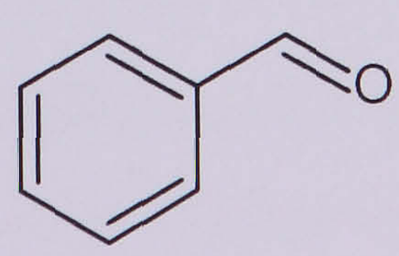
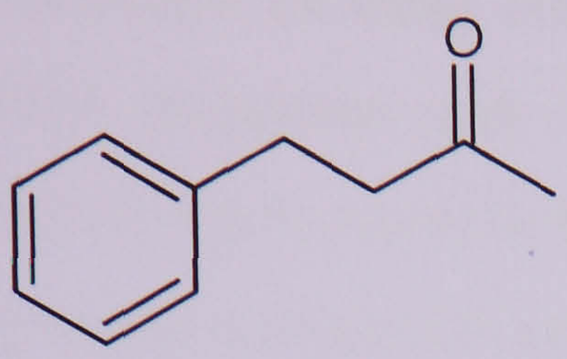


(3)



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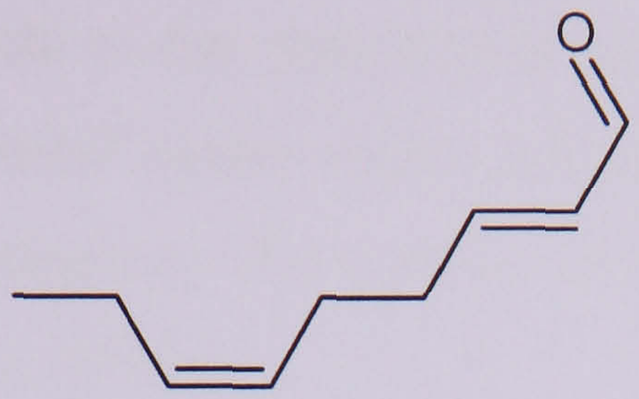

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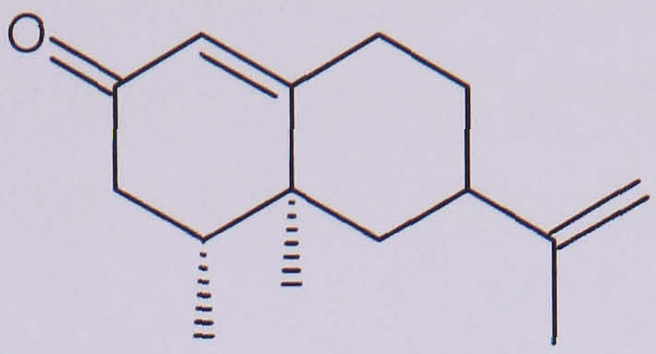
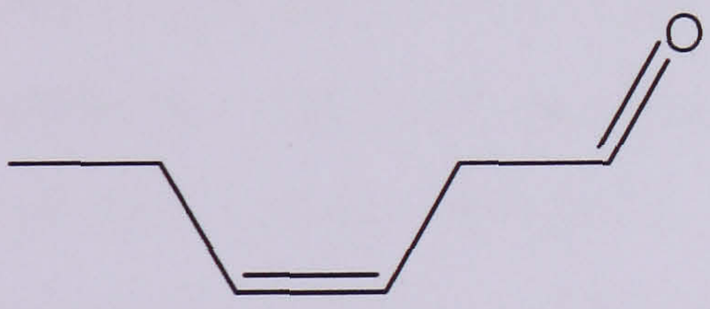
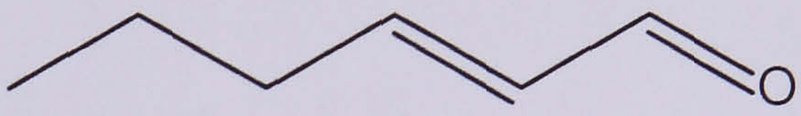


(6)

Unsaturated aldehyde compounds have been shown to be responsible for green, grassy and herbal aromas. Green tissues, when damaged, produce the 'hexenyl' so-called C_6 wound compounds as the result of a peroxide defence mechanism. Linoleic acid acts as a 'trap' for peroxy radicals and in doing so is cleaved to C_6 chain length aldehydes. The initial compound formed is *cis*-3-hexenal (**7**) which rearranges to the stable trans-2-hexenal (**8**) (leaf aldehyde) that characterises freshly mown grass (Oxford Chemicals).

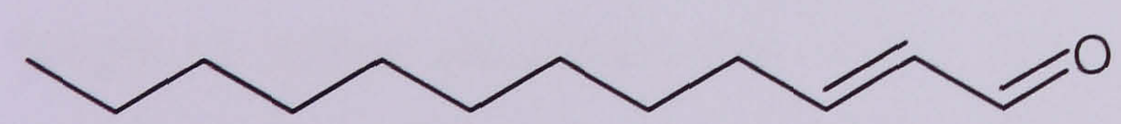


(7)

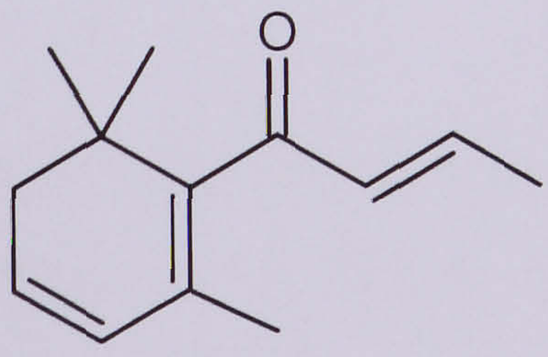


(8)

The long chain aldehyde *trans*-2-dodecenal **(9)** is a key odourant of coriander, whilst the ketone β-damascenone **(10)** is the essence of Bulgarian rose oil, first identified in the 1960's (Ohloff and Demole, 1987). Since then it is has be found to occur in 61 different edible materials (Teranishi, 1999; Williams, 2002).



(9)



(10)

As a general observation, the longer the carbon chain length the more the character of the aldehydes changes. Also associated with a longer chain length is a lowering of the odour threshold, that is, the concentration at which the compound can be detected by the nose.

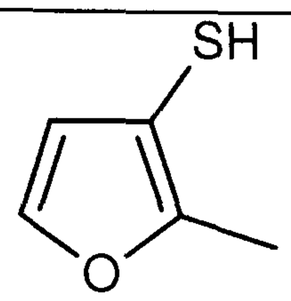
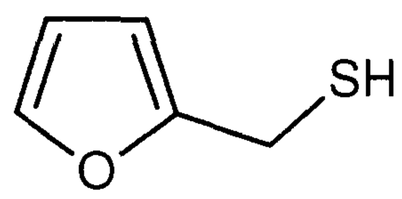
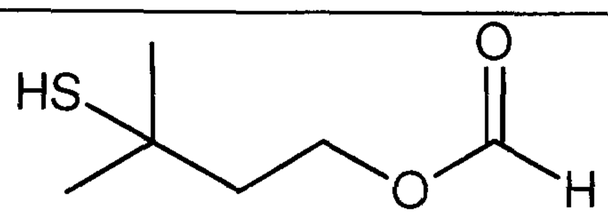
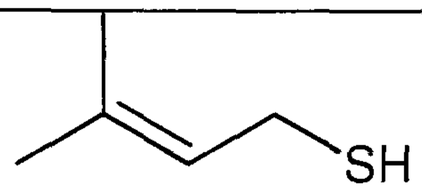
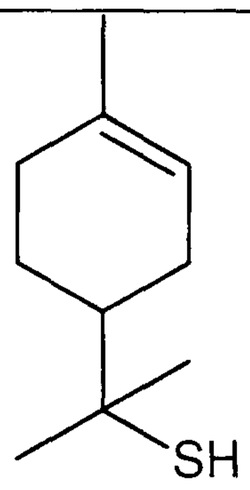
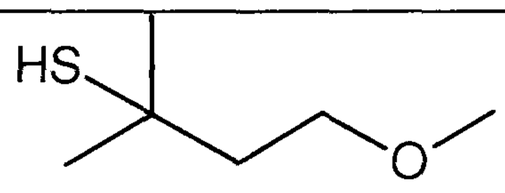
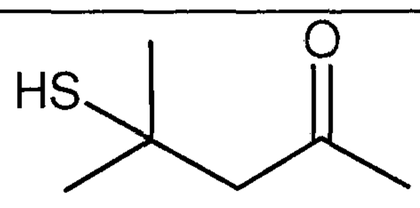
This brief survey of some of aldehyde and ketone aroma compounds illustrates the importance of these compounds to the character of many fragrances and flavours. Their ubiquitous and characterful nature makes them a functional group of high interest upon which to focus attention. For a more thorough review on odour active aldehyde compounds see Rowe (2005).

1.9.2 Sulfur compounds

Sulfur compounds form an important class of aroma compounds found in a wide variety of fragrances and flavourings. They have some of the lowest odour thresholds known and they are generally high impact in nature and, therefore, need only be present in trace amounts to make a big impact on a formulation. Sulfur-containing volatiles are often formed in the course of thermal processing of foods, such as in roasting meat. They are frequently used in savoury flavour applications where a roasted note is required. Volatile organic sulfur compounds obtained by enzymatic and chemical reactions contribute to the aroma of many vegetables, fruits, and food products. (2*R*,3*S*)-3-mercapto-2-methyl-1-pentanol is a character-impact constituent of fresh onions (Widder *et al.*, 2000) and dimethyl sulfide is the primary odour compound for cooked tomatoes (Buttery *et al.*, 1990).

A subset of odourous sulfur compounds are thiols (mercaptans). Thiols are key contributors to blackcurrant, grapefruit, passion fruit and other tropical fruit aromas. They are characteristic of roast meat, coffee and also found in many wines, either as contributors to the odour or as off-notes to the aroma (Mestres *et al.*, 2000). Some common thiols used as aroma compounds are shown in Table 1.5.

In recent years, the development of analytical techniques capable of detecting trace levels of sulfur molecules has led to increasing numbers being identified and used particularly in flavour applications. An indicator of this trend is the Flavour and Extract Manufacturers' Association's (FEMA) list of Generally Recognised as Safe (GRAS) flavours. Between 1993 (GRAS 16 list) and 2001 (GRAS 20 list), a significant number of new materials registered were sulfur compounds (Figure 1.19).

Compound	**Structure**	**Occurrence/ applications**	**Odour threshold ***
2-methylfuran-3-thiol		Roast meat	1×10^{-6}
furfuryl mercaptan		Coffee	4×10^{-6}
3-mercapto-3-methylbutyl formate		Coffee	2×10^{-7}
prenyl mercaptan		Beer/coffee	2×10^{-7} +
1-*p*-menthene-8-thiol (grapefruit mercaptan)		Grapefruit	1×10^{-6}
1-methoxy-3-methyl-3-mercaptobutane		Blackcurrants	8×10^{-8}
4-methyl-4-mercaptopentan-2-one		Sauvignon grape	1×10^{-7} +

* All values are given in mg/m^3 air except those marked '+' which as mg/kg^1 in water.

Table 1.5 Applications and odour thresholds of some common thiols (adapted from Jameson, 2005).

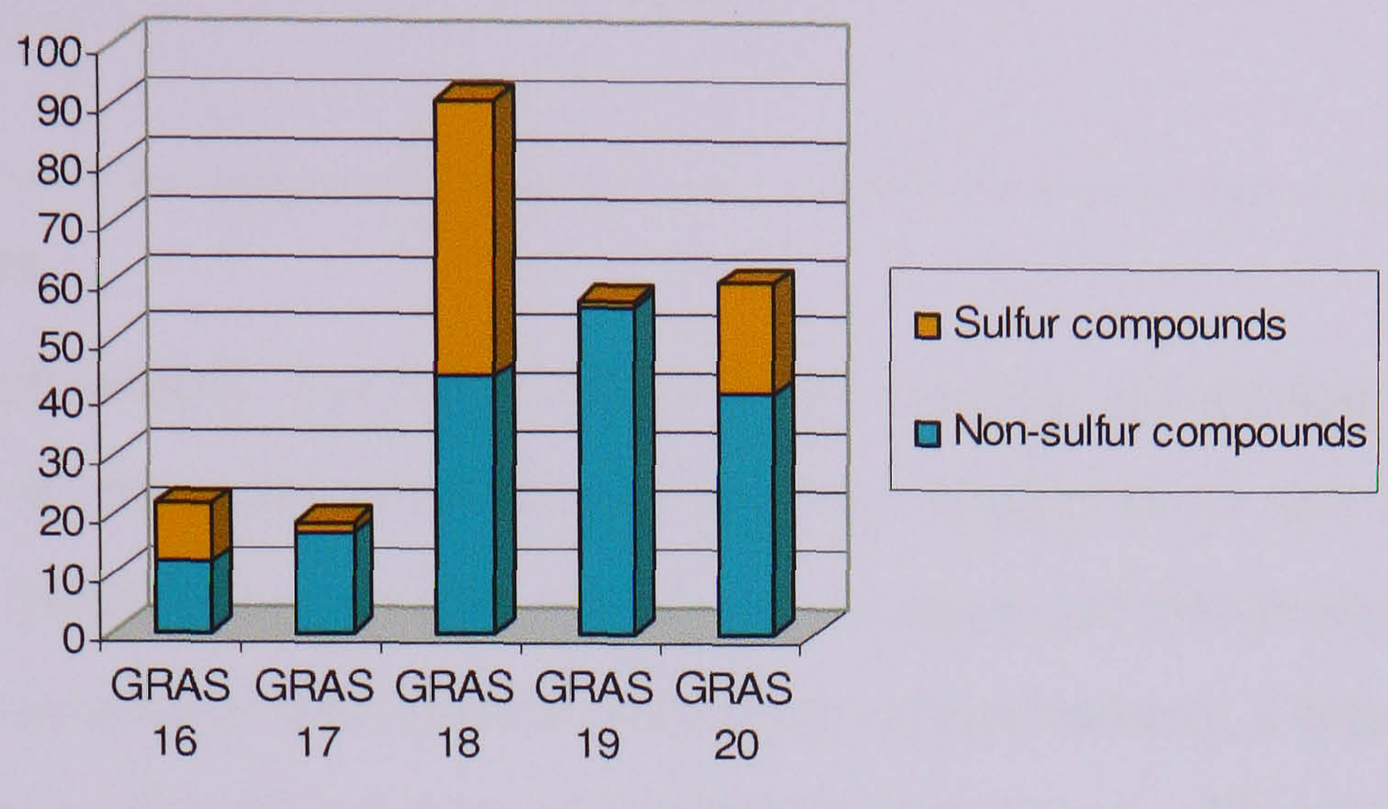
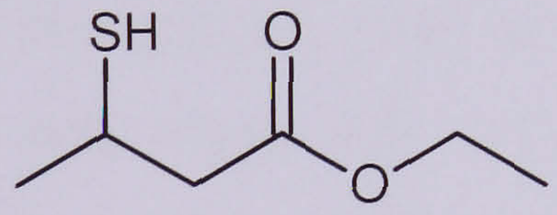


Figure 1.19 Numbers of sulfur compounds recognised on recent FEMA GRAS lists (Jameson, 2006).

Of these new sulfur materials the highest single class of compounds were thiols (Figure 1.20). Many of these species were discovered by extracting large quantities of natural materials and using sensitive techniques include sulfur-specific GC detectors, GC-olfactory and GC-MS. For example, ethyl 2-mercaptobutyrate (**11**) was discovered by extracting large quantities of mango pulp and has been identified as a key mango and tropical fruit component (Dewis and Kendrick, 2002).



(11)

Comprehensive surveys of the role of sulfur compounds as flavouring and fragrance applications have been published (Boelens and Van Gemert, 1993; Rowe, 1998; Rowe, 2000).

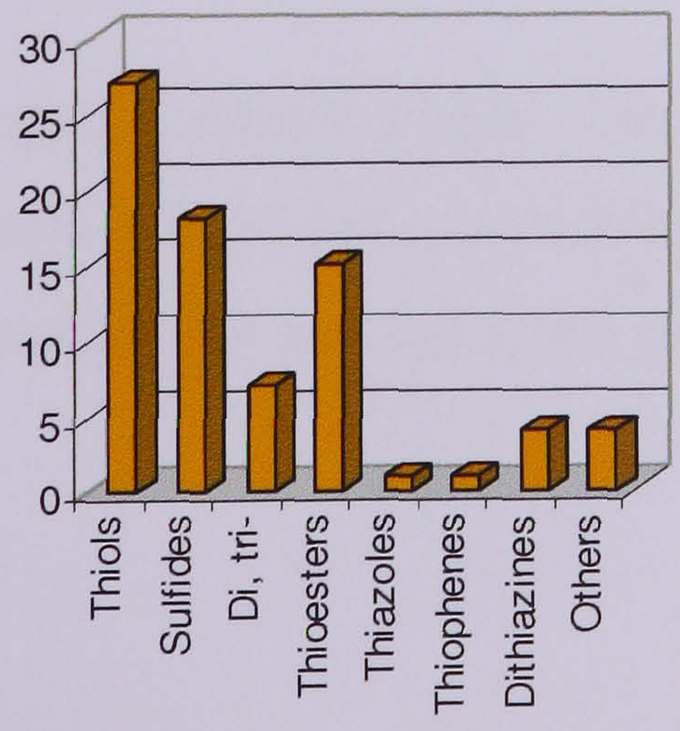


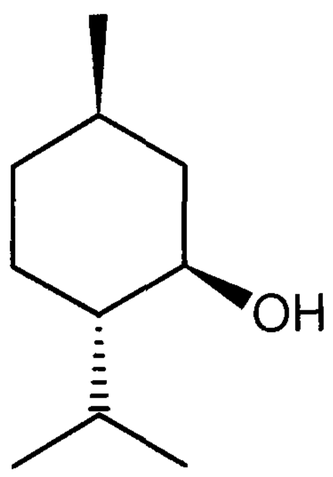
Figure 1.20 Families of sulfur compounds reported on recent FEMA GRAS lists. Thiols make up the highest single class of new sulfur compound registrants (Jameson, 2006).

1.9.3 Menthyl esters

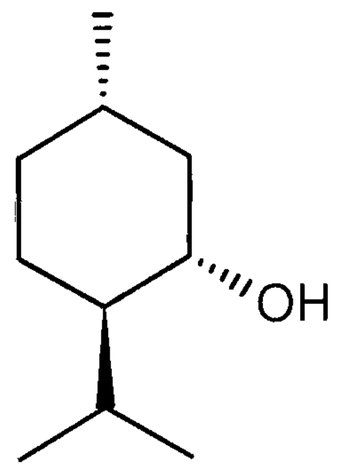
Menthol is the most widely known physiological cooling compound, found abundantly in nature in mint oils from *Mentha piperita* (peppermint) and *Mentha arvensis* (cornmint). Menthol is a classic example of a compound that possesses all three sensory aspects associated with flavour: olfaction in its mintiness; a bitter taste; and trigeminal sensations of both cooling and irritation of the eyes. These qualities have made menthol a very important ingredient in a wide-range of applications including cigarettes, cosmetics, toothpastes, chewing gum, and sweets. These features can also be considered a drawback because they restrict the applications of menthol. The flavour industry is keen to find new compounds that will offer the cooling sensation of menthol but without the bitter taste and strong flavour so they can be used with other flavour systems. In addition, the volatility of menthol means that eye irritation is an undesirable side effect.

There already exist several commercial synthetic compounds that have cooling properties. Research in this area began with an extensive screening program for potential cooling compounds in the 1970's (Watson *et al.*, 1978). Cooling compounds cover a wide range of structures, but only those that are menthol ester derivatives are discussed further here. For a more comprehensive review of several cooling compounds see Dewis (2005). Eccles also provides a detailed review of menthol based compounds (Eccles, 1994).

Menthol is a chiral compound. L-Menthol (**12**) [(1*R*,2*S*,5*R*)-(–)-Menthol] is 45 times more active than D-menthol [(1*S*,2*R*,5*S*)-(+)-Menthol] (**13**) in terms of its cooling effect. Consequently, compounds derived from L-menthol are also considerably more active.

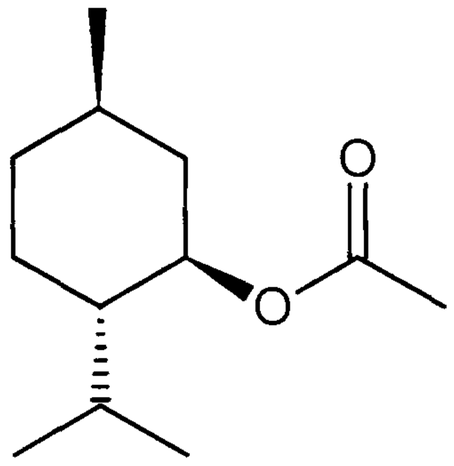


(12)



(13)

There are several esters of menthol that are known to have cooling properties. These include menthyl acetate (**14**), menthyl lactate (**15**), menthyl succinate (**16**), menthyl glutarate (**17**) and dimenthyl glutarate (**18**).



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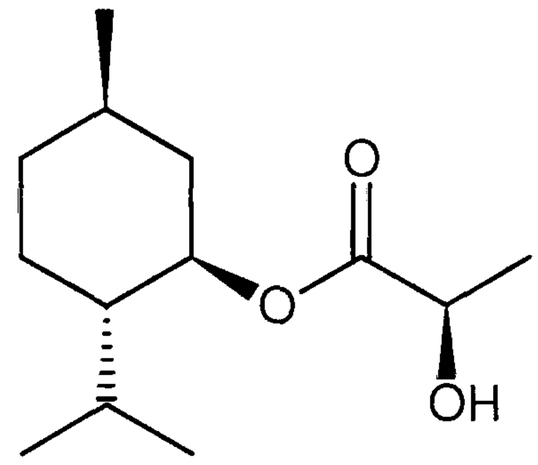

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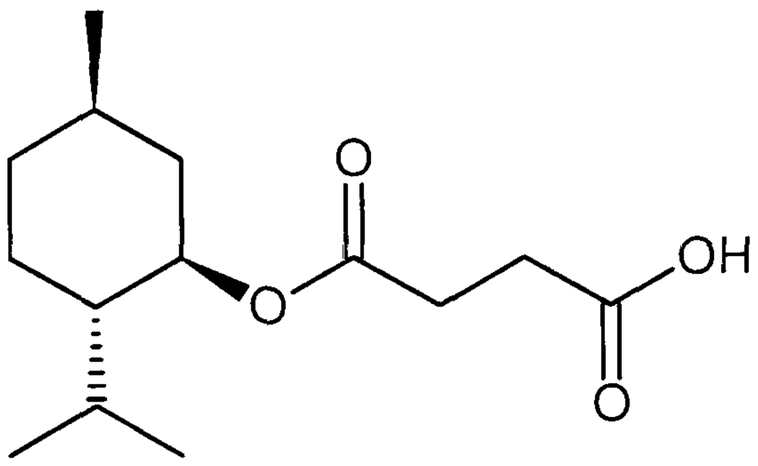

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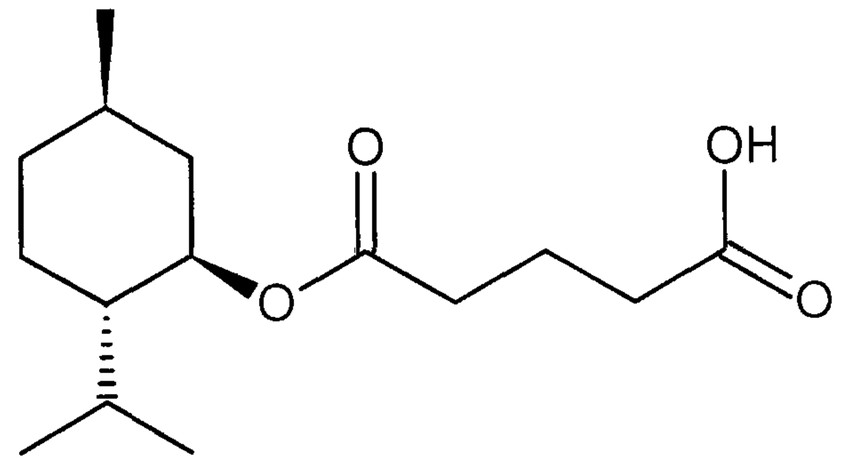

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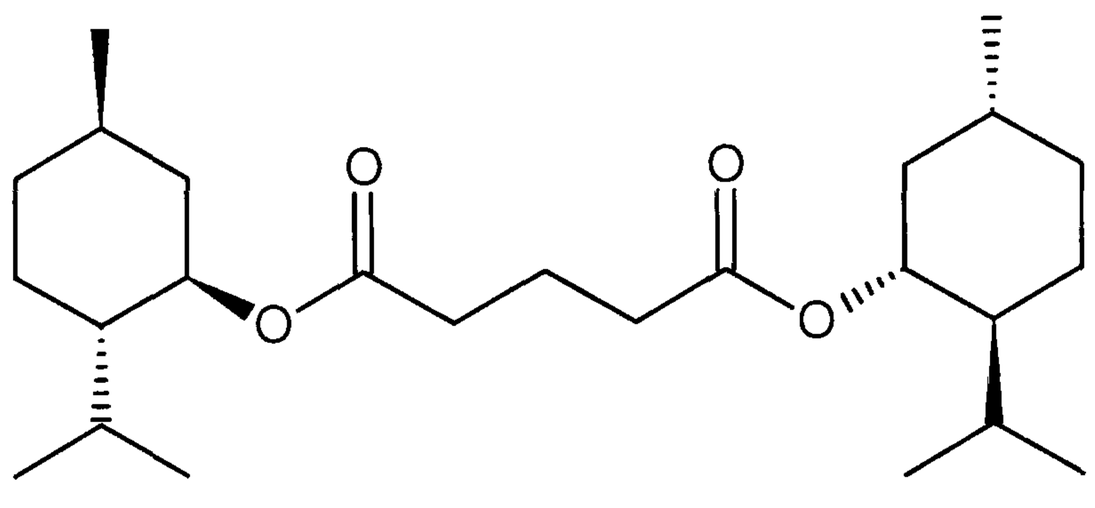

(18)

Menthyl acetate (**14**) occurs naturally in several sources including peppermint and fruits such as raspberry and mango. Menthyl lactate (**15**) has long been GRAS registered as a flavour ingredient. It has only a slight minty odour and faint earthy taste. It does, however, provide a long-lasting cooling effect in the mouth. Monomenthyl succinate (**16**) is one of the better synthetic coolers on the market. It is a ~~popular molecule~~ because of the combination of being virtually tasteless and

having desirable cooling properties. It has been used since the 1960's as a menthol releasing compound in menthol cigarettes. Monomenthyl glutarate (**17**) has a similar sensory profile to the succinate. It is probably the longest-lasting oral cooling agent that is currently commercially available. Despite being a known molecule, the application in flavours is a new development. The occurrence of this compound in nature has been confirmed relatively recently (Hiserodt *et al.*, 2004). This gives it a significant commercial advantage in the traditional European markets. Dimenthyl glutarate (**18**) is also known to have cooling properties and was identified in nature in the same study.

1.10 Aims of this thesis

It has been established that a key aim for both the fragrance and flavour industries is to discover new products, and that a major source of innovation comes from identifying such compounds from nature. This requires isolation from a natural source, identification and subsequent synthesis of the compound.

The primary aims of this work are to develop methods for isolating (from natural sources) specific classes of compounds of interest as flavour and fragrance ingredients. The methods are designed to address particular difficulties associated with unstable analytes, which are potentially present at low concentrations and/or in the presence of up to hundreds of other compounds. For analysis of living plants it is also particular important that any method used is a non-destructive process.

Aroma and taste compounds cover the full spectrum of functional groups; therefore effort has been focussed on targeting three families of compounds with organoleptic properties of particular interest. These are:

- Aldehydes and ketones
- Thiols
- Menthyl esters

The generic structures of these species are shown in Figure 1.21.

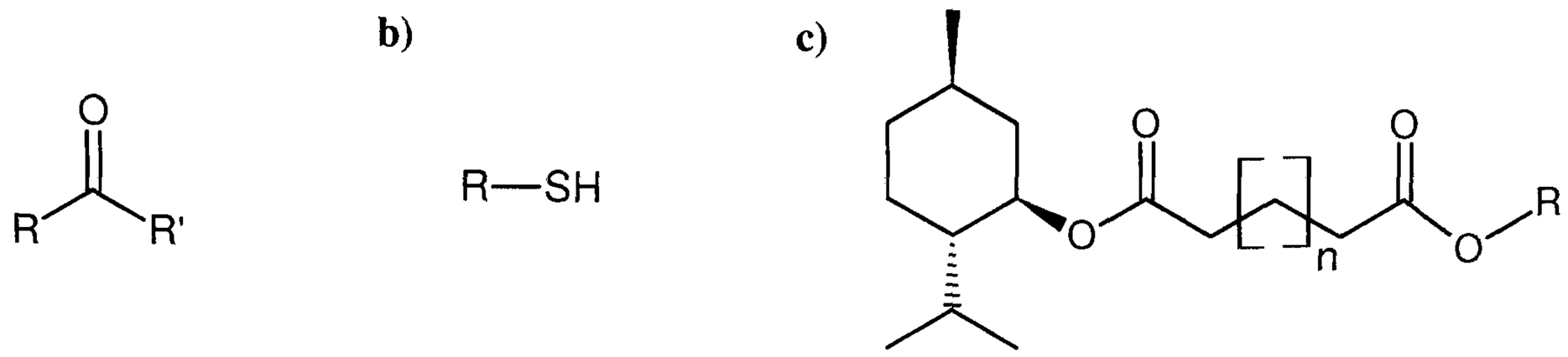

Figure 1.21 Generic structures of a) aldehydes and ketones, b) thiols, c) menthyl esters.

Although many aldehydes and ketones have already been identified as flavour and fragrance compounds, the lighter members of the series are particularly volatile and unstable and have been difficult to isolate from natural sources. The desired outcome from this work was to have a method of trapping these species during field trips. Due to the time and expense involved in many of these expeditions, and their 'one-off' nature, a robust trap is required that would ensure that these species are not missed from the headspace of the plant to be analysed. Chemical derivatisation has been chosen as a tool for isolating these compounds because of the high reactivity of the analyte. Using derivatisation can generate an analyte stable to analysis. In developing the trap to be used in field trips and exploring the parameters of its use, several non-routine novel analytical techniques such as the MS-Nose™ and FTIR and Raman imaging have been used. This work is described in Chapter 3.

For the isolation of thiol compounds, the primary concern is the low concentration and poor stability to conventional analysis of these high-impact components within the complex mixtures that make up many of the more interesting flavour systems. The methods that are explored in this work were designed to concentrate and isolate these species. These methods include utilising polymeric resins, covalent chromatography and metals. This work is described in Chapter 4.

An alternative strategy to new ingredients for both industries is to synthesise a compound chemically with interesting olfactory or gustatory properties based on knowledge of similar structures. In this case, it is important to be able to find these compounds in nature, and provide unambiguous evidence to confirm an isolated

compound is nature identical. Chapter 5 explores the use of advanced chromatographic techniques in conjunction with highly sensitive mass spectrometric detection to confirm the nature identical status of the menthyl ester, L-monomenthyl succinate and to simplify the analysis of a complex food extract. Natural sources often require significant sample preparation before characterisation can be carried out, preferably without the introduction of the artefacts. Extraction techniques (Soxhlet and microwave) are compared to meet this aim.

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2

Materials and Methods

The materials and methods section is sub-divided according to the subsequent results chapters: Section 2.1 applies to Chapter 3, Section 2.2 applies to Chapter 4 and Section 2.3 applies to Chapter 5.

2.1 Characterisation and use of carbonyl specific adsorbent traps

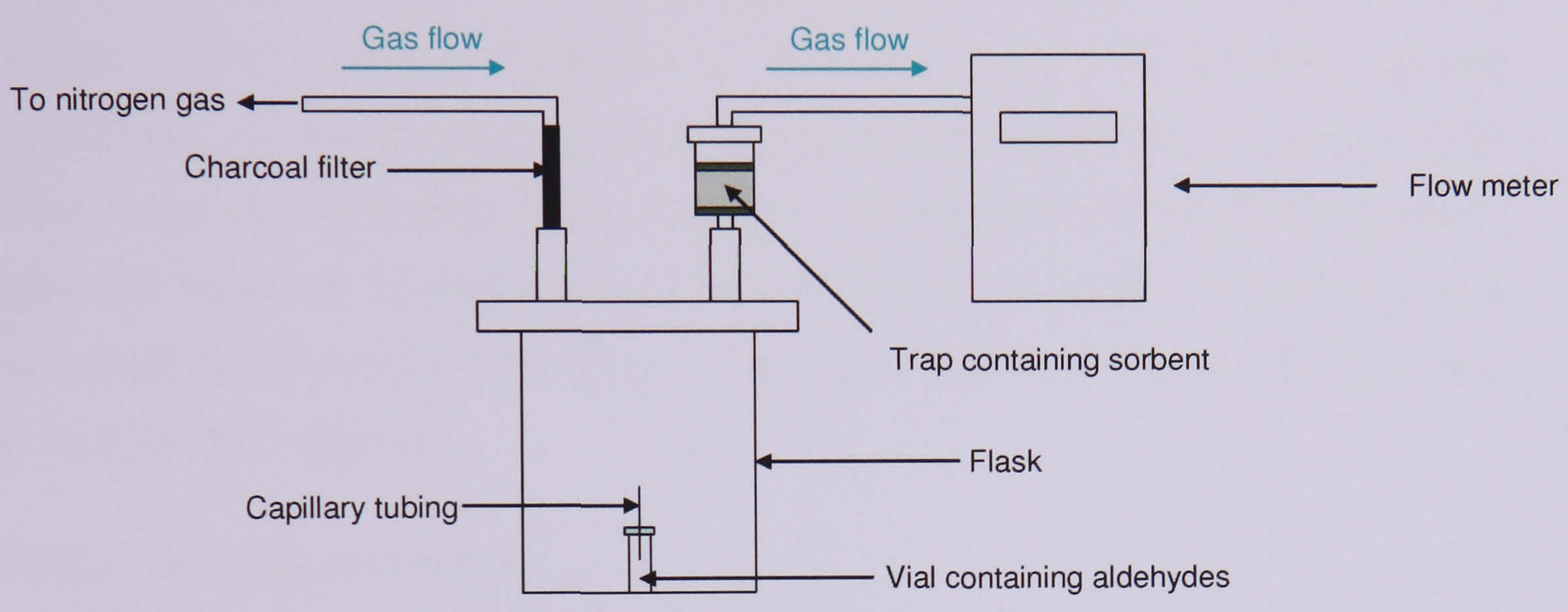
2.1.1 Chemicals and materials

Formaldehyde (37% w/w aqueous solution), acrolein (90% purity), *trans*-2-pentenal (95%), benzaldehyde (99%) and furfural (98%), octane, *O*-(2,3,4,5,6-pentafluorobenzyl)hydroxylamine hydrochloride (98%, PFBHA), sulfuric acid, and anhydrous sodium sulfate were obtained from Sigma-Aldrich (Gillingham, UK). All solvents (methanol, hexane, acetone, ethyl acetate, methyl tert-butyl ether, dichloromethane and diethyl ether) were distol grade (suitable for residue analysis), also purchased from Sigma-Aldrich. Tenax TA (80/100 mesh) was obtained from Supelco (Poole, UK). The PFBHA was purified before use via cold-finger sublimation (Leonard, 1995). Tenax TA was cleaned by Soxhlet extraction with diethyl ether overnight and dried before use (EU Standard, 1999). Polypropylene SPE tubes (1 mL capacity) with polyethylene frits of 20 micron porosity were purchased from Supelco. Personal sampler pumps, model Compur 4903, were from Bayer Diagnostic GmbH (Leverkusen, Germany). Pumps were calibrated before use with an Electronic Flowmeter 5182-0879 from Agilent Technologies (Ipswich, UK).

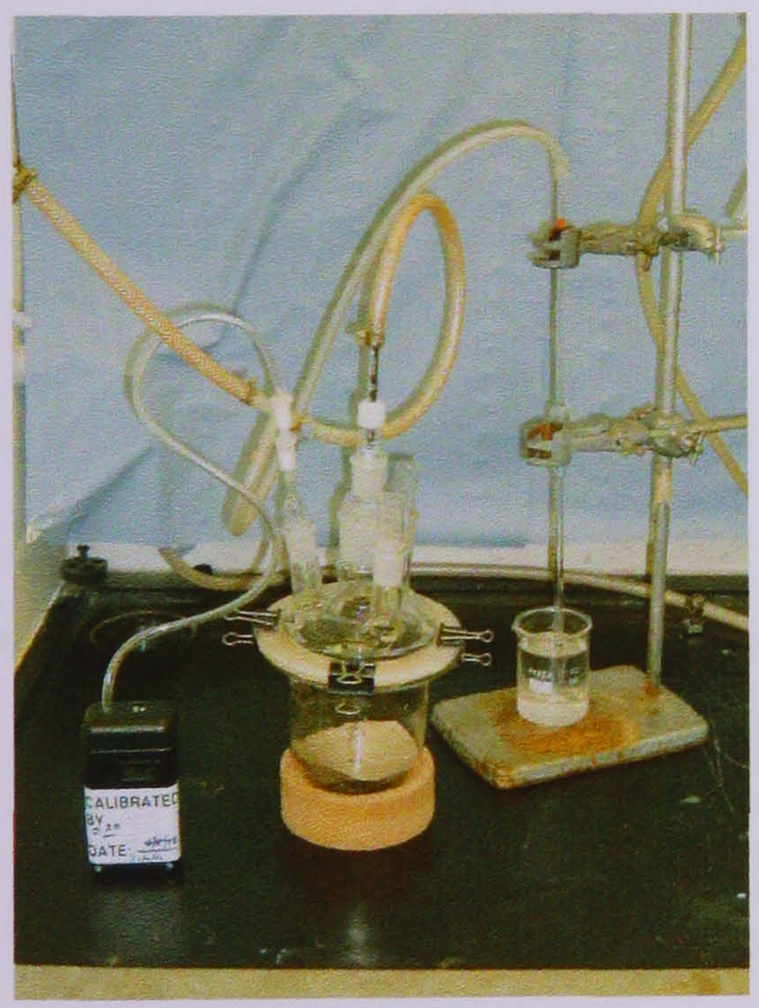
2.1.2 Experimental procedures

2.1.2.1 Preparation of test atmosphere and traps

For the majority of headspace sampling experiments, an artificial test atmosphere of aldehydes was created in a 500 mL flange flask. Figure 2.1 below shows a schematic of the experimental set up with photographs of the equipment



(a)



(b)

(c)

Figure 2.1 Apparatus for generating a test atmosphere: (a) schematic; (b) image of equipment; (c) detail of the aldehyde containing vial.

The following amounts of each aldehyde were measured into a sealed GC vial by microsyringe: formaldehyde (50 μL); acrolein (40 μL); *trans*-2-pentenal (30 μL); benzaldehyde (30 μL) and furfural (30 μL). This was the standard mixture of

aldehydes used to generate a headspace, unless otherwise stated. The lid of the vial was pierced with a short piece of GC capillary column which was inserted into the solution in the vial. This arrangement was used to allow the aldehydes to vaporize into the headspace at low levels (less than 50 ppm) as would be expected in a field test. Nitrogen gas was pumped into the flask through a charcoal filter in order to remove any impurities in the stream. The nitrogen flow was controlled by a simple manometer type arrangement. Polypropylene plastic traps were filled with the sorbent (PFBHA coated Tenax TA or uncoated Tenax TA depending on the experiment) contained between two polyethylene frits. The trap was attached to the flask via custom made glass quick fit adaptors. The pump was attached to the trap to draw the headspace through the trap. After a set amount of time (depending on the individual experiment), sampling was stopped and traps were then desorbed in order to analyse the components.

2.1.2.2 Coating procedure

Purified PFBHA was dissolved in 25 mL methanol. To this solution, 3 g of Tenax TA were added. The amount of PFBHA added depended on the coating level which was varied in some experiments. For a coating weight of 1, 5, 10 and 20% (w/w), the amount of PFBHA added was 37.5 mg, 187.5 mg, 375 mg and 750 mg, respectively. The mixture was shaken on a Gallenkamp flask shaker for approximately 30 min to ensure the Tenax was coated. The excess methanol solvent was removed on a rotary evaporator at 50°C with applied vacuum of approximately 10 mm Hg (these levels were used in order to minimize the risk that PFBHA may be lost from the Tenax via sublimation). The PFBHA coated Tenax was then stored under nitrogen until required. Coated Tenax was usually not kept for use for longer than five days. The coating procedure was based on previously reported conditions (Wu and Hee, 1995).

2.1.2.3 Solvent desorption

Experiments were conducted to investigate the solvent used to desorb the analytes from the trap. The following procedure was followed: 0.1% octane (internal standard) was spiked into the solvents dichloromethane, methyl tert-butyl ether, ethyl acetate, hexane, and the solvent mixtures 5:95 ethyl acetate/hexane, 50:50 hexane/acetone and 80:20 hexane/acetone. One microlitre of a 1% solution of *trans*-2-pentenal was spiked onto a trap filled with 100 mg of PFBHA-coated Tenax. One

hundred millilitres of air was pumped through the trap at a rate of 25 mL.min^{-1} to allow the derivatisation reaction to take place. The analytes were desorbed by feeding 1 mL of solvent into the trap (see Figure 2.2), which was held for 2 min. The solvent was collected in five 200 µL fractions which were then analysed by GC.

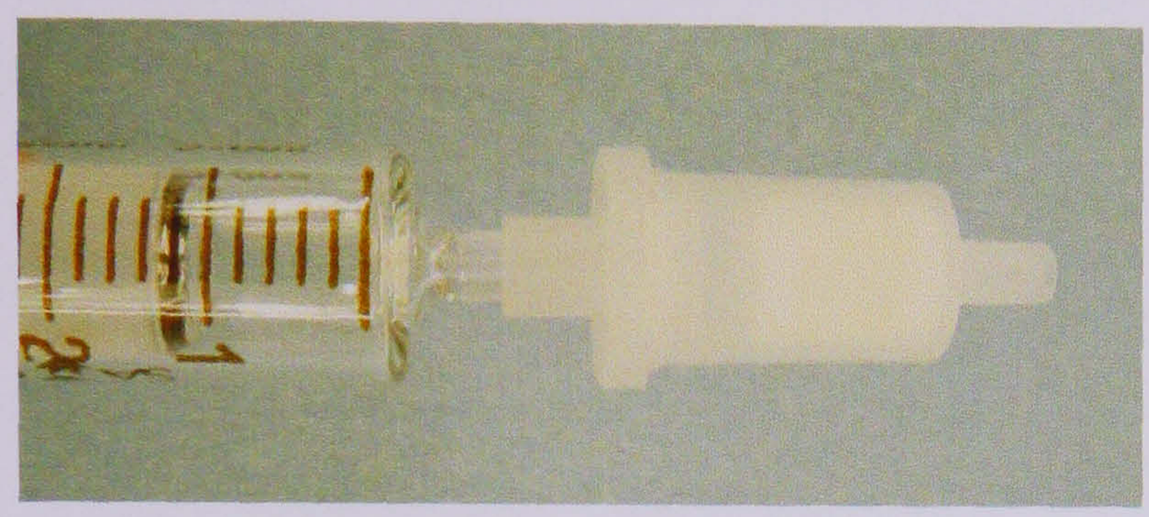


Figure 2.2 Solvent desorption of analytes

2.1.2.4 Conditions for investigating effect of humidity

For the experiment for investigating the effect of humidity, a test atmosphere of 80% humidity was created by adding a 100 mL solution of 80:20 water/sulfuric acid to the reaction flask. This is a modification of an European Standard (EU Standard, 1994). The temperature of the flask rose and condensation formed. The standard headspace trapping experiment was carried out (with the omission of benzaldehyde) for 8 hours. The traps were desorbed with 5% ethyl acetate in hexane (with 0.5% octane internal standard) and analysed by GC.

2.1.2.5 Breakthrough

Measurement of breakthrough was completed on four aldehydes: butanal, propanal, *trans*-2-pentenal and acrolein. Aldehyde gas samples were generated as follows. The volume of a 500 mL flask was accurately determined. A volume of aldehyde was then injected into the flask through a subaseal where it volatilised. Prepared sample atmospheres were all approximately 70 ppm in concentration (known accurately). When the injection system was connected to the MS-Nose, the trace was allowed to settle to a consistent baseline before aldehyde injection. A set volume of aldehyde headspace was drawn from the flask and injected into the system. The time of injection was noted. Experiments were conducted at room temperature. The MS-Nose parameters are described in Section 2.1.3.3.

2.1.2.6 Preparation of aldehyde-PFBO derivative standards

Aldehydes were derivatised with PFBHA in solution in order to provide reference standards for generating libraries of GC-MS spectra. The method was adapted from the method described by Yu *et al.* (1995) An excess amount of PFBHA aqueous solution (1 mL of 25 $mg.mL^{-1}$ solution) was added to 10 µL of aldehyde. Five hundred microlitres of 9M H_2SO_4 were added to adjust the pH to approximately 2. The mixture was allowed to stand at room temp for 24 hours. Four millilitres of hexane were added to extract the PFBHA derivatives. The sample was centrifuged for 10 min. The hexane layer was pipetted off and extracted with portions of 0.05M H_2SO_4 (2 x 30 mL), using a centrifuge for separating the layers. About 50 mg of anhydrous Na_2SO_4 was added to the hexane extract to adsorb trace amounts of water. The residue was concentrated to approximately 1 mL under nitrogen.

2.1.2.7 Headspace experiment on blue hyacinth

A blue hyacinth bloom was contained within a 2 L PET bottle with the base removed. A 'Quickfit' adaptor was used to attach the trap to the bottle and PTFE tape was used to seal the joint. The headspace of the hyacinth was sampled using a 10% w/w PFBHA coated Tenax trap (100 mg), drawing the headspace at a rate of 50 $mL.min^{-1}$ for 2 h. The trap was desorbed with 1 mL solvent (80:20 hexane/acetone containing 0.1% octane as internal standard) and analysed by GC-MS to determine the identity of any aldehydes trapped. An unused trap containing coated Tenax was also desorbed for use as a blank.

2.1.2.8 Headspace experiment on lemon oil

A capped GC vial containing cold pressed lemon oil (Quest International, Ashford, UK) was placed into the test flask (see Figure 2.1). The lid was pierced and a length of capillary column was fed into the vial to allow the oil volatiles to enter the headspace. The headspace of the lemon oil was sampled using a 10% w/w PFBHA-coated Tenax trap (100 mg), drawing the headspace at a rate of 50 $mL.min^{-1}$ for 4 hours. A back trap containing 100 mg of uncoated Tenax was connected in series. The trap was desorbed with 1 mL solvent (80:20 hexane/acetone containing 0.1% octane as internal standard) and analysed by GC-MS to determine the identity of any aldehydes trapped. An unused trap containing coated Tenax was also desorbed for use as a blank.

2.1.3 Instrumentation

2.1.3.1 GC

The GC utilised was a Perkin Elmer Autosystem XL (Perkin-Elmer, Beaconsfield, UK) with a flame ionisation detector (FID). The column used in all experiments was a CP Sil 8CB (Varian, Church Stretton, UK) 25 m x 0.20 mm ID x 0.33 µm film thickness (5% phenyl and 95% dimethylpolysiloxane). The initial oven temperature of 40°C was stabilised before injection of the sample onto the column. One microlitre of sample was injected onto the column and the oven was held at 40°C for 1 min. The temperature was raised at a rate of 10°C.min^{-1} up to 200°C and held there for 5 min giving a total run time of 22 min. The carrier gas used was helium with a velocity of 20 cm.s^{-1} and the split flow was set to 50 mL.min^{-1}. Individual compounds were quantified with respect to the peak areas of the internal standard. When there were two peaks (due to *E* and *Z* isomers of oximes of asymmetric aldehydes), these were both quantified.

2.1.3.2 GC-MS

A GCT TOF mass spectrometer (Waters MS Technologies, Manchester, UK) fitted with an EI source was interfaced to an Agilent 6890 GC (Manchester, UK). The same column type (HP5, 25 m x 0.20 mm ID x 0.33 µm) and conditions were used as for the GC analysis. The exception was for the analysis of the lemon oil headspace where more protracted conditions were employed in order to gain better separation of components. The conditions used were: hold at 50°C for 2 minutes, then a temperature ramp of 4°C.min^{-1} up to 280°C, and hold for 10 minutes, giving a total run time of 69.5 minutes. The injector temperature used was 280°C and the helium flow rate was 0.5 mL.min^{-1}. The injection volume and split ratio depended on the sample. Mass spectra were acquired in EI positive mode over the mass range of 12 to 650.

2.1.3.3 MS-Nose

Breakthrough experiments were conducted using a TOF mass spectrometer (Waters MS Technologies, Manchester, UK) equipped with atmospheric pressure chemical ionization source interfaced with a MS-Nose^{TM} (Waters MS Technologies, Manchester, UK). The positive ion mode was selected for measurements. The scan

range of the mass spectrometer was from 50 to 500. The transfer line was held at 50°C.

2.1.3.4 Raman

Raman images were acquired with a confocal Labram Raman Microscope (Dilor, France). Typical operating conditions were: HeNe laser (633 nm), 10X microscope objective, 800 µm confocal aperture, point mapping mode, sampling interval every 10 µm, acquisition time 10 s per spectrum, spatial resolution (lateral) approximately 10 µm, spectral resolution approximately 5 cm^{-1}. Some images were recorded with better spatial resolution (e.g. 50X objective, 5 µm sampling interval) but over a smaller area.

2.1.3.5 Global IR

Global IR images were acquired using a Bruker FTIR spectrometer (Hyperion) (courtesy of Sergei Kazarian, Imperial College) with a 64 x 64 element MCT Focal Plane Array (FPA) detector operating at 8 cm^{-1} spectral resolution. Surface images were recorded using ATR. Large scale images used Macro ATR (Golden Gate, Specac, diamond element, approximately 0.8 mm x 1.1 mm sampling area). Micro images used Bruker (Coventry, UK) Germanium ATR objective with field of view approximately 50 µm.

2.2 Development of thiol specific trapping methods

2.2.1 Chemicals and materials

<u>Maleimide experiments</u>

N-ethyl maleimide, hexane-1-thiol, hexan-1-ol, sodium sulphate (anhydrous), iodomethane and ^{13}C-labelled iodomethane, sodium hydrogen carbonate, sodium hydroxide solution, hydrochloric acid, sulphuric acid, potassium permanganate, potassium carbonate, *m*-chloroperoxybenzoic acid (mCPBA), 1,8-bis(dimethylamino)naphthalene (Proton Sponge®), 2,6-ditertbutyl-4-methylpyridine (DBMP), 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU), tetrahydrofuran (THF), dichloromethane, hexane, diethyl ether and methanol were obtained from Sigma-Aldrich (Gillingham, UK). Silica-maleimide was obtained from Silicycle (Quebec, Canada). 8-mercapto-p-menthan-3-one (85%) was obtained from Avocado Organics

(Heysham, UK). Buchu leaf oil was supplied by Quest International (Naarden, the Netherlands).

Covalent chromatography

5,5'-dithiobis(2-nitrobenzoic acid), ethane thiol, propane-1-thiol, 2-methylpropane-1-thiol, butane-1-thiol, butane-2-thiol, pentane-1-thiol, hexane-1-thiol, heptane-1-thiol, 3-mercaptobutan-2-one, dithiothreitol and tris (hydroxymethyl)aminomethane were obtained from Sigma-Aldrich (Gillingham, UK). Thiopropyl sepharose B was obtained from Amersham Biosciences (Little Chalfont, UK).

Adsorption onto gold wire

Hexane-1-thiol was obtained from Sigma-Aldrich (Gillingham, UK). Gold wire was kindly supplied by Julian Barwick of ICI MSG's electron microscopy laboratory.

2.2.2 Experimental procedures

2.2.2.1 Maleimide based experiments

Solution phase

Step 1: Formation of the sulfide, 1-ethyl-3-(hexylthio)pyrrolidine-2,5-dione

An equimolar amount of N-ethyl maleimide (1.5 g, 12 mmoles) and hexanethiol (1.8 mL, 12 mmoles) in tetrahydrofuran (10 mL) was placed in a glass screw-top vial. A catalytic amount of 0.1M potassium hydroxide solution (2 mL) was added, the vial was capped (aluminium lining) and shaken for 2 h (Rotashake flask shaker). After shaking for 4 h at room temperature, an additional 2 mL 0.1 M KOH solution was added and the vial shaken for a further 2 h. The THF was removed by overnight evaporation. The residual yellow oil was dissolved in diethyl ether (10 mL) and washed with water (3 x 10 mL portions). The ether layer was dried (Na_2SO_4) and evaporated to dryness under reduced pressure. The reaction was monitored by TLC. The plates were developed in an 80:20 hexane:diethyl ether solvent system. The plates were stained by dipping in a solution of $KMnO_4$ / K_2CO_3 made up thus: $KMnO_4$ (1 g), K_2CO_3 (6.7 g), and 0.5M NaOH aqueous solution. To develop the spots the plates were placed on a hot plate at 100°C for 30 s, or until the plate was dry. The reaction components appeared as yellow spots under visible light. The product was analysed by NMR and FTIR spectroscopies and GC-MS.

Step 2: Oxidation of sulfide to form the sulfone, 1-ethyl-3-(hexylsulfonyl)pyrrolidine-2,5-dione

A solution of mCPBA (18 mmol) in THF (10 mL) was prepared by dissolving the peracid in solvent, cooled using an ice bath. The solution was then slowly transferred to a glass vial containing the crude sulfide, 1-ethyl-3-(hexylthio)pyrrolidine-2,5-dione (5 mmol) (cooled in the ice bath). The vial was shaken for 4 h at room temperature, and the THF removed by rotary evaporation. The residue was dissolved in dichloromethane (DCM). The DCM was washed with sodium sulfite solution (3 x 10 mL) and saturated sodium hydrogen carbonate solution (3 x 10 mL), then dried (Na_2SO_4). The solvent was removed by rotary evaporation yielding the intermediate sulfone, as characterised by NMR, FTIR, and Raman spectroscopies and LC-MS.

Step 3: Elimination of the sulfone, 1-(methylsulfonyl)hexane

A solution of the intermediate sulfone, 1-ethyl-3-(hexylsulfonyl)pyrrolidine-2,5-dione, (0.3 mmol) in THF (1 mL) was placed in a 5 mL screw-top glass vial. A hindered base (DBU, DBMP or Proton Sponge) (0.33 mmol) was added and shaken for 1 h. (Later repeat reactions used DBU only). Iodomethane (0.3 mmol) was added and shaken for a further 2 h. (Note that the iodomethane was either ^{13}C labelled or not, depending on the experiment). The reaction solution was washed with 10% HCl aqueous (2 mL) and partitioned between water and hexane (2 mL). The organic layer was removed and dried (Na_2SO_4) before being evaporated to dryness. Samples were analysed by NMR and FTIR spectroscopies, LC-MS and GC-MS.

Competitive reaction with an alcohol

A solution of N-ethyl maleimide (7 mmol), hexane-1-thiol (7 mmol) and hexan-1-ol (7 mmol) in THF (10 mL) were reacted according to the procedure described in step 1. A second solution containing a 1:5:1.5 ratio of maleimide, thiol and alcohol respectively was prepared and subjected to the same procedure. The products were analysed by NMR spectroscopy.

Solid-phase

Step 1: Reaction of silica-maleimide with hexane-1-thiol

Silica-maleimide powder (1.4 mmol of maleimide) was added to a glass screw-top vial containing a solution of hexane-1-thiol (1.4 mmol) in methanol (10 mL). 0.1M KOH solution (10 μL) was added. After shaking for 4 h, the silica was filtered from the solvent using a Buchner funnel. The silica was washed with fresh solvent (3 x 10 mL) and dried under vacuum. The product was analysed by FTIR and Raman spectroscopies.

Step 2: Oxidation of sulfide to sulfone

A solution of mCPBA (5 mmol) in methanol (10 mL) was prepared by dissolving the peracid in pre-cooled (ice bath) solvent. The solution was slowly added (with cooling) to the product from Step 1 (1.4 mmol of sulfide) in a glass screw-top vial. After 4 h of shaking, the silica was filtered and washed as detailed in the previous step. The product was analysed by FTIR and Raman spectroscopies..

Step 3: Elimination of 1-(methylsulfonyl)hexane

The silica-sulfone (0.4 mmol) was placed in glass screw-top vial containing THF (10 mL). DBU (0.44 mmol) and iodomethane (0.4 mmol) were added and the vial was shaken for 2 hours. Note that the iodomethane was either ^{13}C labelled or not, depending on the experiment. The silica was filtered using a Buchner funnel and washed with THF (3 x 5 mL). The filtrate was collected and washed with 10% hydrochloric acid solution (10 mL). The aqueous solution was extracted with hexane (10 mL), which was removed and dried (Na_2SO_4). The hexane was then removed by rotary evaporation. The residue was analysed by NMR and FTIR spectroscopies and LC-MS.

Buchu leaf oil reactions

Buchu leaf oil was reacted in equimolar amounts according to the reaction protocols described in steps 1-3 of solution phase reaction and solid phase reactions. Solution phase reactions were analysed by GC-MS and LC-MS at each step. The reaction mixture from step 3 of solid-phase reactions was analysed by GC-MS, LC-MS and NMR spectroscopy. The silica residue was examined by FTIR and Raman spectroscopies.

Buchu Leaf oil reference compound

p-Methan-8-thiol-3-one was reacted in equimolar amounts according to the reaction protocol described for steps 1 and 2 for solution phase reactions. The reaction mixture from each step was analysed by NMR and FTIR spectroscopies, GC-MS, and LC-MS.

Direct elimination

A solution of the sulfide, 1-ethyl-3-(hexylthio)pyrrolidine-2,5-dione, in THF (10 mL) was placed in a 30 mL screw top vial with an aluminium lined cap. Iodomethane (2.1 mmol) was added and the vial shaken for 2 hours. DBU (2.3 mmol) was added and the vial shaken for a further 2 hours. The sample was analysed by NMR spectroscopy.

2.2.2.2 Di-sulfide reagent based experiments

Solution phase

Trapping of a mixture of thiols using 5,5'-Dithiobis(2-nitrobenzoic acid)

A buffer solution of tris(hydroxymethyl)aminomethane was prepared by dissolving 0.60 g of tris(hydroxymethyl)aminomethane in 50 mL of distilled water and then altering the pH of the solution to 8.0 by the dropwise addition of sulfuric acid. 5,5'-Dithiobis(2-nitrobenzoic acid) (DTNB) (0.40 g, 0.1 mmol) was dissolved in 20 mL of reaction buffer with stirring. Ethanethiol (740 μL, 0.01 mmol) propane-1-thiol (900 μL, 0.01 mmol), butane-1-thiol (1070 μL, 0.01 mmol), butane-2-thiol (1110 μL, 0.01 mol), 2-methylpropane-2-thiol (1125 μL, 0.01 mmol), pentane-1-thiol (1240 μL, 0.01 mmol), hexane-1-thiol (1420 μL, 0.01 mmol) and heptane-1-thiol (1530 μL, 0.01 mmol) were mixed and stirred together in a conical flask before the DTNB solution was slowly added to the flask with stirring. This solution was left to stir for 30 minutes. The solution was analysed by UV/Vis spectroscopy and LC-MS.

Release of original thiols from DTNB-disulfide

A 2.5 mL sample of the solution from the trapping stage was reacted with dithiothreitol (DTT) (0.13 g, 0.84 mmol) by gradual addition and monitoring the reaction in real-time by UV spectroscopy to determine whether the disulfide had

been totally reduced. This solution was left to stir for 30 minutes. The solution was analysed by LC-MS.

Solid-phase

Trapping of hexanethiol using Thiopropyl Sepharose 6B

The column consisted of a dropping funnel with a diameter of 2.5 cm, plugged with glass wool. Thiopropyl Sepharose 6B was rehydrated according to the manufacturer's instructions by suspension in 15 mL of distilled water and leaving to swell for 20 minutes. One gram of powder resulted in approximately 3 mL of gel. After swelling, the gel was washed for 15 minutes on a sintered glass funnel using 200 mL distilled water, to wash additives off the gel which are present during the freeze drying process. The gel slurry was added slowly to the column and left to settle. After the gel had settled, pressure was used to pack the gel down. The gel was then washed with 50 mL Tris buffer (2.60 g tris (hydroxymethyl)aminomethane in 250 mL distilled water and modified to pH 7.5 using 2.5M hydrochloric acid). 2-Thiopyridone (10 μL, 0.0708mmol) was added to 2 mL of Tris buffer and this was then added to the column. The solution was left in contact with the gel for an hour before the tap of the dropping column was slowly opened so that the solution passed through. The trapped gel was then washed with further portions of 2 mL of Tris buffer. Samples were analysed by UV/Vis spectroscopy and GC-MS.

Release of 2-thiopyridone from Thiopropyl Sepharose 6B using DTT

The gel slurry from the trapping reaction was washed with 5 mL Tris buffer. DTT (0.31 g, 0.20 mmol) was dissolved in 5 mL Tris buffer and passed through the column. The gel was then washed with portions of 5 mL buffer solution. These portions were analysed immediately by UV/Vis spectroscopy to check when the 2-thiopyridone was no longer present in the solution. When none was detectable the washing stopped. The reaction was monitored by UV/Vis spectroscopy and fractions analysed by GC-MS.

2.2.2.3 Adsorption onto gold wire

Two 0.33 m lengths of gold wire (0.2 mm diameter) were heated in a furnace at 300°C for 12 h to desorb any organic species from the gold surface. The wires were coiled and placed in thermal desorption tubes, which were sealed at both ends with

quartz wool. The tubes were connected (in parallel) between air pumps and a flange flask that contained an atmosphere of hexane-1-thiol. The atmosphere of hexane-1-thiol was generated by placing a solution of hexane-1-thiol (0.5 mL) in hexane (1 mL) in a capped GC vial and puncturing the septum with a piece of capillary tubing. The thiol atmosphere was sampled at 20 $mL.min^{-1}$ for 2 h. The tubes were then submitted for immediate thermal desorption-gas chromatography-mass spectrometry (TD-GC-MS) analysis. One length of gold wire was thermally desorbed at 200°C and the other at 300°C. A sample of hexane-1-thiol in hexane was also analysed by GC-MS, to aid in the identification of some of the impurities. The same GC oven temperature programme was used as that in the gold wire experiments. The sample was injected into an isothermal injector at 280°C (final temperature of the thermal desorption experiments).

2.2.3 Instrumentation

2.2.3.1 NMR

^{1}H and ^{13}C NMR data were obtained in a Jeol Eclipse +500 MHz instrument (Welwyn Garden City, UK) at room temperature. Samples were dissolved in $CDCl_3$.

2.2.3.2 LC-MS

A LCT TOF mass spectrometer (Waters MS Technologies, Manchester, UK) fitted with an ESI source was interfaced to an Agilent 1100 gradient pump and autosampler (Manchester, UK). The mass spectrometer was operated over the mass range *m/z* 100 to 600 and calibrated with sodium iodide. The column used was a 2.1 x 100 mm, 3 μm HyPURITY C18 (Thermo Fisher Scientific, UK). Solvent A was water, solvent B was acetonitrile.

For all maleimide based reactions, the gradient for the analysis was 10-100% acetonitirile in 25 min with a 15 min hold at 100% acetonitrile. The flow rate was 0.2 $mL.min^{-1}$. The sample injection volume was 0.5 μL, the column temperature was 40°C and the UV detector was set to 210 nm. Spectra were acquired in ESI positive mode.

For the covalent chromatography, the gradient was 5-100% acetonitirile in 20 min with a 5 min hold at 100% acetonitrile. The flow rate was 0.2 $mL.min^{-1}$. The sample

injection volume was 1 μL, the column temperature was 30°C and the UV detector was set to 220 nm. Spectra were acquired in ESI negative mode at cone voltages of 15 V and 30 V. (The higher cone voltage generated a characteristic fragment ion, the lower more intense protonated molecules).

2.2.3.3 GC-MS

A GCT TOF mass spectrometer (Waters MS Technologies, Manchester, UK) fitted with an EI source was interfaced to an Agilent 6890 GC (Agilent Technologies, Manchester, UK). The column used in all experiments was the same as for experiments described in Chapter 3: an HP5 (Agilent Technologies) (25 m × 0.20 mm ID x 0.25 μm). The column was temperature programmed from 50°C (hold for 5 min) to 300°C at a rate of 20°C.min^{-1} and held for 15 min. The injector temperature used was 280°C and the helium flow rate was 0.5 mL.min^{-1}. The injection volume and split ratio depended on the sample. Data were acquired in EI positive mode over the mass range of *m/z* 12 to 650.

<u>Cool-on column injection</u>

Experiments involving the analysis of sulfones in maleimide experiments were conducted using a modified ZAB-T magnetic sector instrument (Waters MS Technologies Manchester, UK) interfaced to an Agilent 6890 GC fitted with a cool-on column injector (Agilent, Manchester, UK). Cool on-column injection was used in order to reduce the thermal breakdown of any sulfones present in these samples. The GC column was a RTX-1 (10 m x 0.18 mm ID x 0.4 μm) (Thames Restek, Saunderton, UK). The flow rate was 2 mL.min^{-1}. The column was temperature programmed from 50°C (hold for 5 min) to 280°C at a rate of 20°C.min^{-1} and held for 10 min.

<u>Thermal desorption of gold-wire</u>

The TD-GC-MS analysis was performed on a system consisting of a TDU thermal-desorption unit (Gerstel, Bremen Germany) equipped with a CIS 4 programmed temperature vaporization (PTV) inlet (Gerstel) installed on an Agilent 6890 GC interfaced with a GCT TOF mass spectrometer. The GC column was an HP 5 (25 m x 0.2 mm x 0.33 μm). The column was temperature programmed from 50°C (held for 5 min) to 280°C at a rate of 20°C.min^{-1} and held for 15 min. The split ratio was

20:1. The thermal desorber was started at -50°C with injection at -120°C and heated rapidly up to 280°C. Mass spectra were acquired in EI positive mode over the mass range of *m/z* 0 to 650.

2.2.3.4 Raman

Raman spectra were obtained in a Renshaw Raman microscope instrument using a 785 nm wavelength laser. The Raman spectra were collected using 12 scans of 100 seconds for the range 1850 – 200 cm^{-1}.

2.2.3.5 FTIR

FTIR spectra were recorded in the Biorad 375C FTIR spectrometer using the diamond 'Golden Gate' ATR accessory. The FTIR spectrum was collected through the range 4000 – 650 cm^{-1} with accumulation of 1000 scans.

2.2.3.6 UV

UV/Vis spectra were obtained in a Varian CARY UV-Vis spectrometer. The spectra were recorded with water as the blank.

2.3 Identification of a menthyl ester in nature

2.3.1 Chemicals and materials

L-monomenthyl succinate was synthesised from menthol, succinic anhydride and the catalyst, imidazole (all purchased from Sigma-Aldrich) using microwave oven synthesis adapted from the method of Hirose (Hirose *et al.*, 2003). Purity was 96% as determined by GC. Analytical characterisation was carried out by NMR and FTIR spectroscopies and MS. *L. barbarum* dried fruit were obtained from a local health food store. *L. barbarum* tea (tender leaves and bud) was obtained from the Chinese online pharmacy, Qingdao Corner (Qingdao, China). Absolute ethanol (HPLC grade, Riedel de Haën) and acetonitrile (LC-MS grade, Riedel de Haën), diethyl ether, concentrated hydrochloric acid and sodium bicarbonate were purchased from Sigma-Aldrich (Gillingham, UK). Water used for LC mobile phase was filtered before use (0.45 μm filter).

2.3.2 Experimental procedures

2.3.2.1 Preparation of standard of L-monomenthyl succinate

L-Menthol (0.50 g, 3.2 mmoles), succinic anhydride (0.32 mg, 3.2 mmoles) and imidazole (0.22 g, 3.2 mmoles) were combined in a microwave vessel (PTFE). The vessel was irradiated in a microwave oven at a power of 200 W over 18 min up to a temperature of 70°C. After irradiation, the sample was diluted with diethyl ether (10 mL) and extracted with saturated sodium bicarbonate solution (10 mL). The aqueous layer was acidified with hydrochloric acid. The acidic layer was back extracted with diethyl ether (2 x 20 mL). The solvent was evaporated on a steam bath under nitrogen. Further purification was unnecessary as indicated by FTIR, LC-MS (ESI in negative ion mode) and NMR (^{1}H and ^{13}C) spectra.

2.3.2.2 Preparation of plant material extracts

L. barbarum dried fruit and leaves were stored in a freezer overnight at -25°C and then powdered in a blender. The powdered fruit or leaves were extracted in both a glass Soxhlet extractor and in a microwave. For Soxhlet extraction, a total sample of 195 g (fruit) and 46 g (leaves) were extracted. Because of equipment size limitations, this was split into several flasks and each was extracted with 200 mL of 95% ethanol. The extraction was carried out for a total of 13.5 h, as utilised by Hiserodt *et al.* (2004). For microwave extraction, a total sample of 122 g (fruit) and 37 g (leaves) were extracted. Ten grams of sample in 40 mL of 95% ethanol were placed in PTFE vessels. The samples was extracted at 600 W and heated up to 125°C where the sample was held for 3 min. (Conditions adapted from (Barbero *et al.*, 2006)). The extracts from Soxhlet and microwaves extractions were filtered and the filtrate concentrated on a rotary evaporator under vacuum at 55°C. The filtrate from the fruit on keeping overnight, developed sediment that was filtered and discarded. The final masses of concentrated extracts were as follows: 28 g for fruit (Soxhlet extracted); 20 g for fruit (microwave extracted); 10 g for leaves (Soxhlet extracted) and 5 g for leaves (microwave extracted). Samples were prepared for chromatographic separation by shaking 1 g of extract with 2 mL of 15% acetonitrile aqueous solution, centrifuging the resultant solution and filtering (0.2 μm filter).

2.3.3 Instrumentation

2.3.3.1 Microwave oven units

Synthesis of menthyl succinate was carried out in a CEM MES-1000 (CEM Microwave Technology Ltd, Buckingham, UK). The CEM MES was designed for solvent extraction but was found to be suitable for synthesis. The features of the equipment designed for controlling pressure in the vessels was not required. Due to later equipment malfunction, extraction of the plant materials was carried out in a CEM MARS 5 (CEM Microwave Technology Ltd, Buckingham, UK). The CEM MARS system is more commonly used for microwave-accelerated reactions but was also found suitable for extraction under the conditions used.

2.3.3.2 Preparative LC

Reversed-phase HPLC on plant extracts was performed using the RP module of the ProteomeLab™ PF2D Protein Fractionation System (Beckman-Coulter, High Wycombe, UK). The column was a PS-HPRP 2D (4.6 x 33 mm). Solvent A was water and solvent B acetonitrile. Solvents were degassed before use. The column was equilibrated at 15% B, and the sample applied. The column was held for 2 min at 15% B then a linear gradient was run from 15% B to 100% B over 12 min, and returned to 15% B over 1 min, followed by re-equilibration time. The flow rate was 0.75 $mL.min^{-1}$ and the column temperature was controlled at 50°C. The UV absorption profile was monitored at 214 nm. A sample of the standard, L-monomenthyl succinate (20 mM), was run to determine the retention time at which fractions should be collected. Fractions of 375 µL were collected every 30 seconds for the first 3 minutes using a FC 204 Fraction Collector (Gilson Inc, Middleton, WI) into 96-deep-well plates. Blanks were run after each fractionation. Samples were transferred to another 96 well plate (200 µL capacity) for analysis by nano-LC-ESI-MS/MS.

2.3.3.3 Nano-LC-ESI-MS/MS

Nano-LC-ESI-MS/MS was performed in a Q-Tof Ultima Global tandem quadrupole-orthogonal TOF instrument fitted with an in-line Cap LC system (Waters MS Technologies, Manchester, UK). The system was fitted with a C18 cartridge as pre-column (Dionex, USA) and PepMap C18 column, 75 µm id x 150 mm, 5 µm, 5Å

(Dionex USA) as the analytical column. Solvent A was water and solvent B acetonitrile. The column was equilibrated in 5% B and flushed for 3 minutes to remove unbound material before a linear gradient to 100% B over 25 min, held for 2 minutes at 100% B, then back to 5% B in 0.5 min. The flow rate was approximately 200 $nL.min^{-1}$. The column temperature was 20°C and the sample injection volume was 6.4 µL.

The mass spectrometer was calibrated from *m/z* 50 to 300 with a mixture of sodium formate (10 $mg.mL^{-1}$) sodium iodide (0.05 $mg.mL^{-1}$), methane sulfonic acid (1 $mg.mL^{-1}$) and salicylic acid (1 $mg.\mu L^{-1}$). Spectra were acquired using ESI in the negative ion mode. The instrument was operated in data-dependent mode for MS/MS acquisition, switching from the MS to MS/MS mode when an ion of *m/z* 255 was detected of sufficient intensity (60 counts) and then returning to the MS mode when the intensity falls below this threshold. The MS/MS scans were 1 s, and data were acquired for 14 s before switching back to MS mode. MS/MS data were acquired over the range *m/z* 40 to 275. The collision energy was 35 eV and the collision gas was argon. Blanks were run between sample types. The source temperature was 80°C and the capillary voltage set at 3.5 kV.

2.3.3.4 NMR

^{1}H and ^{13}C NMR data for structural confirmation of the standard of L-monomenthyl succinate were obtained in a Jeol Eclipse +500 MHz instrument (Welwyn Garden City, UK). Samples were dissolved in d_6-DMSO.

2.3.3.5 FTIR

FTIR spectra of the synthesised standard of L-monomenthyl succinate were recorded on a Biorad 375C FTIR spectrometer (Hemel Hempstead, UK) using a diamond 'Golden Gate' ATR accessory.

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3

Characterisation and Use of Carbonyl Specific Adsorbent Traps

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The aim of the work discussed in this chapter was to develop a method capable of selectively trapping volatile carbonyl compounds from the headspace around living plants. The trap is primarily for use in field trips. The method chosen was to use *O*-(2,3,4,5,6-pentaflourobenzyl)hydroxylamine (PFBHA) as a derivatising reagent for chemically trapping volatile aldehyde species in a dynamic headspace sampling experiment. PFBHA adsorbed on a support of Tenax® TA was contained within a trap and exposed to generated headspaces of low molecular weight aldehydes. The suitability of these PFBHA coated Tenax traps for headspace trapping in the field was investigated by carrying out a number of characterisation tests. These included studies on the effect of storage on traps (before and after use), humidity levels, breakthrough volume, optimisation of the solvent for desorption and of the coating levels of the derivatising reagent. A trap was successfully created and characterised. It was used to trap aldehyde volatiles in the headspace of a blue hyacinth plant and lemon oil. GC was used in analysis for studies requiring quantification of analytes. GC-MS was used when identification of analytes was required. A MS-Nose™ interface was used for monitoring analytes in the experiments for measuring breakthrough volume. Spectroscopic imaging techniques were used to visualise the PFBHA coating. These latter techniques are not in widespread use and their features are discussed.

3.1 Trap design

Two main criteria were considered when designing the trap: firstly, that it should be suitable for selective trapping of aldehydes and ketone compounds, and secondly,

that is should be suitable for headspace sampling of living plants on fragrance collecting trips outside of the laboratory. This has led to the development of a trap made of plastic housing material, containing a sorbent material coated with derivatising reagent. A review of the literature was made to inform the choice of derivatising reagent which is discussed in Section 3.1.1. Other variables in trap design were the trap housing and sorbent material which are discussed briefly below.

3.1.1 Choice of derivatising reagent

Aldehydes and ketones were established in Chapter 1 as functional groups of particular interest. Aldehydes, and to a lesser extent, ketones are challenging analytes, due to their volatile nature and reactivity. The carbonyls of interest are often found in complex matrices, therefore, selective and sensitive techniques are required to detect these compounds. The strategy for selectively trapping these compounds was based on choosing a derivatising reagent suitable for reacting with these groups. The principle of derivatisation was discussed in Chapter 1. Briefly, this is used to introduce stability and to create species with features which make them easier to analyse.

The chemistry of the carbonyl group is wide-ranging, with a common reaction being the reaction of nucleophiles, particularly amines, with the electropositive carbon of the carbon-oxygen double bond. Derivatising reagents for carbonyls, therefore, commonly incorporate an amine group. Reagents also often include groups that will improve detection such as by UV or fluorescence detectors for HPLC, or specialised GC detectors such as electron capture detectors. Reagents may also incorporate groups that will lead to a distinctive fragment when a mass spectrometer is used as the detector.

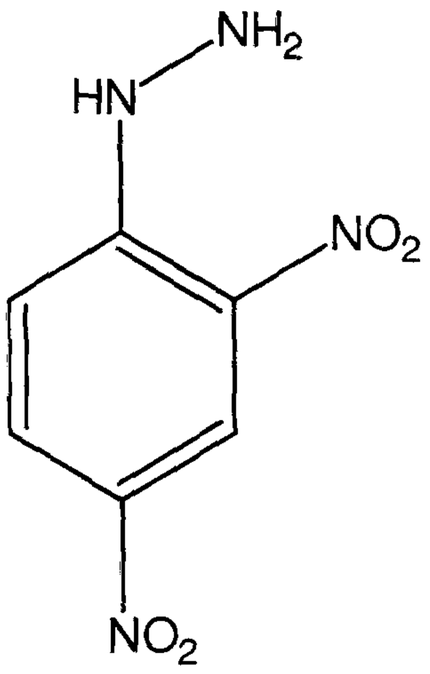
Common HPLC compatible derivatising reagents are hydrazines including:

- 2,4-dinitrophenylhydrazine (DNPH) (**1**);
- 4-(*N*,*N*)-dimethylaminosulphonyl)-7-hydrazino-2,1,3-benzoxadiazole (**2**); and
- dansylhydrazine (DNSH) (**3**).

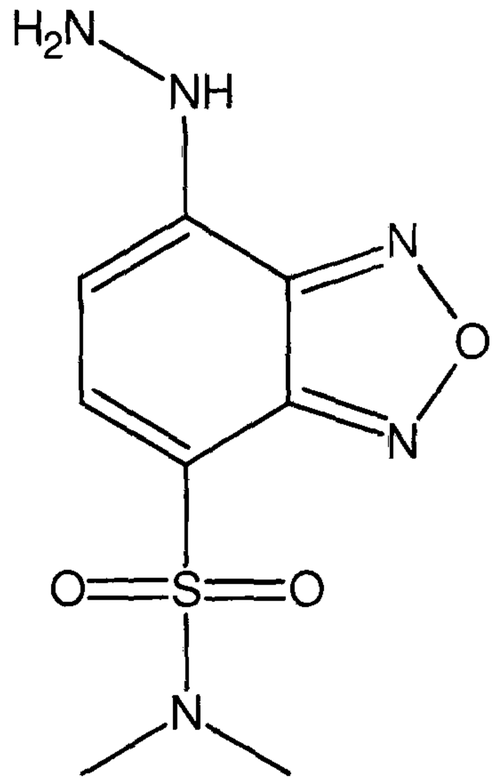
Derivatives suitable for use with GC include:

- cysteamine hydrochloride (**4**);
- pentafluorophenylhydrazine (PFPH) (**5**); and
- O-(2,3,4,5,6-pentafluorobenzyl)hydroxylamine (PFBHA) (**6**).

A summary of the various chemistries used in the assay of carbonyls by gas and liquid chromatography can be found in Nishikawa and Sakai (1995) and the textbook of Blau and Halket (1993).



(1)



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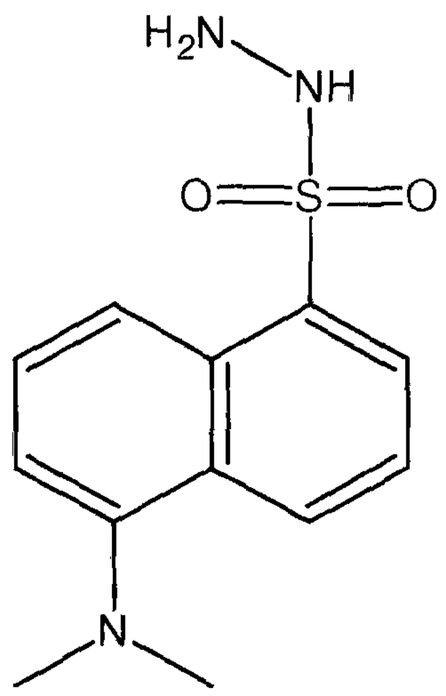

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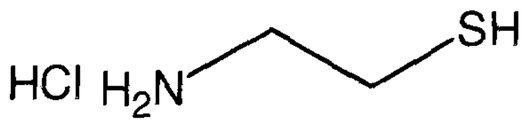

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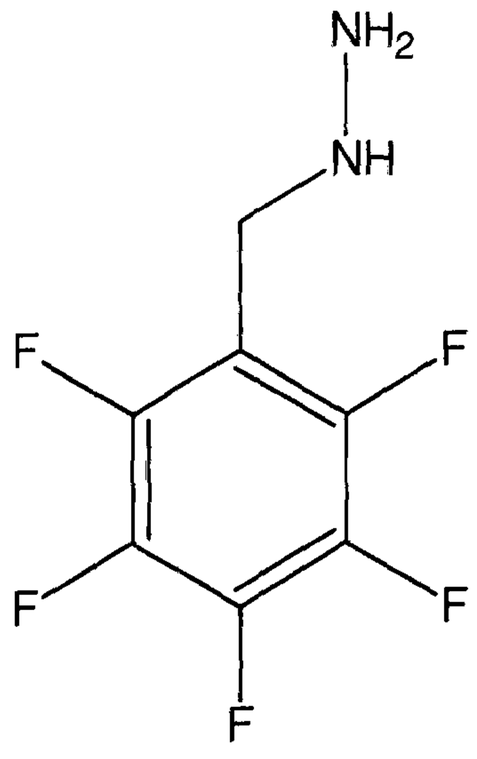

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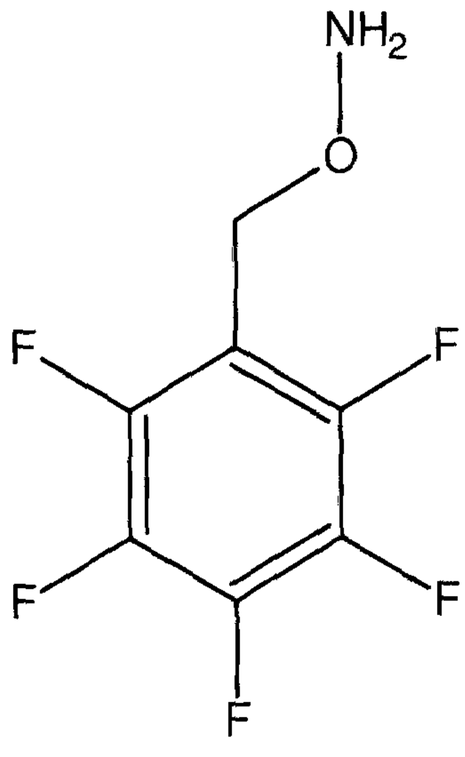


(6)

DNPH is one of the most widespread reagents used for derivatising carbonyl compounds in conjunction with HPLC analysis (Cecinato *et al.*, 2002; Fung and Grosjean, 1981) or LC-MS (Sakuragawa *et al.*, 1999). The main disadvantage of this reagent is that it, and the derivatives, are light sensitive. If GC is used for analysis, the high-temperatures can lead to partial decomposition of the derivatives. As discussed in detail in the Introduction, GC is the most important analytical tool in

volatile aroma compound analysis, therefore, the initial criterion for the derivatising reagent was that it should be GC compatible. Cysteamine is another simple reagent which reacts with aldehydes to form stable thiazolines under mild conditions at room temperature (Umano and Shibamoto, 1987; Yasuhara *et al.*, 1998). This will not react with α,β-unsaturated aldehydes, however, which is a structural motif found in many fragrance and flavour compounds. PFPH has also been used in place of DNPH with GC as the detector (Cecinato *et al.*, 2002).

3.1.1.1 PFBHA

The reagent chosen for use in this particular application of analysis of low molecule weight aldehydes was PFBHA. PFBHA has been used in a wide range of applications. It is frequently used in environmental monitoring of both atmospheric and aqueous aldehydes (Ho and Yu, 2002; Kobayashi *et al.*, 1980; Shen and Hee, 2000; Shimada and Mitamura, 1994; Smith *et al.*, 1989; Wu and Hee, 1995; Yu *et al.*, 1995). This reagent has also been used for biological monitoring of lipid peroxidation products in tissues (Spiteller *et al.*, 1999) and for monitoring aldehydes in human urine (Stashenko *et al.*, 1997). Flavour applications include detecting aldehydes and ketones as off-flavours in beer (Ojala *et al.*, 1994), identifying carbonyls that contribute to the aroma of cognac (Vidal *et al.*, 1993), vodka (Sowinski *et al.*, 2005) and wines (Ferreira *et al.*, 2004). For a review of applications of PFBHA as a derivatising reagent in solution phase reactions in a wide variety of biomedical and environmental applications in matrices such as water, urine and air, see Cancilla and Hee (1992).

The advantages of PFBHA as a derivatising agent are two-fold: the ease of formation of the oxime product and the features of the product, which make it amenable to sensitive detection techniques such as mass spectrometry and electron-capture detection. It has been demonstrated that derivatisation of carbonyl compounds with PFBHA is almost quantitative (yields greater than 80%) in a wide variety of matrices (Cancilla *et al.*, 1992; Cancilla and Hee, 1992).

The reaction between PFBHA and aldehydes is shown in Figure 3.1. It involves nucleophilic attack by the hydroxylamine group on the partially positive carbon of

the carbonyl followed by an acid-catalysed dehydration to from a stable pentafluorobenzyloxime (PFBO) product. The rate of the reaction is pH sensitive and also dependent on the carbonyl substituents, but tends to be at a maximum around pH 4. These oxime products are also formed if ketones are present; however ketones react at a slower rate compared to aldehydes, because of the increased steric hindrance.

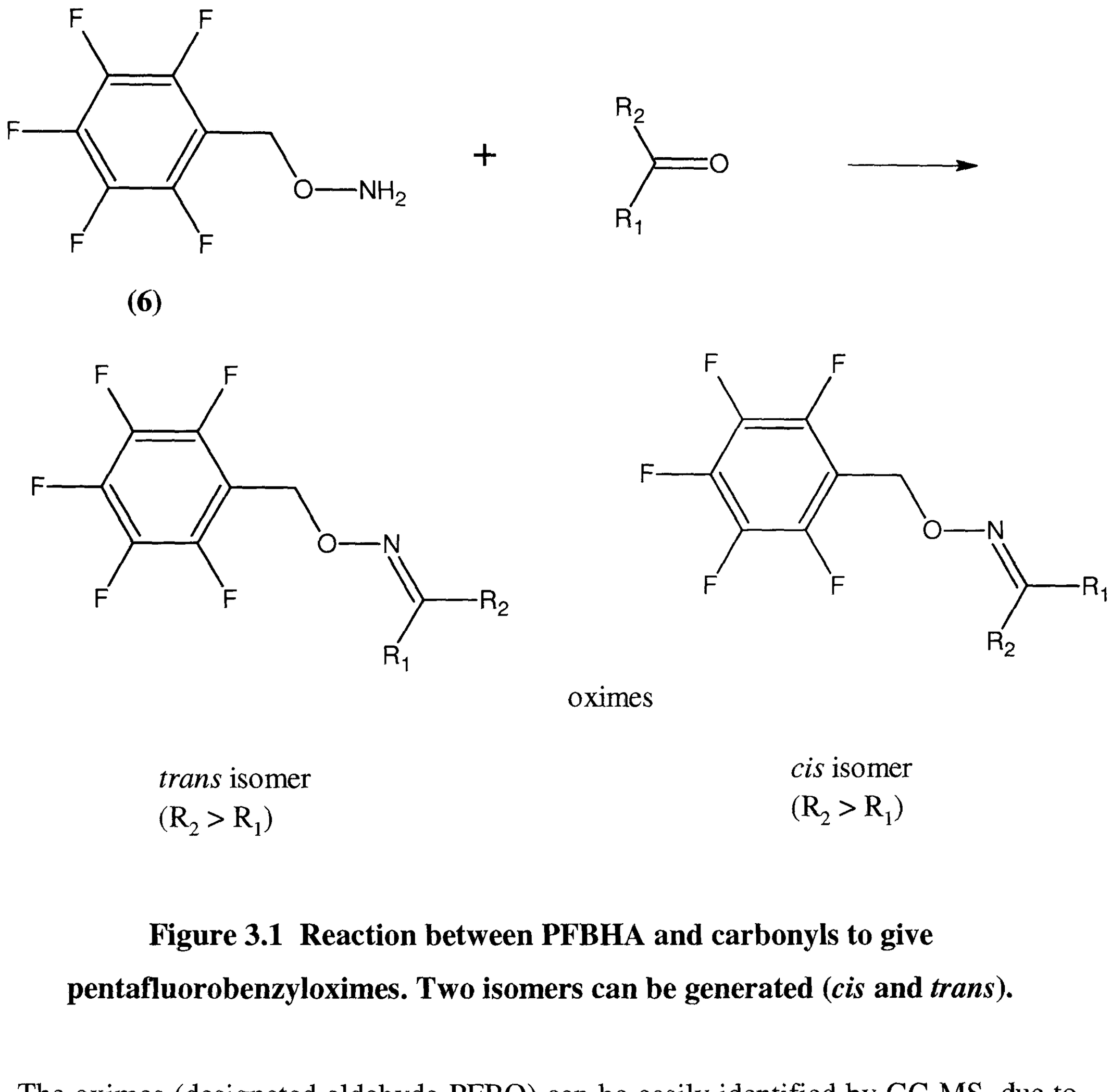


Figure 3.1 Reaction between PFBHA and carbonyls to give pentafluorobenzyloximes. Two isomers can be generated (*cis* and *trans*).

The oximes (designated aldehyde-PFBO) can be easily identified by GC-MS, due to a high abundance *m/z* 181 fragment of the pentafluorotropylium ion, $C_7F_5H_2^+$, which is readily formed on cleavage of the C-O bond. Detection is possible at picogram level in selected ion monitoring (SIM) mode. Alternatively, oximes can also be identified by GC-electron capture detection (GC-ECD) because of the presence of the five fluorine atoms. Asymmetric aldehydes and ketones produce two different

products which are a result of the formation of the *cis* and *trans* isomers of the derivatisation reaction (Figure 3.1).

3.1.2 Choice of trap housing

The trap consisted of a small polypropylene housing shown in Figure 3.2. This is typical of cartridges used for solid phase extraction. The sorbent material is contained within two polyethylene frits. This design was chosen rather than glass tubing because of the desire for a light, portable and unbreakable trap for use in field sampling situations.

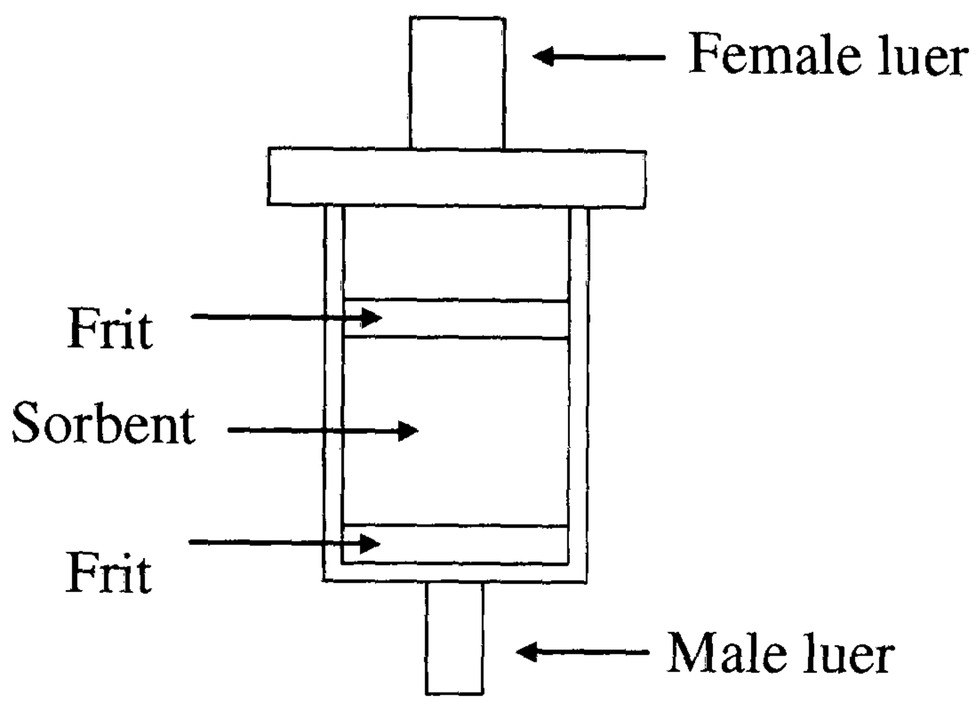


Figure 3.2 Schematic of polypropylene traps used for housing the coated sorbent

3.1.3 Choice of sorbent material

Tenax TA™, often referred to simply as Tenax, was chosen as the material upon which the derivatising reagent was coated. This is a porous polymer based on 2,6-diphenyl-oxide. It is the most widely used adsorbent resin for purge and trap in conjunction with thermal desorption for applications such as trapping volatile organic compounds in air and liquids. Both the US Environmental Protection Agency (EPA) and National Institute of Occupational Safety and Health (NIOSH) specify the use of Tenax in their standard methods. Tenax is suitable for trapping from samples with high moisture content because of the low breakthrough volume for water (Scientific Instrument Services). This is important because living plants generate moisture during headspace trapping experiments due to photosynthesis. Wu and Hee (1995) investigated the compatibility of PFBHA with other common adsorbents Florasil, Chromasorb 101, Chromasorb 106, Chromasorb 102 and Tenax GC. Tenax

GC was found to be the best sorbent based on adsorption/desorption efficiency. Later work by Shen and Hee (2000) utilised Tenax TA, which is considered as a replacement for Tenax G.

3.2 Trap evaluation

The steps for evaluating potential traps are shown in Figure 3.3.

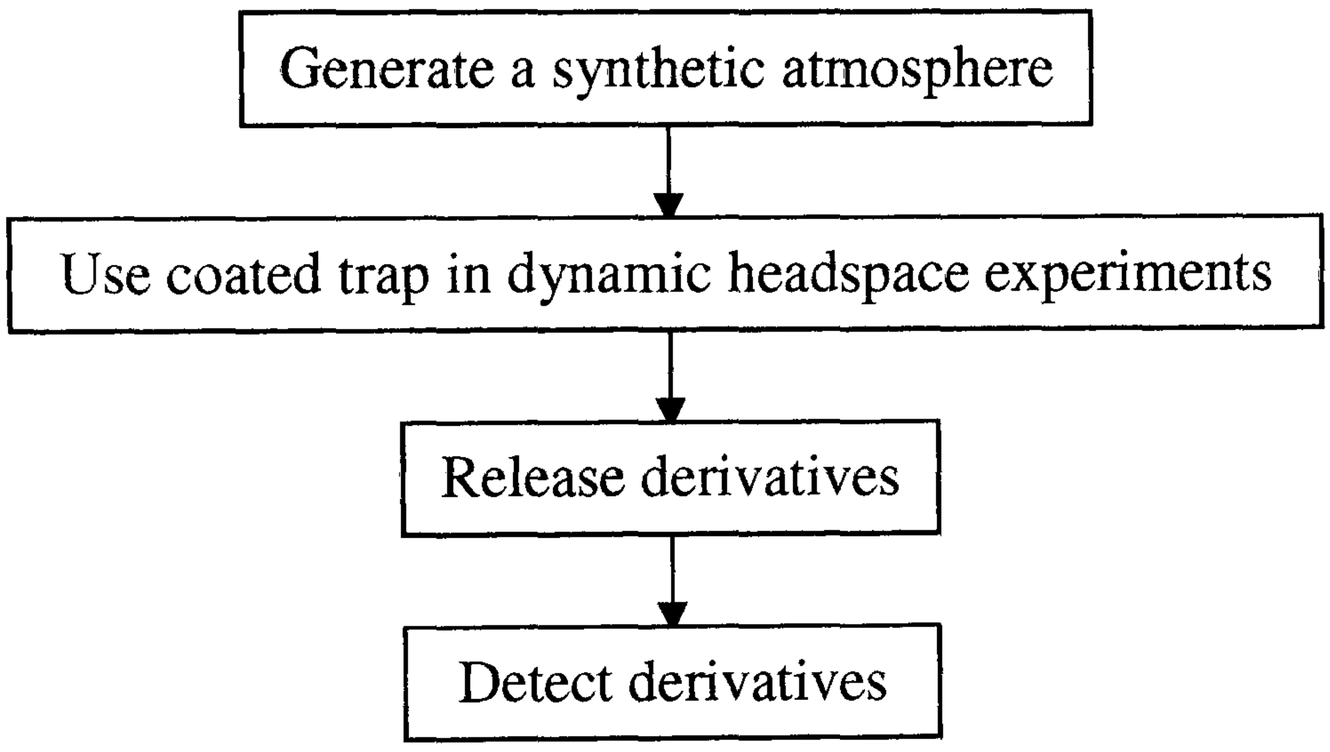


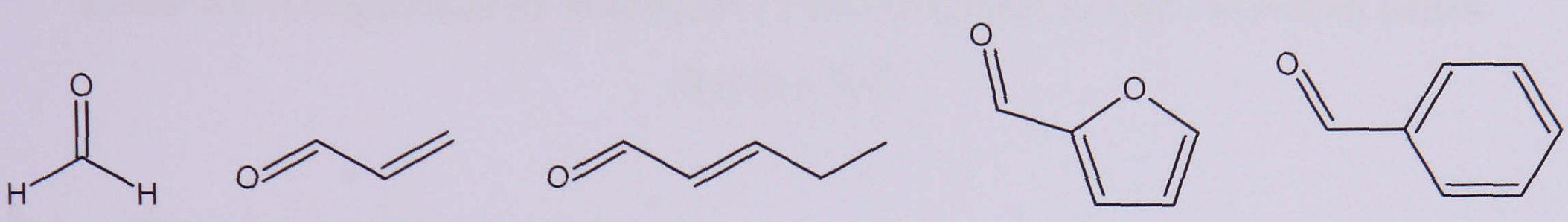
Figure 3.3 Steps involved in evaluating potential traps

Firstly, a synthetic atmosphere of aldehydes is generated (Section 3.2.1). This headspace is used in a series of experiments to explore the operating parameters of the trap (Section 3.3). In the work described here, a solvent is always used to release the derivatives. There are usually two choices for desorbing analytes from sorbents after a headspace trapping experiment: either with solvent, or thermally (Da Costa and Eri, 2004). In this case, solvent desorption was the method of choice because of the opportunity for re-analysis. In thermal desorption, the volatiles from a sample matrix are transferred directly onto a chromatographic column. Because the entire sample is desorbed in 'one-shot', there is no opportunity for repeat analysis. This is an important factor when the samples to be analysed are obtained from a source that is difficult to resample, such as those obtained from a field trip. Solvent desorption dilutes the sample but allows the same sample to be analysed more than once, if necessary, and by more than one analytical technique if further structural information is required to determine the species which are present. The derivatives are detected by the standard techniques of either GC or GC-MS, or the MS Nose™ for

measurement of the breakthrough characteristics of the trap. This instrument and its use are explored in Section 3.3.6.

3.2.1 Headspace generation

For the purpose of testing the suitability of the trap, an atmosphere of low molecular weight aldehydes was generated for use in dynamic headspace experiments. The aldehydes chosen were formaldehyde (**7**), acrolein (**8**), *trans*-2-pentenal (**9**) and furfural (**10**). In some later experiments, benzaldehyde (**11**) was also used as this is a known breakdown product of Tenax.



(7) **(8)** **(9)** **(10)** **(11)**

These aldehydes were chosen, firstly, because of their low molecular weights which are typical of the aldehydes of interest and, secondly, because they contain the motif of an α,β-unsaturated carbonyl (except **7**), a commonly observed structure for aldehydic fragrance compounds. No ketones were used for the development experiments because these are generally more stable than aldehydes. Figure 3.4 shows a typical GC chromatogram of the derivatives of aldehydes **7-11**, with assignments and retention times in Table 3.1.

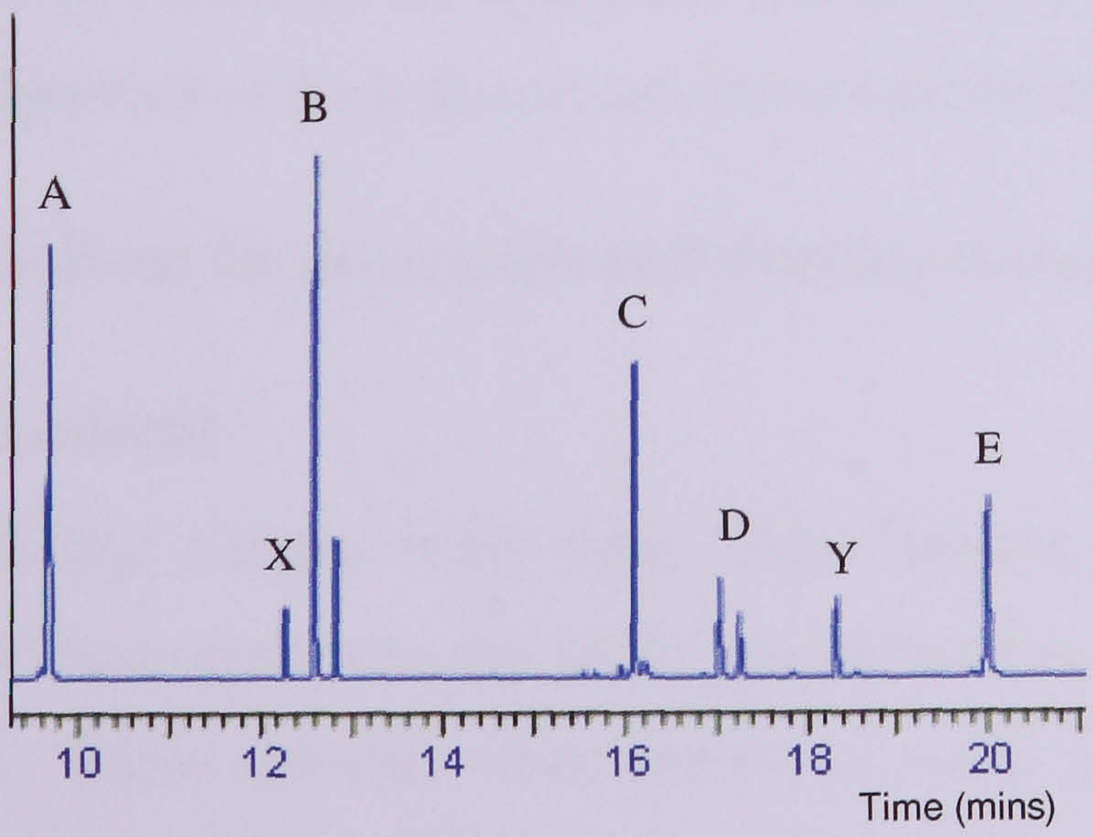


Figure 3.4 A typical GC chromatogram of aldehyde-PFBO derivatives

Annotation	Assignment	Retention Time (min)
A	Formaldehyde-PFBO	9.7
B	Acrolein-PFBO (*cis* and *trans* isomers)	12.6 & 12.8
C	*Trans*-2-pentenal-PFBO	16.1
D	Furfural-PFBO (*cis* and *trans* isomers)	17.0 & 17.2
E	Benzaldehyde-PFBO	20.0
X	Acetone-PFBO (from solvent)	12.3
Y	Unidentified PFBHA related peak	18.3

Table 3.1 Assignment of aldehyde-PFBO derivatives and retention times (Figure 3.4)

3.3 Characterisation parameters

A number of experimental parameters were investigated in order to be able to use the trap in a field situation. It was noted in the introduction that field trips can be expensive expeditions and, therefore, collection equipment should be well-understood before use. The experimental parameters included:

- the amount of PFBHA to use in the coating of Tenax;
- the solvent to be used to desorb the analytes from the trap for analysis;
- the effect of storage on the use of the trap;
- the effect of humidity on the use of the trap; and
- the breakthrough volume of the aldehydes and aldehyde-PFBO derivatives.

The results of investigations of these factors are discussed below.

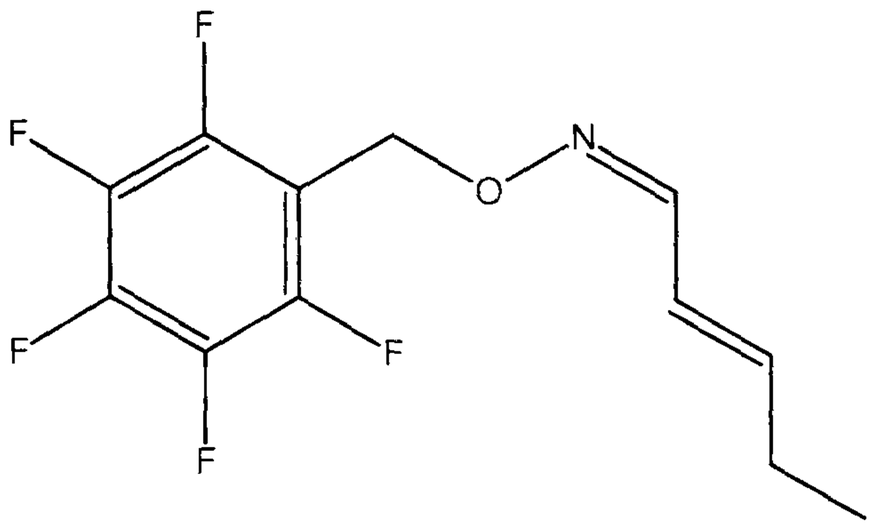
3.3.1 Selection of solvent for desorption and cleaning of traps

3.3.1.1 Desorption solvent

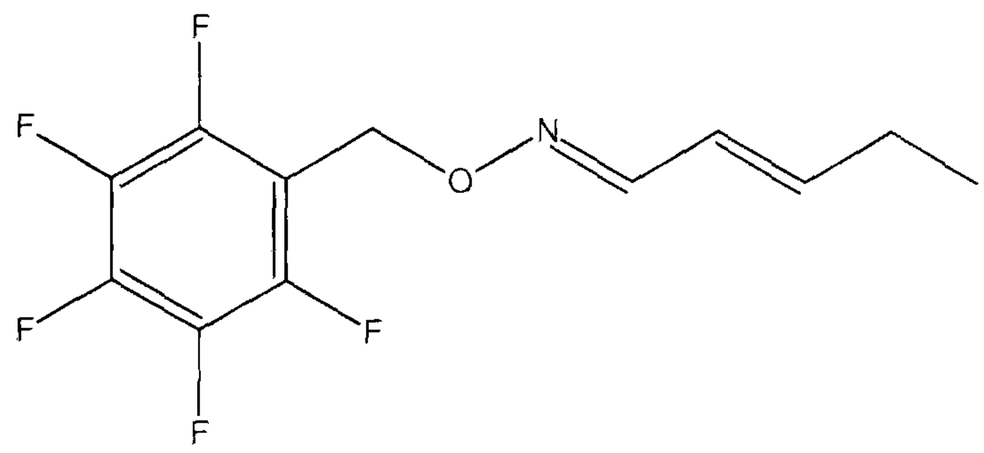
Dichloromethane, methyl tertiary butyl ether, ethyl acetate, hexane, 5:95 ethyl acetate/hexane, 50:50 hexane/acetone and 80:20 hexane/acetone were investigated as desorption solvents. These solvents were chosen to cover a range of polarities. Previous work used hexane as a solvent for desorption (Wu and Hee, 1995). The solvents were compared by measuring the recovery of the derivatisation products with *trans*-2-pentenal spiked on to the PFBHA traps in a dynamic headspace

experiment. An internal standard of octane, added to the solvent, was used for quantification by GC. The experiment was repeated three times for each solvent.

Table 3.2 shows the amount of *trans*-2-pentenal-PFBO recovered with increasing solvent volume for a mixture of 80:20 hexane/acetone, as an illustration. Note that only one peak was observed although two isomers are possible (**12**) and (**13**). Steric hindrance influences the formation of one isomer over the other.



(12)



(13)

For the data shown in Table 3.2, the percentage recovery is calculated by the amount of derivative recovered in each fraction divided by the total amount of *trans*-2-pentenal-PFBO recovered in 1 mL. Peaks were normalised to the internal standard (octane) peak area. These data are represented graphically in Figure 3.5.

	% *trans*-2-pentenal-PFBO recovered		
Volume of solvent (µL)	Run 1	Run 2	Run 3
200	46	62	45
400	83	88	73
600	97	97	89
800	99	98	96
1000	100	100	100

Table 3.2 The recovery of *trans*-2-pentenal-PFBO with increasing solvent volume using a mixture of 80:20 hexane/acetone for desorption.

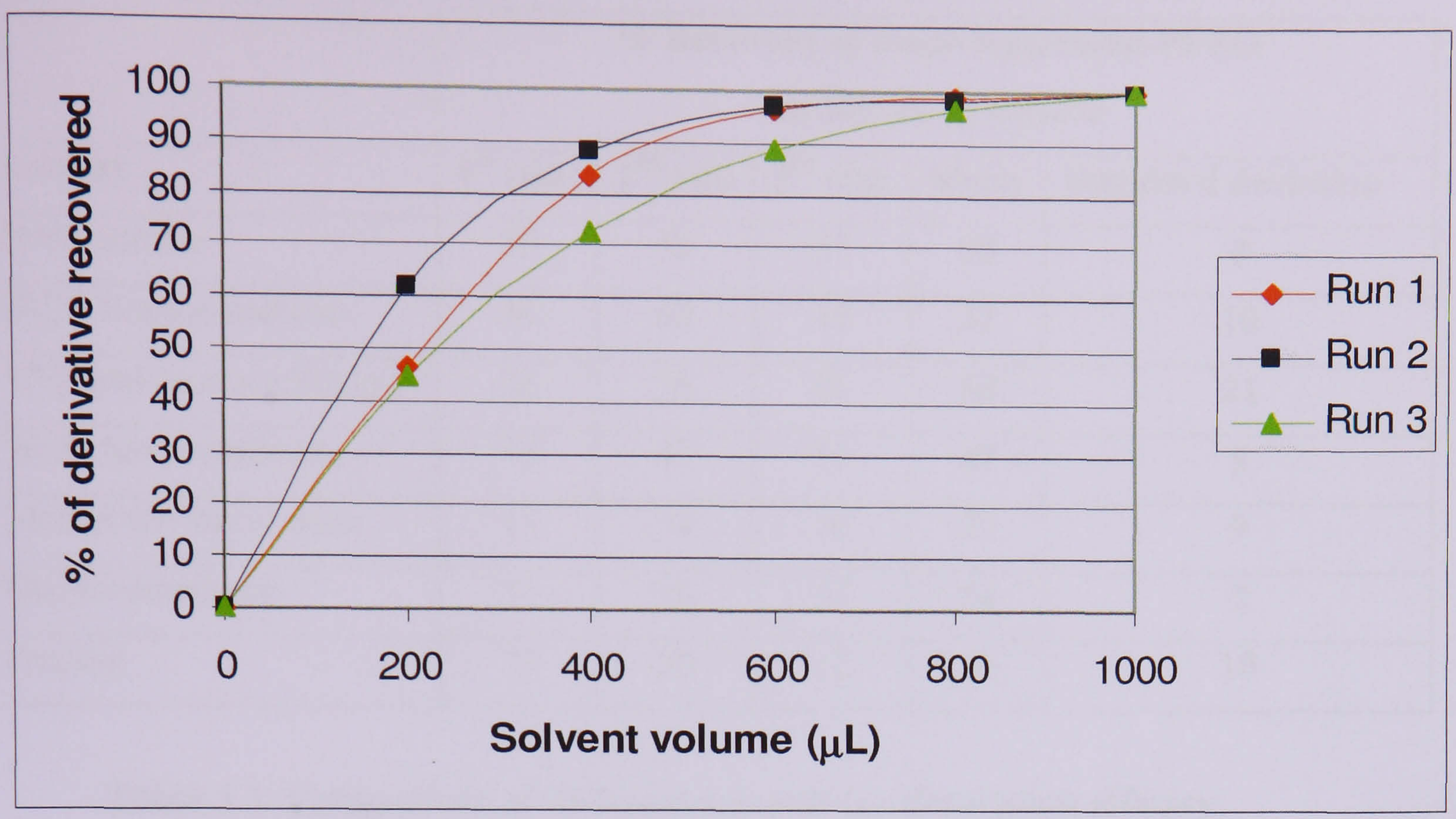


Figure 3.5 Recovery of *trans*-2-pentenal-PFBO using 80:20 hexane/acetone as the desorbing solvent mixture

The most efficient desorbing solvent was determined by comparing the percentage of derivative recovered in the first 200 μL fraction collected. These data are shown in Table 3.3. Ethyl acetate, 5:95 ethyl acetate/hexane and 80:20 hexane/acetone were comparable at desorbing the derivatives from the Tenax. Ethyl acetate gave the best repeatability as measured by the standard deviation. Solvents containing acetone, however, have a distinct advantage: the acetone reacted with the excess PFBHA that was desorbed from the trap and therefore acted to quench the derivatising reagent. This helps prevent contamination of the sample from other sources of aldehydes. 80:20 hexane/acetone removed 94% of the *trans*-2-pentenal-PFBO within 600 μl compared to 90% for 50:50 hexane/acetone (data not shown). Dichloromethane was found to degrade the Tenax. Other chlorinated solvents may be expected to do the same. 80:20 hexane/acetone was chosen as the standard solvent mixture used for trap desorption in subsequent experiments because of the efficient analyte removal and the added advantage of reacting with the derivatising reagent.

	% Recovery of *trans*-2-pentenal-PFBO in 200 µL of solvent				
Solvent	**1st run**	**2nd run**	**3rd run**	**Mean**	**Standard deviation**
Ethyl acetate	50	55	51	**52**	**3**
80:20 hexane/acetone	46	62	45	**51**	**10**
5:95 ethyl acetate/hexane	26	58	67	**50**	**21**
50:50 hexane/acetone	42	47	51	**47**	**5**
Methyl tert-butyl ether	47	34	30	**37**	**9**
Dichloromethane	31	36	34	**34**	**2**
Hexane	19	30	12	**20**	**10**

Table 3.3: Comparison of different solvents for desorption efficacy.

3.3.1.2 Cleaning solvent

The possibility of using one of the solvent systems for cleaning the trap immediately prior to use in the field was also investigated. The criterion for this was that the solvent should remove as little PFBHA as possible when washed with the solvent. Table 3.4 shows the amount of PFBHA washed off the trap as measured by GC (relative to the internal standard peak). Note that the solvents containing acetone led to all PFBHA reacting, and hence no PFBHA was measured.

	% Recovery of *trans*-2-pentenal-PFBO in 200 µL of solvent.				
Solvent	**1st run**	**2nd run**	**3rd run**	**Mean**	**Standard deviation**
Ethyl acetate	71	77	78	**75**	**4**
5:95 ethyl acetate/hexane	46	69	73	**63**	**14**
Hexane	43	40	40	**41**	**2**
Methyl tert-butyl ether	41	29	34	**35**	**11**
Dichloromethane	19	40	16	**25**	**13**

Table 3.4 Comparison of solvents for recovery of PFBHA in 200 µL of solvent.

These data suggest that none of these solvents were suitable for conditioning traps prior to use. They all removed almost 90% of PFBHA within 800 µL. Further studies on more solvents, especially non-polar ones, would be required to find a solvent for this purpose. Two possible solutions to the problem of cleaning the trap before use are either: (a) the trap can be prepared in a super clean manner before use or (b) the trap can be prepared in the field. Case (a) strongly depends on the stability of Tenax especially in the presence of PFBHA. In case (b), there are disadvantages due to the lack of equipment in the field. The PFBHA could be transported to the sampling location as a solution in a highly volatile solvent such as diethyl ether, which would be rapidly lost in the coating process. Difficulties may arise in achieving an even coating of PFBHA, compared to the conditions used in the laboratory (shaking for 30 minutes).

3.3.2 Effect of storage on traps

3.3.2.1 Storage of Tenax

Impurities from the polymer sorbent may arise in headspace trapping experiments and contribute to background noise in GC analysis. These are primarily a problem if they provide interference with analyte peaks. Sorbents are usually thoroughly cleaned immediately before use. As discussed above, a suitable solvent was not found for cleaning the trap immediately before use so the effect of storing Tenax for nine days was investigated. This time period was chosen as indicative of the length of an expedition. Samples were either coated with PFBHA or left uncoated. After nine days, traps were desorbed and examined by GC-MS and compared to freshly cleaned Tenax. Significant levels of contaminants were present in the coated and uncoated Tenax. As a result of this analysis, for all subsequent testing a fresh source of Tenax was used rather than recycling cleaned, used sorbent. There was no evidence for benzaldehyde in the chromatograms which is a known thermal breakdown product of Tenax and a natural fragrance compound.

Cao and Hewitt (1994) have noted the build up of contaminants in blanks on storage with Tenax and other sorbents. Following on from this work, Helmig concluded that contaminant levels could be reduced by conditioning of adsorbents prior to use at high temperature in a thermal desorption tube and preventing the diffusion or air in

to the tubes during conditioning (Helmig, 1996). The same paper suggests storing absorbent tubes capped, under a purified atmosphere and at freezer temperatures. Conditioning samples by thermal desorption is not practicable in this case once the Tenax has been coated with PFBHA.

3.3.2.2 Storage of PFBHA-coated Tenax traps after exposure

The 'shelf-life' of PFBHA-coated Tenax traps after being exposed was examined to give an indication of how long traps could be stored before being desorbed. Traps were exposed to the headspace of a mixture of formaldehyde, acrolein, *trans*-2-pentenal and furfural and stored for three and seven days. Samples were stored with the ends capped, under nitrogen in bags. The level of aldehyde-PFBO derivatives for a trap desorbed after three days compared with the sample left for seven days is overlaid in Figure 3.6, with peak assignments in Table 3.5. The levels of all the derivative species decreased with time indicating loss of species from the trap. It was therefore concluded that traps should de desorbed as soon as possible after exposure. In subsequent work, traps were desorbed immediately after headspace exposure.

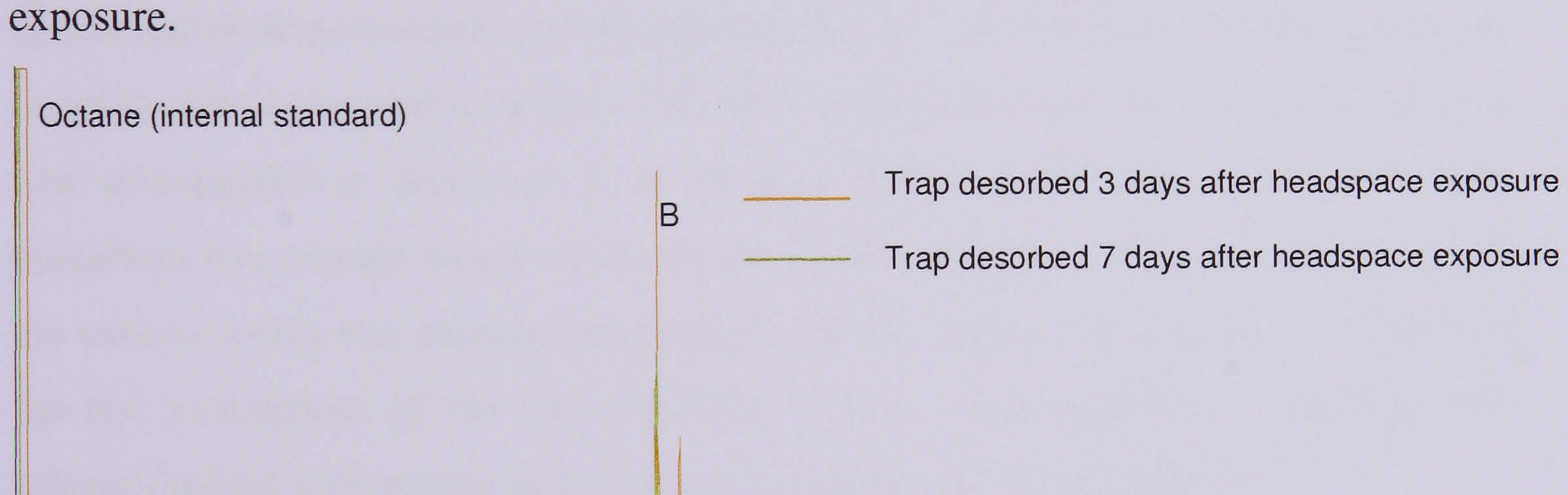


Figure 3.6 GC chromatograms for traps desorbed 3 and 7 days after exposure to an aldehydic headspace.

Annotation	Assignment
A	Formaldehyde-PFBO
B	Acrolein-PFBO (*cis* and *trans* isomers)
C	*Trans*-2-pentenal-PFBO
D	Furfural-PFBO (*cis* and *trans* isomers)
X	Acetone-PFBO (*cis* and *trans* solvent)
Y	PFBHA related impurity

Table 3.5 Peak assignments for species identified in traps desorbed after 3 and 7 days after exposure to an aldehydic headspace (see Figure 3.6).

3.3.3 Selection of coating level

The amount of PFBHA required to coat the sorbent support is an important parameter for the trap. Overloading the Tenax with PFBHA could lead to blocking of the pores of the Tenax, thereby decreasing the effective surface for aldehyde derivatisation and adsorption of the reacted species. Insufficient derivatising reagent could lead to incomplete reaction. The level of PFBHA to be used to coat the Tenax was investigated at levels of 1, 5, 10 and 20% w/w PFBHA. The 20% w/w maximum was chosen based on the results of Wu and Hee (1995). Tenax coated at the various levels was prepared and used to fill the traps. The traps were exposed to the test atmosphere of the five aldehydes (**7-11**). The traps were desorbed with solvent (spiked with octane as an internal standard) and analysed by GC.

Data from this experiment are plotted in Figure 3.7. It shows the peak area ratio for each aldehyde-PFBO product. The peak area ratio is determined by dividing the area of the octane peak by the peak area of the derivative. It is noticeable at 1 and 5 % coating levels that very little higher molecular weight species (*trans*-2-pentenal, acrolein and benzaldehyde derivatives) were detected. This could indicate there is insufficient reagent for reaction. It was concluded from these limited data that a 10% w/w coating level would be used because on average it led to the greatest amount of aldehyde material to be derivatised.

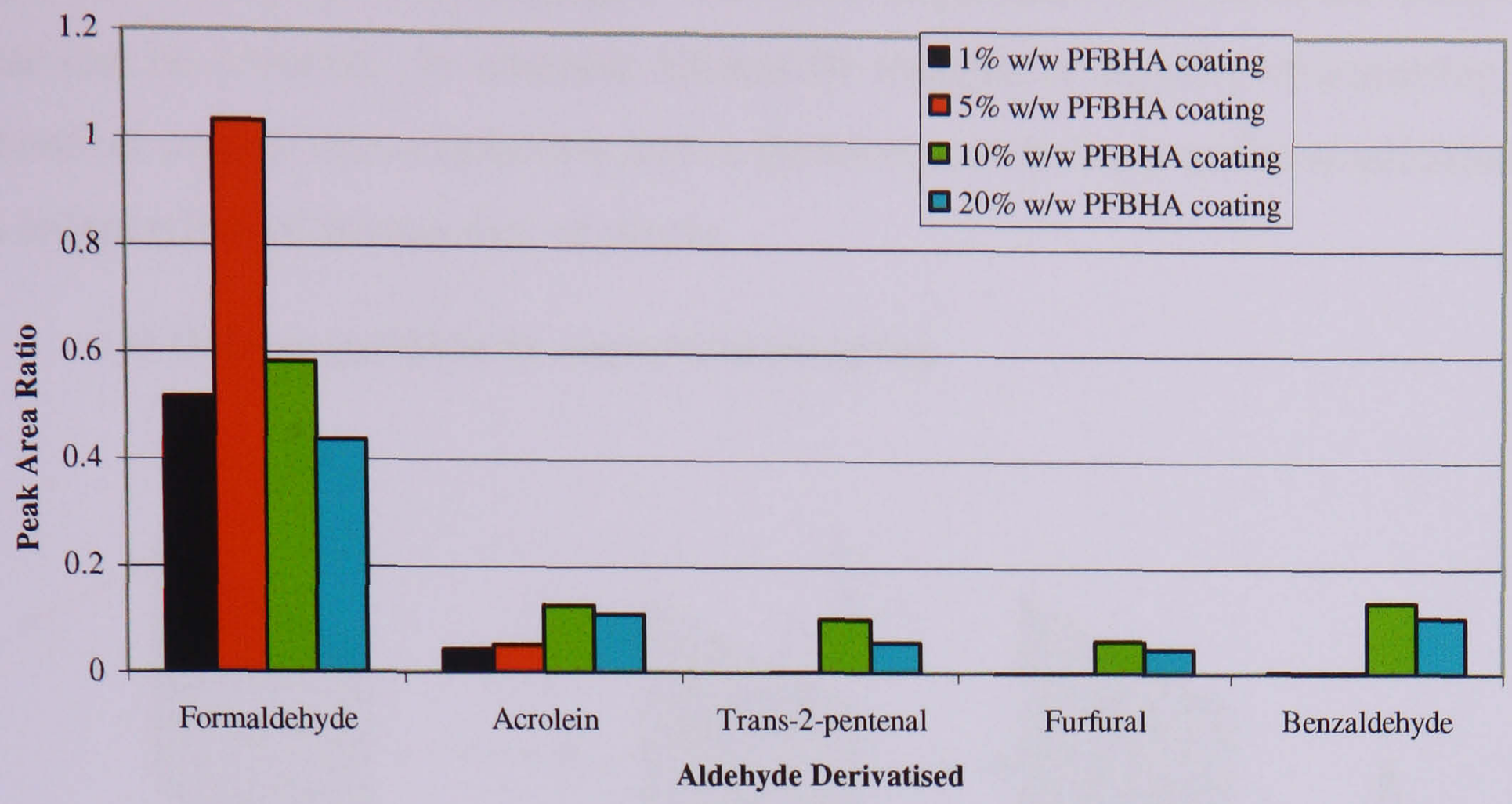


Figure 3.7 Amount of aldehyde-PFBO derivative recovered as the coating level is varied.

3.3.4 Coating distribution

It is important to know the homogeneity of the PFBHA coating over the surface for consistent trapping. This was measured by using spectroscopic-imaging methods which were introduced in Chapter 1. The generation of images and the instrumentation are described in more detail here, as these techniques are not in routine use.

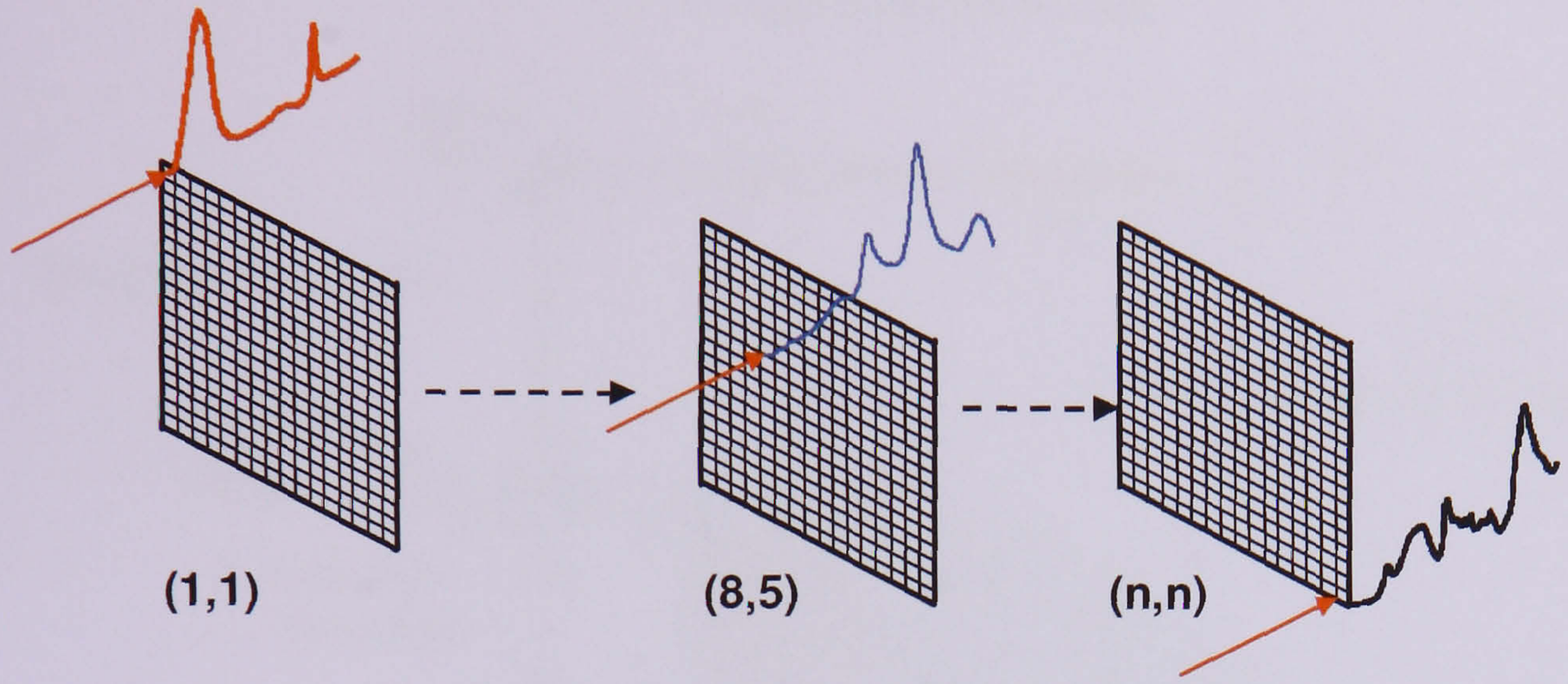
3.3.4.1 Spectroscopic mapping and imaging

Spectroscopic images are acquired by an instrument that can simultaneously record spectral and spatial information of a sample. Most commonly, Raman and IR spectroscopy are used. Every pixel of the image is associated with a spectrum. The spectral image can be obtained in one of two ways. Spatially resolved spectral information can be obtained by scanning the sample point-by-point collecting a spectrum at each point as illustrated in Figure 3.8 (a). Alternatively, if a 2D array of detectors is used, many spectra can be acquired simultaneously as shown in Figure 3.8 (b). Images are based on being able to identify a band that is characteristic for one material that will allow it to be distinguished from others.

The main disadvantage of mapping (whether by using Raman, IR or any other method) is the time taken to acquire an image, which is step-wise and dependent on

the number of pixels in the image. The slow acquisition also limits the sample size that can be covered. In contrast, Global-IR imaging is capable of acquiring a 2D chemical map in one acquisition and is therefore much faster as the acquisition time is independent of the number of pixels.

(a) Data acquisition by sequential mapping



(b) 'Global' data acquisition using a 2-D detector

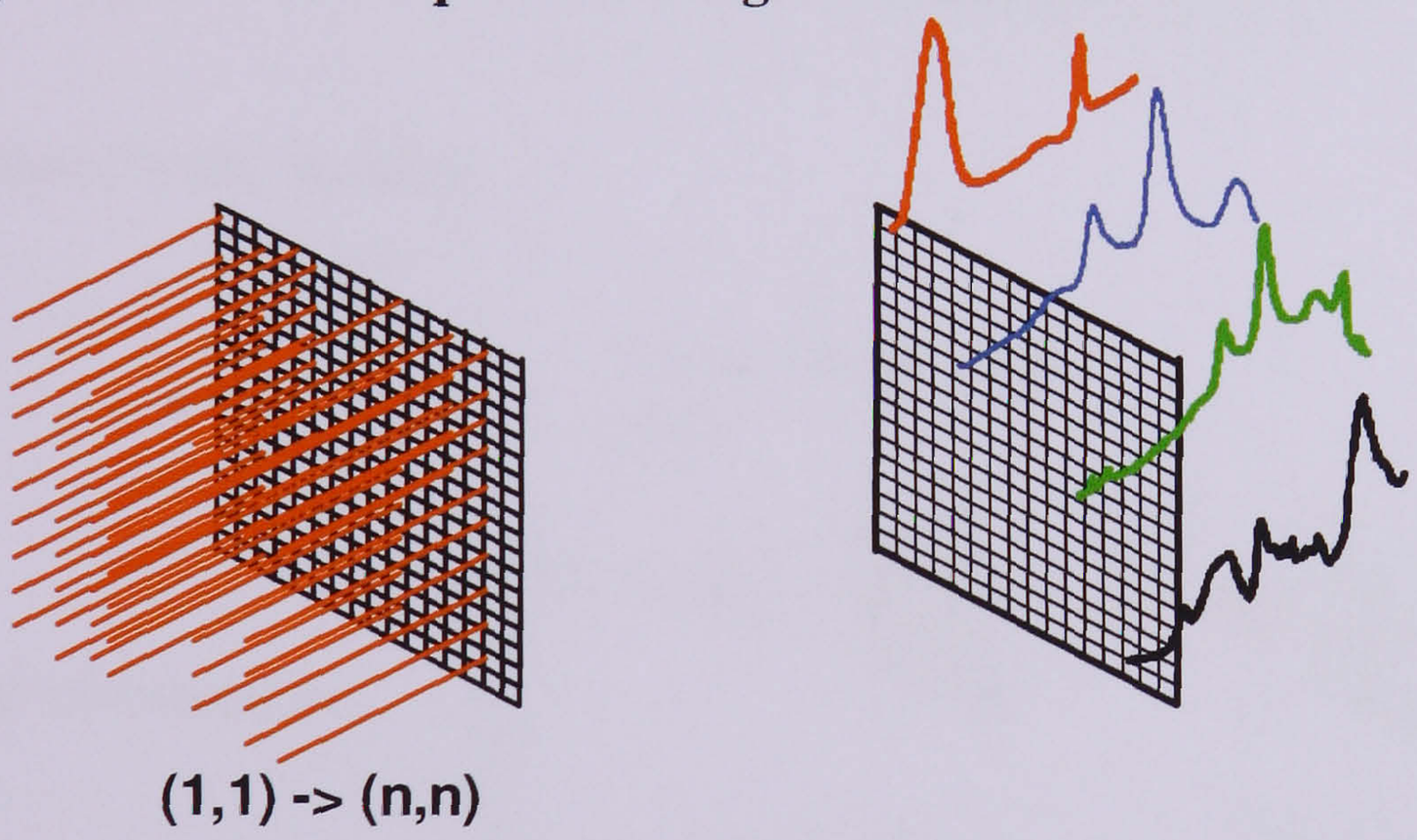
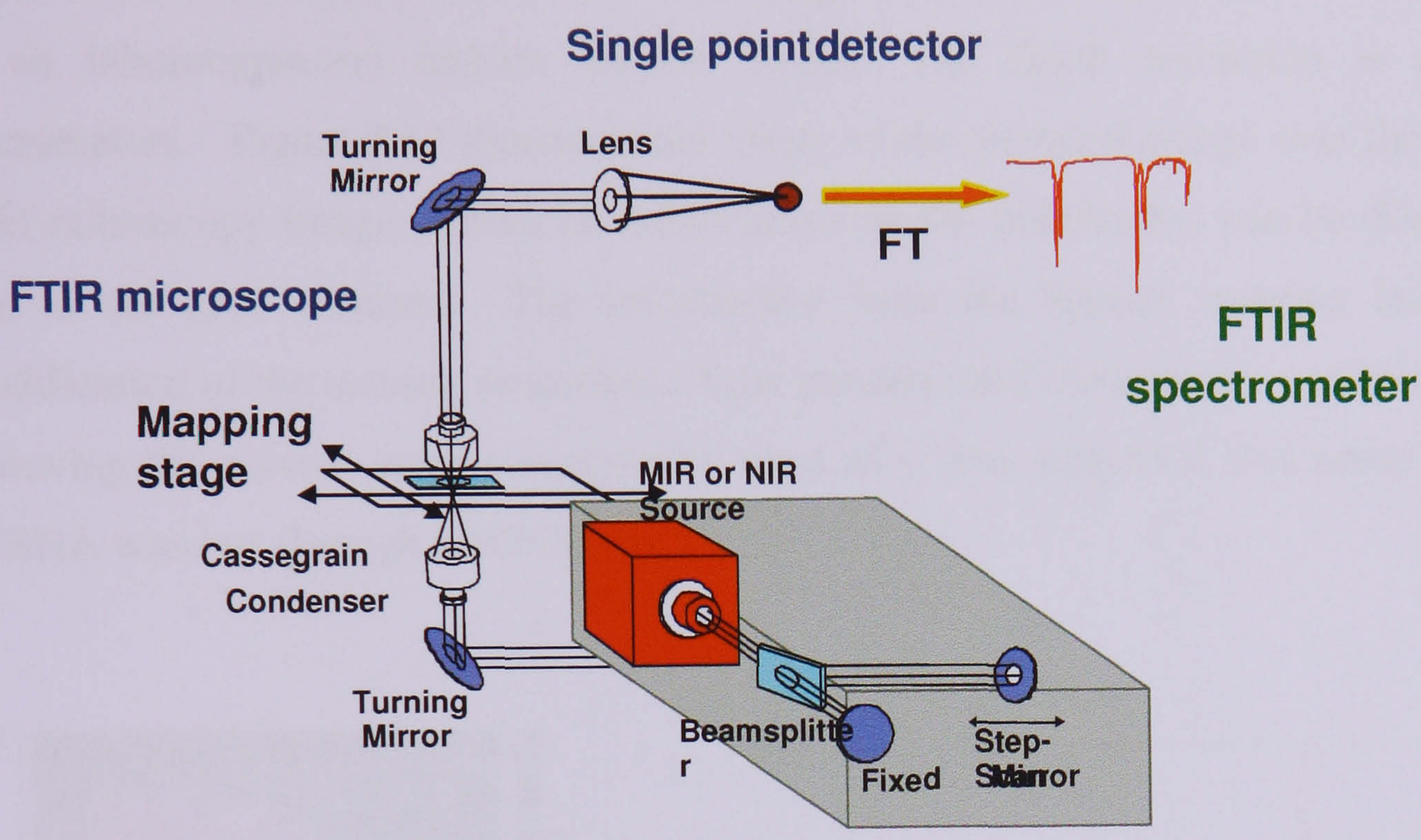


Figure 3.8 Schematic comparison of (a) sequential (mapping) and (b) global image acquisition (Everall, 2002).

The instrumentation for the two methods is shown in Figure 3.9, using FTIR as an example. For mapping, the sample is placed on a conventional FTIR microscope with an automated X-Y sample stage, and spectra accumulated one pixel at a time by incrementally moving the sample. (The same approach is used for Raman mapping). For Global-IR imaging a focal plane array detector is used to capture a spectrum simultaneously at each pixel of the image. A step-scan interferometer is used as the

radiation source. For Global Raman imaging, a 2D detector is used and a tuneable filter that scans the wavelengths of interest, taking 'pictures' at each wavelength. See Kidder *et al*. (2002) and Tran (2003) for reviews of the available technologies.

a) Sequential IR mapping



b) Global FTIR imaging

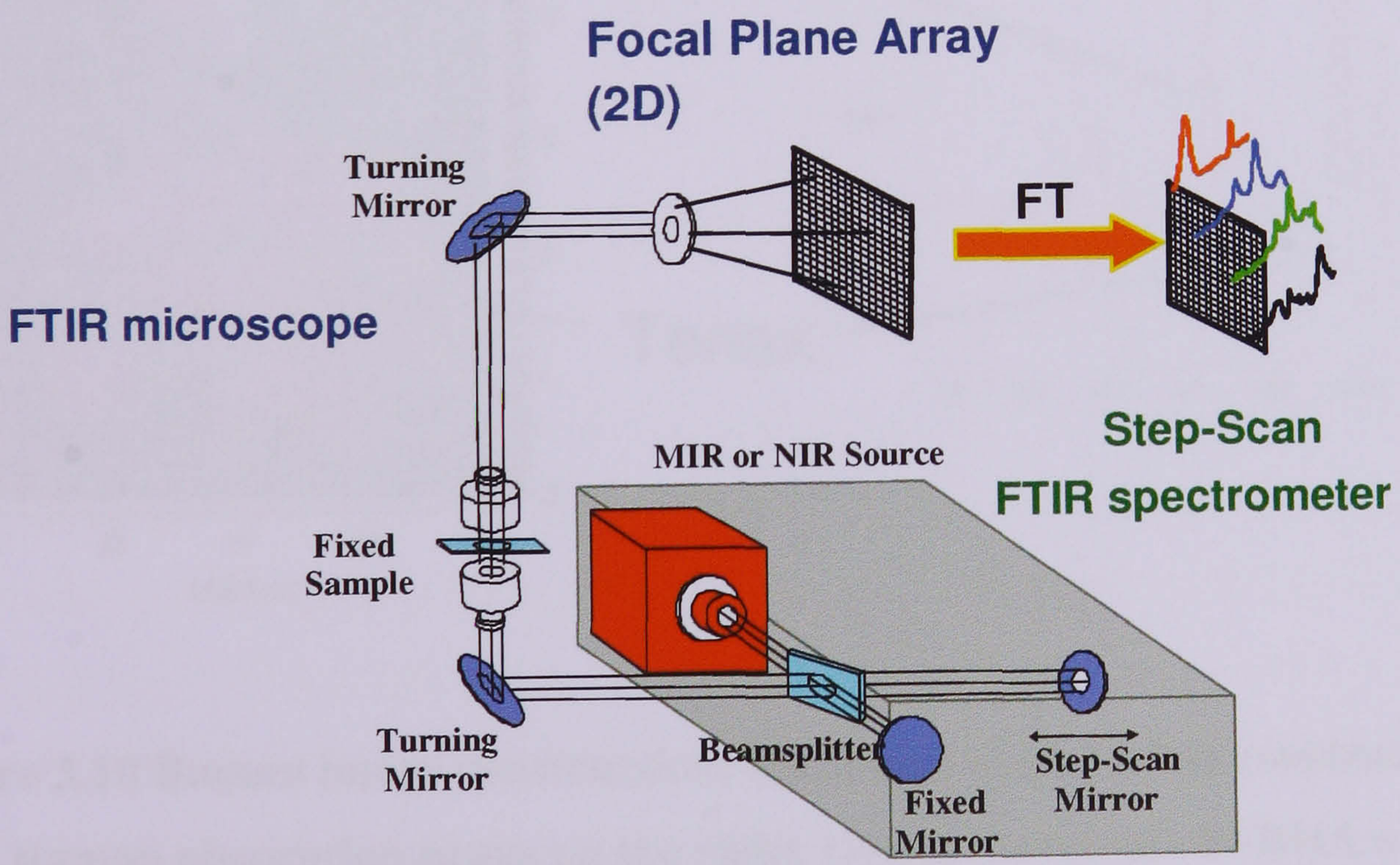


Figure 3.9 FT-IR instrumentation for generating an image (a) sequentially and (b) simultaneously (Everall, 2002).

3.3.4.2 Raman-mapping analysis

Samples of 10% w/w PFBHA coated Tenax were examined initially by Raman microscopy. Figure 3.10 illustrates how the Raman image is constructed for PFBHA coated onto Tenax. The bands of interest are converted into a coloured intensity map (green for PBFHA, pink for Tenax). The image shows that the PFBHA was coated in an inhomogeneous fashion on the Tenax. The depth resolution is a few micrometers. Figure 3.11 illustrates the utility of the chemical image over the white light microscopy image, where no information on the distribution can be discerned due to the poor contrast. The information from the Raman imaging led to a modification of the coating procedure. Low pressure and temperature conditions for removing the solvent were subsequently used as it was suspected that some of the PFBHA was lost through sublimation.

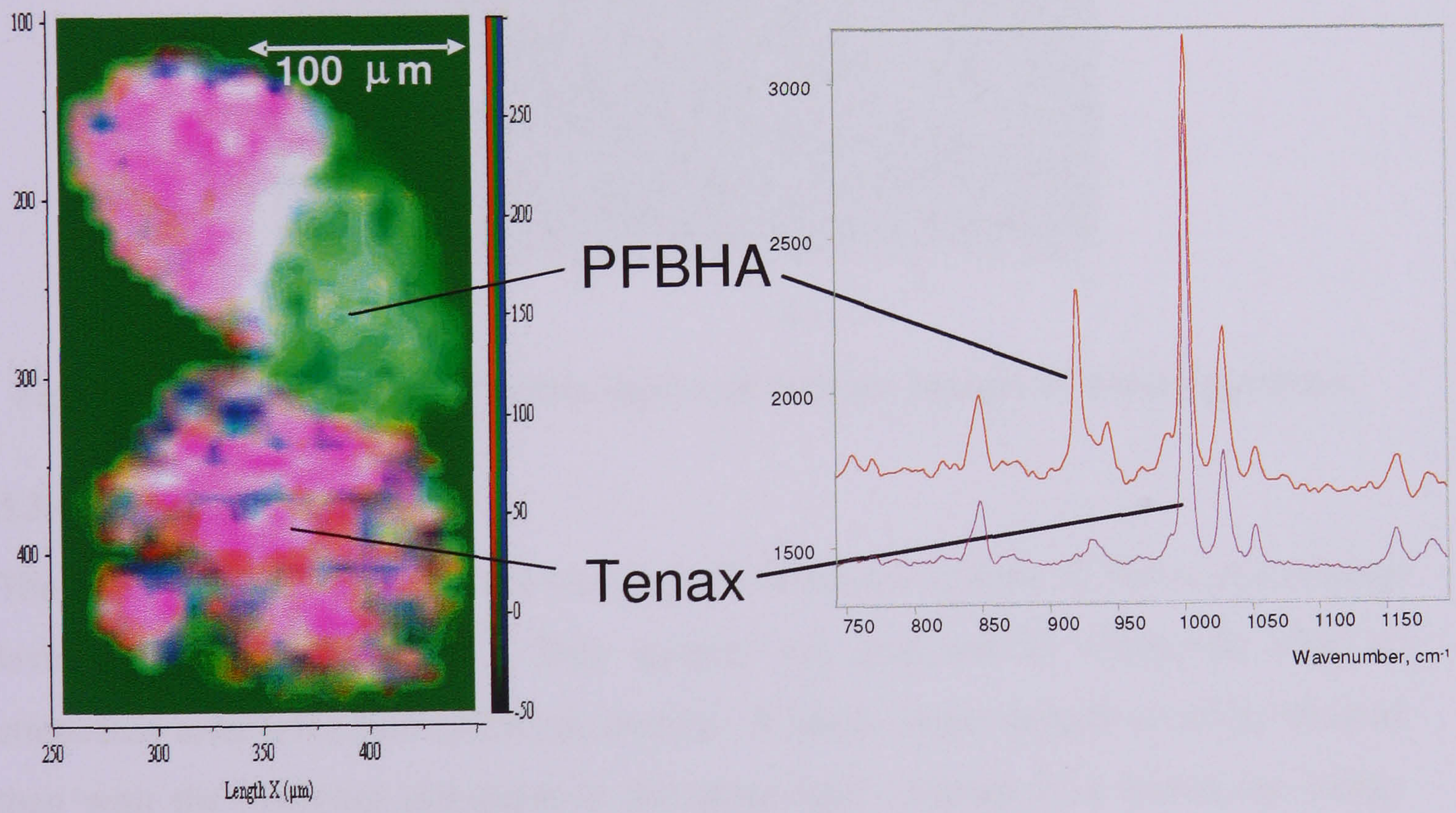


Figure 3.10 Raman image construction: the image on the left is constructed from the Raman absorption peaks on the right. Green represents PFBHA and pink Tenax.

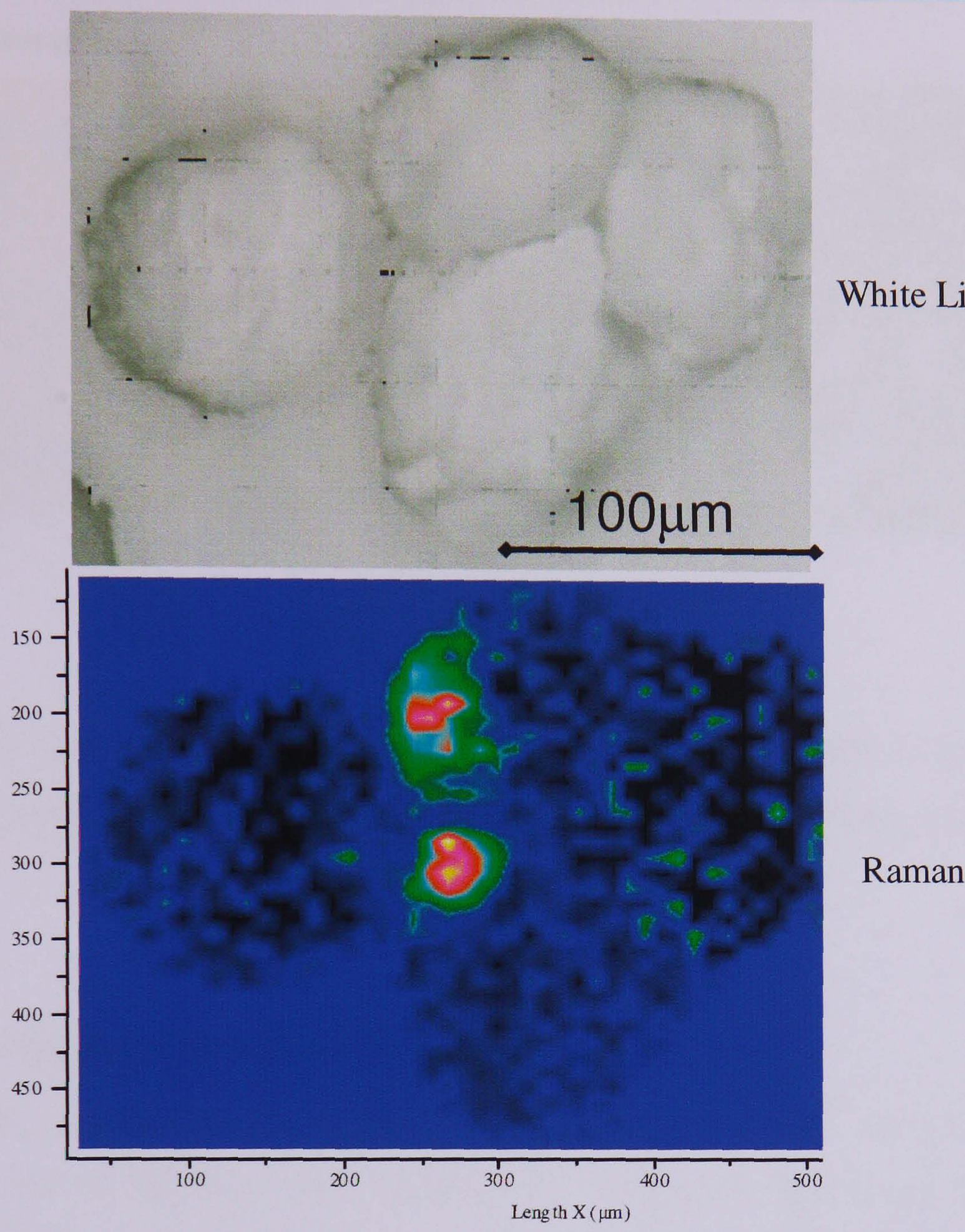


Figure 3.11 Comparison of white light and Raman images of Tenax particles.

3.3.4.3 Global IR imaging

The improved coating procedure was applied to another sample of Tenax at a coating level of 10% PFBHA w/w. This sample was analysed by Global-IR with an attenuated total reflection (ATR) accessory. A much larger sample could be imaged than with the mapping technique in the same time. Figure 3.12 shows the image obtained from Global IR imaging microscope compared with the white light image, The PFBHA-rich areas in the Global IR image are shown in red. The Global IR image indicates that the Tenax is effectively coated with PFBHA. This indicated that the new coating procedure should be used.

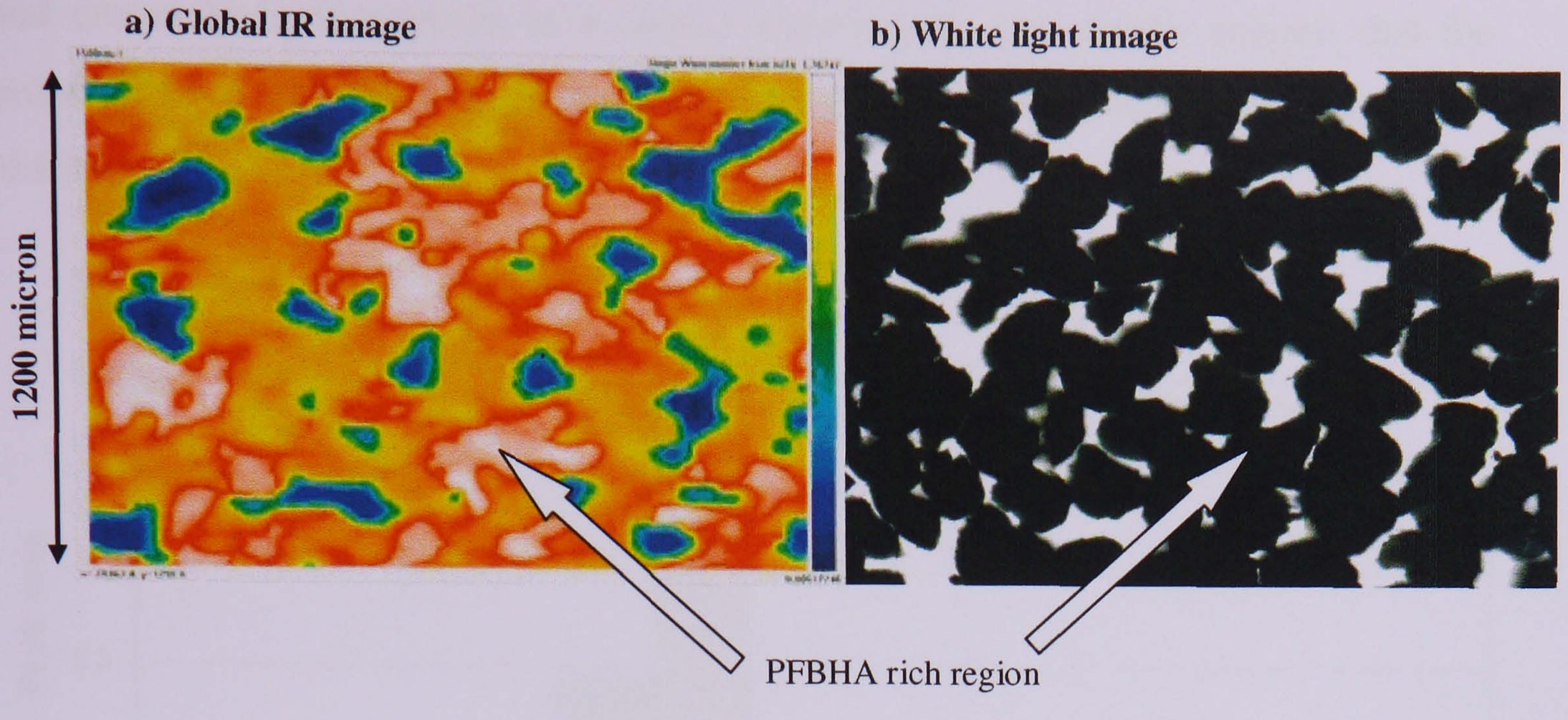


Figure 3.12 (a) Global IR image of Tenax coated with PFBHA (10% w/w coating) compared with (b) white light image of the same sample indicating an even coating of PFBHA on Tenax

3.3.5 Investigation of the effect of humidity

The traps were designed for use in the field where humid climates are likely to be experienced and to be used with living plants which are generating water by transpiration during the headspace trapping. The effects of increased humidity were investigated. The standard trapping headspace experiment was carried out (benzaldehyde was omitted) for 8 hours, in an atmosphere at 80% humidity and in a 'dry' atmosphere (nitrogen gas). Solvent desorbed traps were analysed by GC.

Figure 3.13 compares the amount of aldehyde-PFBO derivative trapped in a dry atmosphere with the humid atmosphere. As with the coating level experiments, the peak areas for the aldehyde derivatives are referenced to the peak areas of the octane internal standard for comparison. The data indicate that on average there is a very slight increase in trap performance at elevated humidity levels. Repeat analyses would be required to confirm this. The reaction between aldehydes and PFBHA generates water; therefore it could be assumed that the presence of water might drive the reaction towards the starting materials. This does not appear to be the case. These results support the work of Wu and Hee (1995) which found no effect of a humidity of 90% on the recoveries of acetaldehyde, acrolein, acetaldehyde, furfural

and crotonaldehyde vapours in a similar experiment The results suggest that the presence of a high humidity atmosphere would not be detrimental to trapping of aldehydes.

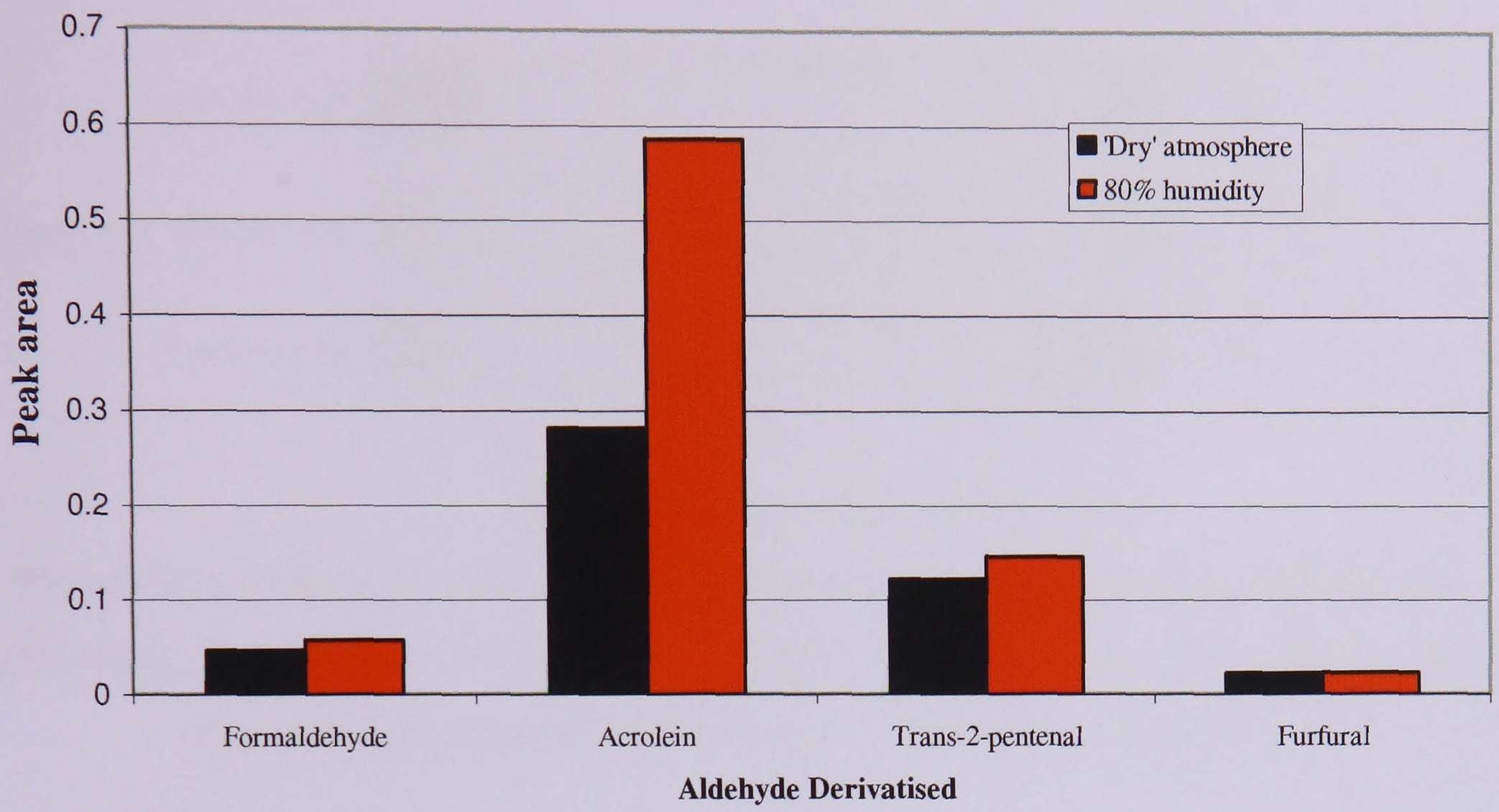


Figure 3.13 Comparison on the effect of humidity on the trapping efficiency of derivatives.

3.3.6 Breakthrough measurement

In a dynamic headspace sampling experiment, breakthrough is said to occur when the sampled species appear in the outlet gas stream, indicating that species are not being retained on the trap and further sampling of the headspace is unproductive. The sorbent-filled trap can be considered as a chromatographic column. The air flow through the tube slowly moves the adsorbed analytes through the sorbent bed as illustrated in Figure 3.14. The breakthrough volume (B_v) of a compound on a specific sorbent is defined as the volume of a vapour in air that can be passed through the sorbent tube before the concentration of the eluting vapour reaches 5% of the applied test concentration.

Breakthrough can occur either because of displacement by another analyte or because of saturation of the adsorbent material. Breakthrough is influenced by a number of factors including, temperature, humidity, the depth of the sorbent bed, and interference from other analytes. It has been shown to be independent of flow rate

(Baya and Siskos, 1996; Harper, 1993; Harper, 2000). Breakthrough studies have been well reviewed by Harper (1993).

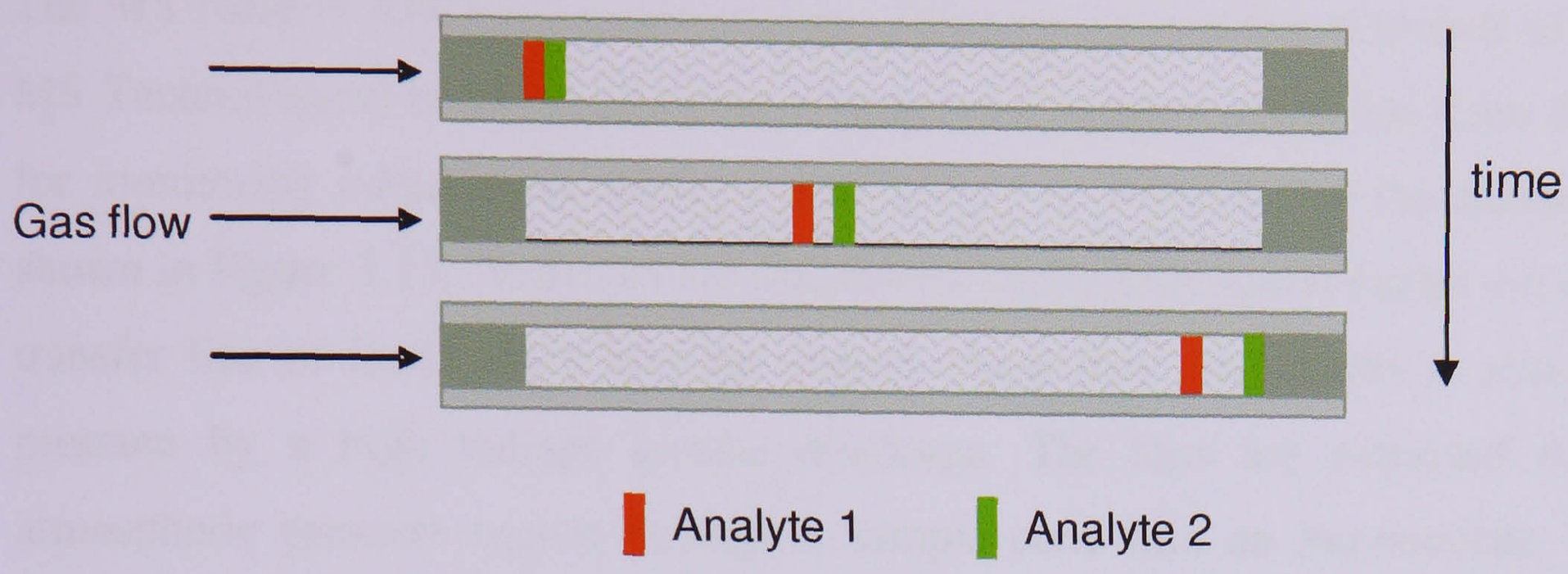


Figure 3.14 Schematic of breakthrough of analytes through a trap filled with adsorbent, which is purged by a gas. Analyte 1 is retained more strongly on the trap and has a higher breakthrough volume than analyte 2.

The simplest method for measuring breakthrough is to treat the trap as a GC column and inject a sample of the analyte as one-shot at the front of the trap. The time for the sample to reach the detector and the flow rate are used to derive the breakthrough volume (Brown and Purnell, 1979). An FID is the usual detector. The ideal method of measuring breakthrough is the one that best matches real sampling conditions, that is, as in a dynamic headspace sampling experiment, where the analytes are continuously added over a period of time. Breakthrough can also be measured by the use of a second trap on the back of the front trap. The back trap can be desorbed and the level of analytes found in the back trap is an indication of whether capacity has been reached (Harper, 1993).

In the experiments described below, breakthrough was measured by on-line real-time measurement with a mass spectrometer as the detector. A single-shot injection was used rather than a continuous gas sample to generate indicative data of the working operation time of the trap. The MS-Nose™ interface was mentioned in Chapter 1 and is described in more detail below. Other researchers have used a different interface, the membrane inlet mass spectrometer (MIMS), to measure breakthrough

of a continuously generated gas sample of chlorinated and non-chlorinated hydrocarbons through a number of sorbents (Peters and Bakkeren, 1994).

3.3.6.1 MS-Nose™ instrumentation

The MS Nose™ APCI-gas phase analyser (built by Micromass, now part of Waters MS Technologies) is an interface for a mass spectrometer which has been designed for monitoring odour components in real time. A schematic of the instrument is shown in Figure 3.15. Volatiles are transferred to the mass spectrometer via a heated transfer line of fused silica into the source where they are ionised at atmospheric pressure by a high voltage corona discharge. The ions are extracted from the atmospheric pressure region through a sample cone into an intermediate vacuum region. From there, they are transported into the analyser region of the mass spectrometer where they are separated on the basis of their mass-to-charge ratio.

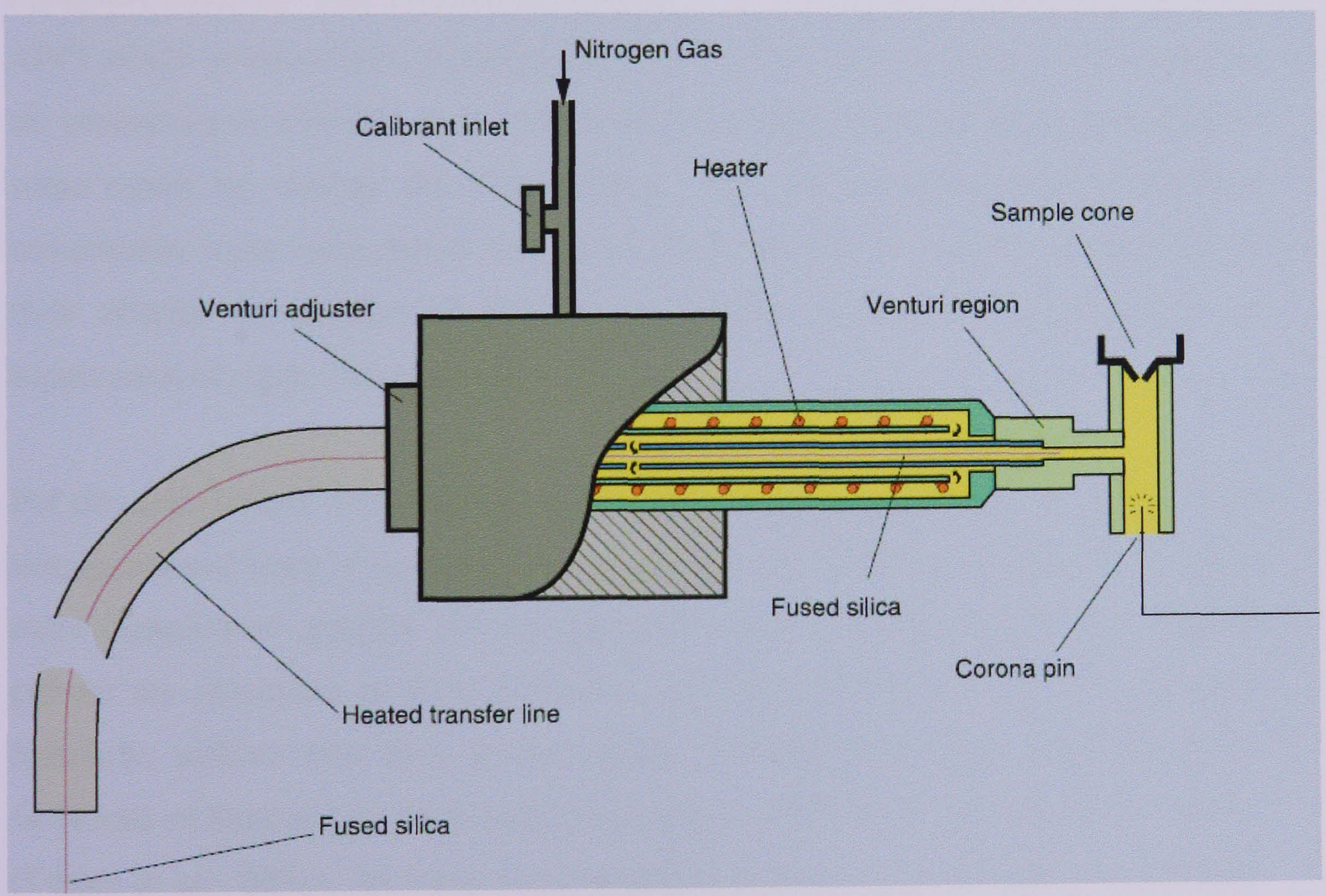


Figure 3.15 Schematic of MS-Nose™ interface (Linforth and Preece, 1998).

This technique has been applied to several studies of the release of odour components from food, both in model release systems and *in vivo*. For example,

monitoring volatiles in the breath (Hodgson *et al.*, 2004), monitoring the flavour release during consumption from beverages (Hodgson *et al.*, 2005; King *et al.*, 2006) and from French fries (van Loon *et al.*, 2006). The system has been shown to be capable of detecting volatiles in the 10-100 ppb range (Taylor *et al.*, 2000). For studies involving the analysis of volatiles from human breath, the speed of data collection needs to be sufficient to see details of volatile release on a breath by breath basis (approximately every 5 seconds). For further discussion of the parameters and principles of this interface, particularly for in vivo analysis see Taylor *et al.* (2000).

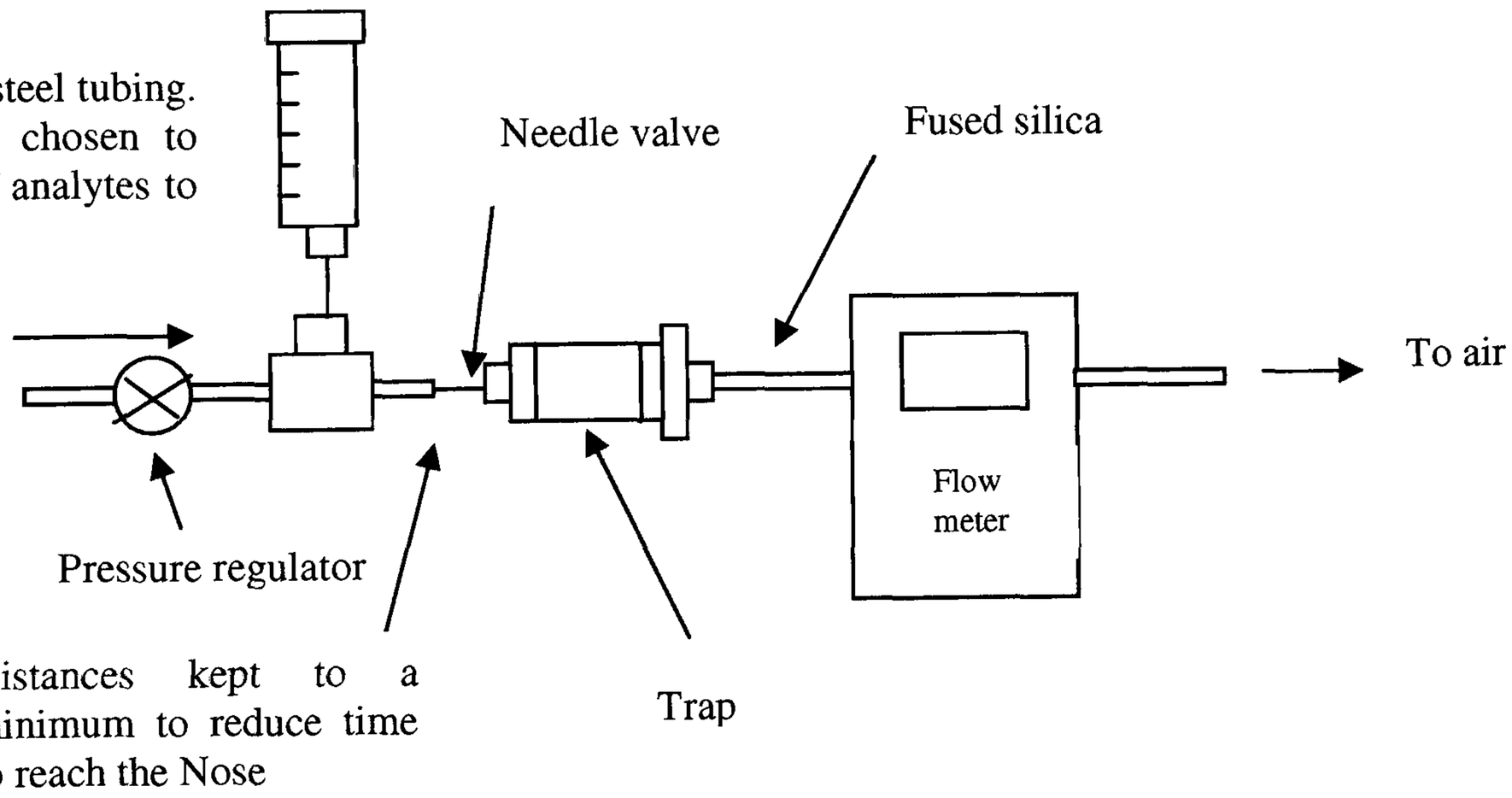
One limitation of the MS-Nose is that it is not ideal for the analysis of complex mixtures because many compounds are introduced simultaneously into the interface, making it difficult to assign ions to compounds unequivocally. For this reason, it is necessary to minimise the amount of fragmentation otherwise the spectra are extremely complex and difficult to interpret. 'Soft' ionisation techniques such as APCI which produce little fragmentation are ideal for this purpose. APCI leads to the production of $[M+H]^+$ ions. The amount of fragmentation can be controlled to some extent by varying the cone voltage. For samples containing mixtures of compounds, some components may ionise preferentially over others (depending on their affinity for protons (Sunner *et al.*, 1988)) leading to the possibility of suppression of signal for some analytes.

For the experiments described below for monitoring breakthrough, the mixture is a simple one and some of the concerns that authors who used this technique to analyse more complicated samples are not applicable. For example, if the only volatiles present are aldehydes or their derivatives, preferential ionisation is not an issue. Similarly, authors who have studied breath volatiles have been concerned about variations of humidity in the breath which could affect the ionisation characteristics (Taylor *et al.*, 2000). In these experiments, headspaces of aldehydes or aldehyde-PFBO derivatives were generated at consistent humidity. The mass analyser was a TOF (LCT instrument).

3.3.6.2 Experiment set-up

Figure 3.16 shows the general set-up of the equipment for measuring analyte breakthrough through a trap as a function of flow rate. A positive flow of nitrogen was maintained through the trap, controlled by a pressure regulator. A flow meter was used to measure the flow through the trap (**a**). The flow meter was removed during measurement (**b**) and the trap was attached to the MS-Nose capillary by insertion of the fused silica through a septum. The sample, a known concentration of aldehyde, was delivered to the system through a syringe inserted into a septum. The time of injection is noted and the species evolved from the trap are detected by the mass spectrometer.

(a) Flow monitoring mode



(b) Sampling mode

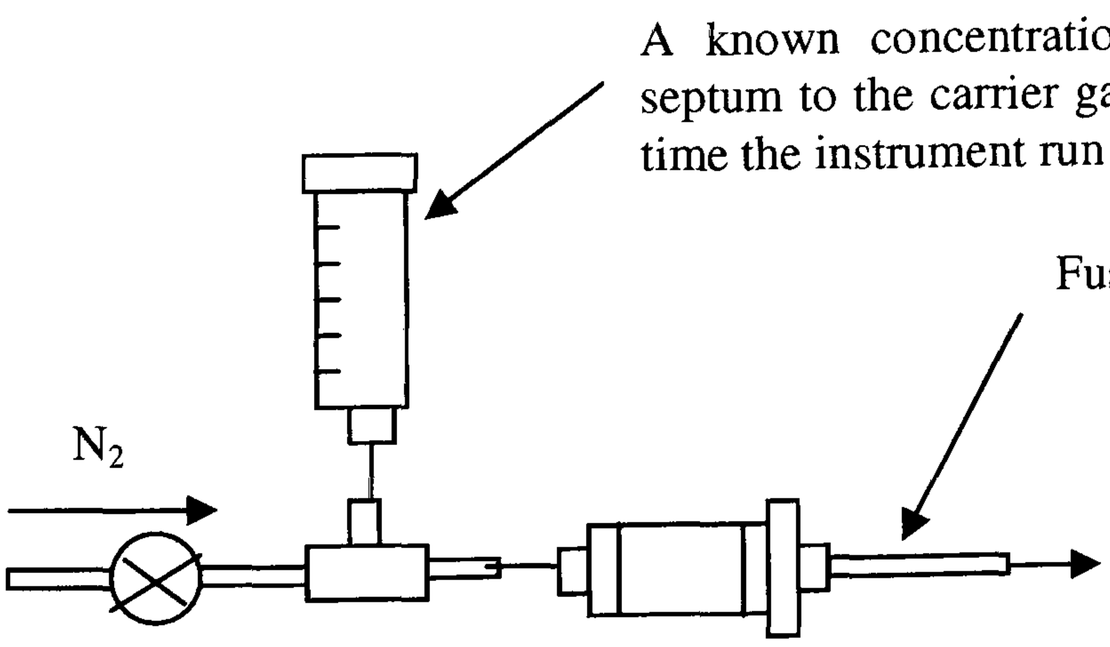


Figure 3.16 Equipment set-up used to test analyte breakthrough. Once the flow rate against the load of the trap is measured with the flow meter (a), the meter is removed and the trap attached to the MS-Nose (b).

A series of experiments were completed.

1. The response of the MS-Nose to aldehydes and aldehyde-PFBO derivatives was investigated to verify that the species ionised and to check that the protonated molecule was detectable by the TOF analyser.
2. The breakthrough behaviour of aldehydes through uncoated Tenax traps was investigated at a flow rate of 20 or 100 $mL.min^{-1}$ and injection volumes of 1 and 50 mL. This was to verify if aldehydes are indeed poorly retained on Tenax.
3. The breakthrough behaviour of aldehydes and aldehyde-PFBO derivatives through PFBHA-coated Tenax traps was investigated at the 20 and 100 $mL.min^{-1}$ and at sample volumes of 1 and 50 mL.

3.3.6.3 Response of MS-Nose to aldehydes and aldehydes derivatives

The response of the MS-Nose to the analytes was first tested by injecting a sample of approximately 2 ppm of each aldehyde vapour through a trap containing no adsorbent. Figure 3.17 shows the MS-Nose trace for the protonated molecules of each aldehyde with time. After injection, there is an intense peak followed by decay of the response.

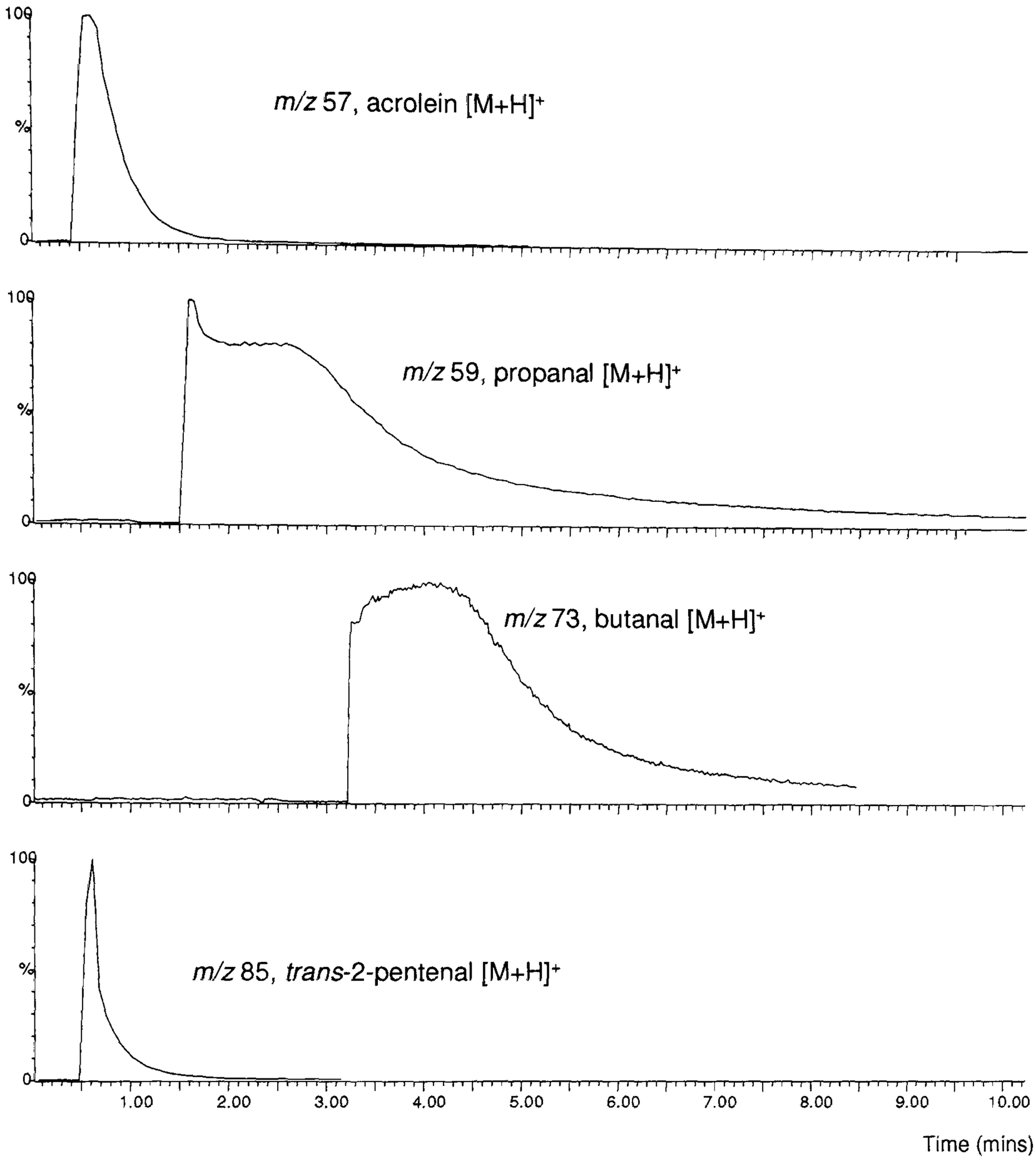


Figure 3.17 MS-Nose trace of protonated molecules for each aldehyde

The mass spectrum for *trans*-2-pentenal acquired at a retention time with an intense signal is shown in Figure 3.18 The most intense peak is due to the $[M+H]^+$ ion at *m/z* 85. Some fragmentation is observed generating peaks at *m/z* 67 and *m/z* 57 due to the loss of water and carbon monoxide respectively from the protonated molecule. Acrolein, propanal and butanal all showed similar mass spectra with the same characteristic ions. Formaldehyde, with a molecular weight of 30, was not detected as the sensitivity of the TOF analyser utilised in these experiments towards low molecular weight ions is low. These experiments indicated that this technique would be suitable for detecting breakthrough of aldehydes by monitoring the $[M+H]^+$ ion.

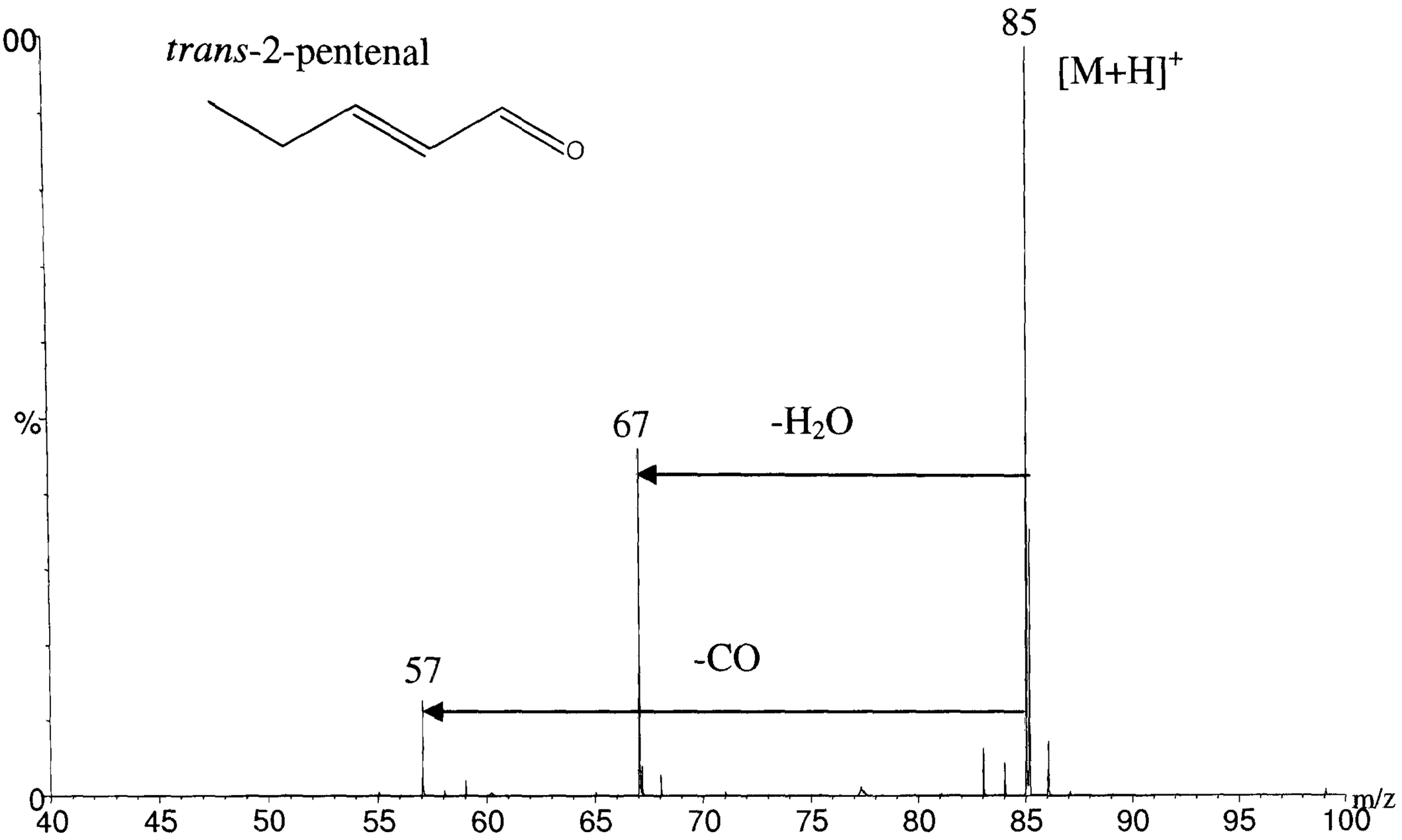


Figure 3.18 Mass spectrum (APCI +ve) indicating the detection of *trans*-2-pentenal by the MS-Nose

Standards of each of the oxime derivatives were synthesised and APCI mass spectra obtained to verify whether the $[M+H]^+$ ion would also be suitable for monitoring breakthrough of these oxime derivatives. Standards were not volatile enough to generate a concentrated enough headspace to be injected through the trap, so standards were analysed by APCI-MS under the same conditions of the MS-Nose. Figure 3.19 shows a typical spectrum for *trans*-2-pentenal-PFBO showing a high abundance $[M+H]^+$ ion. Under these ionisation conditions there was little fragmentation to $[C_7H_2F_5]^+$ at *m/z* 181.

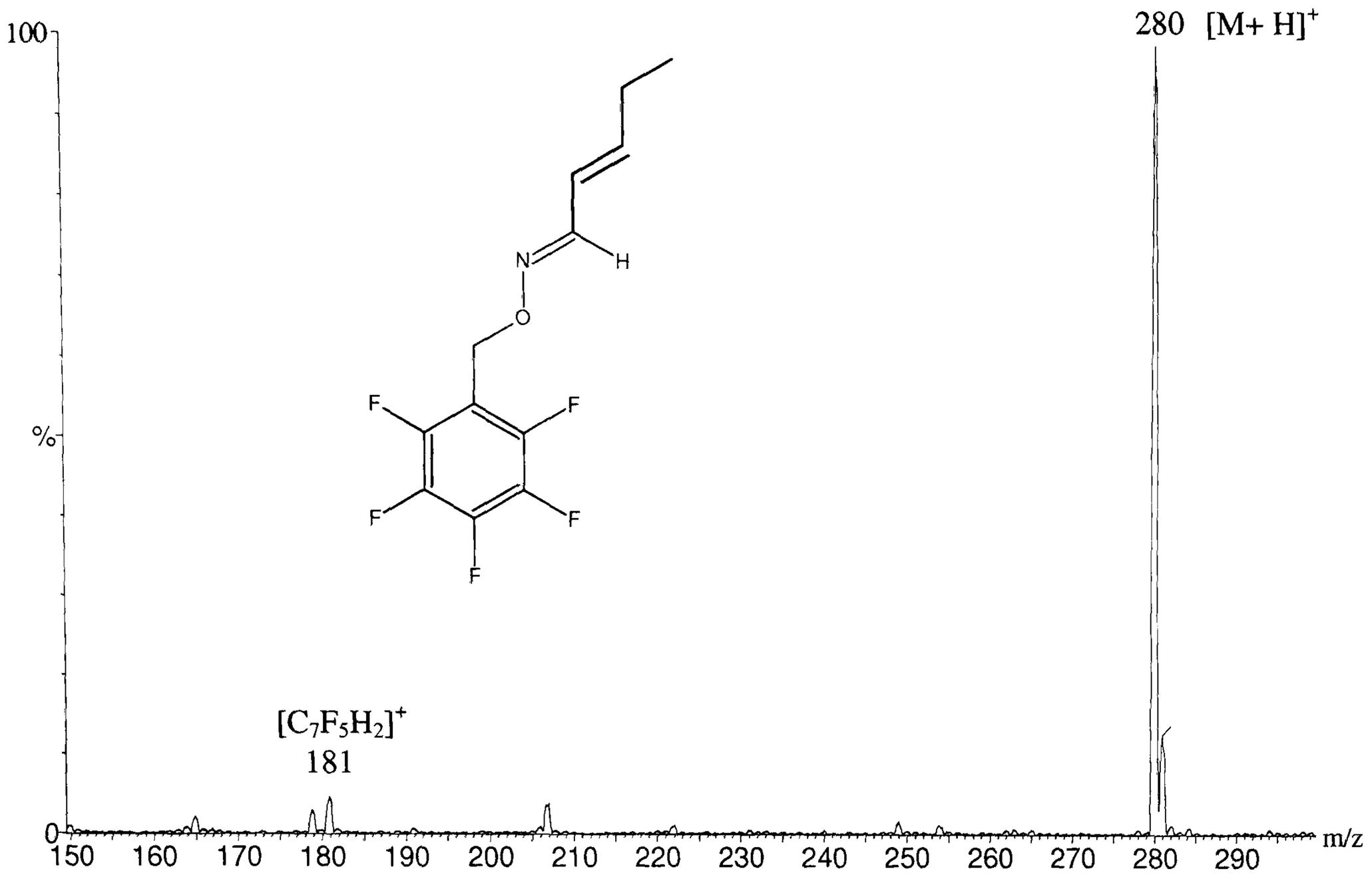


Figure 3.19 Mass spectrum (APCI +ve) for *trans*-2-pentenal-PFBO

Similar spectra exhibiting $[M+H]^+$ are observed for each derivative indicating firstly, that the species ionise and secondly, that the $[M+H]^+$ ion can be used to monitor the presence of the species. Note that *m/z* 181 could not be used to monitor breakthrough because this could also be from PFBHA.

3.3.6.4 Breakthrough behaviour of aldehydes through an uncoated trap

Acrolein, butanal and propanal and *trans*-2-pentenal were injected in turn through a Tenax trap with a flow rate of 100 $mL.min^{-1}$ or 20 $mL.min^{-1}$ and sample volume of 1 mL or 50 mL (concentration of approximately 70 mg/L).

Figure 3.20 shows data acquired at 100 $mL.min^{-1}$ with a 1 mL sample. This can be compared with a sample at 20 $mL.min^{-1}$ with the same size sample (Figure 3.21). Figure 3.22 shows data acquired at 100 $mL.min^{-1}$ with a 50 mL sample. Figure 3.23 shows the data acquired at 20 $mL.min^{-1}$ with the same size sample.

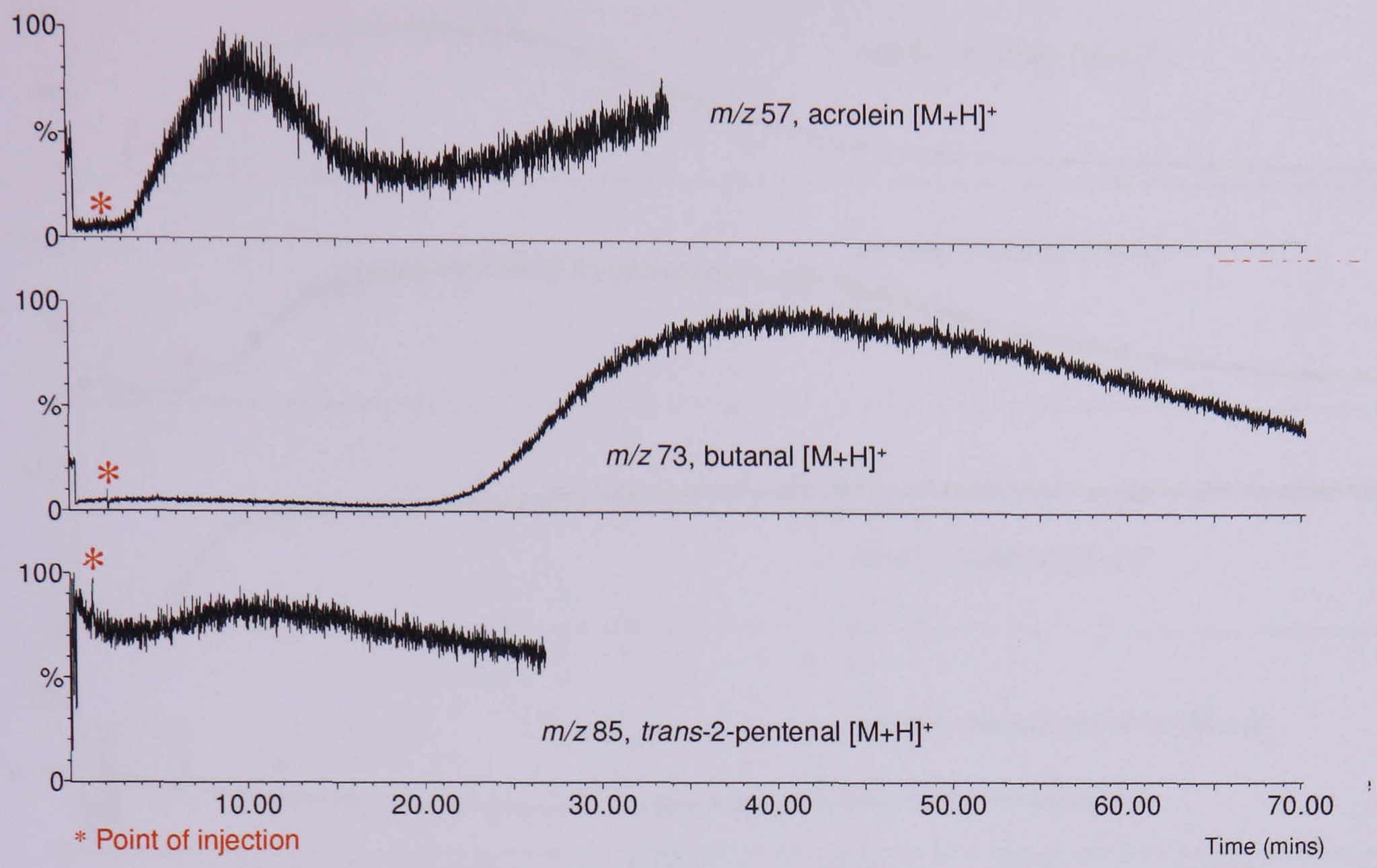
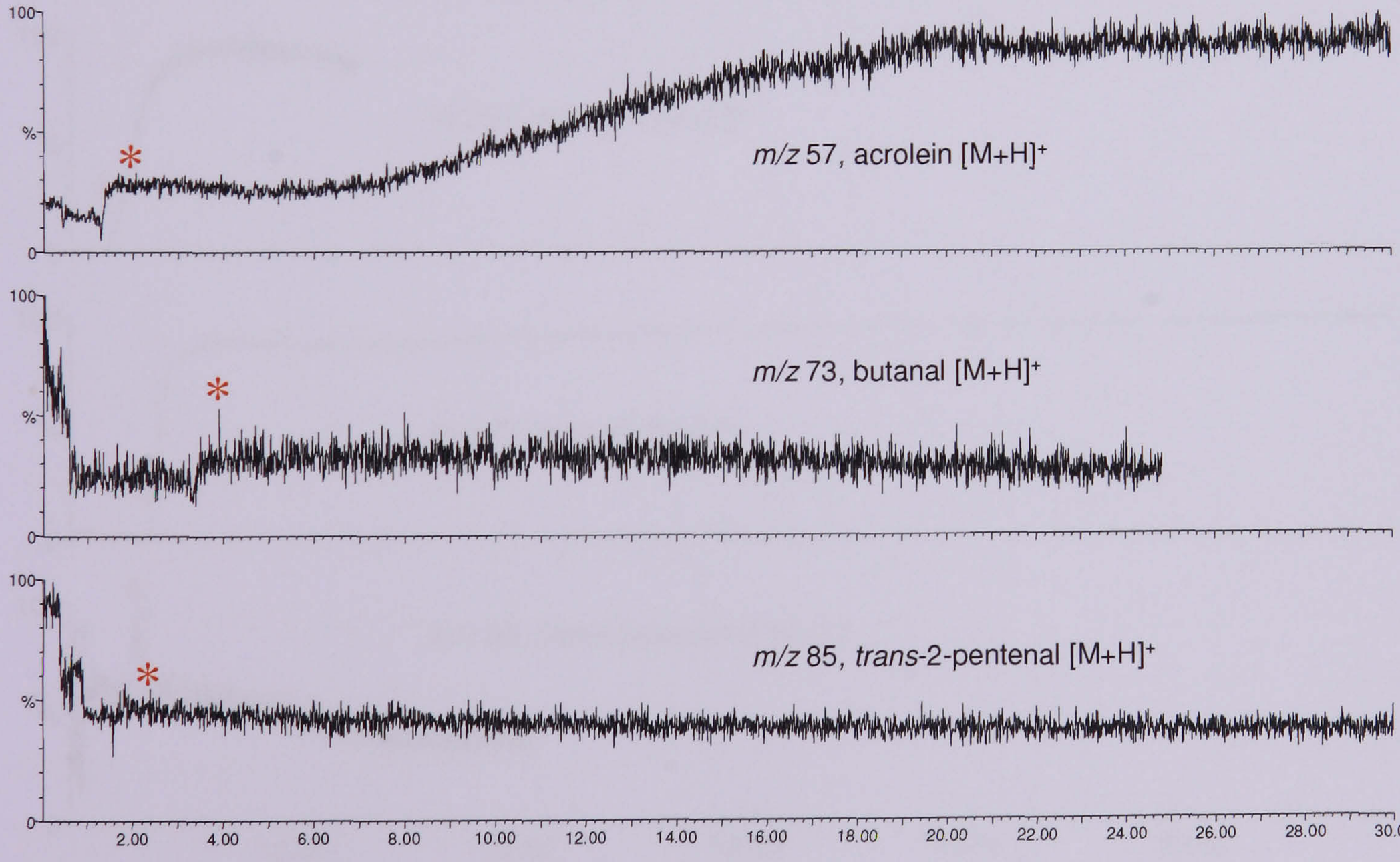


Figure 3.20 MS-Nose traces for the protonated molecule of each aldehyde monitored at a flow rate 100 mL.min^{-1}, injection of 1 mL, uncoated trap



* Point of injection

Figure 3.21 MS-Nose traces for the protonated molecule of each aldehyde monitored at a flow rate 20 mL.min^{-1}, injection of 1 mL, uncoated trap

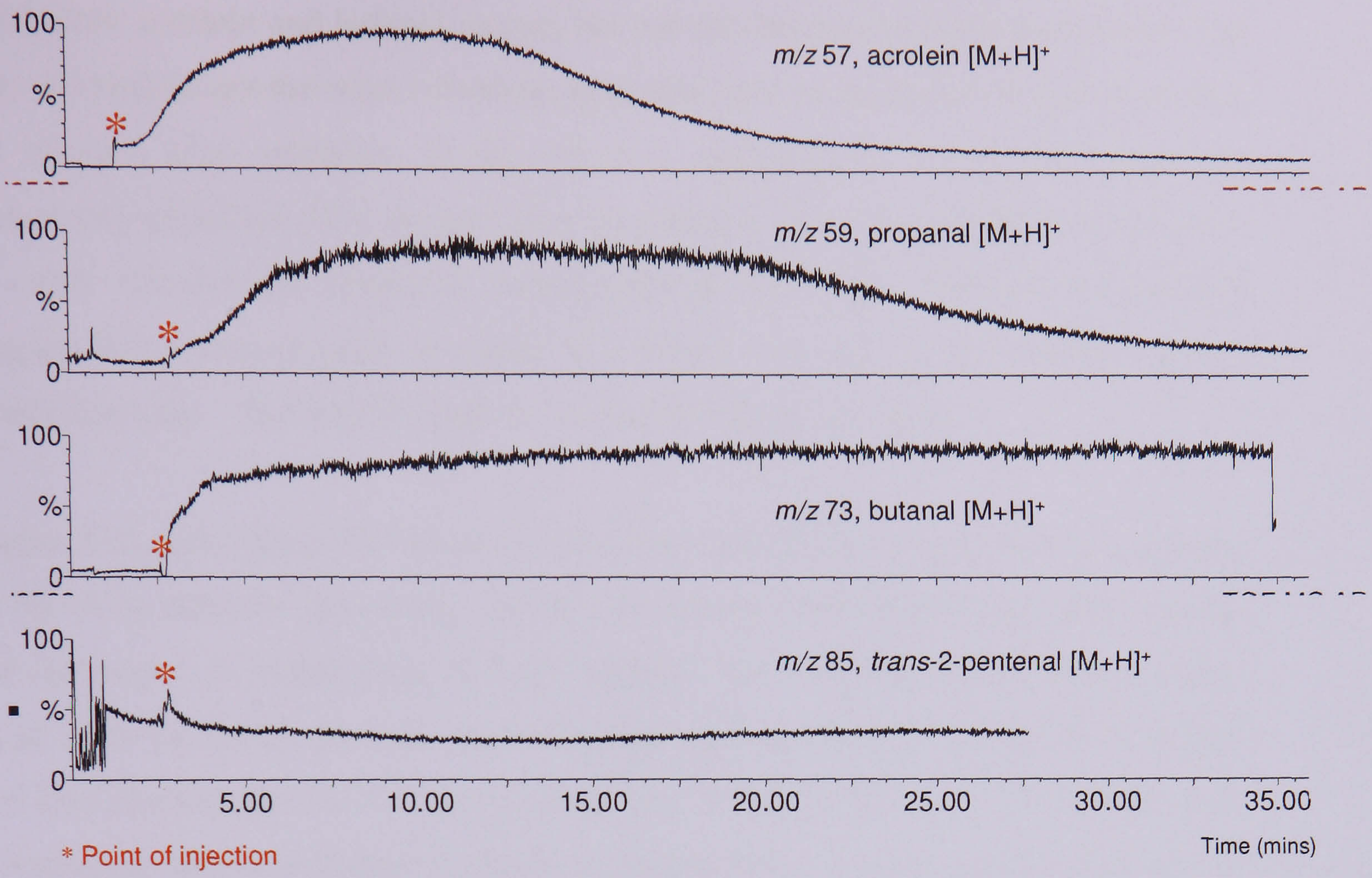


Figure 3.22 MS-Nose traces for the protonated molecule of each aldehyde monitored at a flow rate 100 mL.min^{-1}, injection of 50 mL, uncoated trap

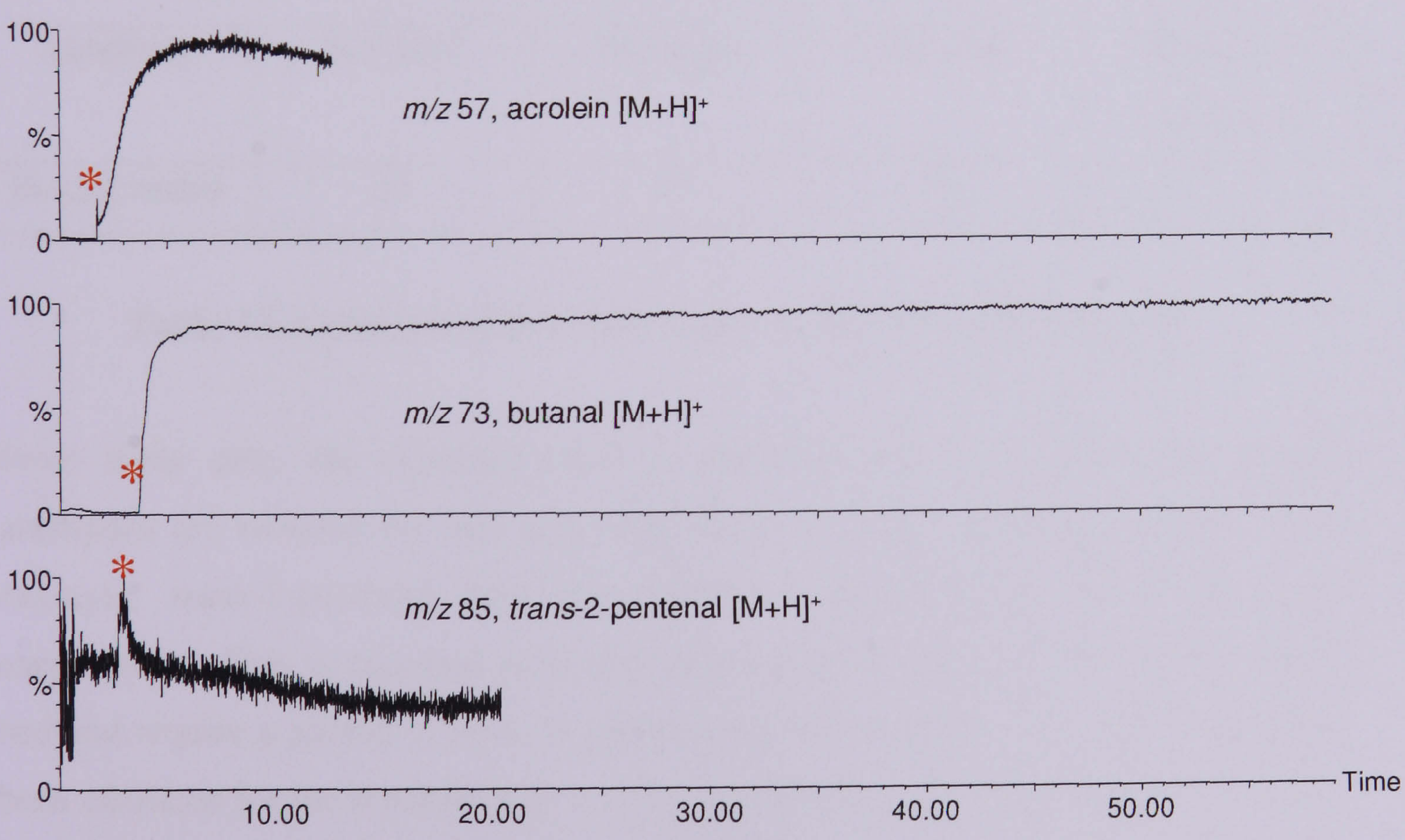


Figure 3.23 MS-Nose traces for the protonated molecule of each aldehyde monitored at a flow rate 20 mL.min^{-1}, injection of 50 mL, uncoated trap

The data in Figures 3.21 and 3.22 show that some breakthrough of the lighter aldehydes, acrolein and butanal, occurs but not for the heavier *trans*-2-pentenal. The data for butanal are the most complete, indicating that breakthrough begins at around 20 minutes after injection. If the curve is extrapolated, all of the butanal is completely expunged from the trap after 90 minutes. There are insufficient data here to verify whether the volume is independent of flow rate. The data for acrolein suggest that a sharper peak is visible at a higher flow rate and a broader one at a lower flow rate. The area beneath the curves should be the same.

Figure 3.22 and Figure 3.23 show the MS-Nose traces for a larger injection volume of 50 mL at different flow rates. As may be expected for a higher injection volume, the ion counts are higher than the 1 mL sample. They are roughly fifty-times higher in all cases except for butanal. Breakthrough is again observed for acrolein, butanal and also propanal but not for *trans*-2-pentenal. Rough estimates of the breakthrough volumes are shown in Table 4.6, based on extrapolation of data at a flow rate of 100 $mL.min^{-1}$.

Aldehyde	Acrolein	Propanal	Butanal	*Trans*-2-pentenal
B_V (l/g resin)	25	35	90	-

Table 3.6 Estimates of breakthrough volumes for each aldehyde

From these data, the expected trend is observed that lower molecular weight aldehydes are retained for less time than those of higher molecular weight. For example, *trans*-2-pentenal does not appear to breakthrough at all in these experiments. This is expected as heavier aldehydes are retained more on the Tenax bed and require a greater volume of gas to purge analytes from the trap. Data have been tabulated for the breakthrough volume (expressed per gram of resin) for a series of aldehydes on Tenax (Scientific Instrument Services). Data were acquired at 20°C on a glass lined stainless steel tube by injecting a small volume of sample on the front of the trap and detecting with a FID. The breakthrough volumes were as follows: propanal (5 $L.g^{-1}$ resin), butanal (30 $L.g^{-1}$ resin) and pentenal (112 $L.g^{-1}$

resin). Direct comparisons between the two techniques are not possible mainly because very different dimension traps were used (1/4" OD x 4.0 mm ID x 100 mm long for the Scientific Instrument Services data).

3.3.6.5 Breakthrough behaviour through an PFBHA-coated trap

The behaviour of the aldehydes through a PFBHA coated-Tenax trap was monitored using a trap of 100 mg Tenax with a 10% coating of PFBHA. The aldehydes were injected in turn through the trap at a concentration of 70 ppm. The flow rate was either 100 mL.min^{-1} or 20 mL.min^{-1} and the injection volume either 50 mL or 1 mL. In each case, the protonated molecule was monitored as a function of time.

The data at a flow rate of 100 mL.min^{-1} are shown in Figure 3.24 and Figure 3.25 shows the data at 20 mL.min^{-1} flow rate. In both cases a 1 mL injection was used. The green traces represent the protonated molecule of the aldehyde-PFBO derivative and red, the aldehydes. The traces for the aldehyde-PFBO derivatives are virtually flat in each case and the ion count level is very low (100's level). This is true for both flow rates. This is taken to indicate that there is little breakthrough of the derivative. The behaviour of the aldehydes through the coated trap is very different from that through the uncoated Tenax. For acrolein there is a very sudden decay curve with a return to the base line within 2 minutes at 100 mL.min^{-1}. Propanal has a blip similar to that of acrolein but the ion count is much lower. The traces for butanal and trans-2-pentenal are essentially noise. A similar pattern is observed at 20 mL.min^{-1} but with lower ion counts. This is taken to indicate that rather than breakthrough, the aldehydes are reacting with the derivatising reagent.

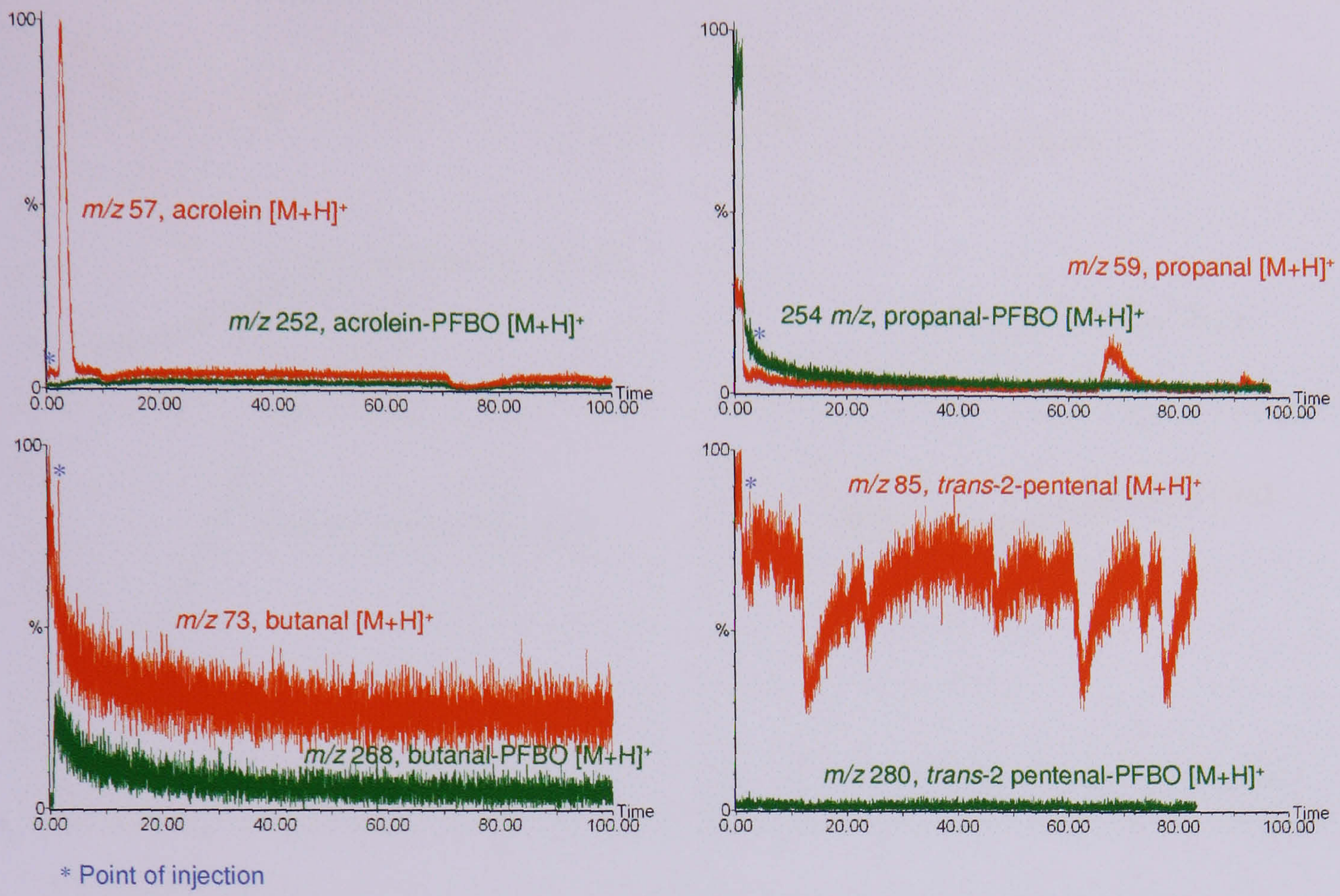


Figure 3.24 MS Nose traces for aldehyde and aldehyde-PFBO derivatives through a PFBHA-coated trap at a flow rate of 100 mL.min^{-1}, with 1 mL sample, monitoring $[M+H]^+$.

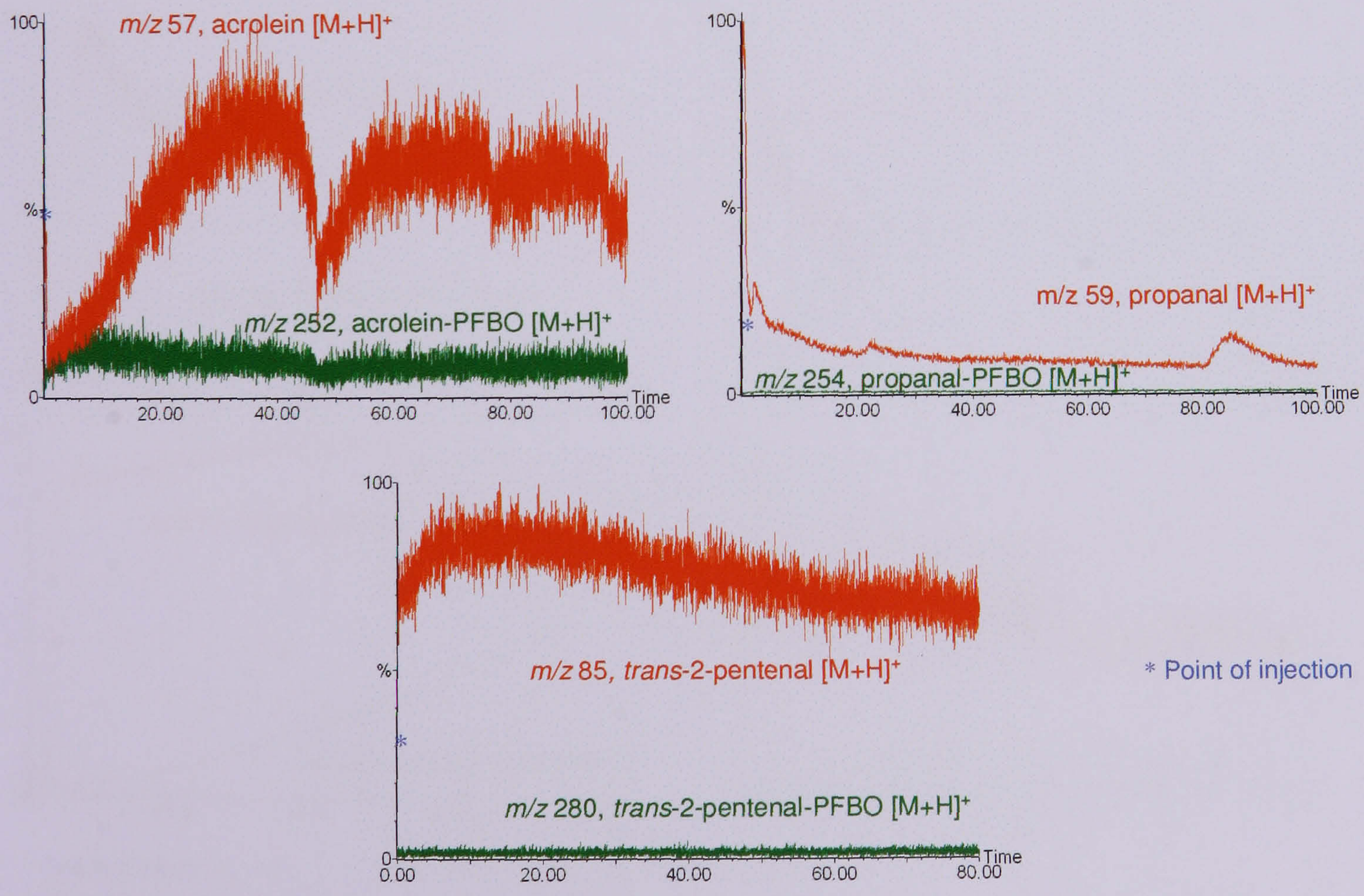


Figure 3.25 MS Nose traces for aldehyde and aldehyde-PFBO derivatives through a PFBHA-coated trap at a flow rate of 20 mL.min^{-1}, with 1 mL sample, monitoring $[M+H]^+$.

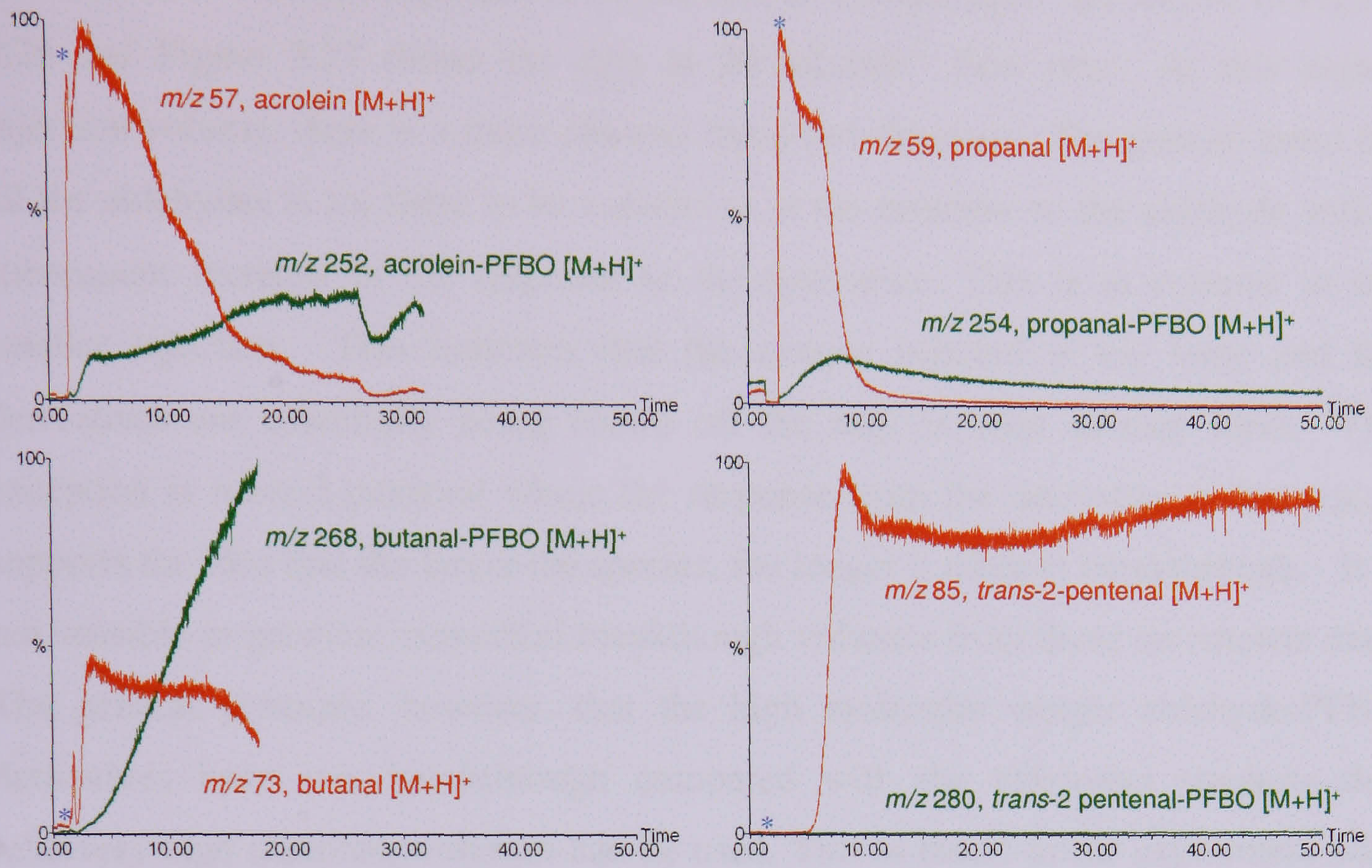


Figure 3.26 MS Nose traces for aldehyde and aldehyde-PFBO derivatives through a PFBHA-coated trap at a flow rate of 100 mL.min-[1], with 50 mL sample monitoring $[M+H]^+$.

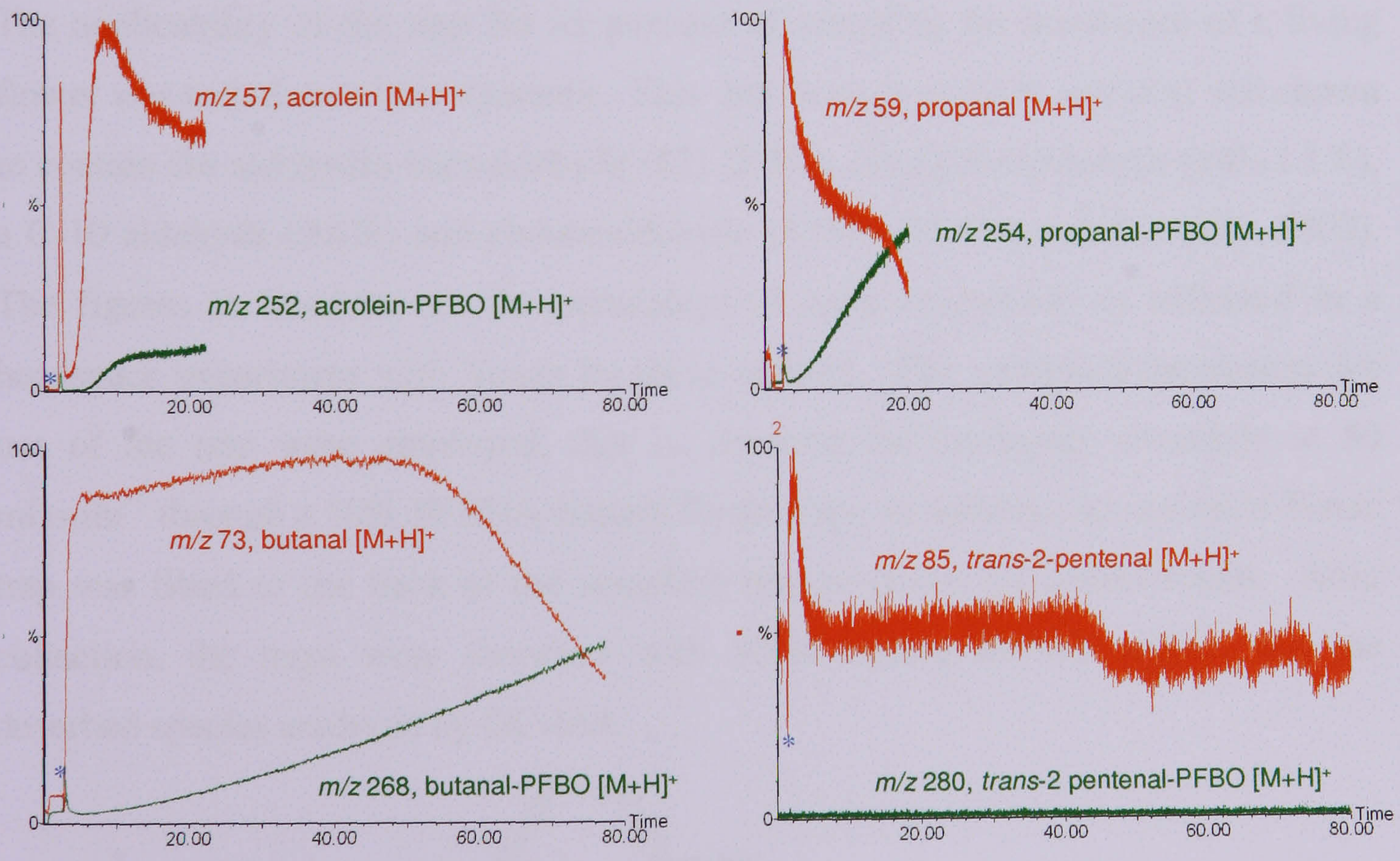
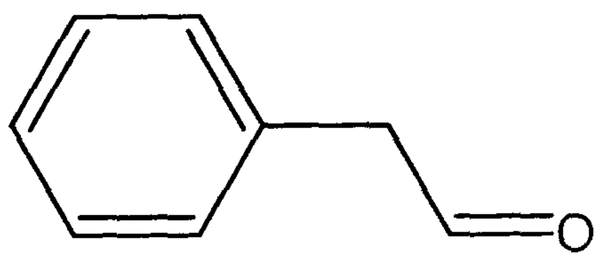


Figure 3.27 MS Nose traces for aldehyde and aldehyde-PFBO derivatives through a PFBHA-coated trap at a flow rate of 20 mL.min^{-1}, with 50 mL sample monitoring $[M+H]^+$.

The data with a 50 mL injection at a flow rate of 100 mL.min^{-1} are shown in Figure 3.26 and Figure 3.27 shows the data at 20 mL.min^{-1} flow rate. At this higher injection volume, there is a more obvious trend in behaviour. The general trend for all the aldehydes is for there to be a decrease in the response to the aldehyde with a subsequent increase in the response to the derivative. This is in contrast to the smaller injection. This indicates that the sample injected is too large and the derivatives are essentially being blown off the trap as soon as they form. The exception is *trans*-2-pentenal where the response from the derivative is flat which supports the idea that the larger the species, the longer it takes to breakthrough. It is not sensible to generate numerical breakthough volumes from these incomplete data. The general principle, however, that the high molecular weight aldehyde-PFBO derivatives have low breakthrough compared with the aldehydes suggests that relatively high sampling volumes can be used. This is tested in the experiment with blue hycanith (described in Section 3.4) where 24 litres of the headspace were sampled and a back trap was fitted to detect any aldehyde-PFBO derivatives.

3.4 Application to blue hyacinth headspace

The applicability of the trap for its purpose of sampling the headspace of a living flower was tested on a blue hyacinth. This has been previously analysed and shown to contain the aldehydes benzaldehyde (**11**) (2.3%), phenylacetaldehyde (**14**) (1.1%), a C-10 aldehyde (0.6%) and cinnamaldehyde (4.6%) (McGee and Purzycki, 2002). The figures in brackets are the percentage of each component as collected in a headspace experiment with Tenax by these authors. The optimised parameters for use of the trap were employed, that is, drawing the headspace overnight at 50 mL.min^{-1} through a 10% PFBHA coated-Tenax trap. In addition, an uncoated Tenax trap was fitted to the back of the sampling trap to check for breakthrough. After collection, the traps were desorbed with 80:20 hexane/acetone solvent and the desorbed species analysed by GC-MS.



(**14**)

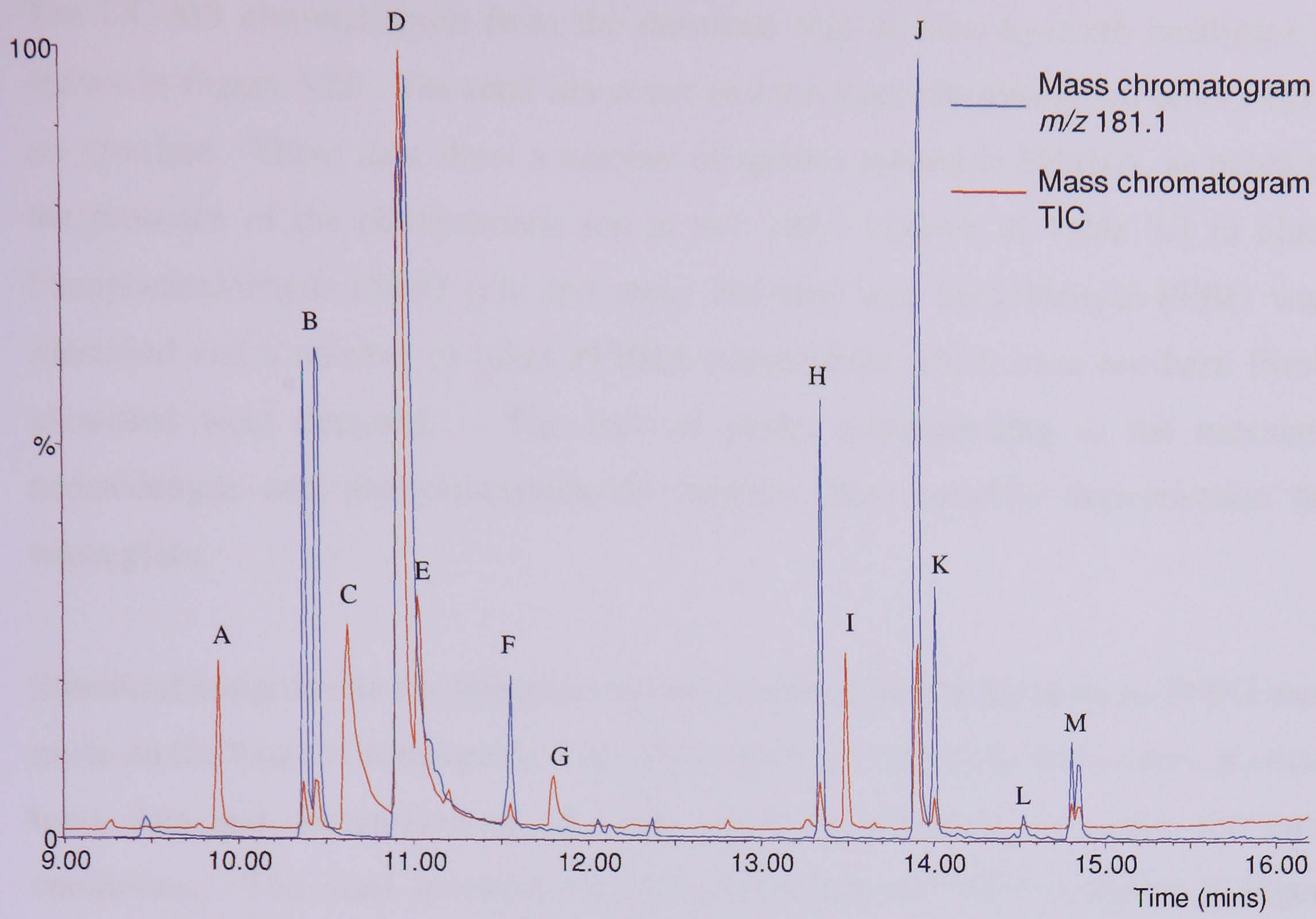


Figure 3.28 GC-MS chromatogram from blue hyacinth headspace

Annotation	Assignment
A	*trans*-Ocimene
B	Acetaldehye-PFBO (*cis* and *trans* isomers, 239 Da mol wt)
C	Phenylethanol
D	Acetone-PFBO (253 Da mol wt)
E	Benzyl acetate
F	methyl-2-butanone-PFBO (267 Da mol wt)
G	Phenylethyl acetate
H	PFBHA related (probably 391 Da mol wt)
I	Possibly alpha-Farnesene (probably 205 Da mol wt)
J	PFBHA related (probably 391 Da mol wt)
K	PFBHA related (probably 393 Da mol wt)
L	Benzaldehyde-PFBO (301 Da mol wt)
M	Phenylacetaldehyde-PFBO (*cis* and *trans* isomers - 315 Da mol wt)

Table 3.7 Assignment for peaks in GC-MS chromatogram of blue hyacinth headspace (Figure 3.28).

The GC-MS chromatogram from the desorbed trap of blue hyacinth headspace is shown in Figure 3.28. The total ion count and the mass chromatogram of *m/z* 181.1 are overlaid. These data show a number of species related to PFBHA, as noted by the presence of the characteristic ion at *m/z* 181.1 (shown in Table 3.7 in blue). Phenylacetaldehyde-PFBO (*cis* and *trans* isomers) and benzaldehyde-PFBO were identified and a number of other PFBHA-components which have not been firmly identified were detected. The lack of peaks corresponding to the unreacted benzaldehyde and phenylacetaldehyde suggests that complete derivatisation has taken place.

Structural assignments for phenylacetaldehyde-PFBO and benzaldehyde-PFBO were made on the basis of comparison with library structure of oxime derivatives, accurate mass data and comparison of retention times for standards run under the same conditions. The mass spectrum for phenylacetaldehyde-PFBO is shown in Figure 3.29 for illustration. The distinctive fragment at *m/z* 181 is dominant. There is also a peak at *m/z* 91 which is due to the tropylium ion from phenylacetaldehyde.

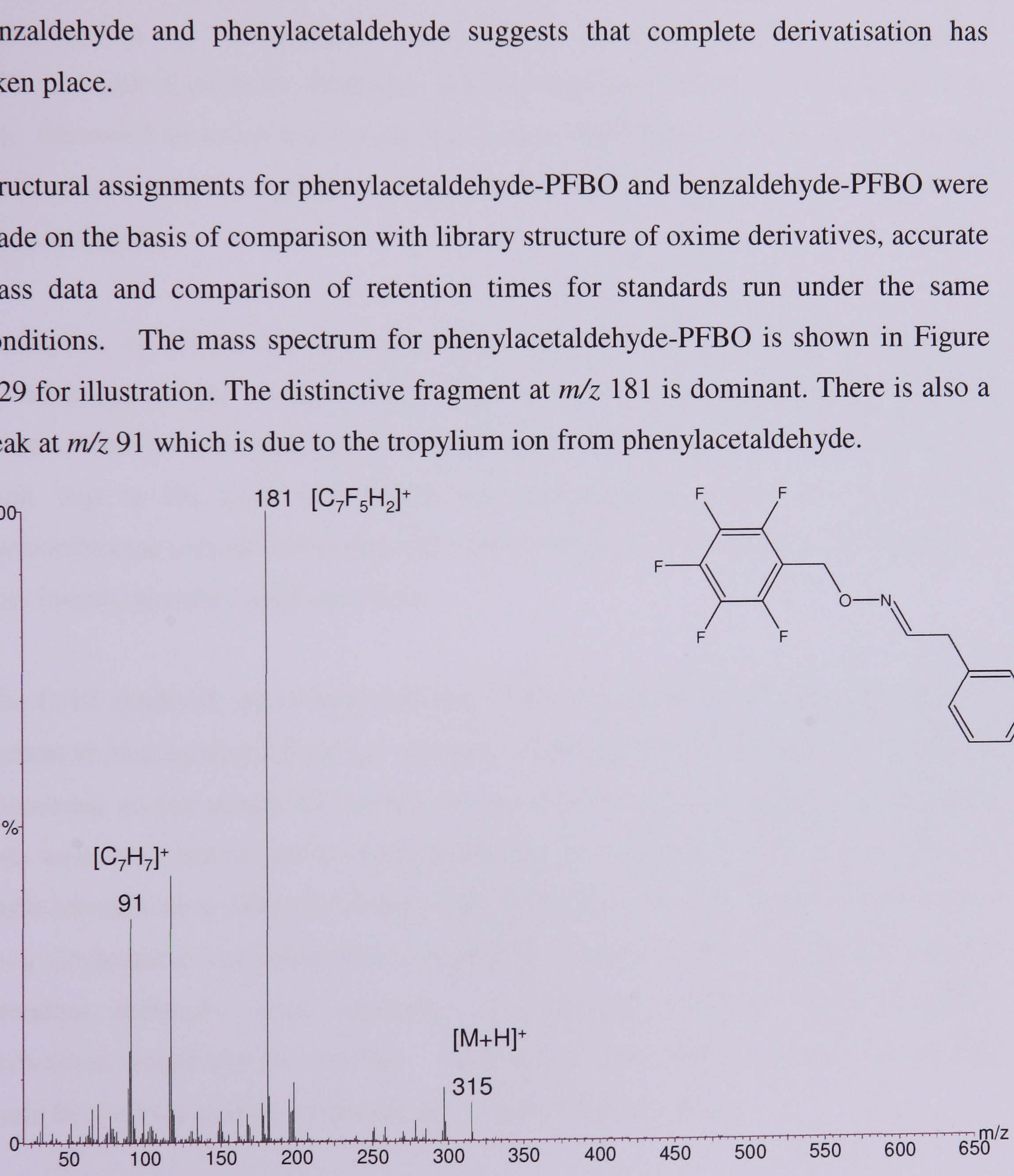


Figure 3.29 TOF mass spectrum of phenylacetaldehyde-PFBO (from GC-MS analysis).

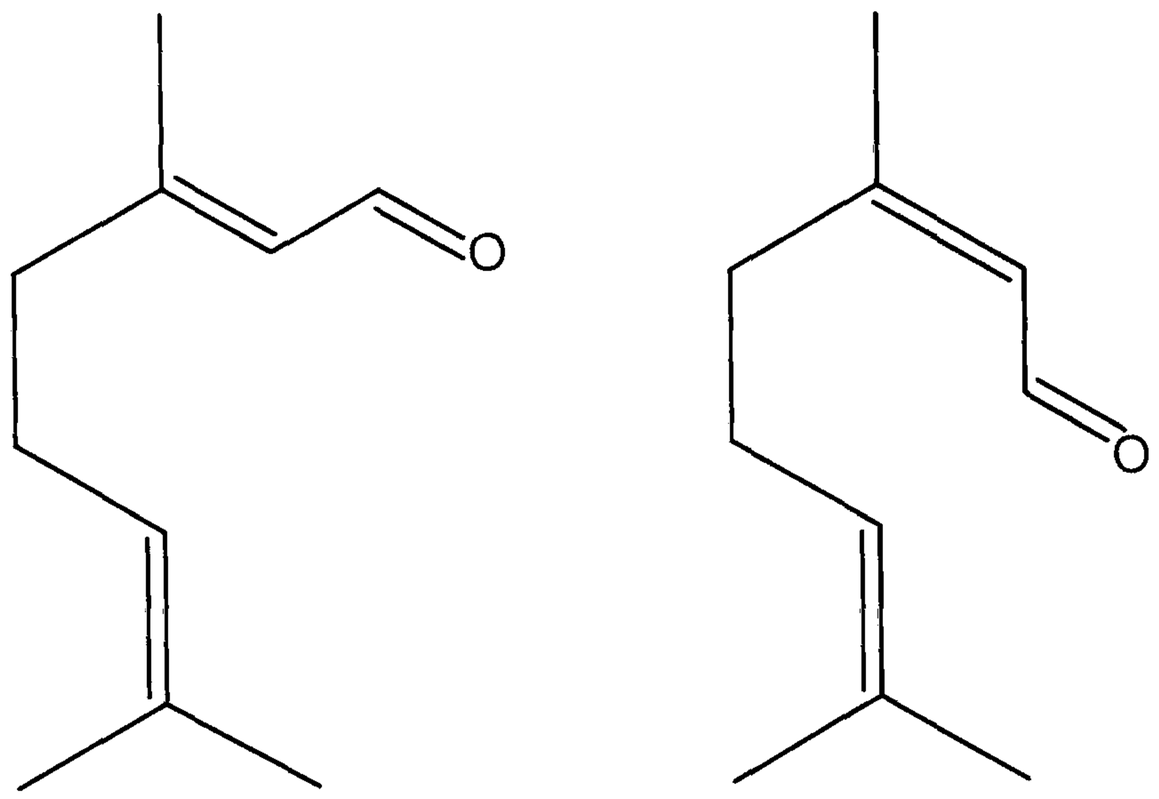
Trans-ocimene (peak A), phenyl ethanol (peak C), benzyl acetate (peak E) and phenylethyl acetate (peak G) (Figure 3.18) are some of the most abundant aroma components of hyacinth bouquet, and these were all detected in this experiment in the same ratio as that presented by McGee and Purzycki (2002). Their presence indicates that the Tenax is still capable of absorbing species that will not react with PFBHA. Acetone-PFBO arises from the solvent desorption of the trap. The presence of the acetaldehyde-PFBO is most likely as a contaminant from the PET bottle used to trap the headspace. Acetaldehyde is a known breakdown product of PET. A blank of the bottle headspace without the plant would be required to confirm this. Methyl-2-butanone has not previously been identified in blue hyacinth. Further verification would be required to check whether this was a contaminant or from the plant headspace.

Desorption of the back trap and analysis on the components indicated that no breakthrough of any trapped species had occurred, although acetone-PFBO was present. This is most likely to have arisen from PFBHA being displaced from the front trap to the back trap which was subsequently reacted with the 80:20 acetone/hexane solvent when this trap was desorbed. This suggests that PFBHA is only loosely absorbed onto the Tenax.

The C-10 aldehyde and cinnamaldehyde, which previous work had suggested are present in blue hyacinth bouquet, were not identified. There are a number of species containing an ion at *m/z* 181 which remain unidentified. Although accurate mass data were obtained for these compounds, the low abundance of the molecular ions made identification difficult. Further work using less energetic ionisation techniques such as chemical ionisation (CI) would give a greater chance of generating more abundant molecular ions. Synthesis of standards of these aldehydes-PFBO derivatives would also be valuable. The results shown here indicate that the trap could be used for analysis of headspace volatiles from flowers.

3.5 Application to lemon oil headspace

The main constituents of lemon oil are hydrocarbons, primarily limonene (between 65 and 95%) and other terpenes. They have been well characterised (Dellacassa *et al.*, 1997; Lota *et al.*, 2002; Starrantino *et al.*, 1997; Verzera *et al.*, 2004). Lemon oil also contains terpene aldehydes typically present at up to 3%. These are the citral isomers, geranial (**15**) and neral (**16**). These compounds are important contributors to the aroma of lemon oil, so much so that the market value of oils is defined according to its citral content. Other aldehydes which have also been identified in lemon include octanal, nonanal, decanal, dodecanal and citronellal.



(15) **(16)**

Uncoated and PFBHA-coated Tenax traps were exposed to a headspace of lemon oil. The solvent desorbed traps were analysed by GC-MS for derivatised aldehydes. Components were identified by the comparison of their mass spectra to previous analysis of lemon oil, with synthesised standards, or with published data, and by comparison of retention indices with synthesised standards.

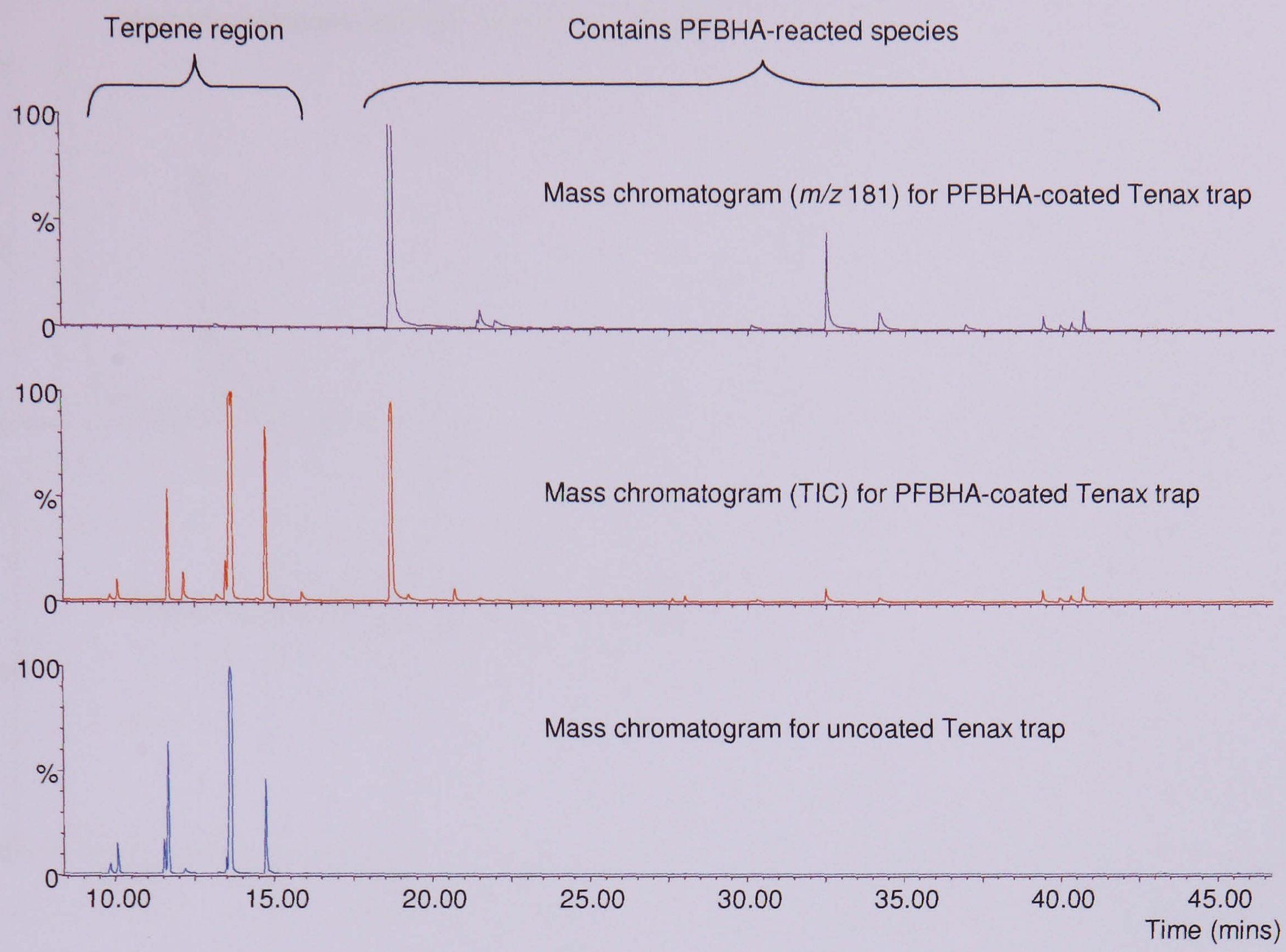


Figure 3.30 GC-MS chromatograms of lemon headspace from an uncoated trap and PFBHA-coated trap.

Figure 3.30 shows the total ion count (TIC) mass chromatograms for the lemon headspace isolated with an uncoated trap and a PFBHA-coated trap. Both traps isolated the main terpene species in lemon oil (α-thujene, α-pinene, β-pinene, sabinene, myrcene, α-terpinene, p-cymene, d-limonene, ocimene isomer, γ-terpinene, terpinolene) which appear at retention times of less than 17 mins. No unreacted aldehydes were identified in either the coated or uncoated traps. Figure 3.30 also shows the region from 20 minutes onwards which shows species reacted with PFBHA, as indicated by the mass chromatogram of the ion of *m/z* 181.

Figure 3.31 shows the mass chromatograms of the ion of *m/z* 181 in the retention time window of approximately 19-43 minutes. The proposed peak assignments are shown in Table 3.7.

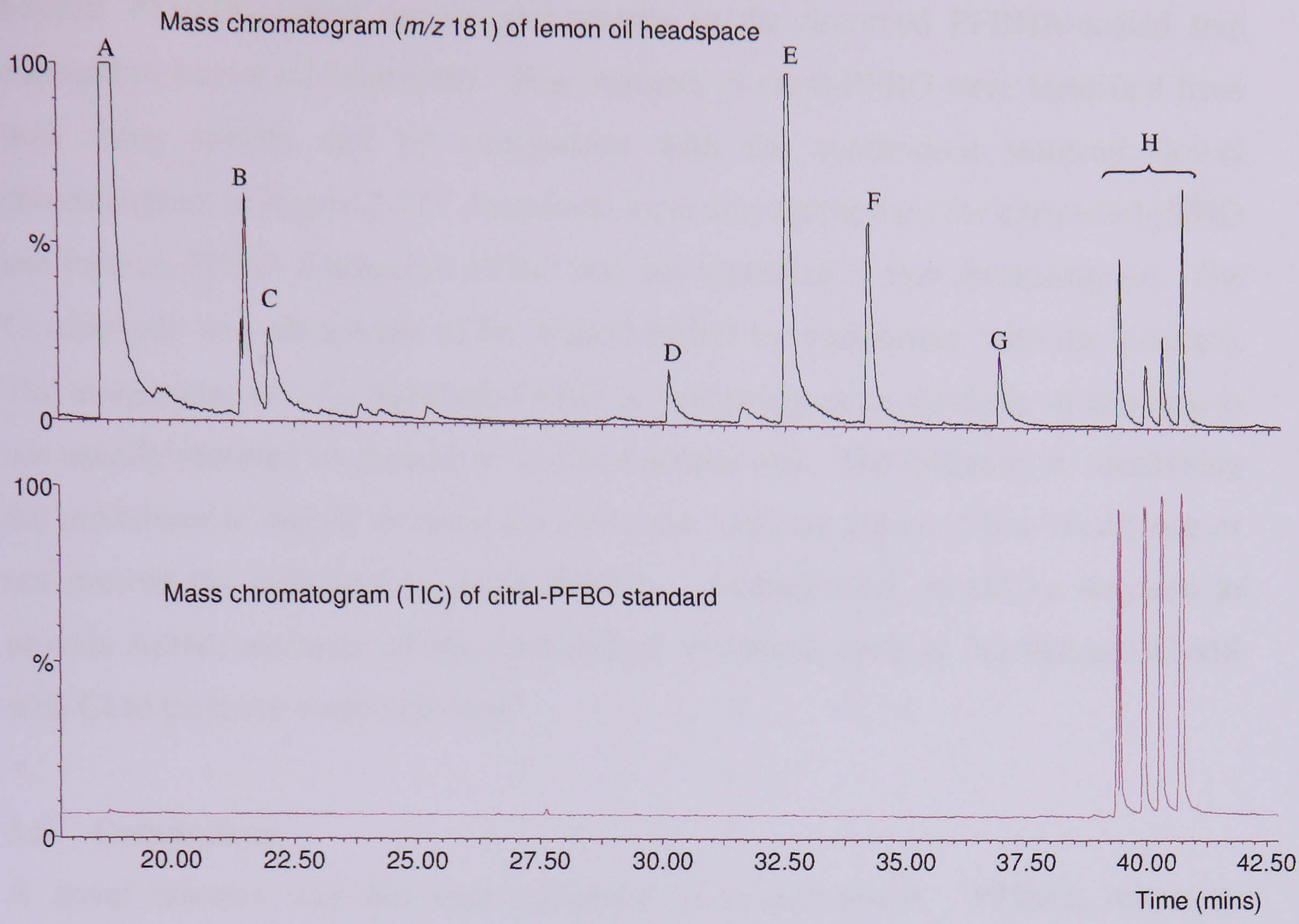


Figure 3.31 GC-MS chromatograms of lemon oil headspace (*m/z* 181) and citral-PFBO standard.

Annotation	Assignment
A	Acetone-PFBO
B	C_4 aldehyde-PFBO
C	C_4 aldehyde-PFBO
D	PFBHA-derivative (*m/z* 181, 193)
E	PFBHA-derivative (*m/z* 181, 193)
F	PFBHA-derivative (*m/z* 181, 239) – probably octanal-PFBO
G	PFBHA-derivative (*m/z* 181, 239) – an aldehyde PFBO
H	Citral-PFBO (neral-PFBO and geranial-PFBO, *cis* and *trans* isomers)

Table 3.8 Assignment for peaks in the GC-MS chromatogram of lemon oil headspace (Figure 3.31).

Several PFBHA-related species are present in the desorbed PFBHA-coated trap exposed to lemon oil headspace. Four isomers of citral-PFBO were identified from their mass spectra and by comparison with the synthesised standard (lower chromatogram in Figure 3.21). Standards were also synthesised for citronellal-PFBO and butanal-PFBO. Citronellal-PFBO was not identified in this chromatogram. The C_4 aldehyde was shown not to be butanal-PFBO by comparison with the standard. The assignment of a C_4 aldehyde-PFBO is interesting as an aldehyde of this size is not usually reported as present in lemon essential oils. The difficulty in identifying the unknowns is mainly because the molecular ions are either of low abundance or not present (as indicated by citral-PFBO). Further work would be required to provide further evidence of the unidentified structures, such as combining GC-MS with CI to generate molecular ions.

3.6 Conclusions

A novel reactive trap has been designed, built and tested. PFBHA has been confirmed as a suitable derivatising reagent for aldehydes. It is compatible with GC and GC-MS analysis. It has been shown to be effective at a coating level of 10 % w/w of Tenax. Sufficient characterisation of the trap has been carried out for it to be taken on a field trip and used in headspace experiments to isolate fragrances from exotic species that may contain aldehyde (or ketone) compounds. The final operating conditions of the trap were to sample at 50 $mL.min^{-1}$ for 8 hours and desorb with 80:20 acetone/hexane. Samples should be desorbed immediately after trapping has taken place. The data presented here support a tentative conclusion that the high humidity that may be experienced in field trips would not be detrimental to the isolation of aldehydes. It is recommended that a back trap is used as a precaution guard against breakthrough.

The data from the MS-Nose for measuring analyte breakthrough are encouraging for demonstrating the utility of this technique. Provided that a compound will ionise, the use of the MS interface is certainly capable of detecting PFBHA derivatised analytes and could be applied to other analytes. Use of a mass spectrometer as a detector has the distinct advantage of that it allows one to monitor ions from different species. Further work on the experimental design and repeat studies would be required to

make this technique more robust for this purpose. The experimental set-up could be improved to replicate a dynamic headspace experiment as used in other sampling experiments described in this chapter, continually drawing the headspace through the trap to the MS-Nose.

Spectroscopic imaging techniques have also been shown to illustrate the distribution of the PFBHA on the Tenax. This information could not easily have been obtained by other techniques.

In both hyacinth and lemon oil headspace a ketone and an aldehyde were identified which have not previously been reported. More experimental work would be required to confirm these observations. Results from the analysis of flower and fruit oil samples suggests that using GC-MS with EI alone is not always sufficient to identify the structures of the derivatised species due to the low intensity of many molecular ions peaks. The following protocol is suggested to provide more information:

- GC-MS used with EI (preferably with accurate mass) to check for the presence of derivatised aldehydes, with *m/z* 181 as a diagnostic ion;
- GC-MS with CI (again preferably with accurate mass) used to aid the determination of unknowns, and.
- Analysis of a standard of the derivatised aldehyde if unknowns are provisionally identified, to check the retention time along with mass spectral data.

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Yu, J. Z., Jeffries, H. E. and Lelacheur, R. M. (1995). Identifying Airborne Carbonyl-Compounds in Isoprene Atmospheric Photooxidation Products by Their Pfbha Oximes Using Gas-Chromatography Ion-Trap Mass-Spectrometry. *Environmental Science & Technology* **29**, 1923-1932.

4

Development of Thiol Specific Trapping Methods

It was established in the introduction that thiols make a significant contribution to the flavours of foodstuffs and thus are of interest to flavourists who seek to emulate natural tastes in synthetic food products. They are high-impact chemicals, that is, they produce a noticeable aroma when present in quantities as low as parts per trillion and the odours are often the key contributor to the overall flavour. Species present at these low levels in complex mixtures can be very difficult to analyse. The added difficulty for thiols is that they are highly reactive, easily undergoing oxidation, complexing with metals and to a lesser extent undergoing nucleophilic reactions. They are also easily adsorbed on surfaces, such as chromatographic columns. An approach is described here that utilises the chemical reactivity of thiols in order to extract the materials selectively from complex mixtures of natural origin.

In this chapter, an introduction is made to thiol chemistry as a basis for determining a thiol-specific isolation technique. This is followed by a review of current and potential methods for isolating thiol compounds. The majority of the chapter is devoted to the exploration of three methods chosen for further development, namely maleimide-functionalised solid-phase silica beads, disulfide functionalised gels and gold wire. The results of testing on model systems of thiols and on flavour extracts are presented.

4.1 Introduction to thiol chemistry

The thiol group is capable of a undergoing a wide range of chemical reactions, summarised in Figure 4.1. Thiols are nucleophiles and much of their chemistry is based on this reactivity. For example, thiols can react with organohalides in nucleophilic substitution reactions to generate thioethers. Thiols can also attack carbonyl compounds (RCOX) to generate thioesters (RCOSR'). In compounds where there is a double bond conjugated to a carbonyl group, the preferred reaction is for the thiol to add across the double bond (conjugate or Michael addition). Another common reaction is the oxidation of thiols to disulfides. These chemical reactions are well documented in any standard organic chemistry textbook (Clayden *et al.*, 2001; McMurry, 2000; Patrick, 2003; Vollhardt and Schore., 2006). Thiols also have a high affinity for metals and metal oxides, particularly with mercury, gold, copper and their compounds. Gold, palladium and platinum can chemisorb sulfur gases.

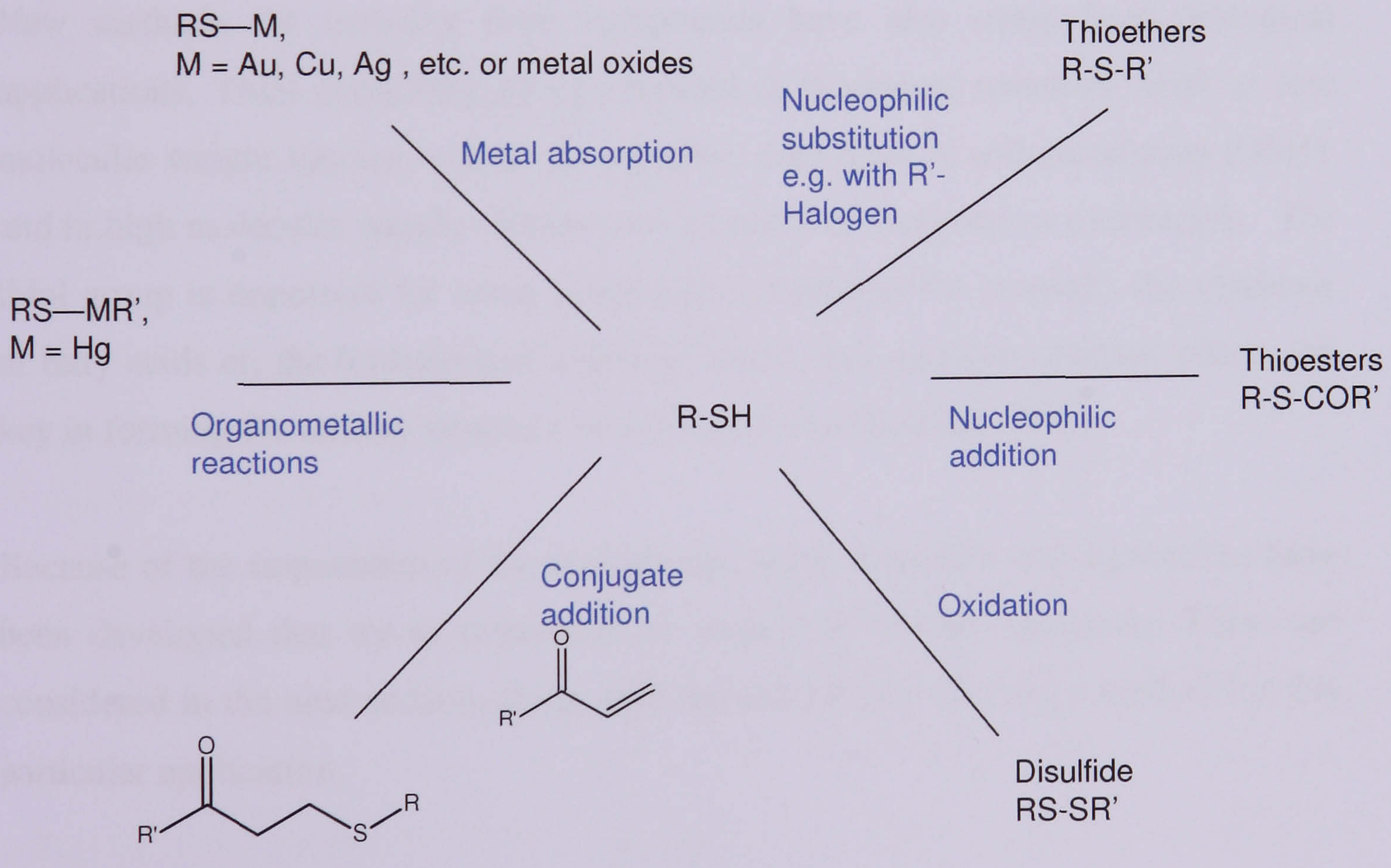


Figure 4.1 Selected generic reactions of thiol groups.

The thiol group is important to many areas of research, including synthetic chemistry, biological research and environmental applications. In synthetic chemistry, several protecting groups have been designed for the reactive thiol group.

For an overview of thiol protecting groups see Greene and Wuts (1999). Other excellent reference texts for synthetic reactions involving thiols have been published (March, 1992; Patai and Rappoport, 1974).

In the realm of environmental research, volatile sulfur compounds are of concern for a number of reasons. Release of sulfur compounds in the environment can be responsible for environmental damage such as contributing to acid rain, destroying forests and causing corrosion. In petrochemical applications, the presence of trace levels of sulfur impurities can be detrimental to the performance of the oil. As well as being desirable flavour compounds, thiols also cause odour problems such as contributing to malodour in sewage systems. Considerable effort has been directed at finding methods preventing emission of volatile sulfur compounds into the environment. A review of environmental sulfur compound analysis has been published (Wardencki, 1998).

New methods for isolating thiol compounds have also come from biological applications. Thiol containing groups abound in biological materials, both as low molecular weight species such as the cysteine, coenzyme A and glutathione (GSH), and in high molecular weight species such as peptides, enzymes or membranes. The thiol group is important for many biochemical reactions, for example, the synthesis of fatty acids or, the formation of disulfide bonds from cysteine residues which are key in forming the tertiary structure of many proteins (Jocelyn, 1972).

Because of the importance of the thiol group, many strategies and approaches have been developed that try to overcome, or make use of their reactivity. These are considered in the next section, along with the criteria for selecting a method for this particular application.

4.2 Method selection

A survey was made of the literature in order to assess suitable methods for isolating thiols from flavour matrices. All aspects of the chemistries of thiol groups were considered including organic synthetic methods, inorganic chemistry, organo-metallic chemistry and commonly used biologically techniques.

In searching for a suitable method for selective isolation of thiol compounds, the following points were considered:

- Is the method selective for thiols? For example, alcohols and amines can undergo some of the same reactions as they are also nucleophilic.
- Will the thiols be concentrated?
- Is the regeneration of the thiol possible? This is desirable because organoleptic analysis requires the thiols in their original form.
- Will the reaction stabilise the thiol to the conditions required for analysis?
- Will the method make the thiols easier to detect?

Two of these criteria are in some respects incompatible: that is, the desire to have a thiol which can be released in the original form suitable for assessment of its aroma, and at the same time have a sample that is stabilised and easily identifiable for analysis which implies a chemical transformation.

Selectivity can be achieved in a number of approaches: through reactivity, separation and detection. A review of isolation methods that are selective toward thiols in different ways is now presented. Their relative merits and limitations in the light of these criteria are considered.

4.2.1 Synthetic organic chemistry methods

Conjugate (or Michael) addition is one of the most common and important reactions in organic synthesis (Perlmutter, 1992). In this reaction, nucleophiles add, in the presence of bases, to the double bond of compounds of the form C=C—EWG where EWG is an electron-withdrawing group. Commonly EWG is a carbonyl group giving α,β-unsaturated carbonyl compounds or a nitrile group (C≡N). The base-catalysed mechanism for the reaction is shown in Figure 4.2. The site of attack is dependent upon the nature of the nucleophile: a 'hard' nucleophile (such as RO^-) attacks the carbonyl group (1,2-addition), whereas a 'soft' nucleophile (such as RS^-) attacks the double bond (1,4-addition). A comprehensive discussion of 1,2-versus 1,4- additions has been published (Oare and Heathcock, 1989).

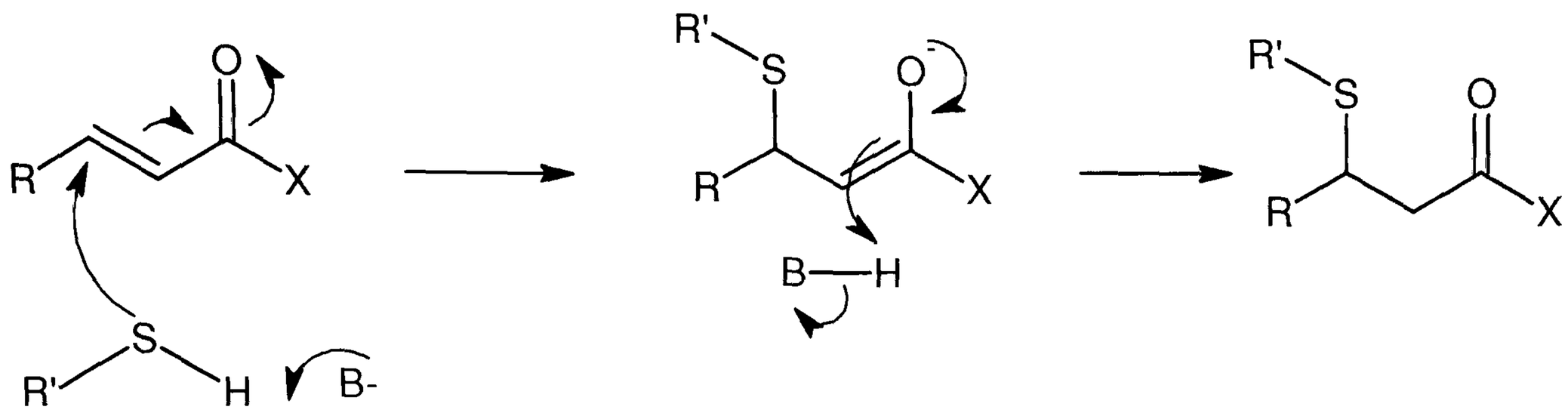
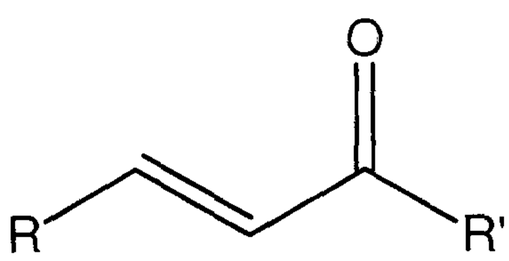
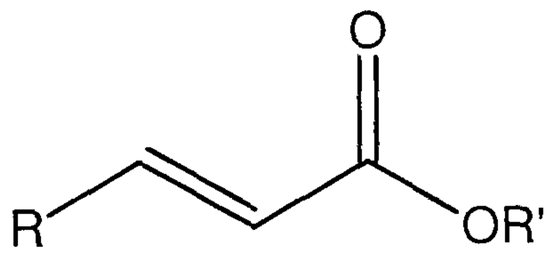


Figure 4.2 The base–catalysed (B) mechanism for 1,4-addition of a thiol to an α,β-unsaturated carbonyl compound. The 'soft' sulfur nucleophile reacts preferentially with the 'soft' electrophile, the double bond.

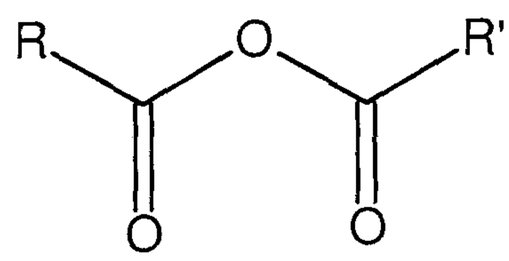
Reagents typically used in organic synthesis include vinyl ketones (**1**) (Choudary *et al.*, 1999), conjugated esters (**2**) (Matsuura and Takashina, 1993) and anhydrides (**3**) (Emori *et al.*, 1998). Vinyl ketones have the disadvantage of a tendency to polymerise, depending on substituent groups. The esters and anhydrides have the advantage of containing groups where the charge of the intermediate can be delocalised which stabilises the reaction intermediate. The product is stable, and the reaction reversible under the right conditions.



(1)

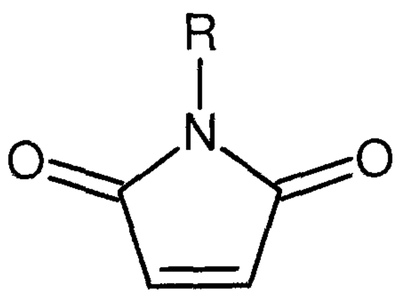


(2)

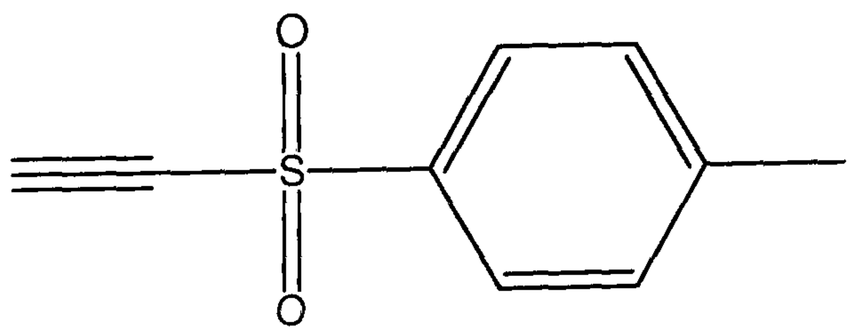


(3)

One of the most frequently used reagents is based on maleimide (**4**). The nature of the maleimide ring makes direct attack less favourable, as the carbonyl carbon would need to become tetrahedral, which would increase the strain in the 5-membered ring. Maleimide reacts with thiols under mild conditions. (See Section 4.2.2 for further applications).



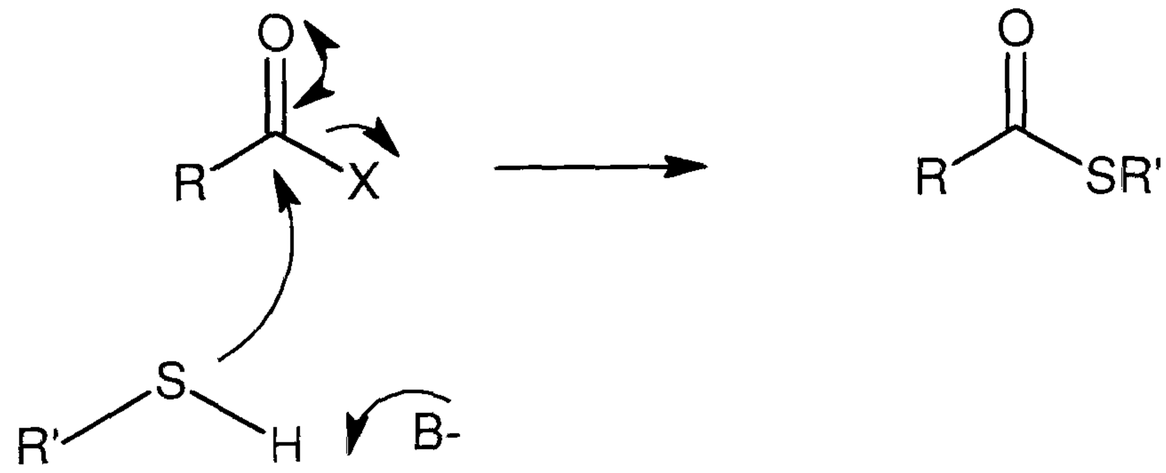
(4)



(5)

Conjugate addition to an alkyne, for example tosylacetylene (**5**), forms an alkene derivative which is stable to acidic and basic conditions. Removal of the thiol is possible at room temperature with an excess of pyrrolidine (Arjona *et al.*, 1999; Arjona *et al.*, 2003). This method, however, is reported as being unsuitable when alcohols are present and so would not be applicable to all food matrices. Conjugate addition is an attractive reaction method for the isolation of thiols, because of the relatively high selectivity. Amines and alcohols, being hard nucleophiles, are more likely to react at the carbonyl group.

Thiols can undergo reactions with carboxylic acid derivatives such as esters, acid chlorides and anhydrides (Figure 4.3). The advantage of this reaction is that the thiol is easy to regenerate under mild hydrolysis conditions. Poor selectivity is the key concern as the presence of other nucleophiles, such as alcohols and amines, will lead to competitive reactions. Both amines and alcohols are common in food matrices.

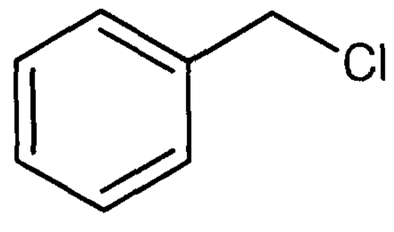


X = OR", Halogen, CH_2CO_2R"

Figure 4.3 Reaction of a thiol with a carboxylic acid derivative by nucleophilic addition and substitution.

The reaction of thiols with organohalides to generate thioethers (sulfides) in a nucleophilic substitution reaction is frequently used, particularly for protecting the thiol group during synthesis. Protection with benzyl chloride (**6**) is common (Dymicky and Byler, 1991; Vogtle and Klieser, 1982; Yin and Pidgeon, 1997) and it is relatively facile to deprotect using ammonia (Corrie *et al.*, 1977). Other para-substituted benzyl derived groups have also been used. For a detailed review of the protection of thiols as thioethers see Greene and Wuts (Greene and Wuts, 1999).

Iodoalkyl reagents are most commonly for reaction with thiols. Other nucleophiles can also react in a competitive manner.



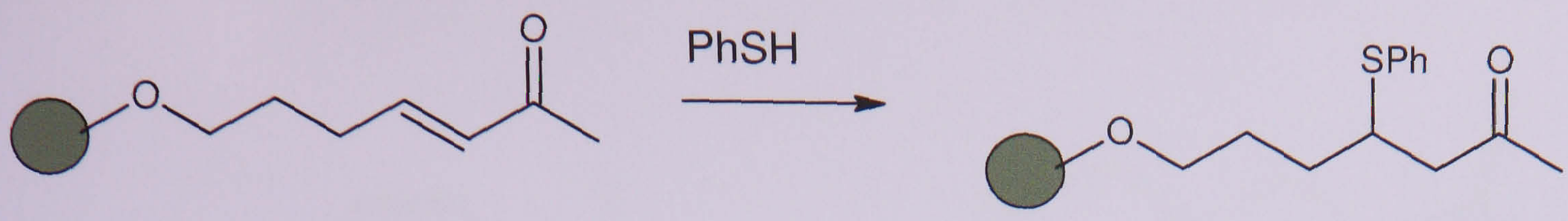
(6)

Disulfides are also used to protect sulfur groups. A common reagent is the disulfide, 5,5'-dithiobis(2-nitrobenzoicacid) (DTNB). This is discussed in Section 4.2.3, as this is also used in several biological applications.

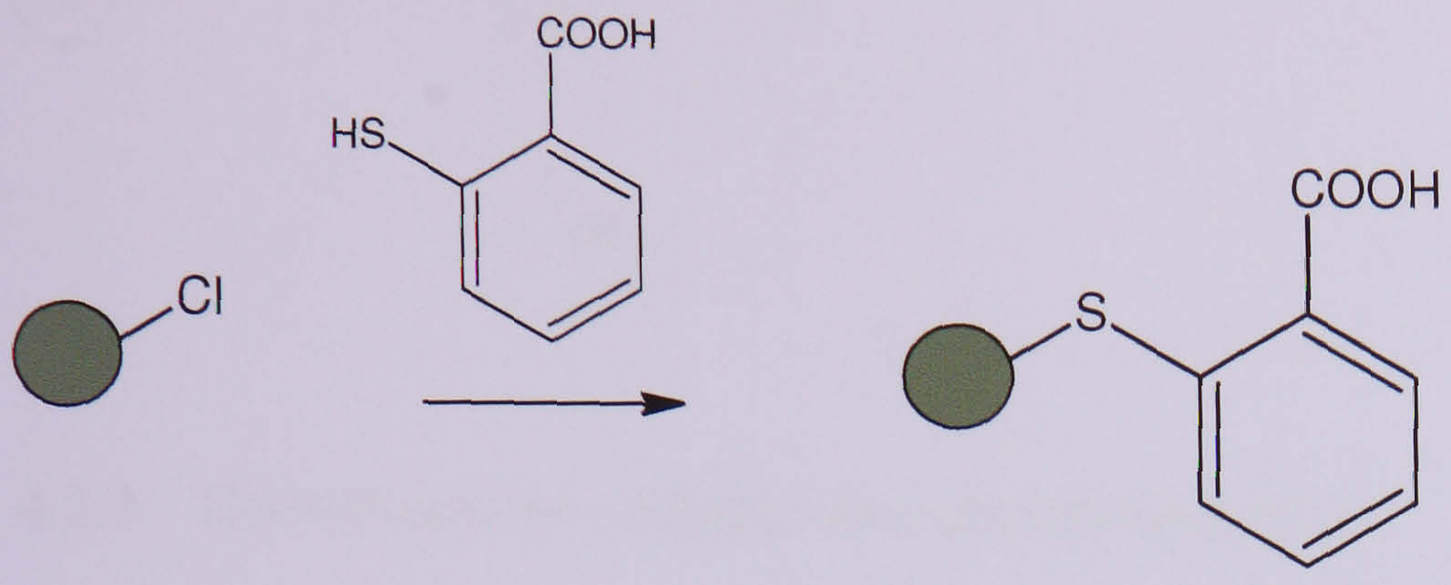
4.2.1.1 Solid-phase reactions and reagents

Products of reactions with thiols carried out in solution usually require purification steps before most analytical techniques can be successfully applied to determine their molecular structure. For a simplified method of purification, solid-phase reagents have been considered. Solid-phase chemistry was introduced in Chapter 1. Solid-phase reactions are particularly attractive because a simple filtration is all that is required to separate the reacted species from everything else in the matrix. It has also been discussed in Chapter 1 that such reagents can be used to concentrate the thiols because a large volume of a thiol-containing matrix can be reacted with the solid-phase reagent.

Many of the synthetic reactions that have been described above have been conducted using solid-phase resins. A conjugate addition and nucleophilic substitution are shown in Figure 4.4. Solid-phase chemistry has been extensively reviewed (Booth *et al.*, 1998; Brown *et al.*, 1998; Brown, 1998; Hermkens *et al.*, 1996).



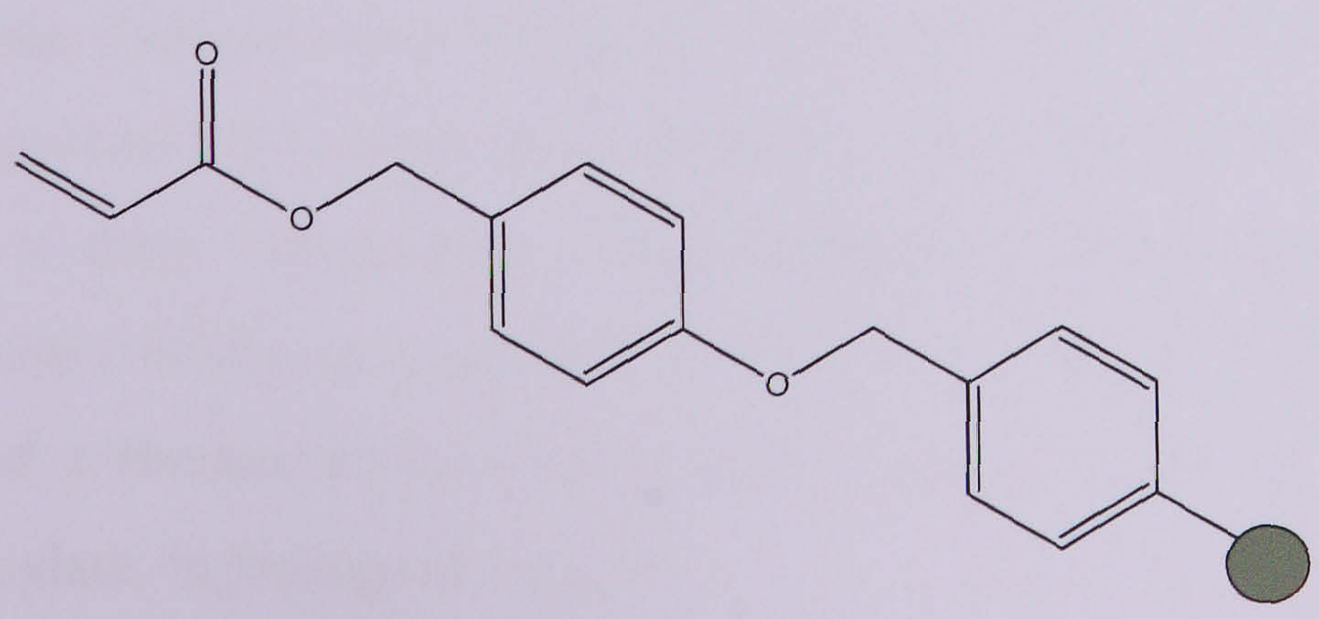
(a) (Chen *et al.*, 1994)



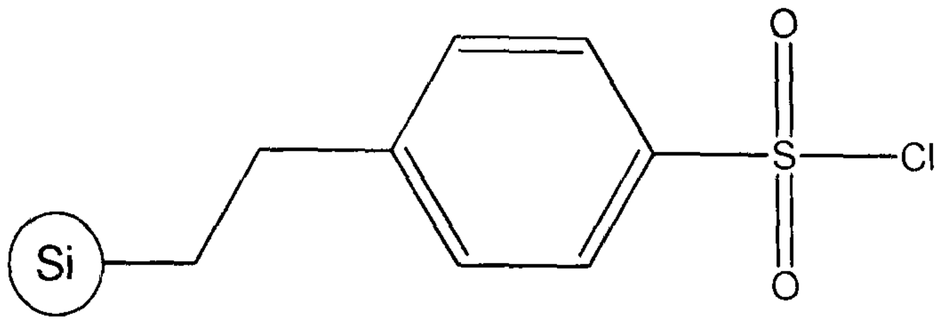
(b) (Cody *et al.*, 1994)

Figure 4.4 Reactions of thiols using solid-phase reagents (a) conjugate addition, (b) nucleophilic substitution.

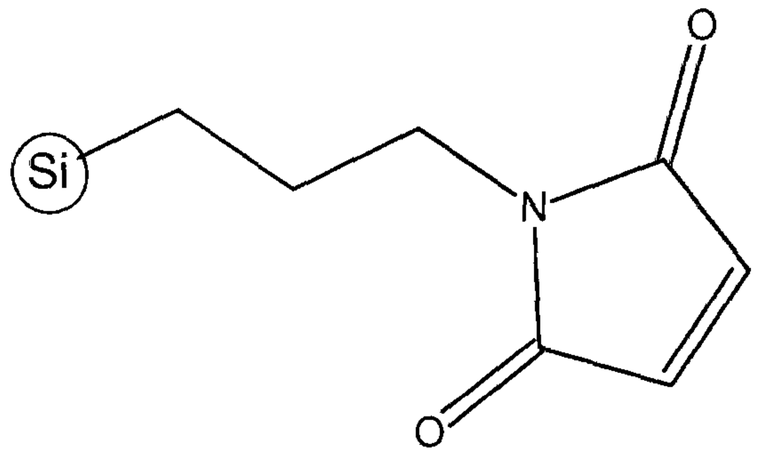
Several commercial resins are available that have been designed to react with thiols, or more usually, other nucleophiles such as amines. The acrylate (REM Rink resin) (**7**) is bound to polystyrene and can be used for conjugate addition reactions with nucleophiles. Silica bound-chlorosulfo (**8**) can be used to immobilise alcohols and scavenge nucleophiles. Silica bound-maleimide (**9**) was designed as a thiol scavenger. The use of the silica-maleimide reagent is investigated in Section 4.3.2. The other two reagents were not used in this work: the acrylate because it is polystyrene based which is not compatible with an aqueous system. The silica-chorosulfo reagent was not considered to be specific enough for thiols. A literature search revealed that there were no published reports of using these types of reagents to isolate compounds from food matrices.



(7)



(8)

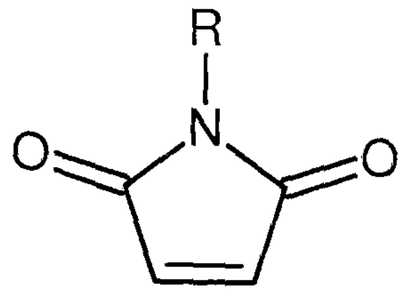


(9)

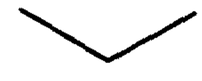
4.2.2 Derivatisation reagents for chromatography

Most analyses of complex matrices require the involvement of chromatography. Thiols generally do not have good chromatographic properties: they have a tendency to absorb onto the column materials and this leads to intense peak tailing. They also have poor detectability so that, for example, there may be no characteristic ions in a mass spectrum. Derivatising reagents can, therefore, make two improvements to the detection of thiols, firstly stabilising the molecule, making it less reactive, and secondly introducing a species which generates a higher detector response. There are several commercially available derivatising reagents designed primarily for liquid chromatography. Many of these reagents are compatible with UV/Vis detection, a common detector for HPLC, or include fluorescent tags. The majority of these have been developed with the analysis of biological systems in mind.

As derivatisation is simply a specialised micro-synthesis reaction it is not surprising that many of the commercially available derivatising reagents use the same chemistries as standard organic synthesis. Use of the maleimide group is prominent and several commonly used N-substituted maleimides are shown in Figure 4.5. The substitution of the nitrogen atom frequently utilises a fluorophore to aid detection. Biologically important thiols have been identified using N-subsitituted maleimides. For example, a thiol antioxidant, N-acetylcysteine, was reacted with N-(1-pyrenyl)maleimide (NPM) and identified by HPLC (Ercal *et al.*, 1996). Ercal *et al.* (2001) also used a fluorescent maleimide-based reagent to identify the thiol drug, sodium thiosalicylate, in biological samples.

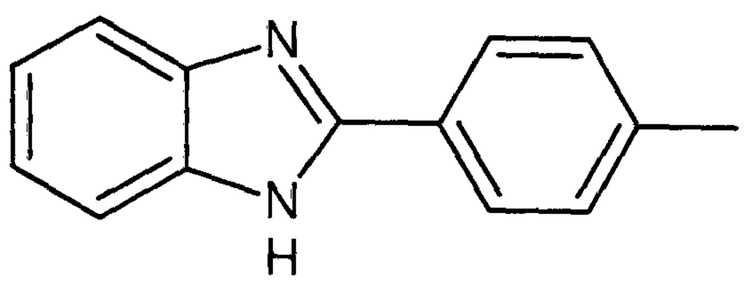


R =



N-ethylmaleimide (NEM)

R =



N-[4-(2-Benzimidazolyl) phenyl]-maleimide (BIPM)

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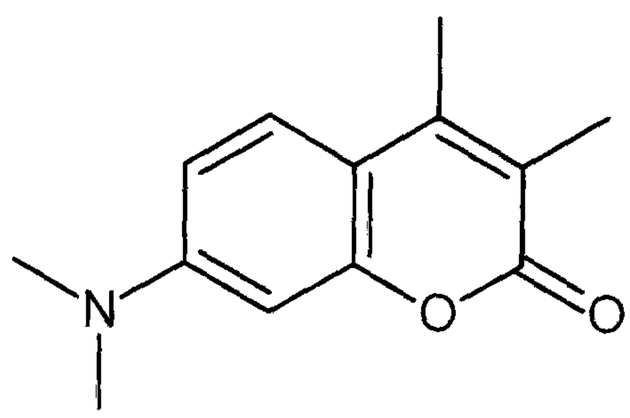
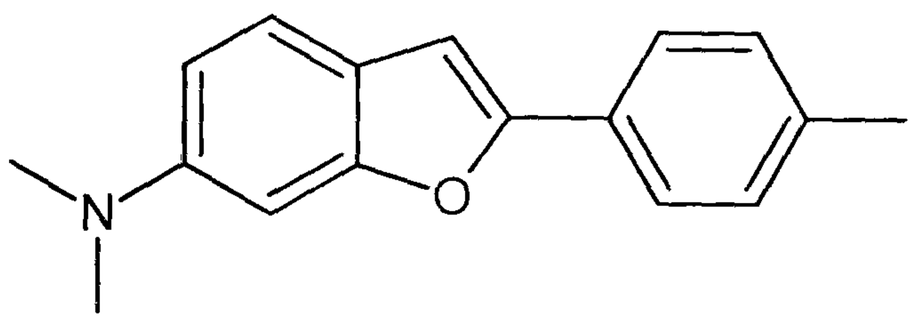


N-[4-(6-Dimethylamino-2-benzofuranyl)phenyl]maleimide (DBPM)

R =



N-(7-Dimethylamino-4-methyl-2-oxo-3-chromenyl)maleimide (DACM)

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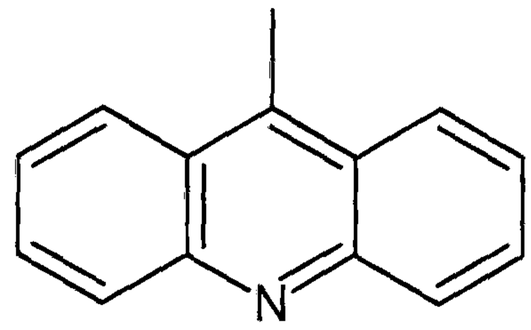


N-(9-Acridinyl)maleimide (NAM)

Figure 4.5 N-substituted maleimides used as derivatising reagents.

Other derivatising reagents used in conjunction with fluorescence detection include those based on bimanes, dansyl aziridine, halogenosulfonylbenzofurazans and *o*-phthalaldehyde. The use of these reagents has been reviewed (Shimada and Mitamura, 1994).

A reagent used frequently with UV/Vis detection is 5,5'-dithiobis(2-nitrobenzoicacid) (DTNB) also known as Ellman's reagent. This disulfide reacts with thiols forming a new disulfide bond. The reaction results in the release of 2-nitro-5-sulfhydrylbenzoic acid (TNB) which absorbs at 412 nm. The reaction is shown in Figure 4.6. The advantage of this reaction is the specificity to thiols.

DTNB has been used to detect trace levels of thiols bubbled through aqueous media at pH 8. The resulting disulfides were determined by HPLC. The thiol vapours were detected in the low ppm or ppb range (Kuwata *et al.*, 1982). DTNB has also been used with solvent extracts of various food sources (Voldrich *et al.*, 1995).

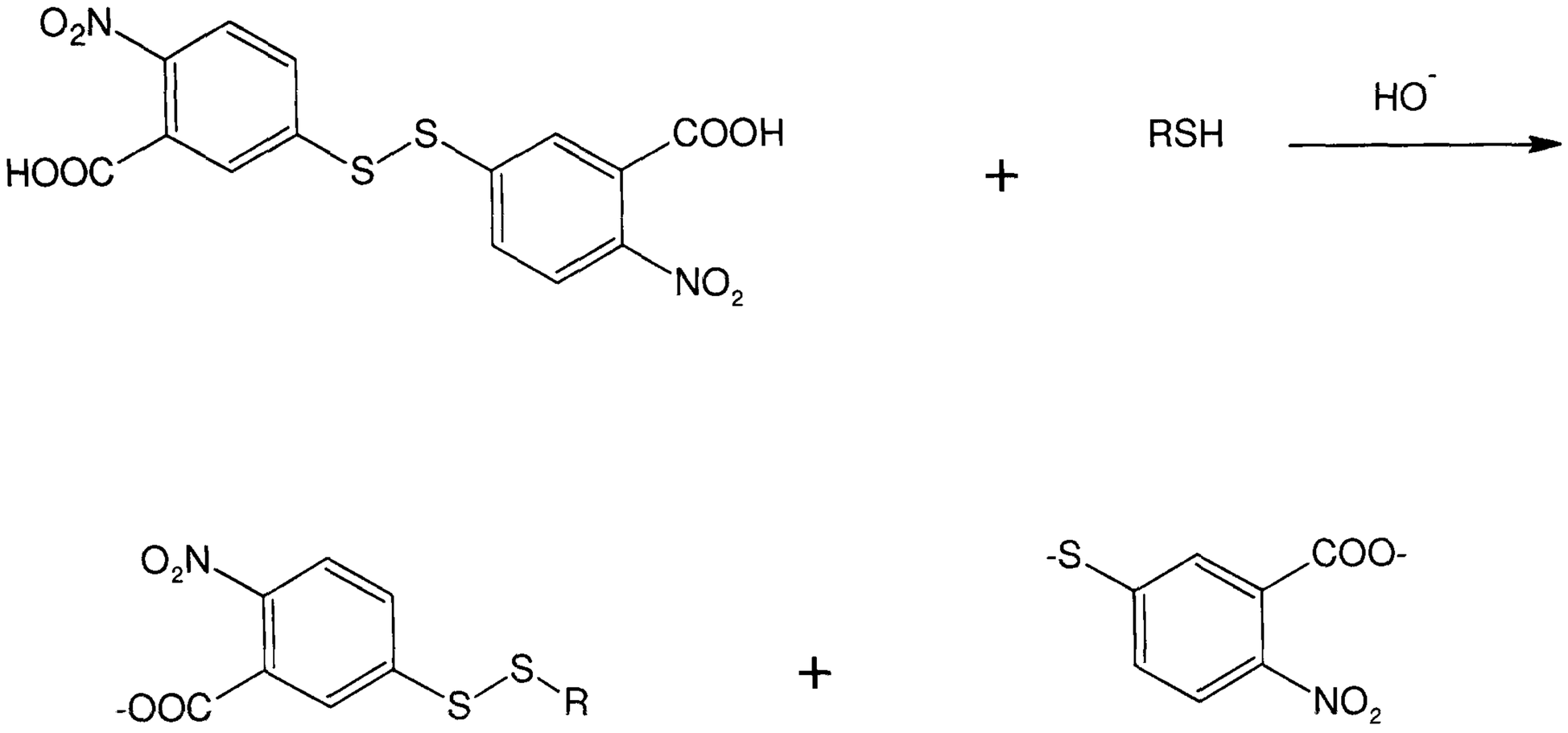
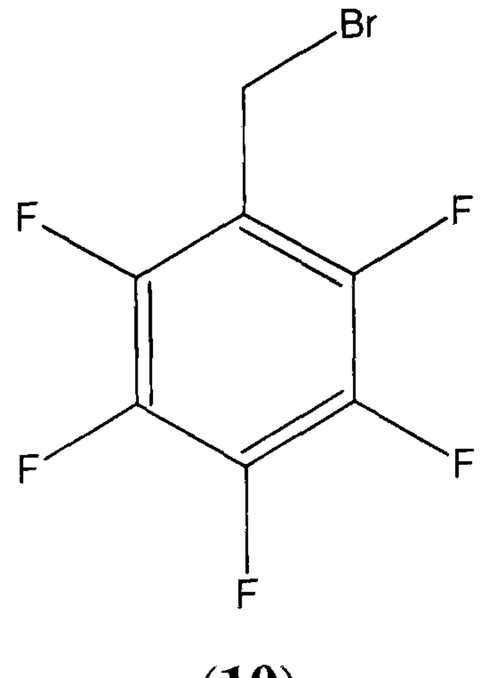


Figure 4.6 Reaction of a thiol with 5,5'-dithiobis(2-nitrobenzoicacid) (base-catalysed).

A suitable reagent for GC analysis is pentafluorobenzyl bromide (PFBBr) (**10**). This reagent converts thiols to halogenated derivatives that are easily detected by electron capture. A recent study used SPME fibres coated with PFBBr in conjunction with gas chromatography-negative chemical ionisation-mass spectrometry to detect the thiol derivatives, which were found at ppt levels in wine (Mateo-Vivaracho *et al.*, 2006).

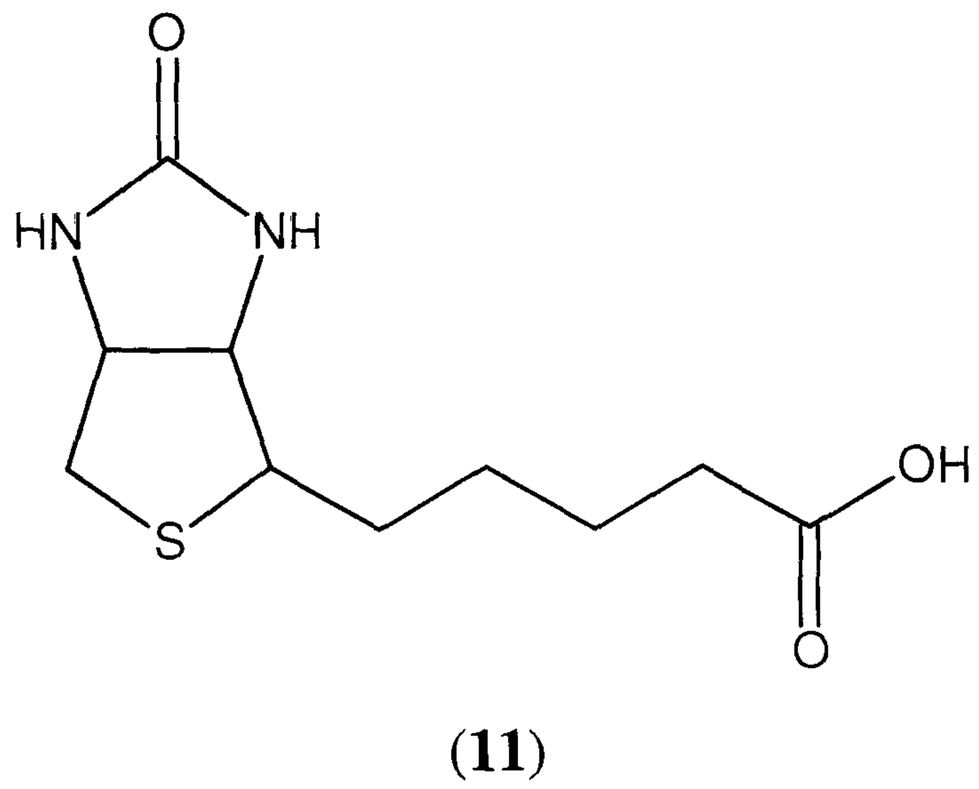


(10)

The disadvantage of using these derivatising reagents is that, whilst they provide a method of tagging the reacted species, they do not provide for a physical isolation step and do not allow for concentration of the species of interest.

4.2.3 Biotin-avidin affinity chromatography gels

The use of biotinylated reagents in conjunction with affinity chromatography was another possibility investigated for isolation of thiols. Affinity chromatography was introduced in Chapter 1. This is used to separate species based on bio-recognition of molecules. The interaction is reversible. The vitamin, biotin (**11**), has a strong affinity for the egg-white glycoprotein avidin (or its bacterial counterpart streptavidin). The biotin can be coupled to low and high molecular weight compounds and still be recognised by avidin.

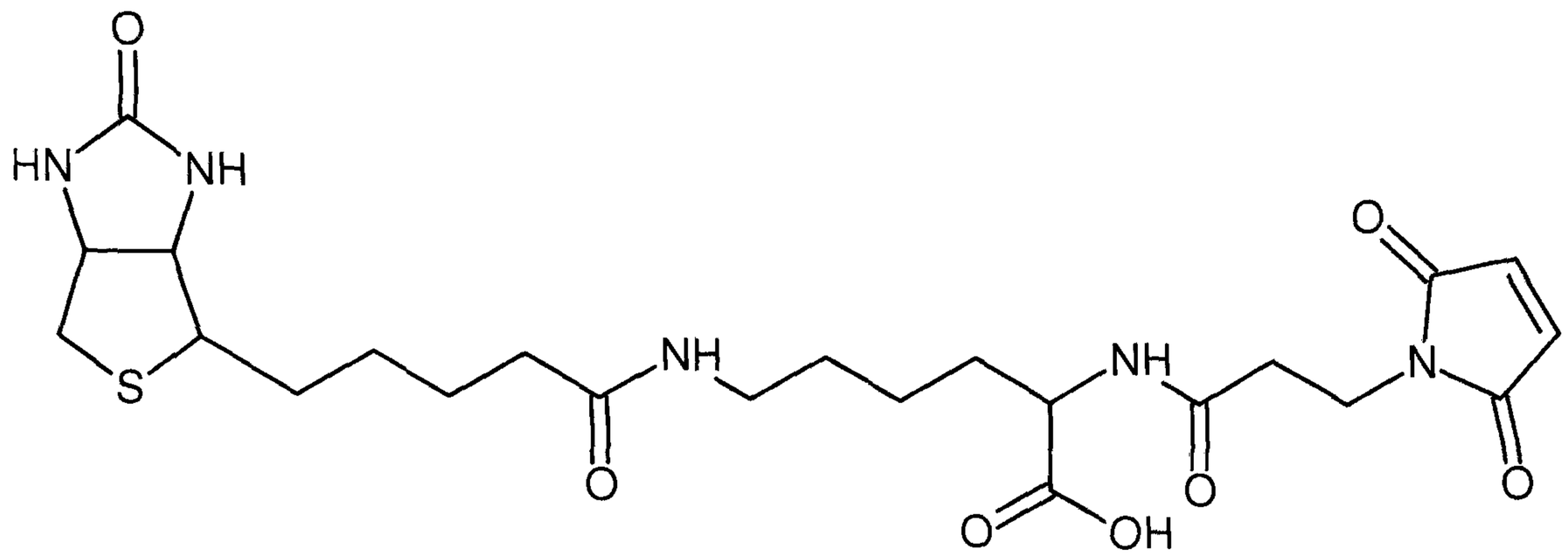


(11)

Biotinylated reagents consist of:

- the biotin moiety, for affinity binding;
- a linker group – usually included to increase solubility or optimise the binding with avidin; and
- a reactive group that will react with the molecules of interest.

Several commercially available reagents have been designed to react with thiol compounds, particularly the cysteine moiety found in proteins. These include reagents which are maleimide, iodoacetyl and disulfide based. Three examples are N-(3-maleimidylpropionyl)biocytin (**12**), N-(biotinoyl)-N'-(iodoacetyl) ethylenediamine (**13**) and N-[6-(Biotinamido)hexyl]-3'-(2'-pyridyldithio) propionamide (**14**).



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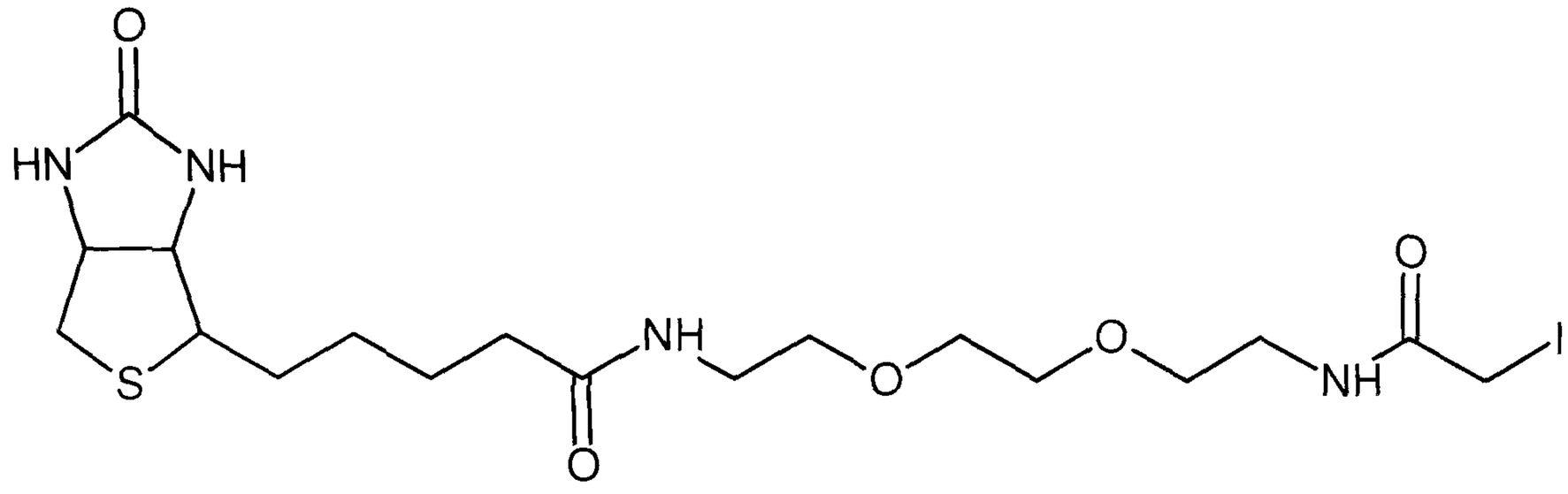

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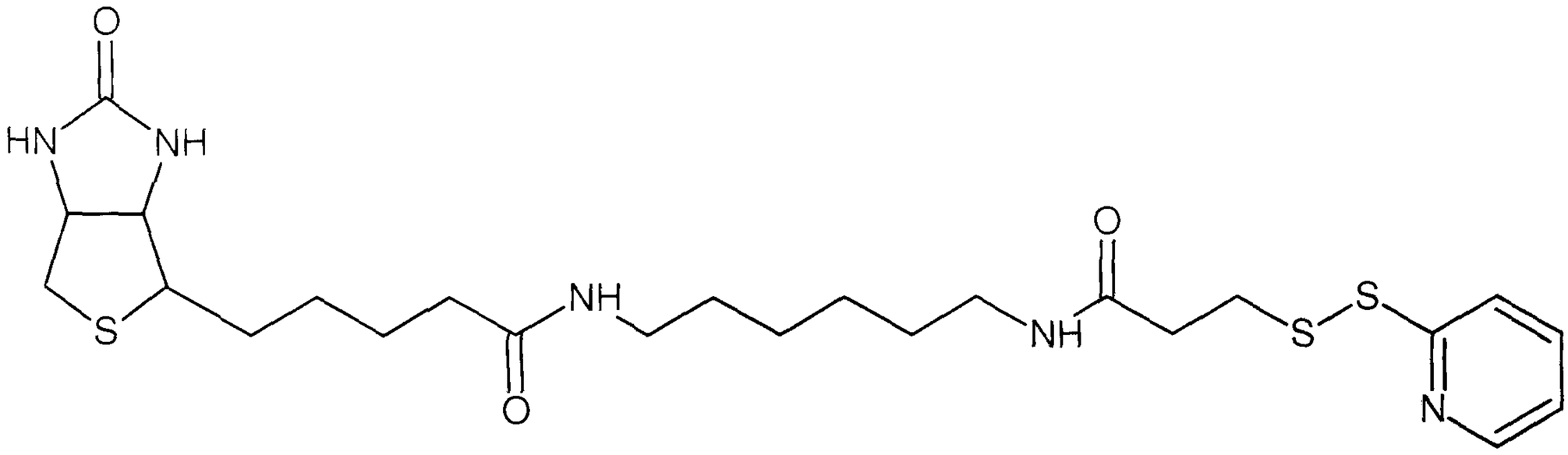


(14)

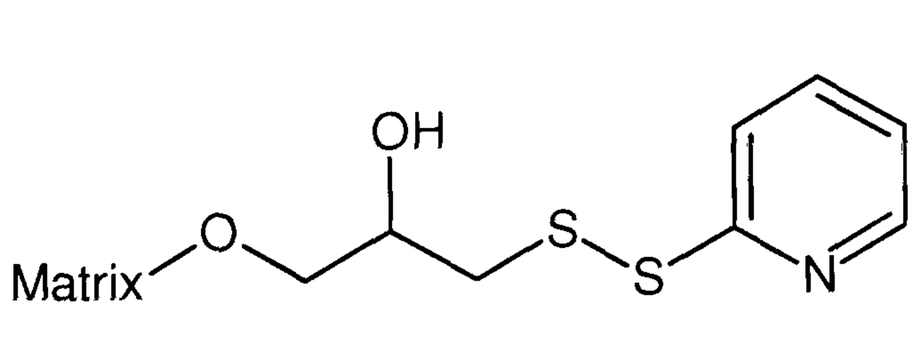
Maleimide based reagents have been designed (Bayer et al., 1985) and used for an enzyme-immunoassay (Fujiwara and Saita, 1987) and for labelling membrane proteins (Roffman *et al.*, 1986). Isotope-coded affinity tags (ICAT reagents) are specialised biotinylated reagents used to label proteins. They incorporate an isotope-labelled flexible linker and the iodoacetyl group for reaction with cysteine. They have found widespread use in quantitative proteomics experiments (Gygi *et al.*, 1999).

The isolation step takes place by passing the reacted biotinylated reagent through an affinity chromatography column containing (strept)avidin where it binds. The main difficulty of this technique is then to release the species of interest from the column. The binding of the native protein to biotin is so strong that the conditions required for the removal ligand require denaturation of the protein. Using immobilised monomers of avidin or streptavidin means less harsh conditions can be used to overcome this difficulty (Henrikson *et al.*, 1979). Another way to ensure release of the bound molecule is to incorporate a linker which is cleavable under milder conditions. Photo-cleavable linkers (Thiele and Fahrenholz, 1994) and fluoridolyzable linkers (Lin and Morton, 1991) have been synthesised. For reviews of avidin-biotin technology see Bayer and Wilchek (1990; 1988).

Despite the number of biotinylated reagents available for thiols, this method was not seen as having any significant advantages over the use of a solid-phase thiol specific bound reagent, and therefore was not investigated further.

4.2.4 Thiol-specific covalent chromatography gels

Thiol-specific gels for carrying out covalent chromatography have also been used for isolating thiols. There are several commercially available reagents consisting of the chromatography matrix, a linker group and a disulfide group, for example, thiopropyl sepharose 6B (**15**). These gels have been used for purifiying proteins and peptides (Krieger and Hook, 1991; Orford *et al.*, 1991). They depend on displacement reactions of disulfides. The thiol to be captured displaces the aromatic sulfide, the loss of which can be monitored by UV/Vis spectroscopy. The captured thiol is displaced by adding another thiol, usually dithiothreitol, DTT (**16**).



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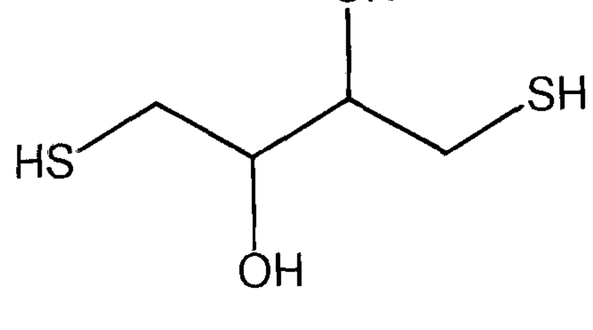


(16)

The advantage of this technique for the purposes of this research is that large quantities of food matrix could also be passed through the column to concentrate any thiols present. This technique also depends on the thiol used for the displacement having a higher reactivity than the attached thiol.

4.2.5 Metal and metal compound affinity

The affinity of sulfur with heavy metals such as platinum, gold, copper and mercury is well known. There are a number of commercially-available immobilised thiol compounds on solid-phase resins which are used as scavengers for metals in organic synthesis reactions (Mathew and Pillai, 1992; Saegusa *et al.*, 1978). The affinity between gold and sulfur is also exploited in electrochemistry in assembling mono-layers on surfaces (Hoang *et al.*, 2002; Takehara *et al.*, 1992). Much knowledge of this area has arisen because of investigations into how sulfur poisons certain catalysts, particularly platinum (Fischer and Kelemen, 1977). There are also a number of metal-based commercially available systems to remove hydrogen sulfide and other thiols from gas streams. Johnson Matthey's, Puraspec 2000 and 6000 series are based on copper, zinc and aluminium oxides reacting to form metal sulfides. These methods, whilst suitable for trapping thiols, are not designed for re-release of the sulfur compounds for analysis.

Studies on the use of metals for thiol isolation and release have been carried out by Kagel and Farwell (1986). Foils of silver, platinum, palladium, nickel, tungsten and ruthenium were used to collect seven sulfur-containing gases including two thiols, which were then thermally desorbed by passing a large controlled current through the foil and detecting compounds by flame photometry. Palladium, platinum and silver proved most successful at trapping thiol compounds with a detectability of less than 50 parts per trillion. As this study was focussed on the analysis of simple gaseous samples, the effect of other compounds from a complex matrix are not known.

Mercury salts have been used to extract sulfur compounds in the past, but these also react with sulfides, disulfides and thioesters (Jocelyn, 1972). An organo-mercury compound, *p*-hydroxymercuribenzoic acid, has been successfully used to extract thiol compounds from wine (Darriet *et al.*, 1993; Tominaga and Dubourdieu, 2006;

Tominaga *et al.*, 1998) and from heated meat products (Kerscher and Grosch, 1998). Mercury compounds have also been used in covalent chromatography where a cross-linked agarose gel containing phenylmercuric chloride was used to isolate the thiols from solvent extractions of food matrices (Full and Schreier, 1994; Schneider *et al.*, 2003). The use of mercury compounds for isolating thiols was not pursued in this research due to safety concerns.

4.2.6 Ionic liquids

A novel potential approach that was also considered for this work was the use of ionic liquids for the selective isolation of sulfur compounds. Ionic liquids are low volatility solvents made of ions rather than molecules which can give distinct selectivity and reactivity when compared to conventional organic solvents. This is due to the wide range of cations and anions that can be combined to give solvents with varied chemical and physical properties. Ionic liquids have been used as replacements for traditional volatile organic solvents in organic synthesis, solvent extraction, and electrochemistry. Several reviews have been published on this rapidly expanding field (Koel, 2005; Olivier-Bourbigou and Magna, 2002; Pandey, 2006).

Ionic liquids have been used as novel solvents for liquid-liquid extractions (Huddleston *et al.*, 1998), including for the extraction of azo dyes from waste water (Vijayaraghavan *et al.*, 2006), extracting heavy metal ions from aqueous solutions (Wei *et al.*, 2003), for extraction of amino acids from pharmaceutical samples and fermentation broth (Smirnova *et al.*, 2004) and phenols from environmental waste water (Ye *et al.*, 2007). Salts of gold compounds could potentially be used to separate thiol compounds from other organic compounds. Gold based ionic liquids already exist (Deetlefs *et al.*, 2002; Hasan *et al.*, 1999). One group of researchers have recently used a non-gold based ionic liquid for desulfurization of diesel and oils (Esser *et al.*, 2004). Although promising, the use of ionic liquids was rejected because of the time involved in possibly synthesising the ionic liquids, and the inexperience of this author using these materials.

4.2.7 Non-chemical methods

Thiols and other volatile sulfur compounds have also been analysed by methods which do not involve any chemical reactions of the thiol group, despite the difficulties associated with this 'direct' analysis. As mentioned in the introduction, adsorbents are commonly used to collect volatiles. Sorption methods followed by gas chromatography and detection with sulfur specific detectors such as flame photometric detection (FPD) have been used. Examples include isolation of isopropane thiol, methanethiol and sulfides in gas samples by SPME (Nielsen and Jonsson, 2002a; Nielsen and Jonsson, 2002b) and detection of low-volatility sulfur species and of thiols with PDMS from air and gaseous samples followed by thermal desorption-GC-MS analysis (Baltussen *et al.*, 1999). These authors also showed the limitations of traditional adsorbents such as Tenax and Carbotrap 300, which gave poor recoveries of certain sulfur compounds and artefact formation. Another study looked at the application of SPME to wines. Although several disulfides and sulifides were found and quantified, the one thiol studied could not be quantified due to interference from other early eluting peaks (Mestres *et al.*, 1998). Other authors have identified thiol compounds from simple solvent extraction of foods. For example, 2-heptanethiol was found in bell peppers extracted in this way (Simian *et al.*, 2004).

For techniques where derivatisation has not been used, a sensitive detection method is required, such as those that have been designed specifically for sulfur compounds, or a mass spectrometer. The most common of the sulfur specific detectors is the flame photometric detector. Other options include sulfur chemiluminescent, electron-capture, atomic emission, electrochemical and photoionization detectors. A review of sulfur-specific detectors has been published (Wardencki and Zygmunt, 1991).

4.2.8 Techniques chosen for further development

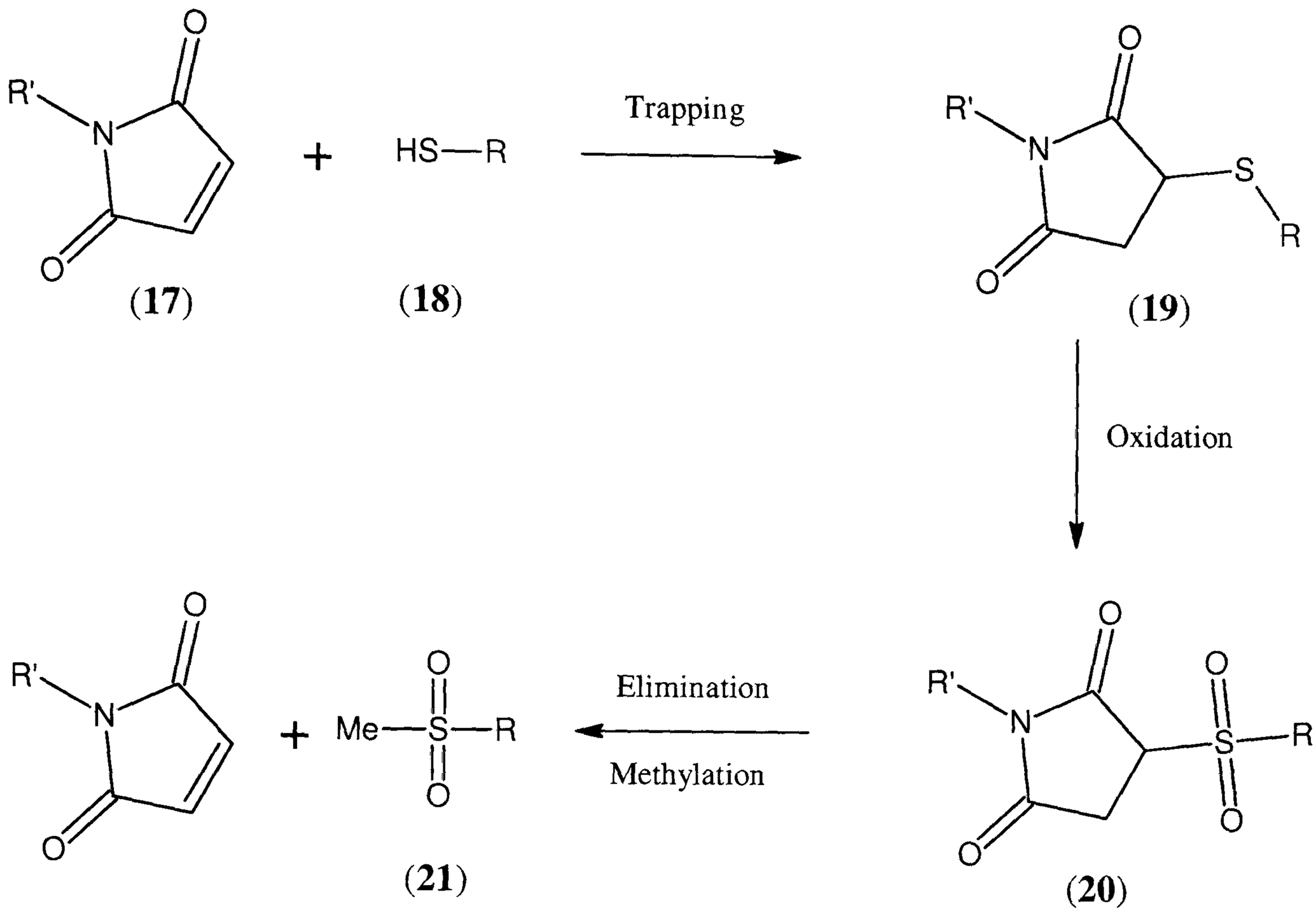
Three of the reviewed methods of isolating sulfur compounds were investigated further. Table 4.1 shows the methods chosen and the attractive features of each of the techniques. All of these methods have a component which is immobilised, which offers the possibility of simplified isolation procedures and concentration of analytes when compared with solution-phase reactions.

Maleimide functionalised silica beads	**Dilsufide based covalent chromatography**	**Gold wire**
Simple isolation step	Simple isolation step	Simple isolation step
Concentration of analytes	Concentration of analytes	Concentration of analytes
Selective towards thiols	Very high selectivity towards thiols	Selective towards thiols
Well-established reaction chemistry	Well-established reaction chemistry	Simple procedure
Novel application	Possibility of regenerating thiols for organoleptic analysis	

Table 4.1 Comparison of thiol trapping techniques chosen for further development and advantages of using these methods.

4.3 Maleimide reactions

A reaction protocol was developed to trap thiols and release a stabilised species. The chemistry was first optimised in stages by studies on a model thiol in the solution-phase before application to a solid-phase reagent. The synthesis steps are shown in Figure 4.7. The first step is addition of the thiol to the maleimide (see Figure 4.2 for mechanism). Rather than eliminating the thiol again once isolated, the second step involves oxidation (of the sulfur) to a sulfone. This is to allow for formation of a highly stable species for characterisation which is formed on elimination in conjunction with methylation. This step also introduces an extra selectivity: if other nucleophiles have reacted, only sulfones will be oxidised. The disadvantage of this is that the original thiol is not available for assessment of organoleptic qualities, and it introduces more steps, increasing analysis time and complexity.



(**a**) R' = Ethyl (solution-phase reactions) or (**b**) silica-bead (solid-phase reactions).
R = $(CH_2)_5CH_3$

Figure 4.7 Overall reaction protocol for reacting thiol and releasing reacted species, (a) solution-phase method development reagents (b) solid-phase reagents.

For the solution-phase reactions, structural determination was carried out using a variety of analytical tools, including NMR, Raman and IR spectroscopies, GC-MS and LC-MS. Analysis of solid-phase reactions is more difficult (as described in Section 1.8) and this limits the analytical tools available. Raman and FTIR spectroscopies were used to monitor the solid-phase reactions.

Hexane-1-thiol was chosen as a model compound because of its simple structure and because it is of a similar molecular weight to the type of thiols usually found as flavour compounds. N-ethyl maleimide was used for solution-phase reactions. A silica-based reagent was chosen for the solid-phase reagents. This was selected rather

than other polymer based solid supports for a number for reasons as shown in Table 4.2.

Silica based solid-phase reagent	**Organic polymer based solid-phase reagent**
Active sites are on the surface where they are accessible, giving fast kinetics and high yields	Active sites are inside the matrix so the rate of reaction is largely dependent on the rate of diffusion through the polymer.
Solvents are not required to swell the silica.	Solvents must be used to swell the reagent which also means that products can get trapped inside the material, thus affecting the yield.
The silica used is a porous, irregular system to maximize the loading and is not affected by organic solvent since the pore structure is rigid and permanent.	Can be affected by the organic solvent.

Table 4.2 Comparison of silica based and organic polymer based solid-phase reagents.

The functionalised silica is supplied as 'end capped' where residual non-functionalised groups are reacted with trimethylsilylchloride to form Si-O-Si$(CH_3)_3$. This gives an overall structure as shown in Figure 4.8. The end-capping process renders the silica non-acidic and non-polar, to prevent non-specific binding with the surface and maximise the reaction with the grafted function. The functionalized silica has a pH stability range between 2 and 12 because the residual OH's are end-capped. Silica is also compatible with aqueous matrices: this is important for analysing some food matrices. Like other polymer supported reagents, the materials can easily be packed into solid-phase extraction cartridges or other cartridges used in automated synthesis.

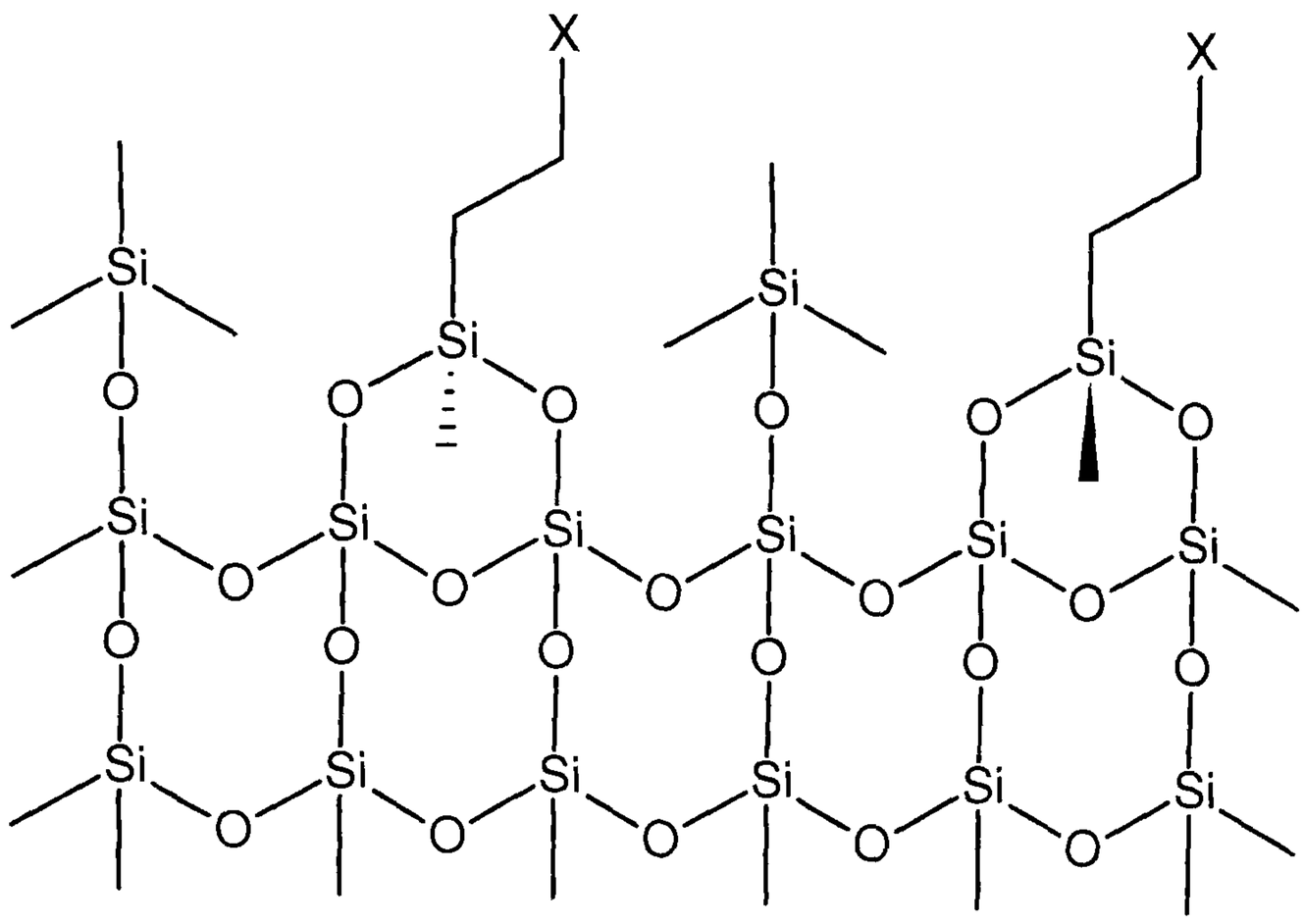


Figure 4.8 Representation of the surface structure of functionalised silica (Silicycle, 2003).

4.3.1 Solution-phase method development

4.3.1.1 Trapping

The first step in the derivatisation procedure is the trapping of the hexane-1-thiol (**18**) by N-ethyl maleimide (**17a**). The reaction was optimised for the reaction time and the amount of base used to catalyse the reaction. The reaction was monitored by thin layer chromatography. The optimised reaction conditions used a low concentration of base and a reaction time of 4 hours at room temperature. The reaction was repeated several times to demonstrate consistency. The product sulfide, 1-ethyl-3-(hexylthio)pyrrolidine-2,5-dione (**19a**), was obtained in high yield (typically greater than 90%).

Several techniques were used for structural characterisation of the product (but not for every repeat experiment). Proton NMR found the sulfide to be consistently of greather than 95% purity. FTIR data indicate the loss of an absorbance due to the C=C bond (1586 cm^{-1}) and shift in C=O symmetric and asymmetric vibration peaks (1697 cm^{-1} and 1774 cm^{-1}). The GC-MS (EI) chromatogram of the reaction mixture is shown in Figure 4.9. The main species was identified as the expected product from the fragment ions, as no molecule ion was observed. (Note also the presence of the impurity 1,1'-dithiodihexane, which may have been formed during the reaction or

chromatographic analysis). The ^{1}H NMR data were as follows: (500 MHz, $CDCl_3$) δ ppm 0.87 (t, J=6.6 Hz, 3 H) 1.10 - 1.21 (m, 1 H) 1.16 (t, J=7.2 Hz, 2 H) 1.23 - 1.41 (m, 6 H) 1.53 - 1.69 (m, 2 H) 2.50 (dd, J=18.6, 3.7 Hz, 1 H) 2.62 - 2.79 (m, 1 H) 2.85 (dt, J=8.3, 6.3 Hz, 1 H) 3.10 (dd, J=18.6, 9.2 Hz, 1 H) 3.56 (q, J=7.33 Hz, 2 H) 3.68 (dd, J=9.2, 3.7 Hz, 1 H)

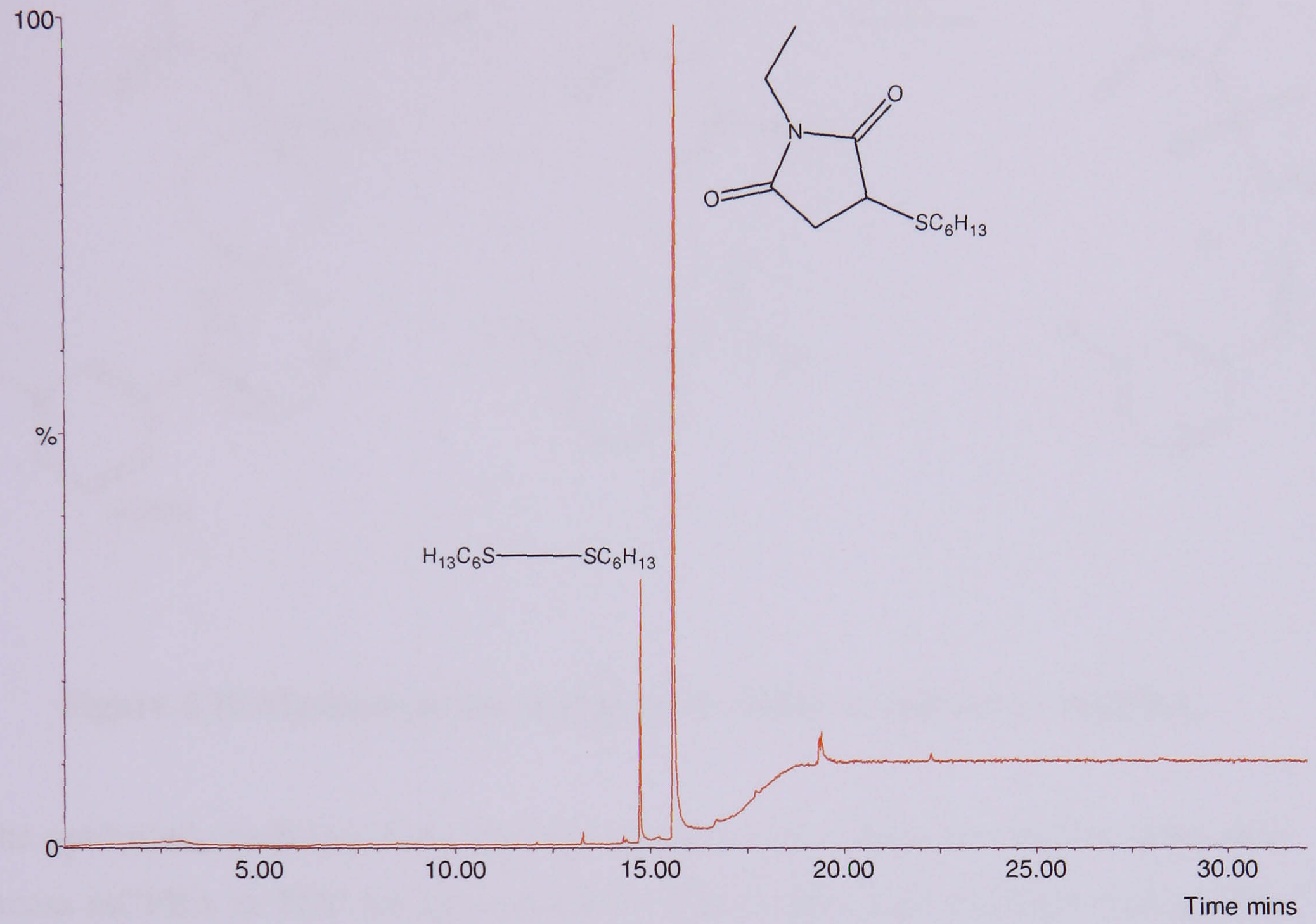


Figure 4.9 GC-MS (EI) chromatogram of reaction mixture showing the expected product, 1-ethyl-3-(hexylthio)pyrrolidine-2,5-dione, and the impurity 1,1'-dithiodihexane.

4.3.1.2 Oxidation

In the next step, the sulfide (**19a**) is oxidised to the sulfone, 1-ethyl-3-(hexylsulfonyl)pyrrolidine-2,5-dione (**20a**). The strong oxidising agent *m*-chloroperoxybenzoic acid (mCPBA) generates a sulfoxide (S=O) with one equivalent of reagent and the sulfone with two equivalents $(S=O)_2$. This step is to generate a more stable species for elimination. The mechanism for the reaction is shown in Figure 4.10. mCPBA is a well-known oxidising reagent (March, 1992; Paquette and Carr, 1990; Pelter *et al.*, 1987). Yields of sulfones and sulfoxides have been shown to be excellent, even in the presence of other reactive moieties such as

amino, olefinic or acetylenic moieties (Craig and Purushotharman, 1970; Delia *et al.*, 1965).

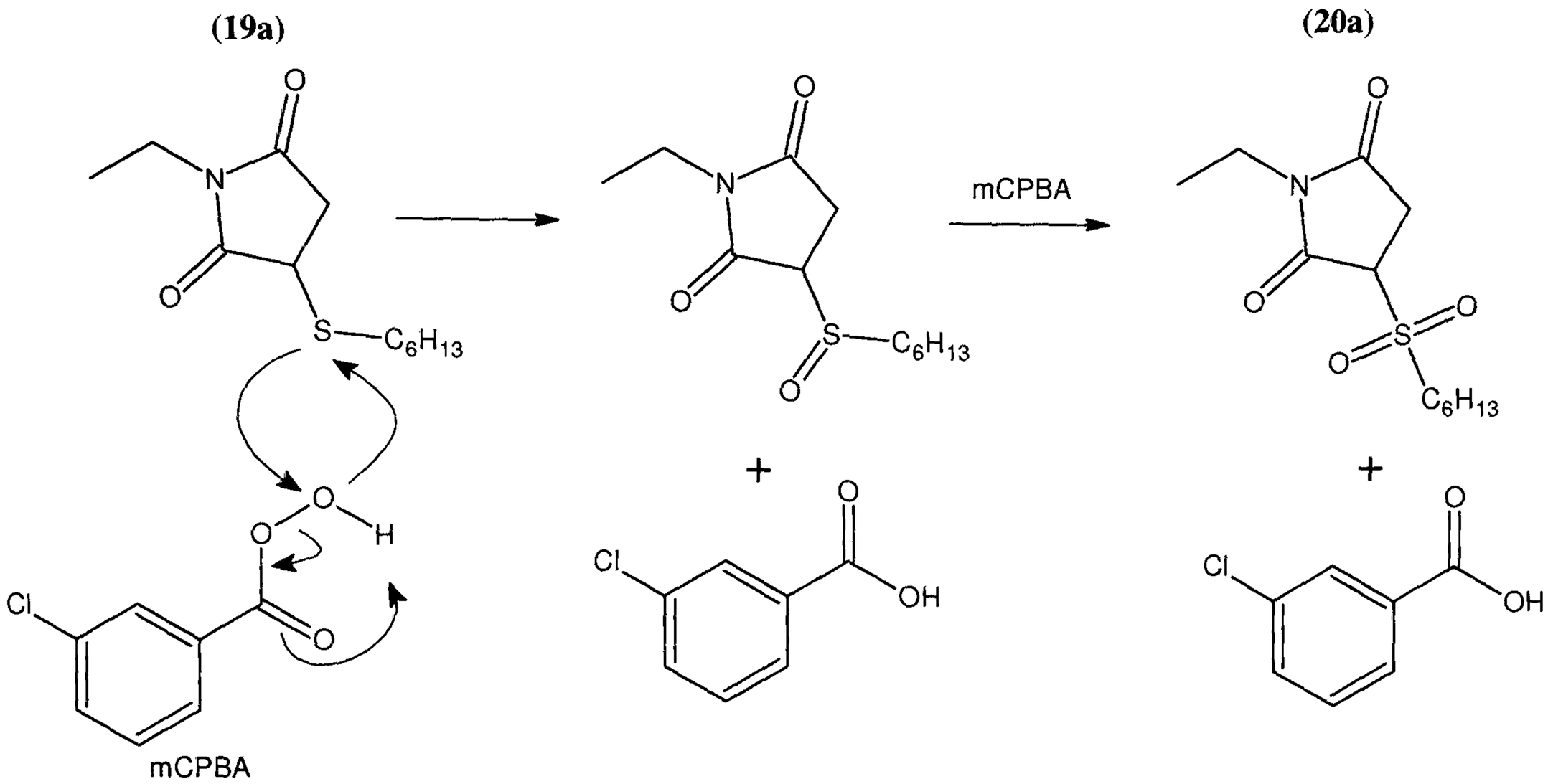


Figure 4.10 Mechanism for oxidation of sulfide to sulfone by mCPBA.

The optimised conditions found for this reaction were to react the sulfide (**19a**) with excess mCPBA in THF for approximately 4 hours with ice-water bath cooling. The product was purified by extraction into dichloromethane. The product sulfone (**20a**) was obtained at greater than 99% purity as determined by ^{1}H NMR in the most effective reaction. ^{1}H NMR (500 MHz, $CDCl_3$) δ ppm 0.89 (t, J=6.9 Hz, 3 H) 1.18 (t, J=7.3 Hz, 2 H) 1.23 - 1.37 (m, 5 H) 1.47 - 1.55 (m, 2 H) 1.9 (dt, J=15.8, 7.83 Hz, 1 H) 2.99 (dd, J=19.1, 9.6 Hz, 1 H) 3.30 (dd, J=19.3, 4.0 Hz, 1 H) 3.40 - 3.46 (m, 1 H) 3.60 (d, J=7.3 Hz, 1 H) 4.19 (dd, J=9.5, 4.0 Hz, 1 H).

The sulfone was also analysed by LC-MS (ESI +ve mode, low cone voltage). The mass chromatogram for the protonated molecule (*m/z* 276), the UV chromatogram and the TIC are shown in Figure 4.11. Figure 4.12 shows the mass spectrum of the sulfone. $[M + Na]^+$, $[M + NH_4]^+$ and $[M + Na + ACN]^+$ adducts were observed.

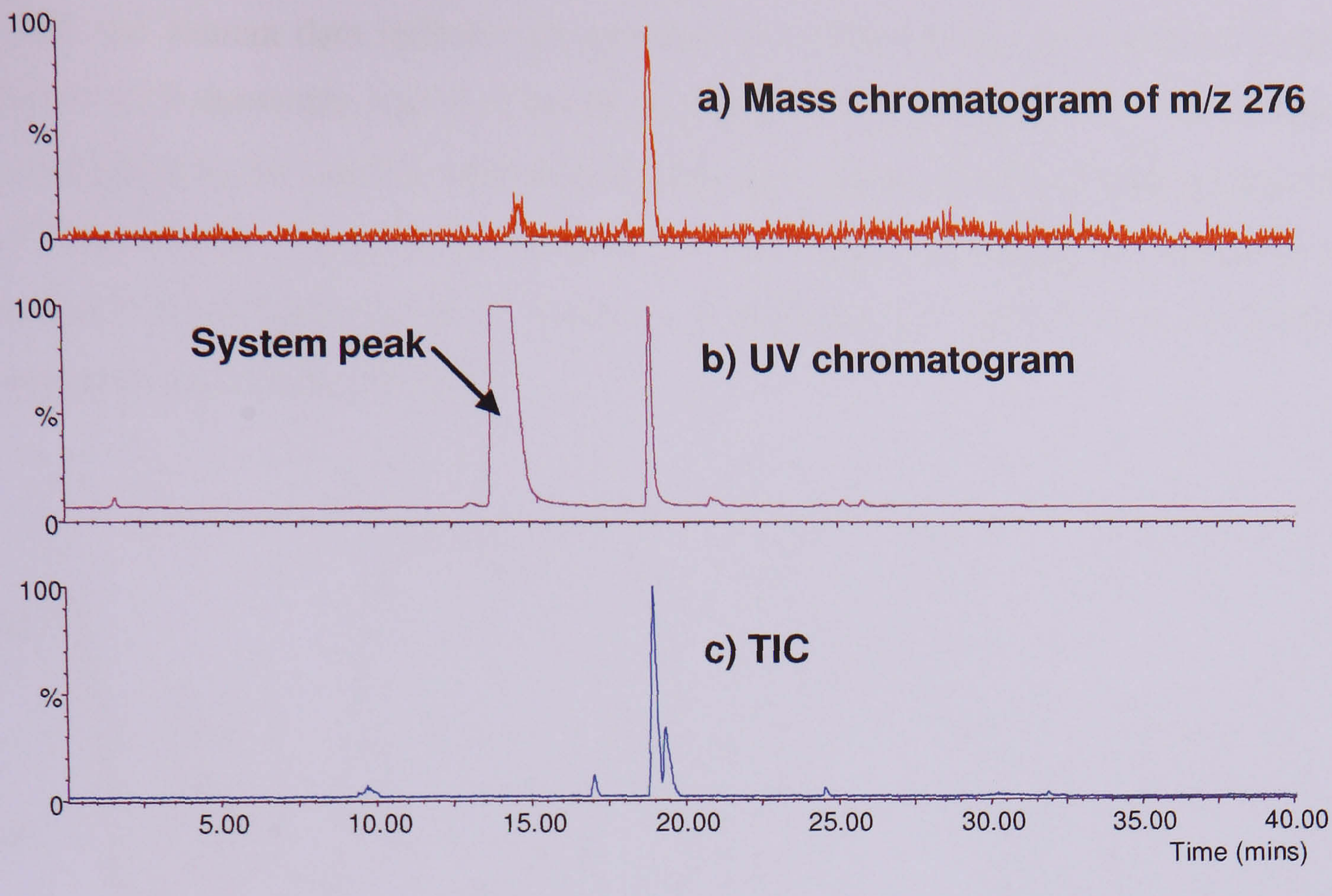


Figure 4.11 LC-MS chromatograms for product of oxidation reaction.

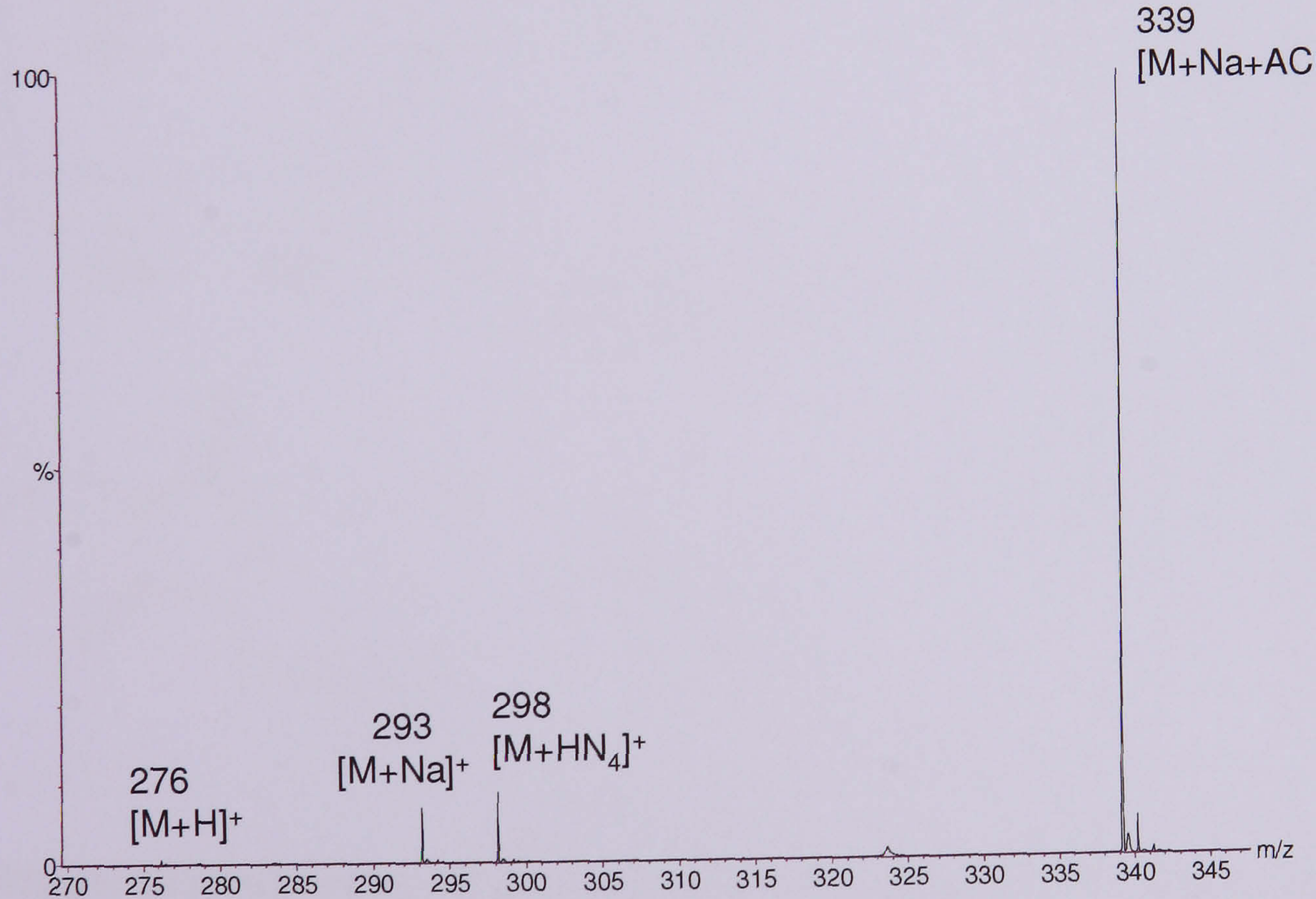


Figure 4.12 Mass spectrum of product sulfone (19a).

FTIR and Raman data indicate the presence of sulfone bonds and carbonyl bands. Figure 4.13 shows the region of the IR spectrum for the sulfone bands. The product is compared to the starting material and a standard sulfone to aid with the assignment of these bands. There is no evidence of incomplete oxidation to sulfoxide or hydroxyl functionality in the IR spectra. A weak band at 1171 cm^{-1} in the Raman spectrum was assigned as S=O.

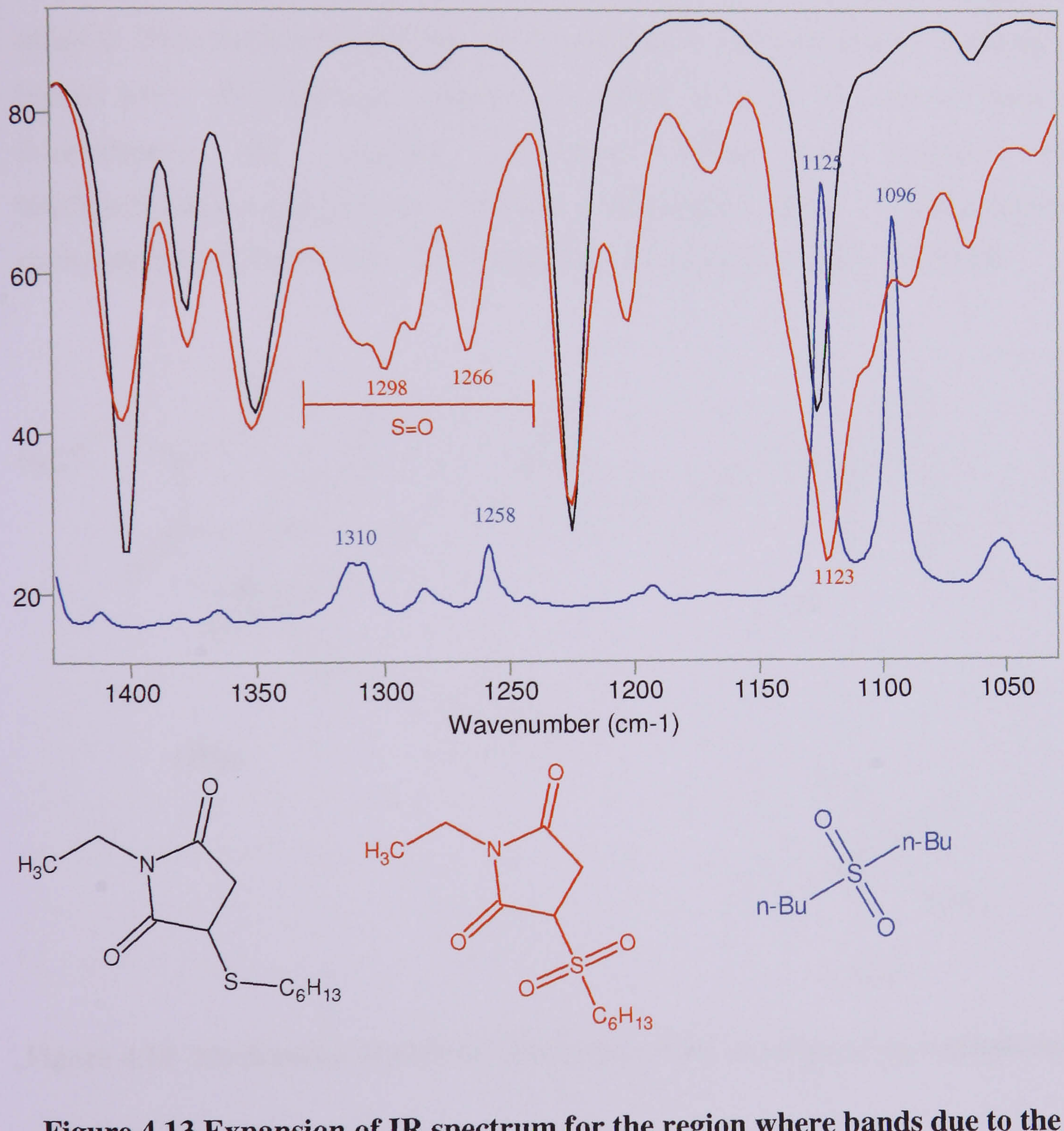


Figure 4.13 Expansion of IR spectrum for the region where bands due to the S=O bonds should appear. The starting materials (black), sulfone product (red) and a standard sulfone (blue) are shown.

4.3.1.3 Elimination

The final step is the release of the sulfone from the maleimide ring. The elimination can be achieved with heat, very strong base such as butyl lithium, or moderate base. The last was chosen for the mildest conditions, given that there are potentially unknown functional groups in the thiol to be trapped that could be affected by the reaction conditions. A hindered, non-nucleophilic base is required because an unhindered amine base could attack the carbonyl groups, resulting in nucleophilic addition. The reaction mechanism for β-elimination and methylation is shown in Figure 4.14. An alkylating reagent is required to 'trap' the sulfone, such as iodomethane. The suitability of three bases was assessed: 1,8-bis(dimethylamino)naphthalene (Proton Sponge®) (**21**), 2,6-ditertbutyl-4-methylpyridine (DBMP) (**22**), 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU) (**23**).

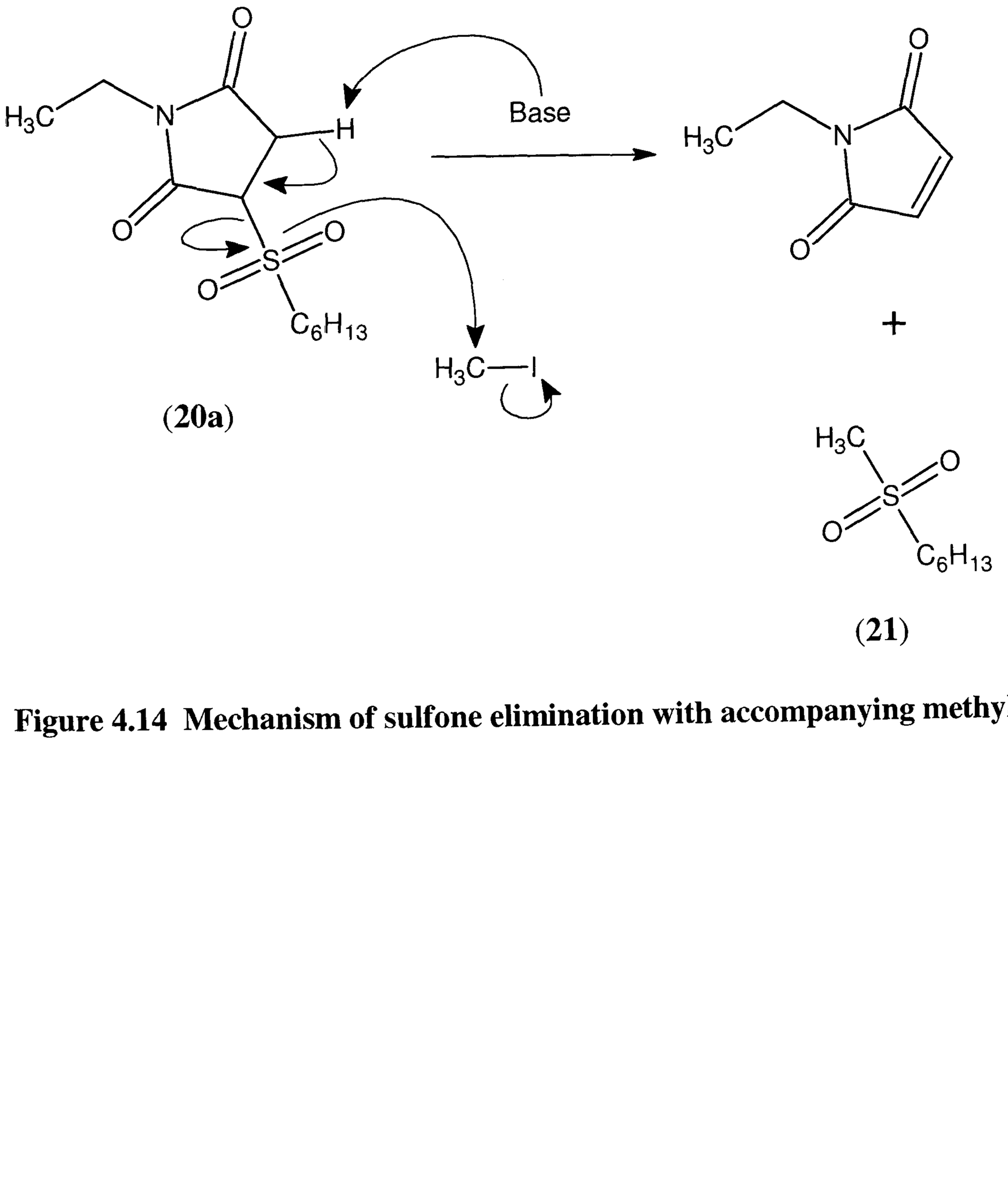
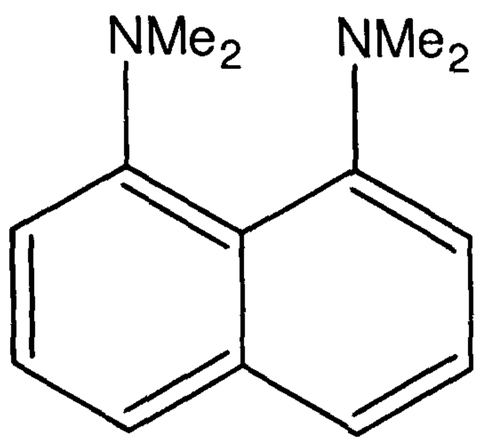


Figure 4.14 Mechanism of sulfone elimination with accompanying methylation.



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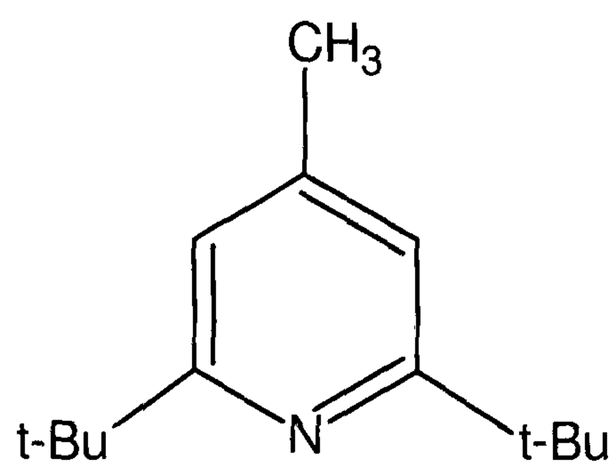

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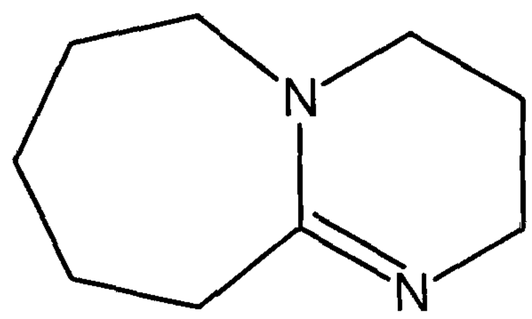

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In trial reactions, each base was reacted with the sulfone (**20a**) in the protic solvent, THF, in the presence of iodomethane for four hours at room temperature. The reaction solution was washed with 10% aqueous HCl to remove any excess base and the aqueous layer extracted by hexane. Only DBU was found to give the desired sulfone, 1-(methylsulfonyl)hexane (**21**), as indicated by LC-MS, GC-MS and ^{1}H NMR data. The LC-MS chromatograms of *m/z* 165.1 and the total ion count are shown in Figure 4.15 illustrating the identified compound with a retention time of 15.2 minutes. Note that this compound was found to ionise poorly. The TIC also shows that there are several impurities. The ^{1}H NMR data for the sulfone was as follows: (500 MHz, $CDCl_3$) δ ppm 0.88 (dt, J=8.9, 7.0 Hz, 4 H) 1.17 - 1.36 (m, 5 H) 1.39 - 1.47 (m, 1 H) 1.43 (d, J=7.6 Hz, 1 H) 1.84 (dt, J=15.8, 7.8 Hz, 1 H) 2.89 (s, 2 H) 3.00 (d, J=16.2 Hz, 1 H)

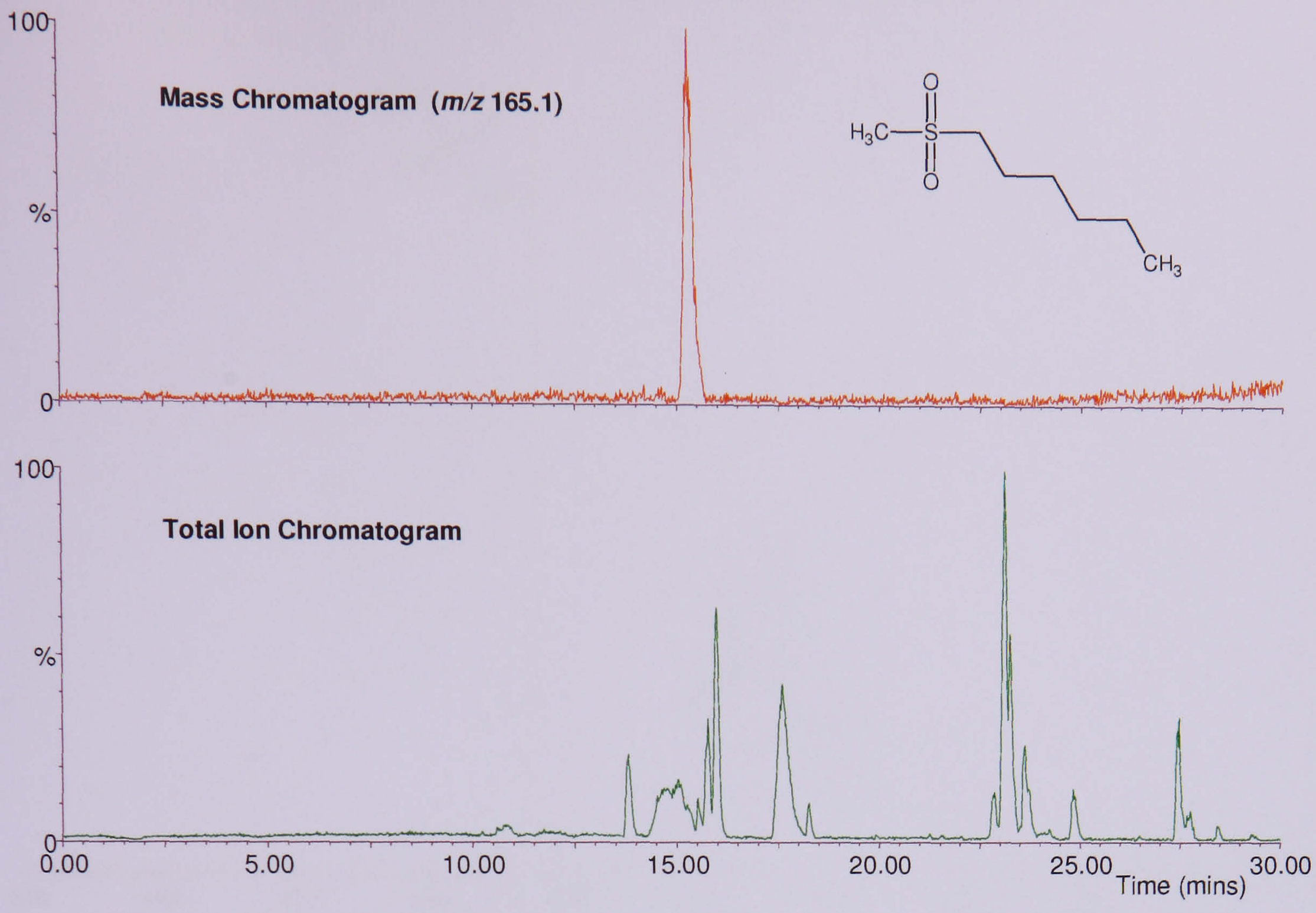


Figure 4.15 LC-MS (ESI +ve) chromatogram of the reaction product from the elimination reaction (non-^{13}C labelled)

The elimination reaction was repeated using DBU, and ^{13}C-labelled iodomethane, to aid identification by ^{13}C NMR and MS. There is evidence for the desired ^{13}C-labelled sulfone from ^{1}H and ^{13}C NMR data and GC-MS data. The ^{13}C NMR data was dominated by one peak due to the ^{13}C labelled methyl group indicating that methylation had taken place as expected. ^{13}C NMR (100 MHz, $CDCl_3$) δ ppm 54.5 (CH_2), 40.5 ($^{13}CH_3$), 31.3 (CH_2), 28.2 (CH_2), 22.5 (CH_2), 22.4 (CH_2), 14.0 (CH_3).

GC-MS data also show a small amount of unreacted starting sulfone (**20a**) (Figure 4.16). Later repeats of this experiment appear to have generated some ring opening, catalysed by the DBU. This is suggested by FTIR data where each carbonyl vibration (symmetric and asymmetric) is a doublet suggesting there are two slightly different imides present. There was no evidence of the carbonyl resonances from maleimide but some evidence for ethyl residues by ^{1}H NMR. It does not appear that N-ethyl maleimide was regenerated as expected.

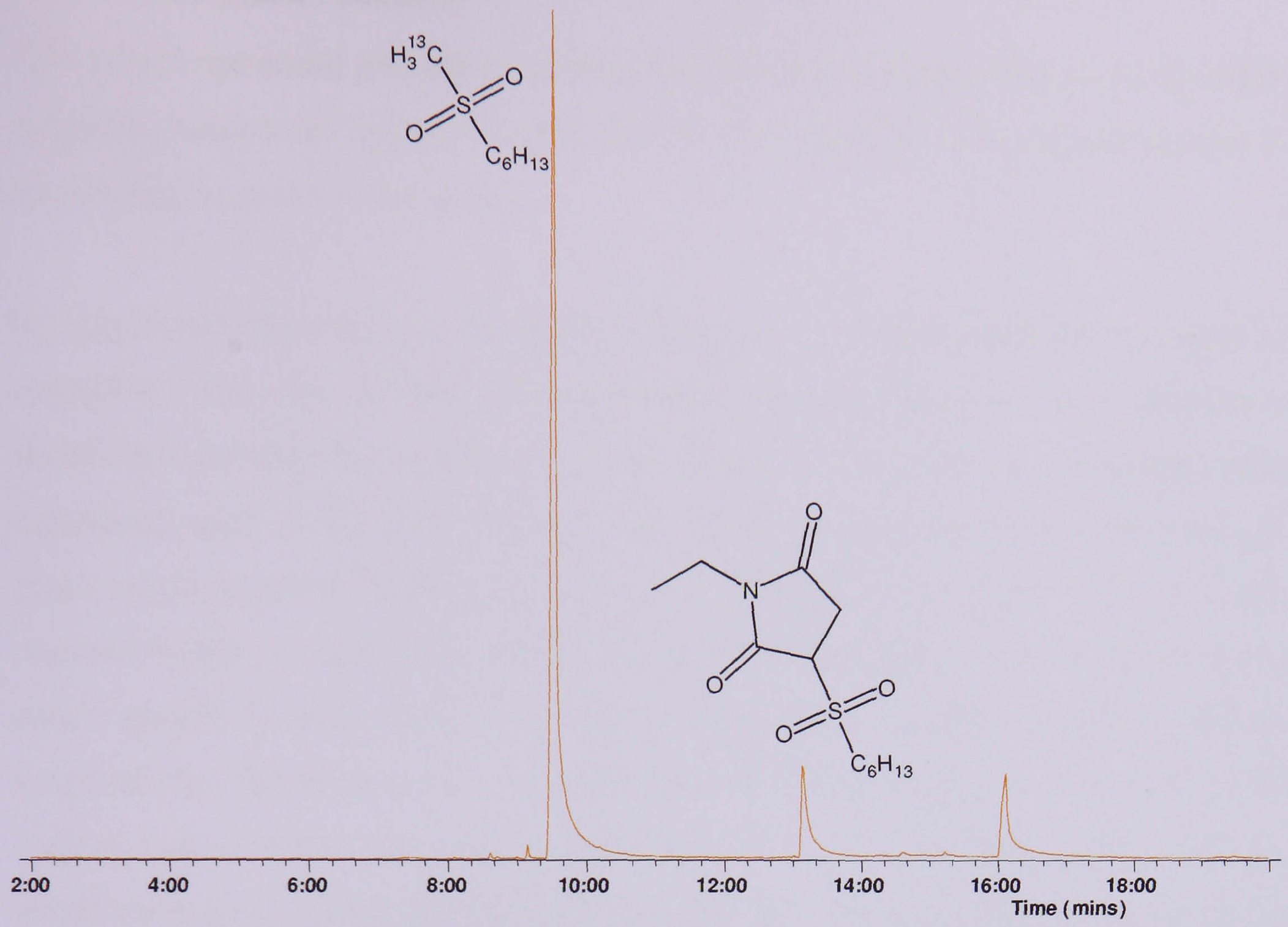


Figure 4.16 GC-MS chromatogram of the reaction product from the elimination reaction (^{13}C labelled).

During the course of the reaction, a black solid was precipitated. ^1H NMR and LC-MS analysis indicated that the major component was a methylated form of DBU with a molecular weight of 169. The methylation of DBU is an undesirable side-reaction which prevents the complete formation of the desired sulfone. It was speculated that the formation of methylated-DBU occurs when DBU and iodomethane are present in solution together. To minimise this possibility in later reactions, DBU was added to the sulfone and allowed to react before iodomethane was added. It was found that this problem was not entirely overcome by this two stage process, as DBU and methylated DBU were also identified from the reaction mixture in a later experiment when the materials were added separately (identified by ^1H NMR and LC-MS).

Although the elimination reaction was not fully optimised, the chemistry developed in the solution-phase was next applied to the silica supported maleimide, to demonstrate the principle.

4.3.2 Solid-phase reactions

The overall optimised procedure (as outlined in Figure 4.7) was applied to the solid-supported reagent the silica-maleimide (R' = Silica-$(CH_2)_3$). Once again, hexane-1-thiol is used as a model compound.

By using supported reagents, the product work-up and isolation procedures should be simplified. The use of this silica-supported reagent, however, also presents a significant problem for monitoring the progress of the reaction. Solution-phase techniques such as LC-MS, GC-MS and NMR are not applicable and although magic-angle-spinning NMR may be used, it was not available for this work. Analysis therefore is limited to FTIR and Raman spectroscopy, both of which can be easily applied to solids analysis. During analysis of samples by use of Raman spectroscopy, fluorescence was also identified as a major problem, and thus useful analysis was mainly restricted to FTIR. Further complications arose from the relative levels of organic and inorganic material because the loading of maleimide on silica is only 0.74 $mmol.g^{-1}$. This means that weak spectral bands (such as the C=C stretch) can be obscured by the intensity of the silica absorbance.

4.3.2.1 Trapping

The conditions developed in the solution-phase reactions (4 h reaction time, 0.1M base for catalysis) were used for the trapping step. The silica was analysed by FTIR and Raman spectroscopy, after a 4 hour period of shaking in the presence of hexane-1-thiol and filtering. The most useful indicator of reaction was a slight shift in the change in position of the major carbonyl absorbance from 1706 cm^{-1} in the starting maleimide to 1700 cm^{-1} in the product. This suggests there is a change in the imide carbonyl environment and, although it is consistent with loss of the maleimide double bond, it is not conclusive. Note that this shift of the carbonyl absorbance to lower wavenumber does not match the observed changes in the solution-phase reactions. The C=C stretching vibration (which can just be resolved at 1585 cm^{-1} in the starting material) is too weak in the FTIR spectrum to be used as an indicator for the extent of reaction. The silica absorbance was found to hinder the analysis of the spectra.

Raman spectra were gathered from different areas of the sample. These data revealed differences that indicate that the product is not homogenously dispersed on the silica adsorbent. One sample showed no evidence of the maleimide component either reacted or unreacted, the spectrum being dominated by the silica. Another sample, however, did show the presence of organic components, with evidence of the imide carbonyl at 1771 cm^{-1} and a weak absorption at 1583 cm^{-1}, consistent with the maleimide unsaturation (Figure 4.17). This is at much lower levels than in the starting material; indicating that some, but not all, of the unsaturation has been lost.

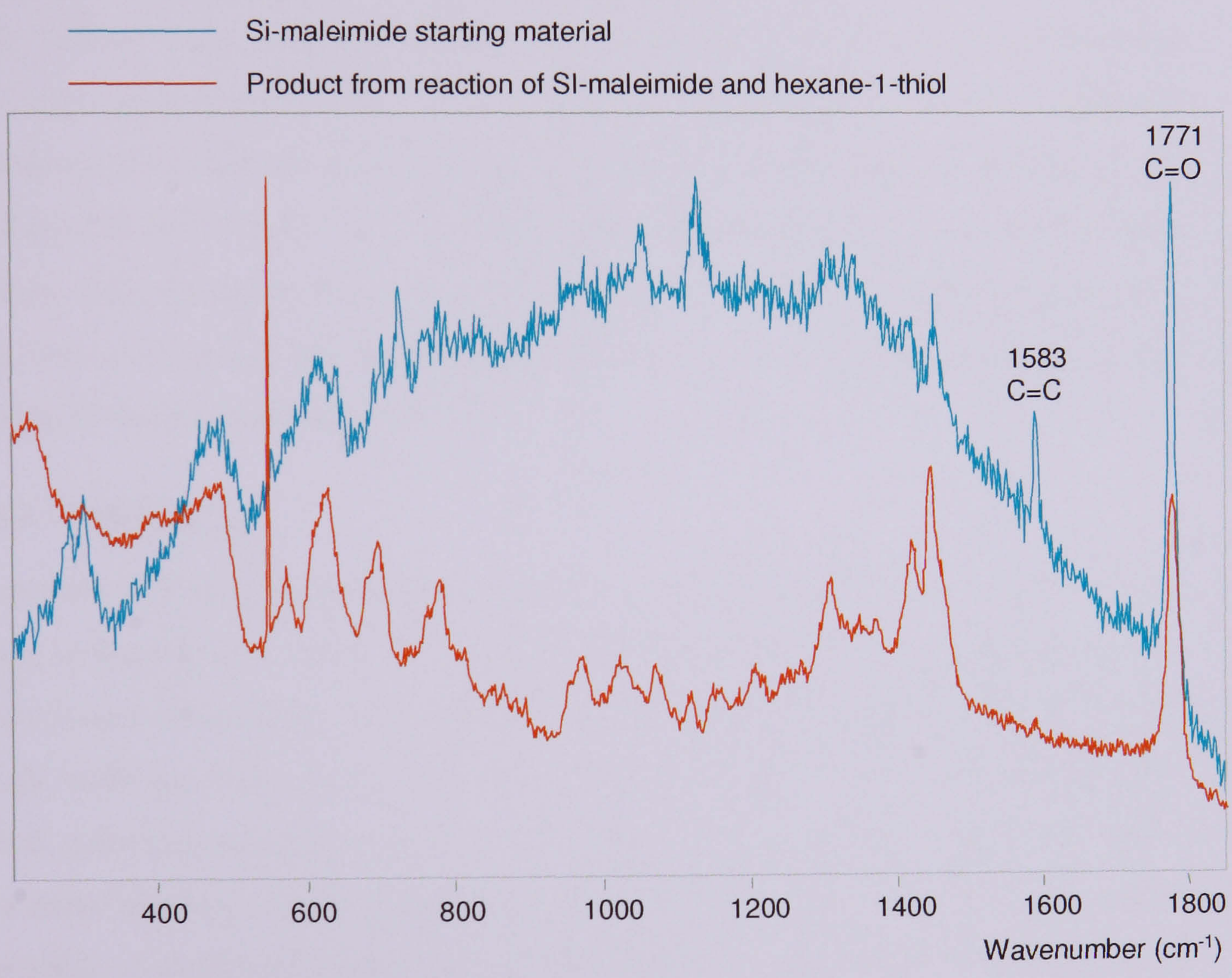


Figure 4.17 Raman spectra of Si-maleimide starting material (blue) and product from trapping reaction (red).

To conclude, the evidence suggested that the reaction had been partially successful, although there is still some C=C unsaturation present. Some loss of material during the filtration process was also observed. The finest silica particles passed through the filter paper, and may have carried attached thiol with them. To counter this, silica maleimide was pre-filtered before use.

4.3.2.2 Oxidation

The reaction conditions developed in the solution-phase were applied to effect the transformation of sulfide (**19b**) to the sulfone (**20b**) using mCPBA. Assuming that the sulfide was made in the first step, FTIR analysis of the product from the oxidation reactions is inconclusive. The FTIR spectrum indicates that the there has been a change in the environment of the maleimide carbonyl stretch during the reaction. The carbonyl band has moved from the position it was in for the sulfide sample from 1698 cm^{-1} to 1706 cm^{-1}, which is more similar to the original silica-maleimide starting material. There is a broad peak at 1325 cm^{-1}, which is too high to be the sulfone bands seen previously in the spectrum of standards of sulfones (at 1170 cm^{-1} (symmetric) and 1311 – 1269 cm^{-1} (asymmetric) and is currently unassigned. The lack of these bonds may be due to the intensity of the silica absorption in that region. This similarity was not observed in the solution-phase reactions. The Raman spectral analysis is inconclusive but the FTIR suggests that some sort of reaction has taken place. There is no conclusive evidence of the presence of sulfone functionality.

4.3.2.3 Elimination

The elimination step was attempted despite the uncertainty of the formation of the sulfone in the previous stage. The same reaction conditions were used as for the solution-phase experiment. As the product is released into solution, NMR, FTIR and LC-MS analysis could be used. Proton NMR analysis of the residue from the reaction solution (once the silica had been removed by filtration) showed that there was a small amount of sulfone (**21**) present, but that the majority of the sample was either DBU or methylated-DBU. The NMR assignment for the sulfone is as follows: ^{1}H NMR (500 MHz, $CDCl_3$) δ ppm 0.82 - 0.94 (m, 3 H) 1.06 (t, J=7.2 Hz, 1 H) 1.15 - 1.36 (m, 5 H) 1.44 (ddd, J=14.7, 7.3, 7.1 Hz, 1 H) 1.77 - 1.96 (m, 2 H) 2.71 (s, 1 H) 2.95 - 3.06 (m, 2 H).

Figure 4.18 shows the LC-ESI (positive ion mode) mass chromatograms of the reaction product. The mass chromatogram of *m/z* 182 for the ammonium adduct of 1-(methylsulfonyl)hexane is shown in Figure 4.18(a) and TIC in Figure 4.18(b). The two large peaks in the TIC are system peaks. Note the poor ionisation of the sulfone as shown by the small peak in the TIC at retention time of 14.6 mins. The mass

spectrum for the compound assigned as 1-(methylsulfonyl)hexane contains a number of adduct ions: *m/z* 182 $[M+NH_4]^+$, *m/z* 203 $[M+K]^+$, *m/z* 206 $[M+H+ACN]^+$, *m/z* 223 $[M+NH_4+ACN]^+$, *m/z* 228 $[M+Na+ACN]^+$.

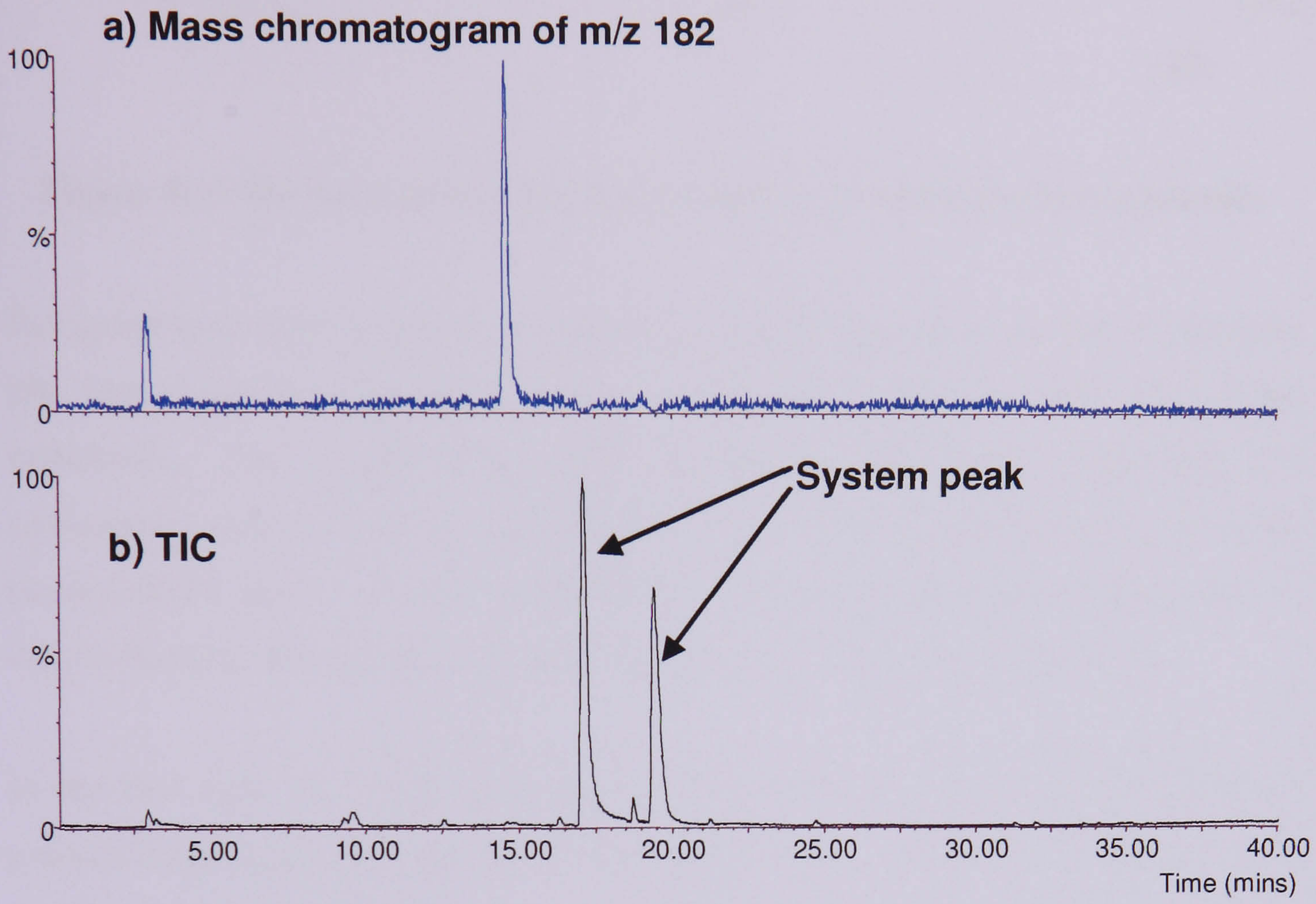
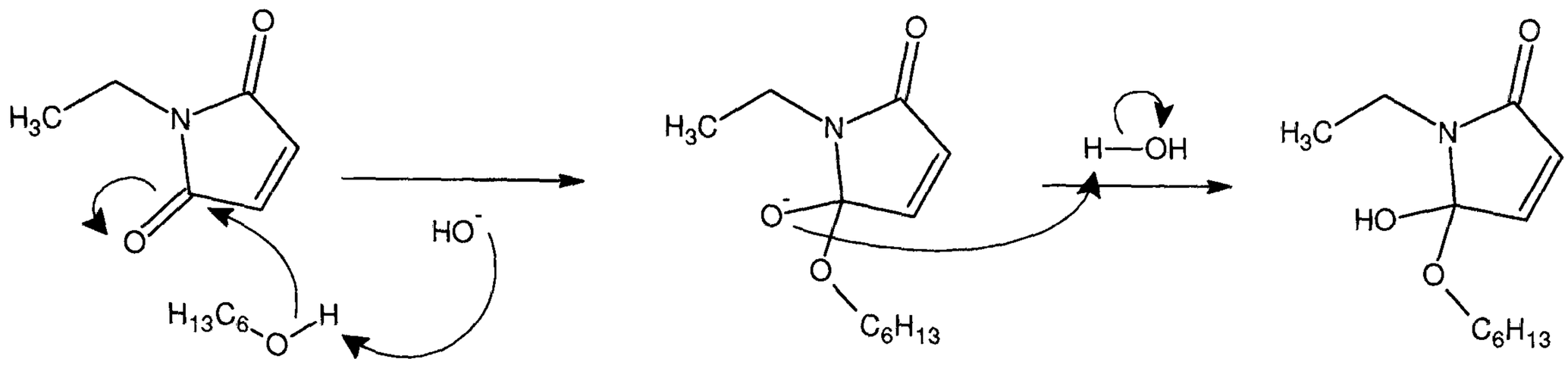


Figure 4.18 LC-ESI+ mass chromatogram (low cone voltage) of products from solid-phase elimination reaction.

4.3.3 Investigation of selectivity of reagents

It was stated earlier that one of the key requirements for a successful derivatisation method is selectivity. To confirm that maleimide is unreactive towards alcohols the first step of the derivatisation procedure was applied to solutions containing both hexane-1-thiol and hexan-1-ol. The most probable competitive reaction of the alcohol as a hard nucleophile is to react via 1,2-addition to the carbonyl to form a hemiacetal (**25**) (Figure 4.19).



(25)

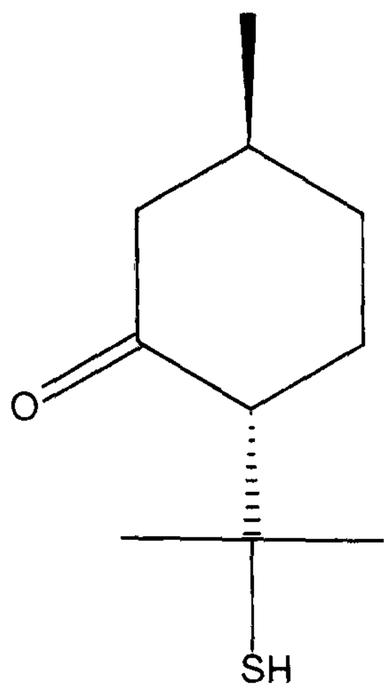
Figure 4.19 Mechanism for most likely reaction of alcohols with maleimide.

The trapping reaction was carried out using two different concentrations of reactants. The first experiment contained equimolar amounts of thiol, alcohol and N-ethyl maleimide. The second experiment contained excess N-ethyl maleimide (1.5 equivalents) and alcohol (5 equivalents). This would indicate firstly, if excess alcohol might lead to a higher likelihood of reaction and secondly, if all hexane-1-thiol is reacted, whether excess alcohol would react with excess maleimide.

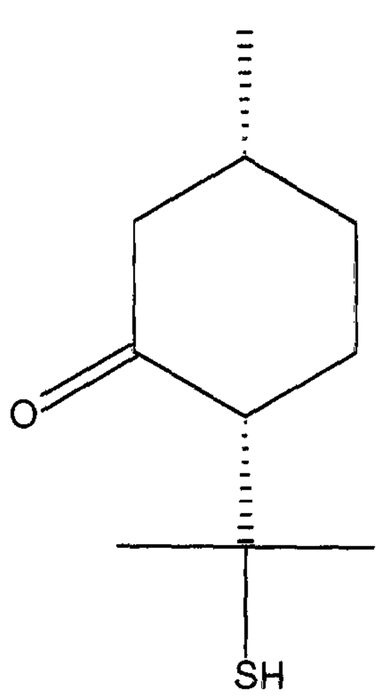
In the first case, ^{1}H NMR data showed only evidence of the expected reaction product between the thiol and maleimide. The sulfide product (**19a**) and hexan-1-ol were present in approximately 1:1 ratio, indicating that the reaction between hexane-1-thiol and N-ethyl maleimide is not adversely effected by the presence of hexan-1-ol. There was no evidence of unreacted maleimide or the hemiacetal (**25**) (expected from reaction of the alcohol) in the ^{1}H NMR spectrum. The product mixture from the second experiment contained unreacted maleimide, the sulfone product (**19a**) and unreacted hexan-1-ol in the expected ratios (^{1}H NMR data not shown). These experiments demonstrate the selectivity of this reaction towards thiols.

4.3.4 Reaction with Buchu leaf essential oil

The reaction chemistry was next investigated using an essential oil commonly referred to as Buchu leaf oil, an extract of the South African shrubs *Agathosma betulina* and *A. crenulata.* The extract is known to contain the isomers trans-*p*-mentha-8-thiol-3-one (**26**) and cis-*p*-mentha-8-thiol-3-one (**27**) at approximately 3% and many other components such as terpenes (major components), alcohols, ethers, ketones, hydroxyketones and thioesters (Kaiser *et al.*, 1975; Posthumus and Beek, 1996).



(26)



(27)

The reactions developed in the solution and solid phases were applied to trap the thiols present in this essential oil (Figure 4.20). The reaction was carried out in the solution-phase despite the potential complexity due to the presence of all the other species in Buchu leaf oil. The reaction mixture at each stage was analysed by LC-MS and GC-MS to search for components with the correct molecular weight of the species as shown in Table 4.3.

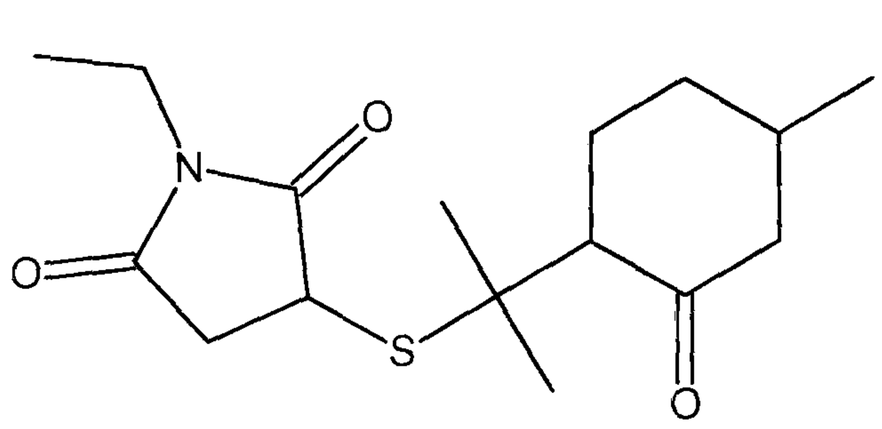
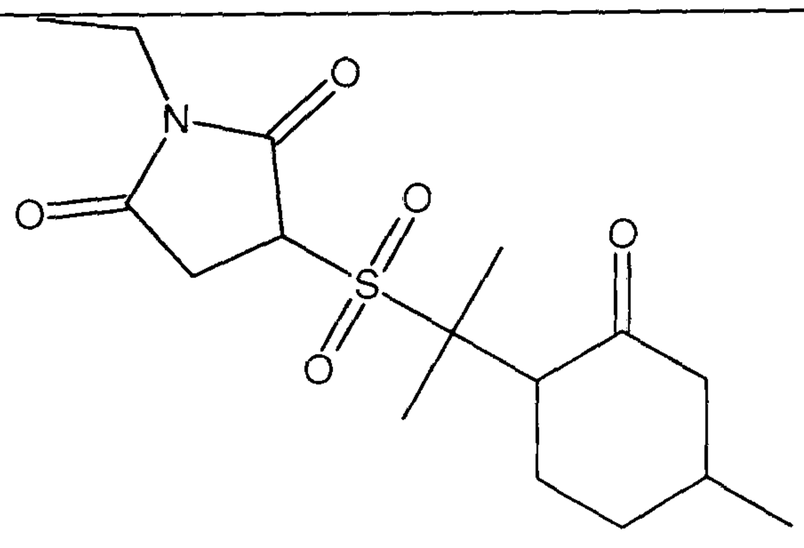
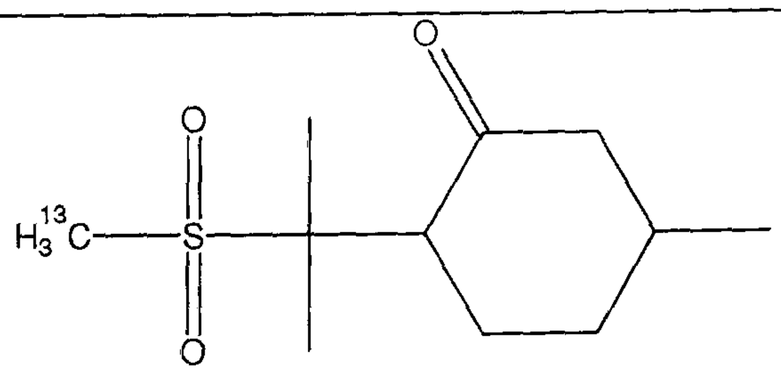
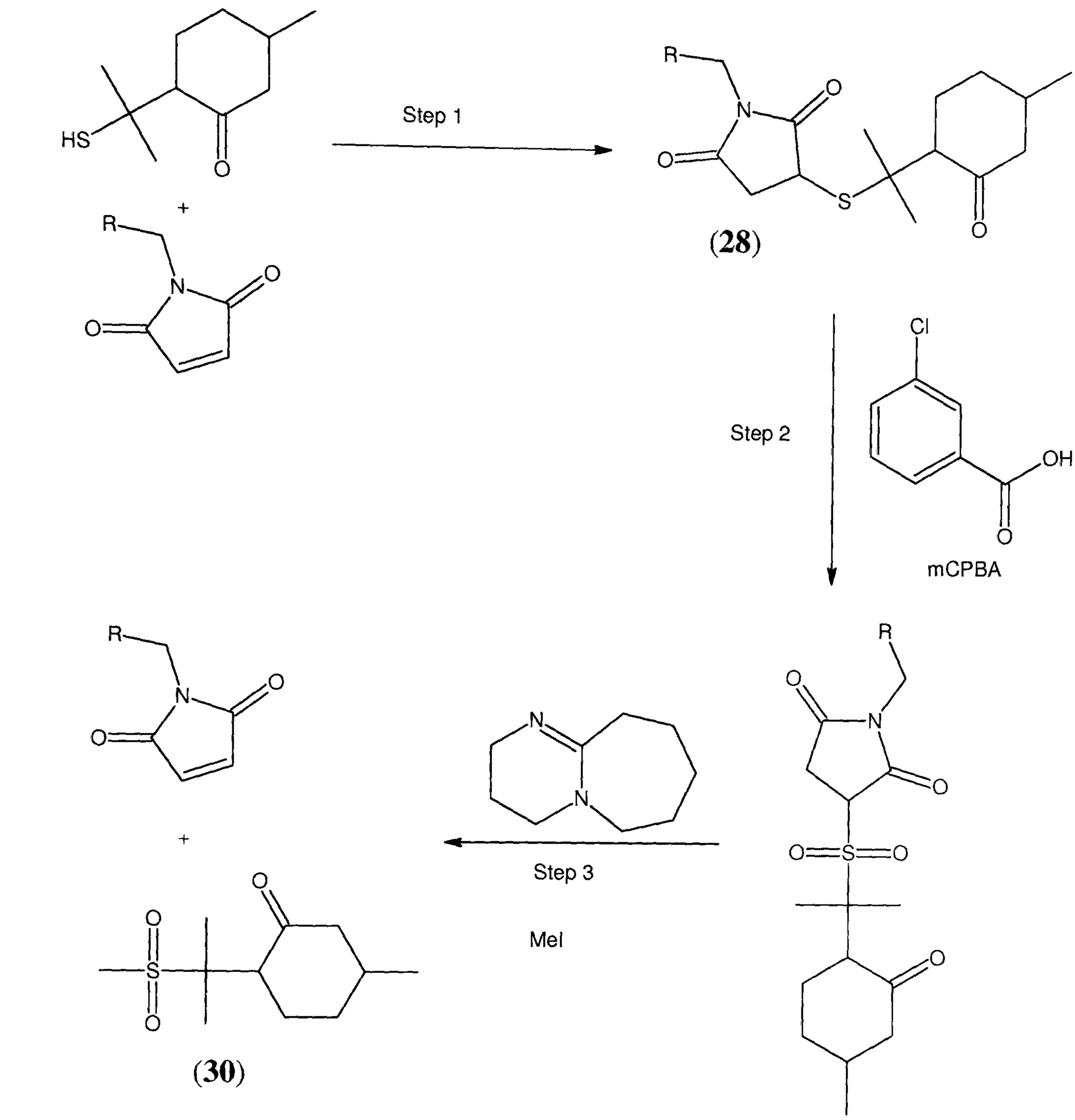
Step	**Expected product**	**Formula weight**
1		311.4
2		343.4
3		233.3

Table 4.3 Structures and formula weights of the expected reaction products after each step of the solution-phase reaction.



(**a**) R = Ethyl (solution-phase reactions) or (**b**) silica-bead (solid-phase reactions).

Figure 4.20 Reaction scheme for the isolation of the thiols found in Buchu leaf oil using the devised reaction scheme.

It was found that no additional solvent was required for solution-phase reactions. The solid-phase reaction was only analysed at the final step, which if successful, should theoretically contain only the compound of interest by the end of the elimination and methylation step.

4.3.4.1 Solution-Phase

The LC-MS data (in positive ESI mode) for the Buchu leaf oil before reaction and after each synthetic step are shown in Figure 4.21. The GC-MS data on the same samples are shown in Figure 4.22, with species assigned in Table 4.4. Cool on-column injection was used in order to reduce the thermal breakdown of any sulfones present in these samples. This was previously shown to be applicable to the characterisation of the types of sulfur-based components that were expected to be present in these samples.

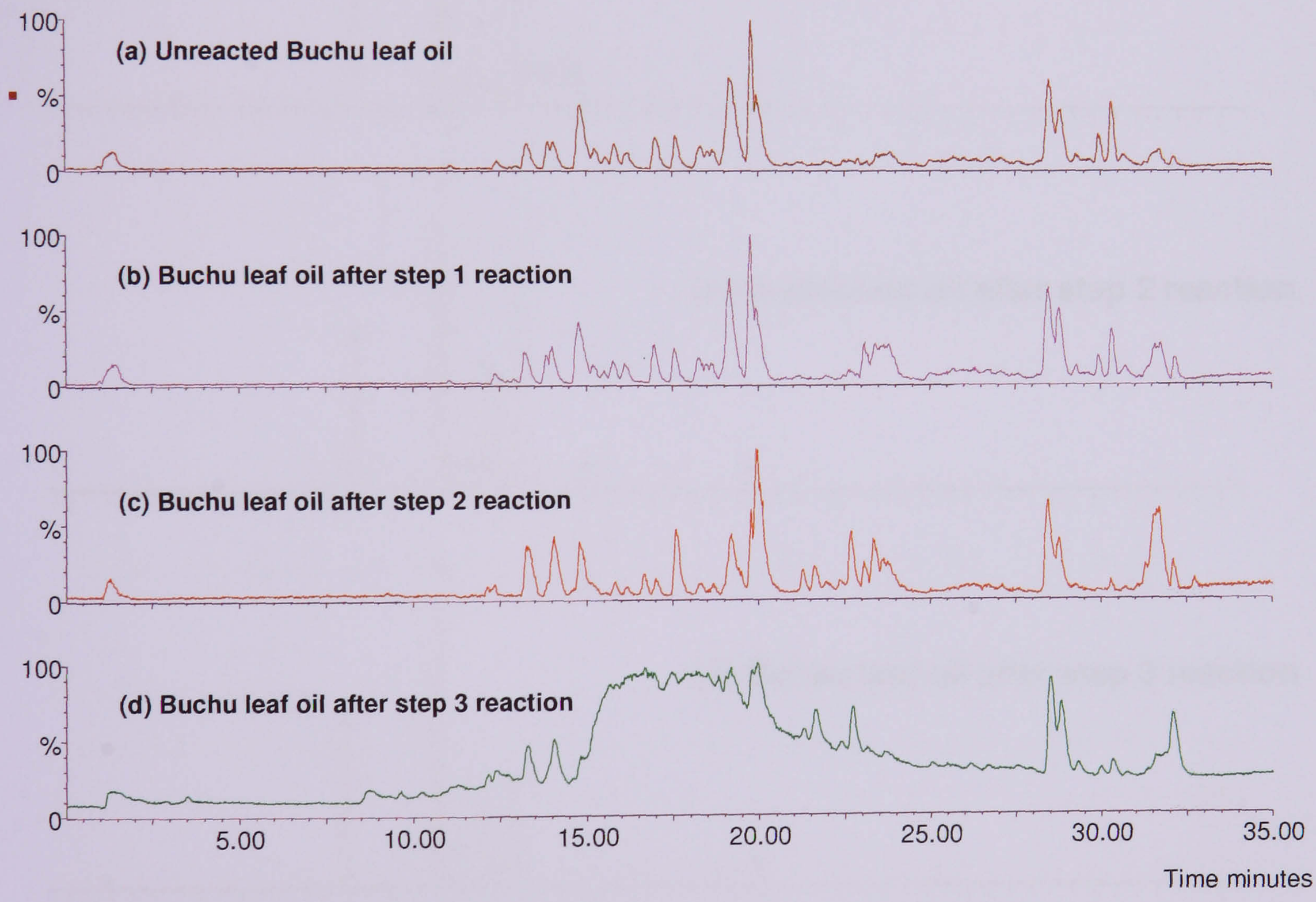


Figure 4.21 LC-MS chromatograms (ESI +ve) for Buchu leaf oil before reaction and after each synthetic step.

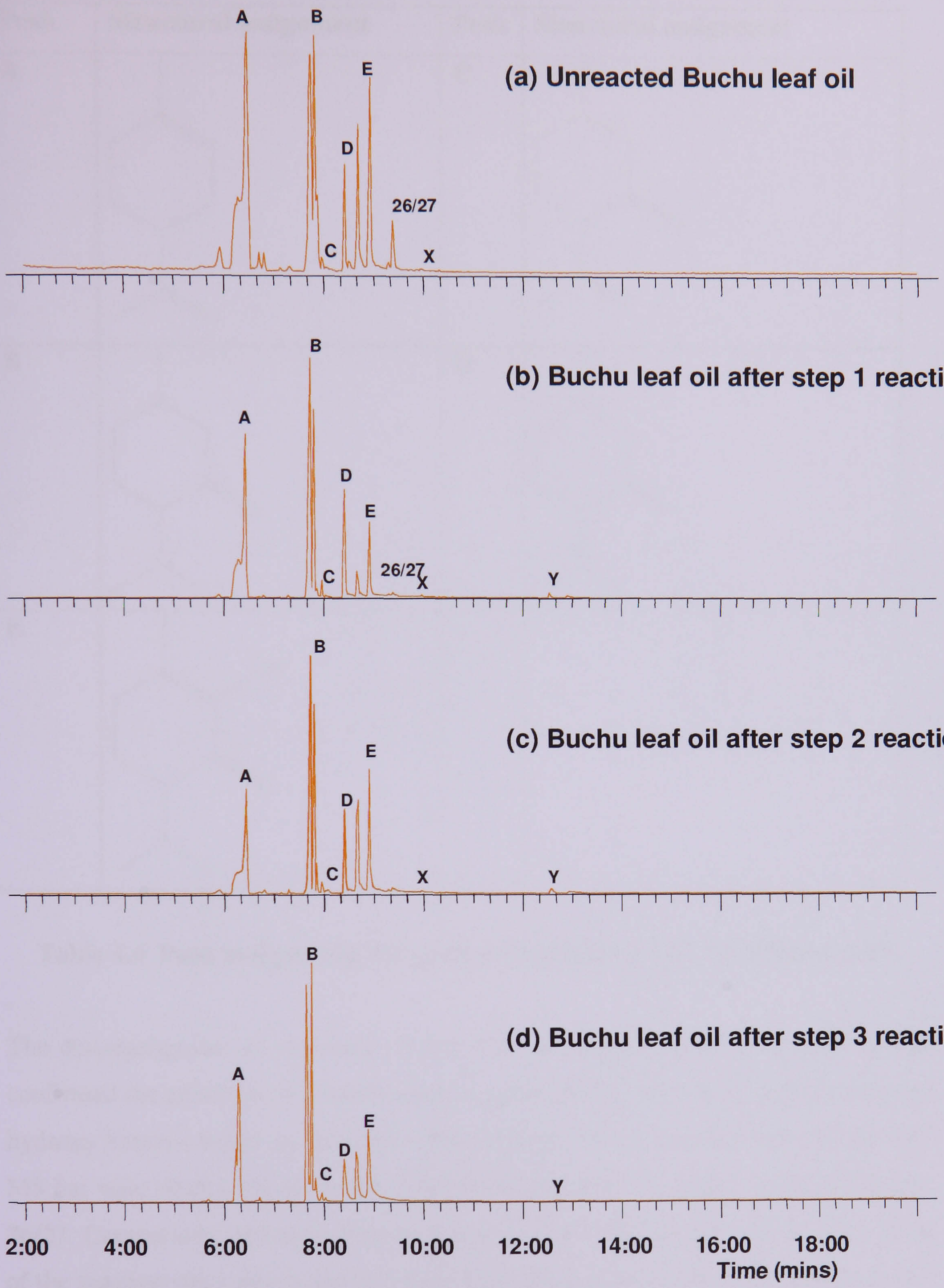


Figure 4.22 GC-MS Chromatograms for Buchu leaf oil before reaction and after each synthetic step.

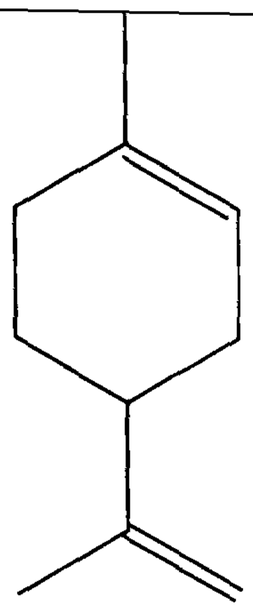
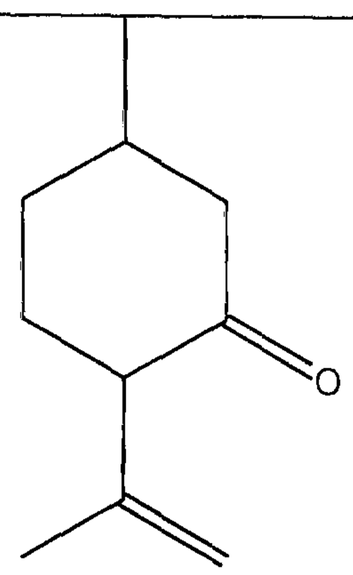
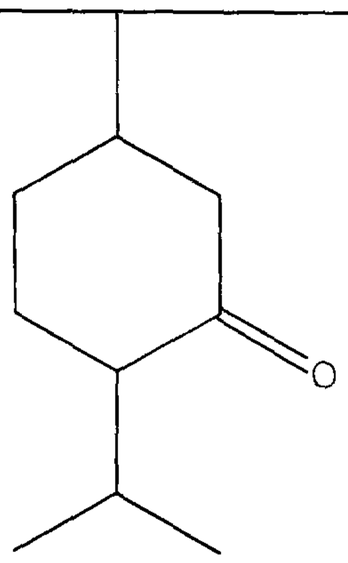
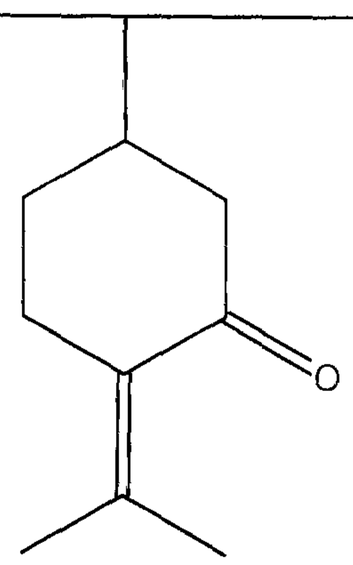
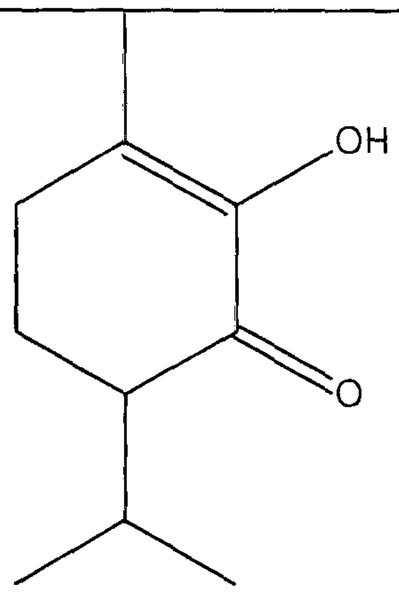
Peak	Structural assignment	Peak	Structural assignment
A		**C**	
B		**D**	
E			

Table 4.4 Peak assignments for species identified by GC-MS (Figure 4.22).

The chromatograms of unreacted Buchu leaf as analysed by LC-MS and GC-MS confirmed the presence of several isomeric terpenes (GC data only) and ketones and hydroxy ketones based on terpenes. The isomeric thiols were not detected by LC-MS but were observable in the GC-MS chromatograms (see Figure 4.22) labelled as **26/27**. Comparison between chromatograms of unreacted Buchu leaf oil with those of the reaction steps shows that the Buchu leaf oil components remain present as the main species in each of the 3 reaction steps. Some of these have been identified as isomeric and the structures of the proposed components may be slightly different to those identified as **A**-**E** (Figure 4.22 and Table 4.4).

Step 1

There was no evidence in either GC-MS or LC-MS data of the presence of a species of the correct molecular weight of the expected product (**28**), although it was observed that the thiols (**26**) and (**27**) are present at much lower levels than those in the original Buchu leaf oil sample. The GC-MS data indicates that after the first reaction, an additional pair of peaks (**Y**) (possibly due to a component with a molecular weight of 261 Da) is proposed to be from a species not present in the Buchu leaf oil, but there is no evidence that is the expected reaction product (**28**) (Figure 4.22 b).

Step 2

The GC-MS and LC-MS chromatograms from this sample have a very similar profiles to that from the reaction mixture from Step 1, which is mostly unreacted components from the Buchu leaf oil (Figures 4.21 c and 4.22 c). There is no evidence for the presence of the expected sulfone (**29**). The peaks labelled **Y**, in addition to those found in the Buchu Leaf Oil, are also present in this sample.

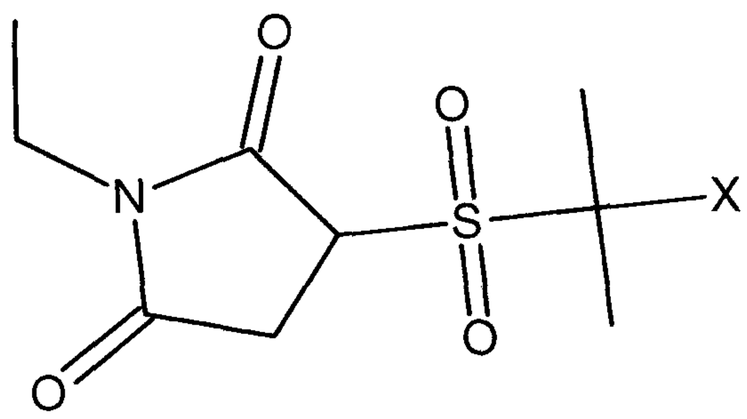
Step 3

The LC-MS chromatogram of the final step reaction products is shown in Figure 4.21d. They consist mainly of DBU and $^{13}CH_3$ modified DBU. There is good evidence for the presence of several compounds with molecular weight of 233 Da in the Step 3 products. It is possible that one of these components is the expected sulfone (**30**).

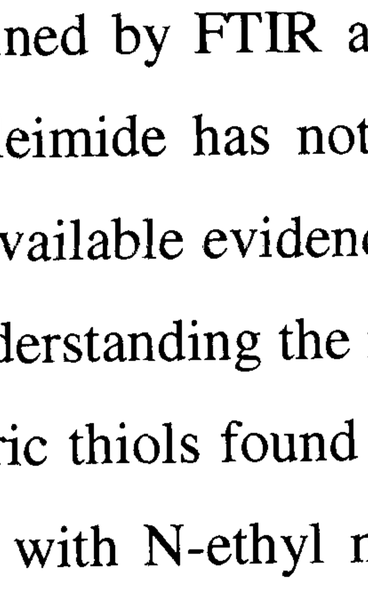
To summarise, there is no evidence for the presence of the expected reaction products from the first two reactions by either LC-MS or GC-MS data. There is some evidence in the LC-MS data that a peak arising from a species of the correct molecular mass was observed. The evidence from the data acquired previously suggests that both GC-MS and LC-MS would be expected to detect the expected species. This indicates, therefore, that these reactions have not taken place.

4.3.4.2 Solid-Phase

The silica-maleimide was reacted with Buchu leaf oil using the designed reaction protocol. Only the reaction product at the end of Step 3 was analysed by ^{1}H NMR, GC-MS and LC-MS. GC-MS data showed no evidence of the expected product and LC-MS chromatograms were once again dominated by the presence of DBU and $^{13}CH_3$ modified DBU. The LC-MS data, however, does suggest the presence of a component of molecular weight 233 which has a similar retention time to that of one of the species of the same molecular weight in the solution-phase reaction mixture. Proton NMR data confirmed the presence of DBU and $^{13}CH_3$ modified DBU. Resonances were also assigned to a structure below.



The silica residue was also examined by FTIR and Raman spectroscopies. These data suggest that the original maleimide has not been regenerated, as there is no evidence for a double bond. The available evidence again does not point strongly to a successful reaction. To aid in understanding the results of the reactions with Buchu leaf oil, the standard of the isomeric thiols found in Buchu leaf oil, *cis* and *trans* p-mentha-8-thiol-3-one was reacted with N-ethyl maleimide and also underwent the oxidation reaction. Note that the standard was 85% thiol and the major impurity was named as pugelone (**31**). The reaction products were analysed by NMR, FTIR, GC-MS and LC-MS.



(31)

4.3.4.3 Reaction of standard of p-Mentha-8-thiol-3-one

After step 1 of the reaction, a component of the expected molecular weight of 311 Da was obtained as shown in the GC-MS chromatogram (Figure 4.23). NMR and IR data however, show little evidence of the ketone functionality. The ^{13}C NMR data indicate a very low level of carbonyl that has the right chemical shift for ketone, however, this could be from the pugelone impurity (**31**) known to be present in the starting material. Again, by IR there is only a weak band in the correct position to be ketone. The maleimide carbonyls are present.

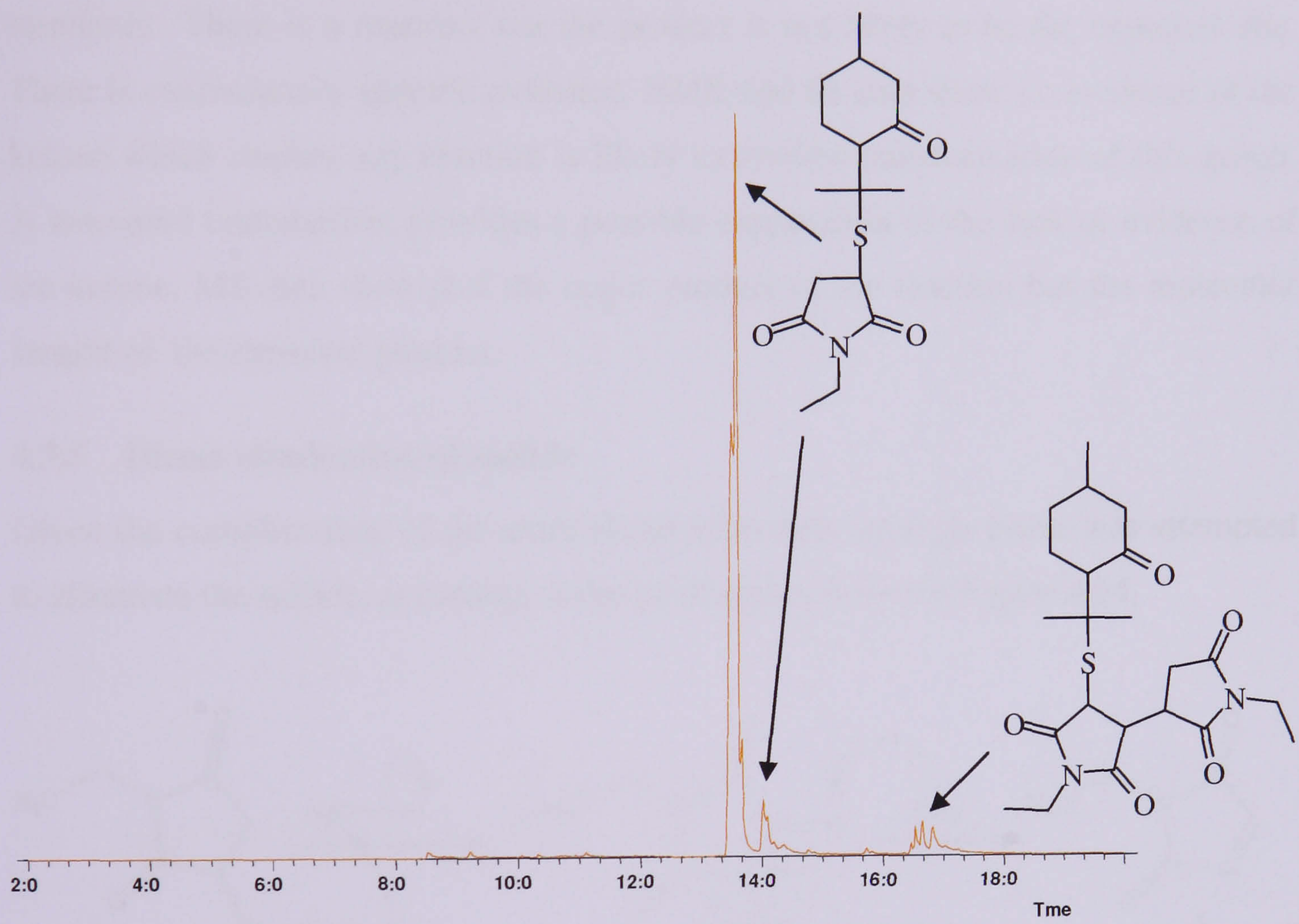


Figure 4.23 GC-MS chromatogram from product from reaction of N-ethyl maleimide and p-Mentha-8-thiol-3-one.

The next step was oxidation of the sulfide to sulfone. There was no evidence for species of the correct mass in the GC-MS trace. Again by ^{1}H NMR and FTIR spectra there was no evidence of the ketone. ^{1}H NMR data suggest that there could be evidence of oxidation of sulfur to sulfone but it is not consistent with the expected product.

Because the first reaction did not appear to produce the required intermediate it is not surprising that no evidence could be found that either the oxidation or elimination reaction had occurred.

To summarise, the reaction with the thiols in Buchu leaf oil did not progress as anticipated. The evidence suggests that a reaction has taken place with the thiols, as indicated by the GC-MS data for the solution reaction showing reduction in these components. The difficulty appears to be the first step (shown from studies on the standard). There is a reaction, but the product is not likely to be the expected one. There is contradictory spectral evidence. NMR and IR data show no evidence of the ketone which implies any reaction is likely to involve transformation of this group. A keto-enol tautomerism provides a possible explanation of the lack of evidence of the ketone. MS data show that the major product of the reaction has the molecular weight of the expected product.

4.3.5 Direct elimination of sulfide

Given the complications of the extra elimination step, an experiment was attempted to eliminate the sulfide, according to the mechanism shown in Figure 4.24.

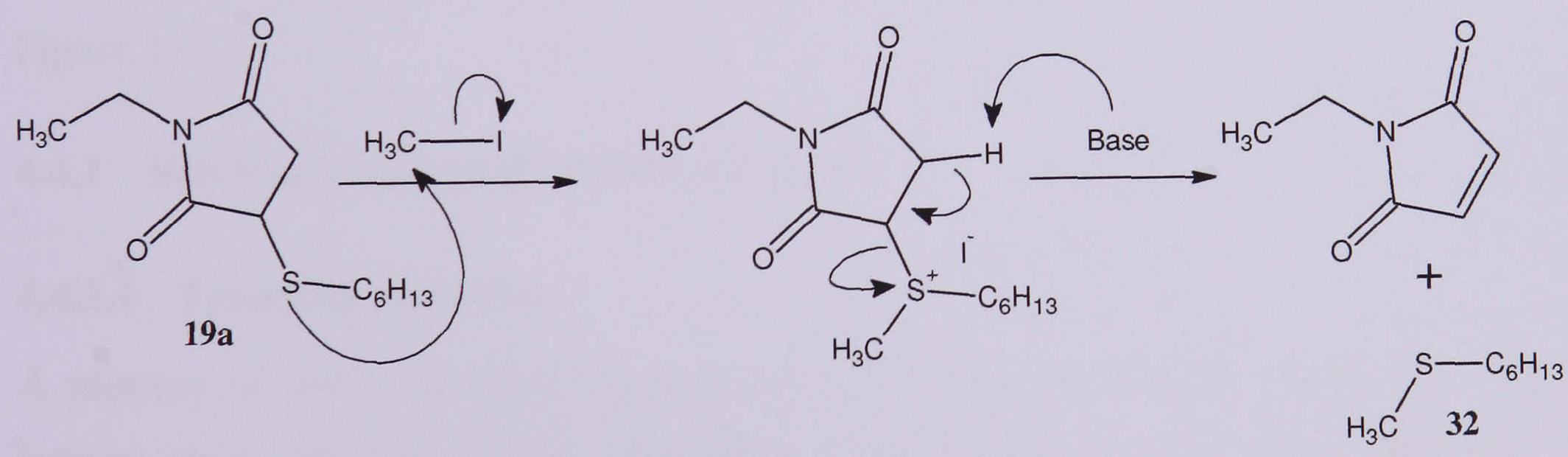


Figure 4.24 Mechanism for direct elimination of a methylatedsulfide.

The reaction was carried out at room temperature to minimise side reactions in the alkyl chain. In a test solution containing unknown compounds the reaction conditions need to be as mild as possible to prevent isomerism/degradation of the thiol side-chains. Proton NMR analysis gave no indication of the presence of the desired sulfide (**32**). The major product formed was methylated-DBU, as has been

observed before for elimination reactions. A longer reaction time or higher temperature may be required to facilitate the initial methylation of the sulfur atom.

4.4 Covalent chromatography with disulfide reagent

In parallel with the investigation using malemide-based species, an alternative strategy using disulfide reagents was investigated. This method is attractive for a number of reasons:

- Selectivity towards thiols in forming S-S bond.
- Mild reaction conditions.
- Simple trap and release steps by displacement reactions.
- Easy to follow reaction by UV/Vis spectroscopy.
- Possibility of coupling the thiol active reagent to a solid-support.

The use of a disulfide reagent is complementary to the silica-bead reaction in that the original thiol is regenerated with the consequent potential for organoleptic analysis. Reactions were first investigated in the solution-phase with 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB) for method optimisation before applying to the commercial covalent chromatography material, thiopropyl sepharose 6B (**15**), designed for isolating thiols. (The reaction of DTNB with thiols was shown in Figure 4.6).

4.4.1 Solution-phase method development

4.4.1.1 Trapping reactions

A mixture of low molecular weight alkyl thiols of carbon chain length C_2 – C_7 (ethane thiol, propane-1-thiol, 2-methylpropane-1-thiol, butane-1-thiol, butane-2-thiol, pentane-1-thiol, hexane-1-thiol and heptane-1-thiol) in equimolar amounts (0.01 mmoles) was reacted with DTNB in a buffer solution (pH 8) for 30 minutes at room temperature. A dark orange colour was observed after less than a minute. No further colour change was observed after 10 minutes. The UV/Vis spectra of the starting material (DTNB) and the product reaction mixture are shown in Figure Figure 4.25, illustrating the shift in absorption as the new disulfide species are formed. The absorption maximum at 324 nm is characteristic of DTNB. The new thiol derivatives formed with DTNB on trapping have an absorption maximum at

340 nm. A new absorption peak is seen at 412 nm due to the release of 2-nitro-5-sulfhydrylbenzoic acid (TNB).

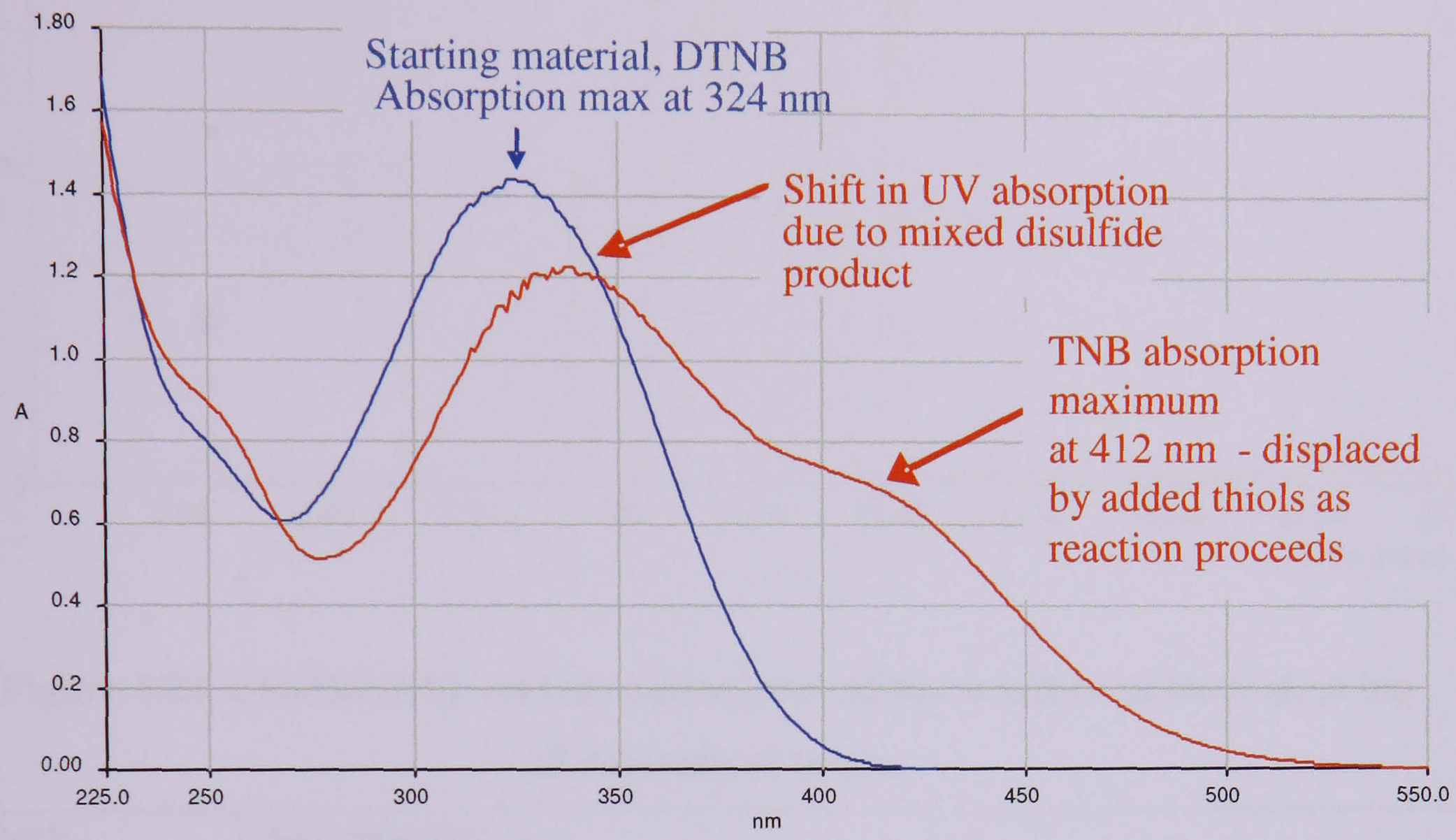
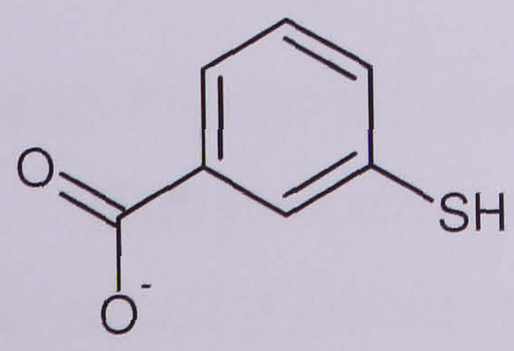


Figure 4.25 UV/Vis spectra of starting material and during reaction, indicating trapping reaction taking place.

The reaction mixture was also characterised by LC-MS, with ESI in negative ion mode. The chromatogram is shown in Figure 4.26 which along with the structural assignments in Table 4.5 illustrates that all thiols reacted with DTNB. The species were identified by their molecular ions. A characteristic fragment ion of *m/z* 153 (generated by fragmentation of molecular species in the source region of the mass spectrometer) was found that could be used to determine if a derivatised thiol is present in solution. This ion most likely has the empirical formula $C_7H_5O_2S$. It is probably formed by loss of the nitro and alkyl functionality from the derivatised thiols, to form an ion similar to that shown below (**33**). The UV detector also indicated that all the species shown in Figure 4.25 were also UV active.



(**33**)

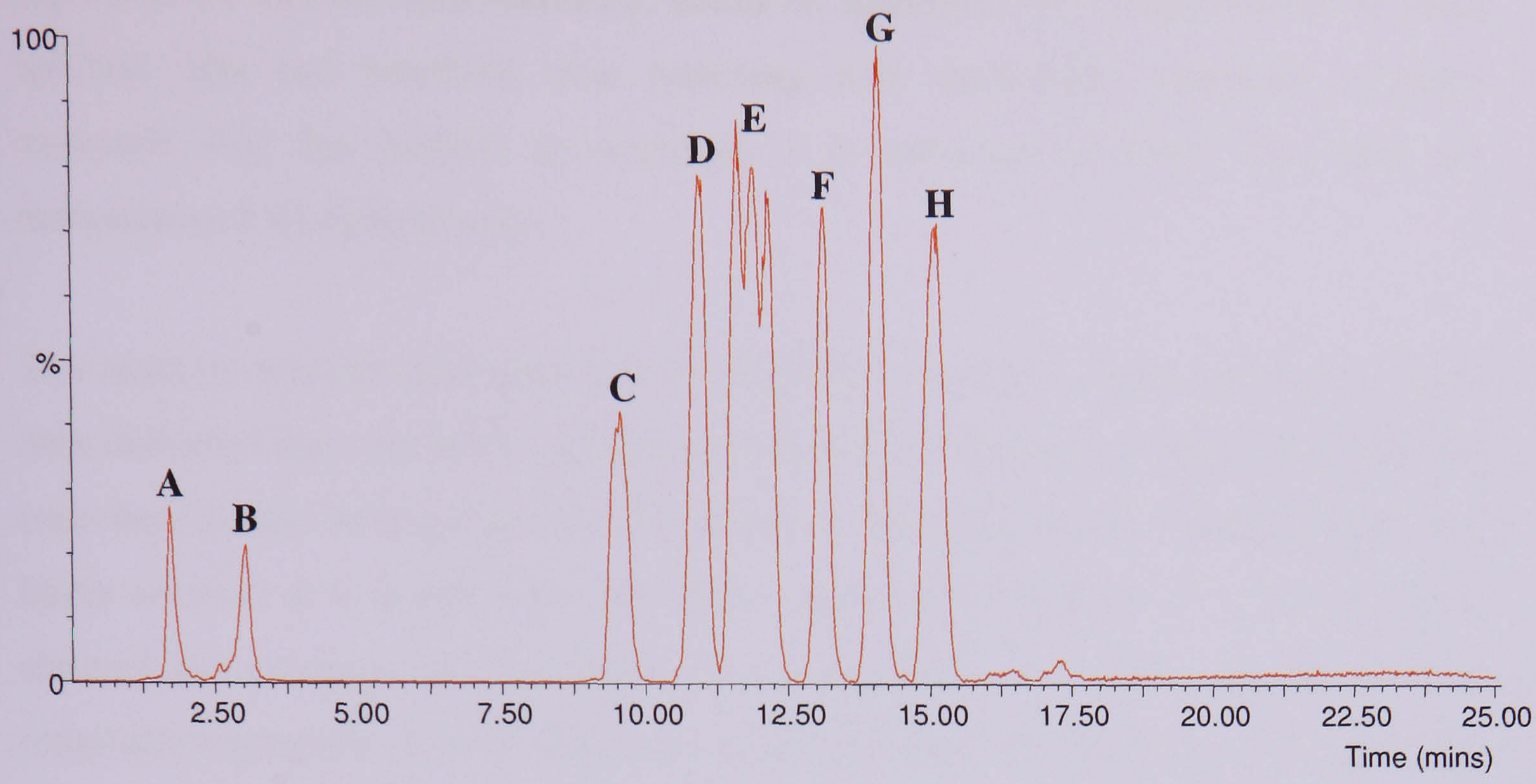


Figure 4.26 LC-MS (ESI –ve) chromatogram of the reaction mixture showing all derivatised thiols.

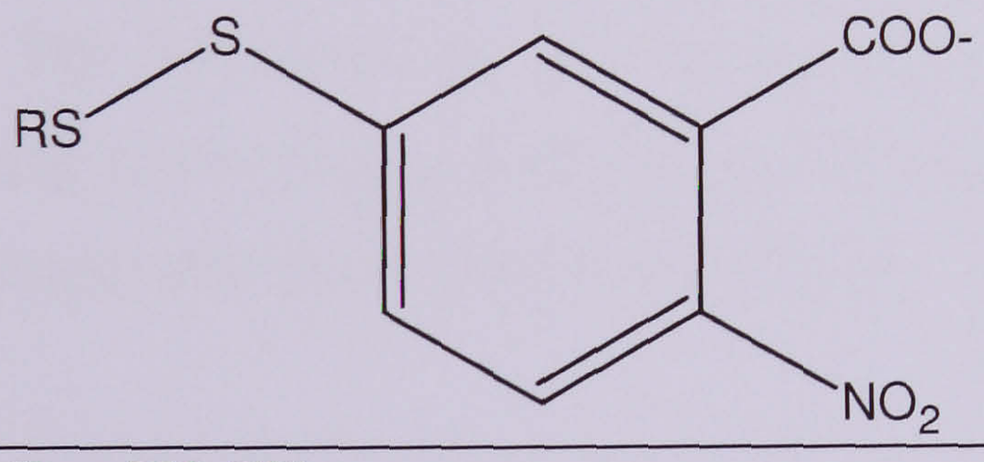
Peak	Assignment
A	TNB (deprotonated)
B	DTNB
Thiol derivatives	
C	$R = CH_2CH_3$
D	$R = (CH_2)_2CH_3$
E	$R = (CH_2)_3CH_3$, $CH_2CH(CH_3)_2$, $CH(CH_3)CH_2CH_3$ (3 isomers)
F	$R = (CH_2)_4CH_3$
G	$R = (CH_2)_5CH_3$
H	$R = (CH_2)_6CH_3$

Table 4.5 Table of assignments for species identified by LC-MS (ESI –ve) (Figure 4.26).

TNB and some unreacted starting material, DTNB, were identified. The three butane thiols (i.e. 2-methylpropane-1-thiol, butane-1-thiol and butane-2-thiol) were not

distinguishable from the mass spectral data alone. These three thiols are, however, separated by LC-MS and therefore, could be identified by a combination of mass spectral data and retention time matching with derivatised standards of these materials. For this method development, it is sufficient to know that there are derivatives of all three isomers.

The reaction mixture was analysed by GC-MS to look for unreacted thiols. These data indicated that low levels of butane-2-thiol and 2-methylpropane-2-thiol (i.e. the branched thiols) were present in the solution. These sterically hindered thiols are likely to react at a slower rate. No other thiols were observed. GC-MS data also showed the presence of low levels of the disulfides, 1,1'-dithiodiethane and 1-(ethyldithio)propane. It is not known at what point these disfulides formed: they may have been present at any point from the combination of starting materials up to formation during the chromatographic analysis.

4.4.1.2 Release reactions

The second step for this reaction was to show that the thiols can be regenerated which would allow for organoleptic analysis. This is achieved by addition of the strong reducing agent, dithiothreitol (DTT), to the mixed disfulides displacing the original thiol. The mechanism for this displacement reaction is shown in Figure 4.27. The driving force of the reaction is the stability of the species formed (highly delocalised structure) and the entropically favourable release.

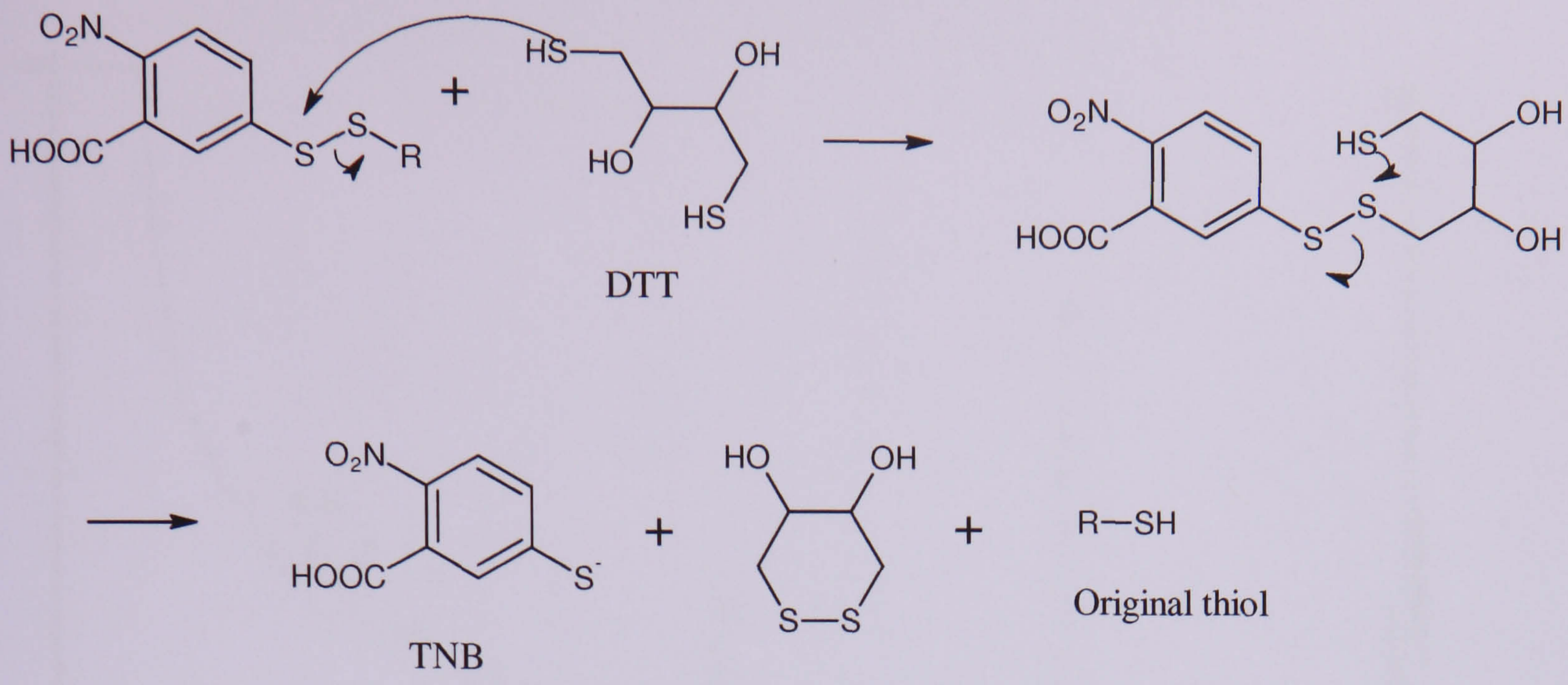


Figure 4.27 Reaction of dithiothreitol with the 'trapped' disulfide.

DTT was added in excess to the solution of mixed disulfides and left overnight. On examination, a colour change from light to dark orange was observed. The reaction was monitored by UV/Vis spectroscopy and the reaction mixture analysed by GC-MS. The UV spectra of the starting mixture and the reaction products are shown in Figure 4.28. The peak at 340 nm due to the mixed disulfides decreased, indicating a reaction had taken place.

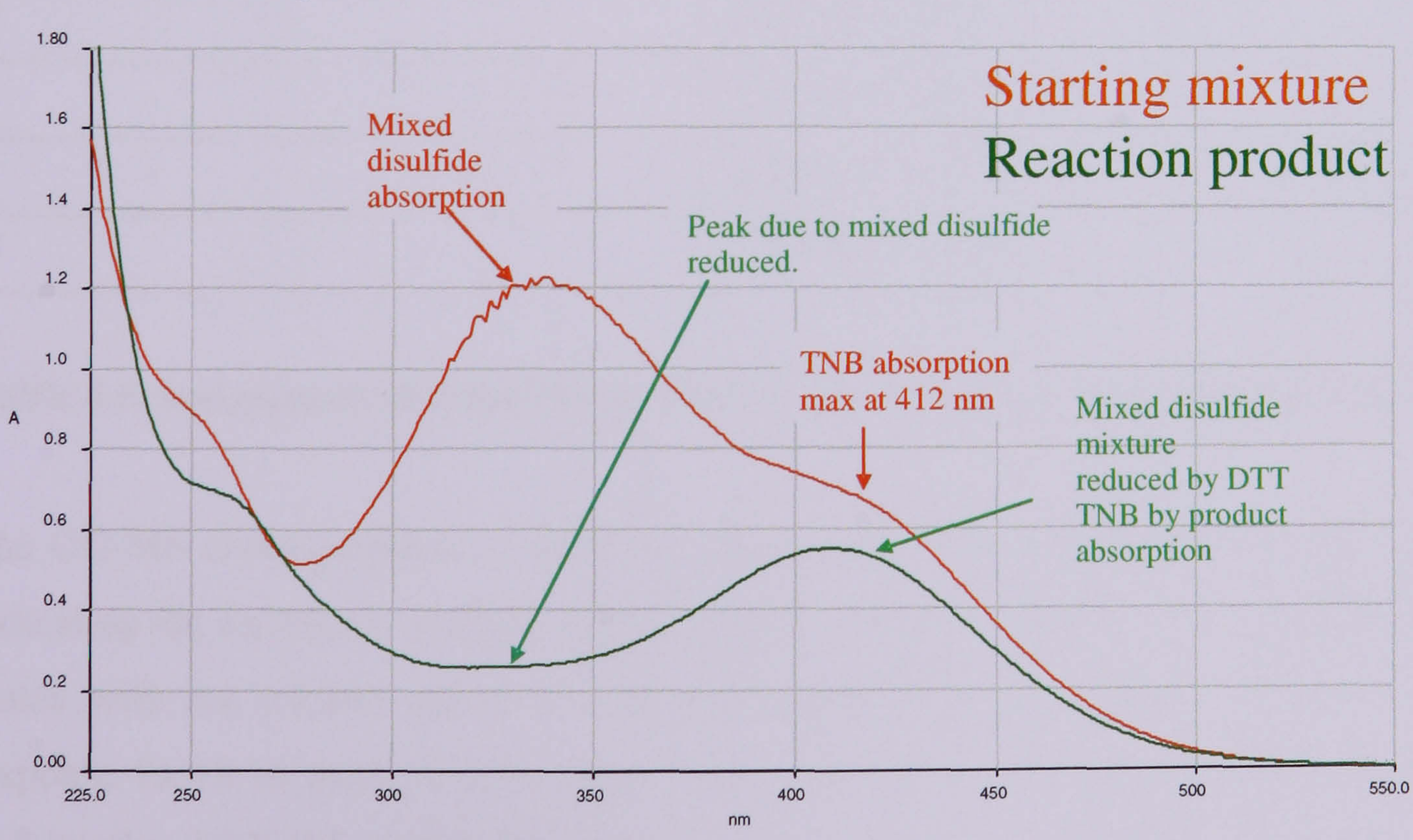


Figure 4.28 UV/Vis spectra of mixed disulfide and product after reduction with DTT.

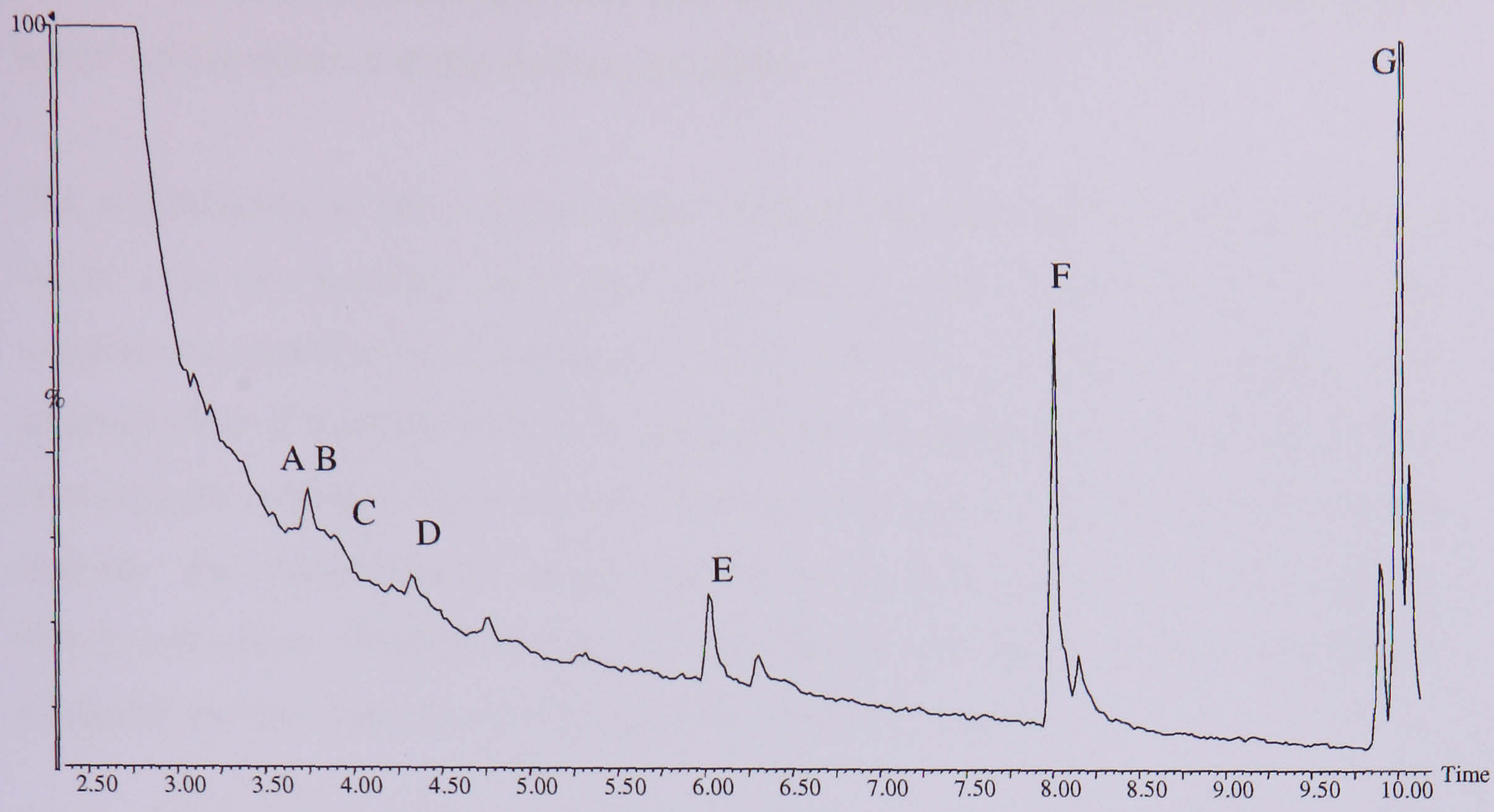


Figure 4.29 GC-MS chromatogram of reaction mixture after reduction with DTT.

Peak	Assignment
A	Propane-1-thiol
B	Butane-2-thiol
C	2-Methylpropane-2-thiol
D	Butane-1-thiol
E	Pentane-1-thiol
F	Hexane-1-thiol
G	Heptane-1-thiol

Table 4.6 Assignment of thiols from reaction mixture after reduction with DTT.

The GC-MS chromatogram is shown in Figure 4.29 with assignments in Table 4.6 indicating the re-release of all the thiols, with the exception of ethanethiol which co-elutes with the solvent and is therefore not observed. If it is assumed that the response factor of each thiol is similar, then the relative peak sizes show that the heavier the thiol, the greater the yield. This could be for a number of reasons: it could reflect the relative reactivity of the species, or the lighter thiols could be absorbed on the chromatography column to a greater extent than heavier thiols, or

the more volatile species could have been lost prior to analysis. Finally, the response factors of the detector to the thiols could differ.

The experiments in the solution-phase indicate that the reaction in this media is facile, easy to monitor, and takes place under mild conditions (pH 8, room temperature, quantitative in minutes). The LC-MS analysis is also encouraging as it illustrates that if reaction were to be carried with a complicated mixture in solution a characteristic fragment ion could be observed, which would help to identify reacted species. The comparison of straight and branched chain species, however, suggests that in a situation where there may be secondary or tertiary thiols, these structurally hindered and less reactive species will not be trapped as easily.

4.4.2 Solid-phase covalent chromatography

The reaction was then applied to the solid-phase, utilising the covalent chromatography gel, thiopropyl sepharose 6B from Amersham Biosciences. Ideally, the reaction would have been carried out with a covalent chromatography gel based on DTNB for direct comparisons with the solution-phase results. This material was no longer commercially available, so the related reagent was used instead. The principle is the same, with the released species in this case being 2-thiopyridone. This has UV/Vis absorptions at 343 nm and 247 nm. The mechanism for trapping is shown in Figure 4.30. Just as in the solution-phase reaction, it is the stability of the released species which drives the formation of the disulfide from the added thiol in preference to remaining as the original disulfide.

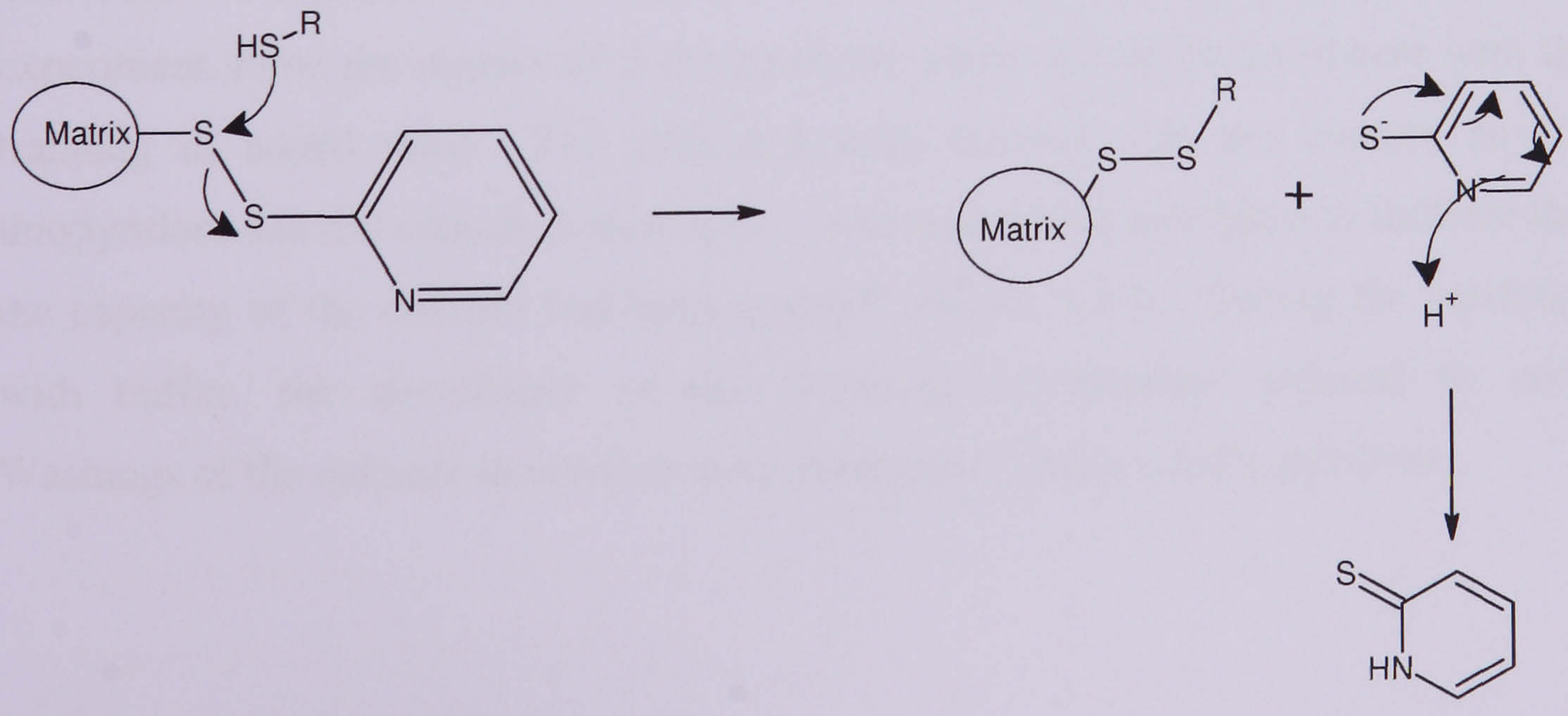
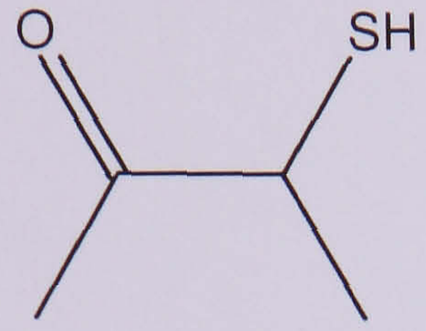


Figure 4.30 Mechanism for reaction of thioproyl sepharose 6B (simplified structure) with thiols.

For simplicity, only one thiol was used in these reactions. A UV-active thiol was substituted for the straight chain species because its presence could also be monitored by UV/Vis spectroscopy. 3-mercaptobutan-2-one (**34**) has a UV absorption at 295 nm which does not overlap with absorptions from the other UV/Vis active reagents.



(34)

The gel was prepared according to the manufacturer's instructions and used to pack a glass dropping column.

4.4.2.1 Trapping reaction

Excess 3-mercapto-2-butanone in buffer was added in portions to the gel, previously washed with buffer. The solution was left in contact with the gel for an hour before the tap of the dropping column was opened so that a slow flow was achieved. The effluent from the column was collected as fractions and these were analysed by UV/Vis spectroscopy to monitor the release of 2-thiopyridone. The gel was then washed with distilled water in 5 mL portions to rinse off any unreacted thiol.

Figure 4.31 shows the UV spectra for the consecutive washing of the gel after trapping, with the absorption maxima at 343 nm and 274 nm. The amount released decreases on addition of more thiol. The first four fractions of this trapping experiment show the release of 2-thiopyridone which would be consistent with the trapping of added thiol. The fifth and sixth fractions did not contain any 2-thiopyridone but did contain 3-mercapto-2-butanone which was taken to indicate that the capacity of the column had been reached (Figure 4.32). During the washings with buffer, the absorbance of the 3-mercapto-2-butanone reduced to zero. Washings of the column showed no more presence of thiol or 2-thiopyridone.

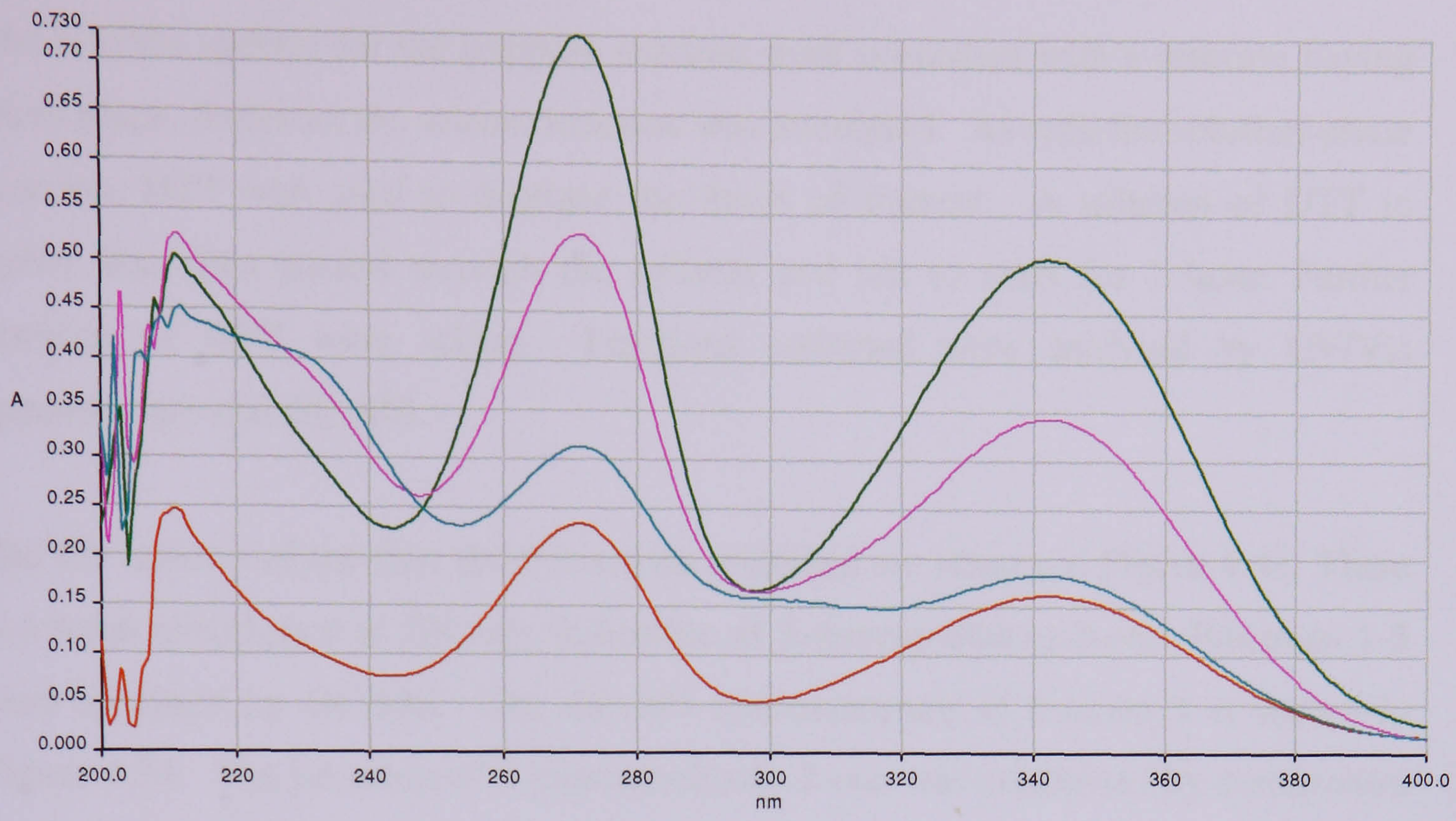


Figure 4.31 UV/Vis Spectra of the first four fractions collected on addition of 3-mercapto-2-butanone to the gel indicating 2-thiopyridone is being released.

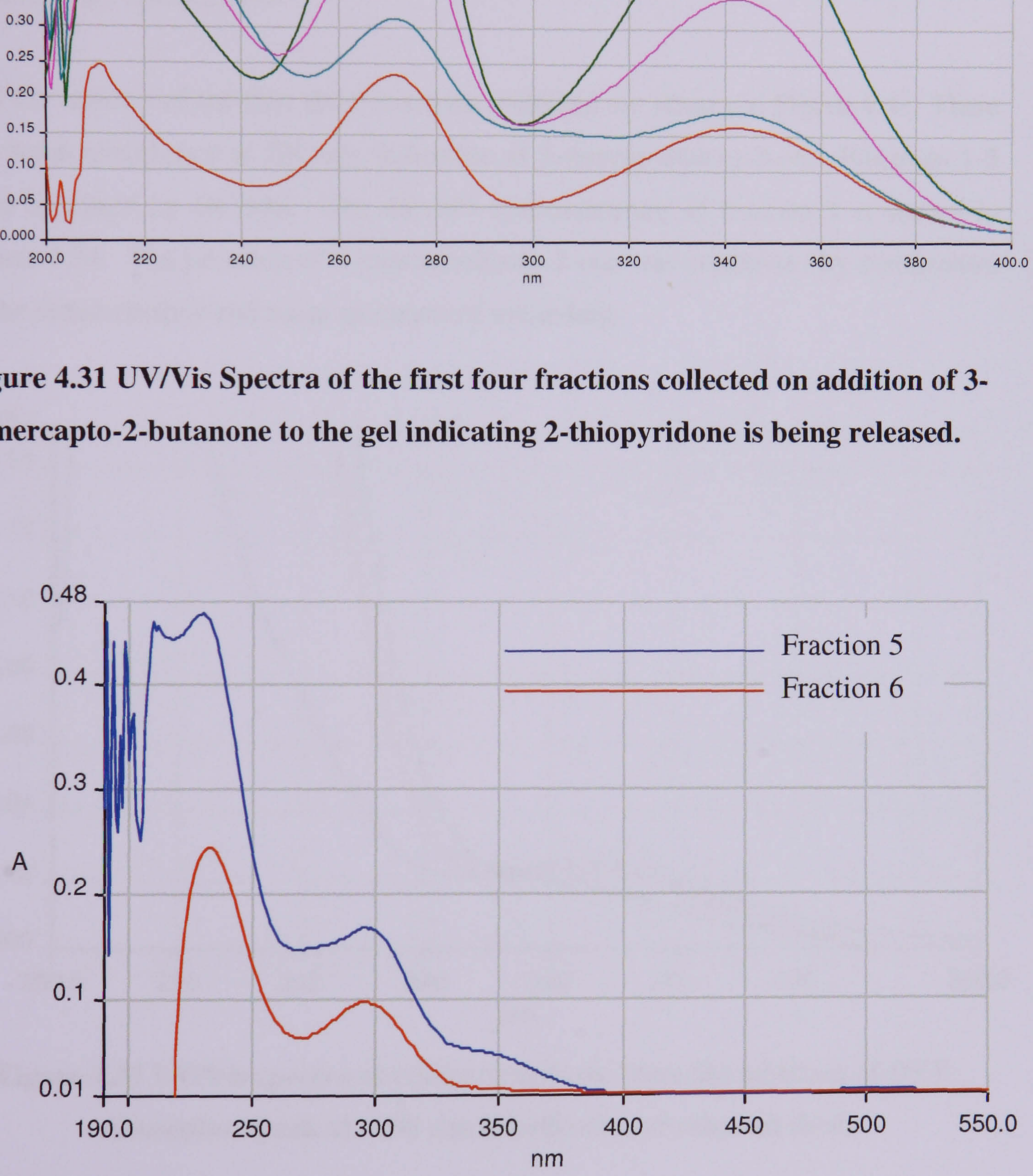


Figure 4.32 UV/Vis Spectra of the final two fractions from the column. The absorbance maxima at 290 nm are indicative of 3-mercapto-2-butanone.

4.4.2.2 Release reactions

The UV/Vis spectra for the trapping reaction were consistent with a reaction having taken place, therefore the release reaction was attempted. As with the solution-phase reaction, DTT was used to displace the thiols of interest. A solution of DTT in buffer was then passed through the column and left to react for 1 hour. Further portions of DTT were added. Fractions collected were analysed by UV/Vis spectroscopy and GC-MS.

The UV spectra of the first three fractions collected are shown in Figure 4.33. There is a weak absorbance at 290 nm, indicative of 3-mercaptobutan-2-one. Fractions 1-3 were analysed by GC-MS. The GC-MS chromatogram of fraction 1 is shown in Figure 4.34. The presence of 3-mercaptobutan-2-one was confirmed by comparison of the retention time and mass spectrum of a standard.

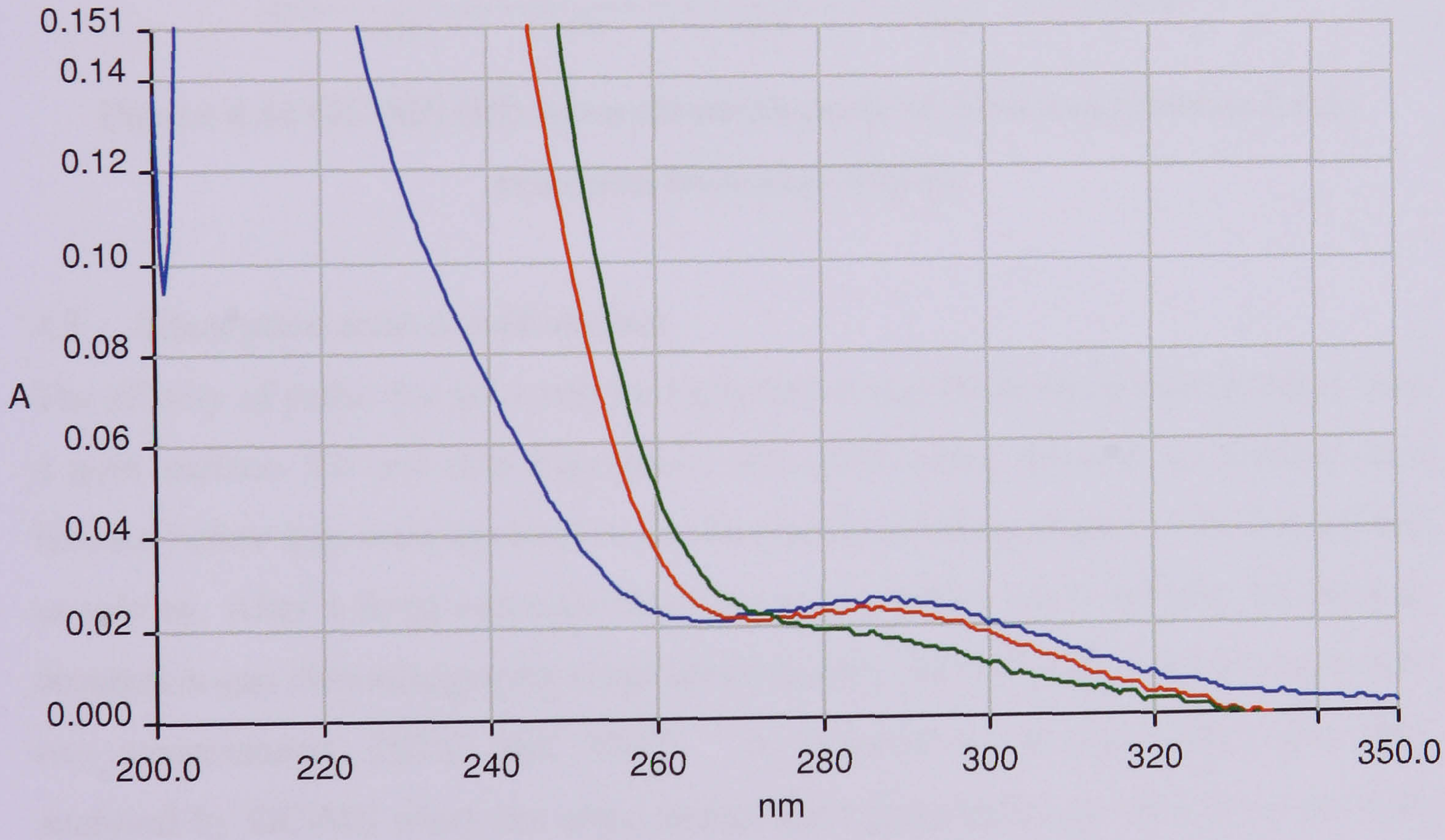


Figure 4.33 UV/Vis spectra of release fractions, from the addition of DTT. Absorption peak at ~290 nm is indicative of released thiol.

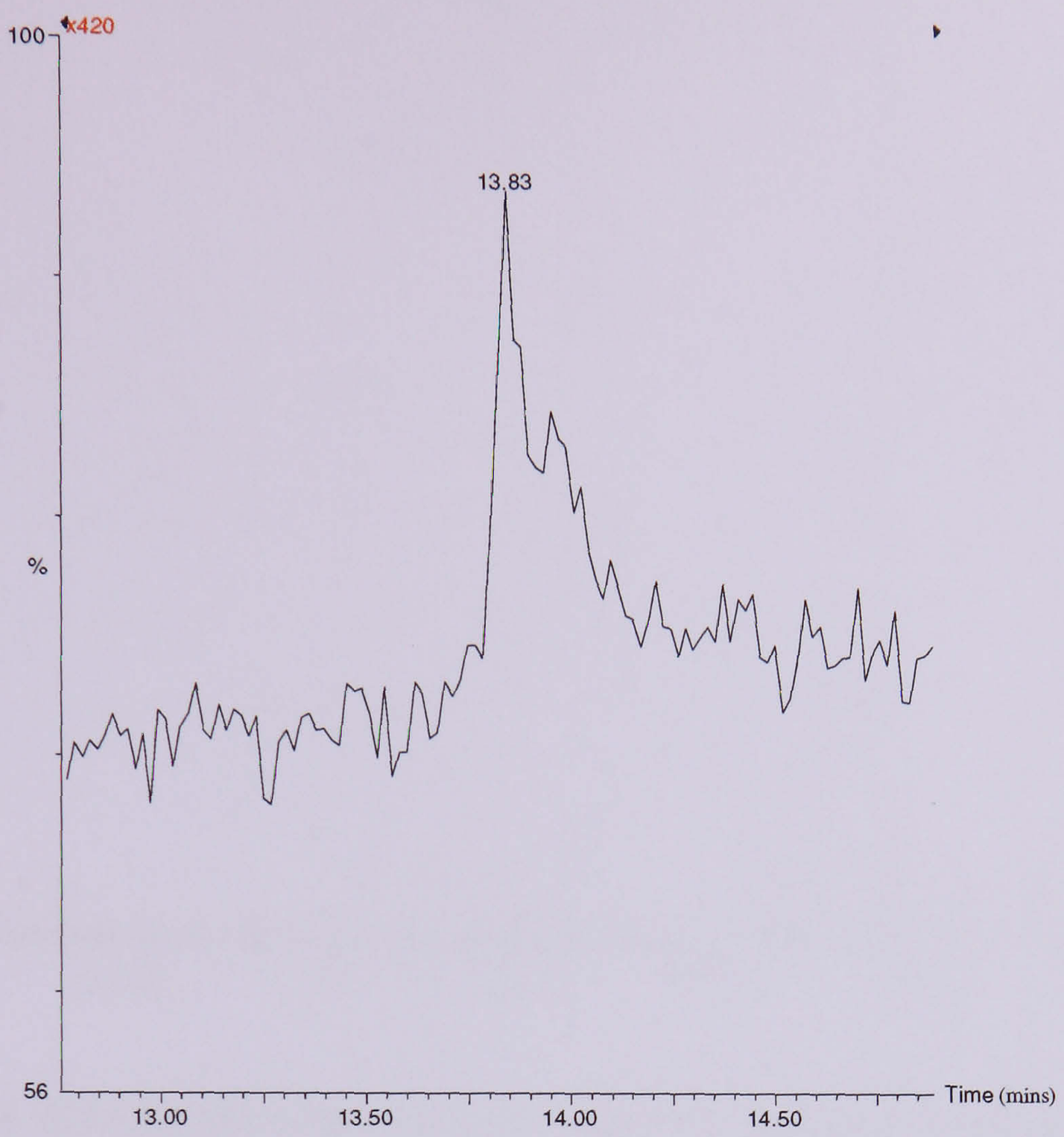


Figure 4.34 GC-MS (EI) mass chromatogram of 3-mercaptobutan-2-one rreleased from the column.

4.5 Adsorption onto a gold surface

The affinity of sulfur for gold may be exploited to trap thiols by adsorbing them onto a gold surface. To test this hypothesis, thin gold wires, cleaned by heating in a furnace before use, were exposed to a headspace containing hexane-1-thiol in hexane as solvent. After a fixed exposure time, the wire samples were analysed by thermal desorption-gas chromatography-mass spectrometry (TD-GC-MS) with desorption at two temperatures, 200°C and 300°C. A standard of hexane-1-thiol was also analysed by GC-MS using the same temperature programme conditions to aid with the identification and origin of analytes. The chromatograms from thermal desorption at 200°C and 300°C are shown in Figure 4.35 and Figure 4.36 respectively, with peak assignments as shown in Table 4.7 and Table 4.8. Sulfur containing species are shown in blue. Assignments were made on the basis of comparison to library spectra.

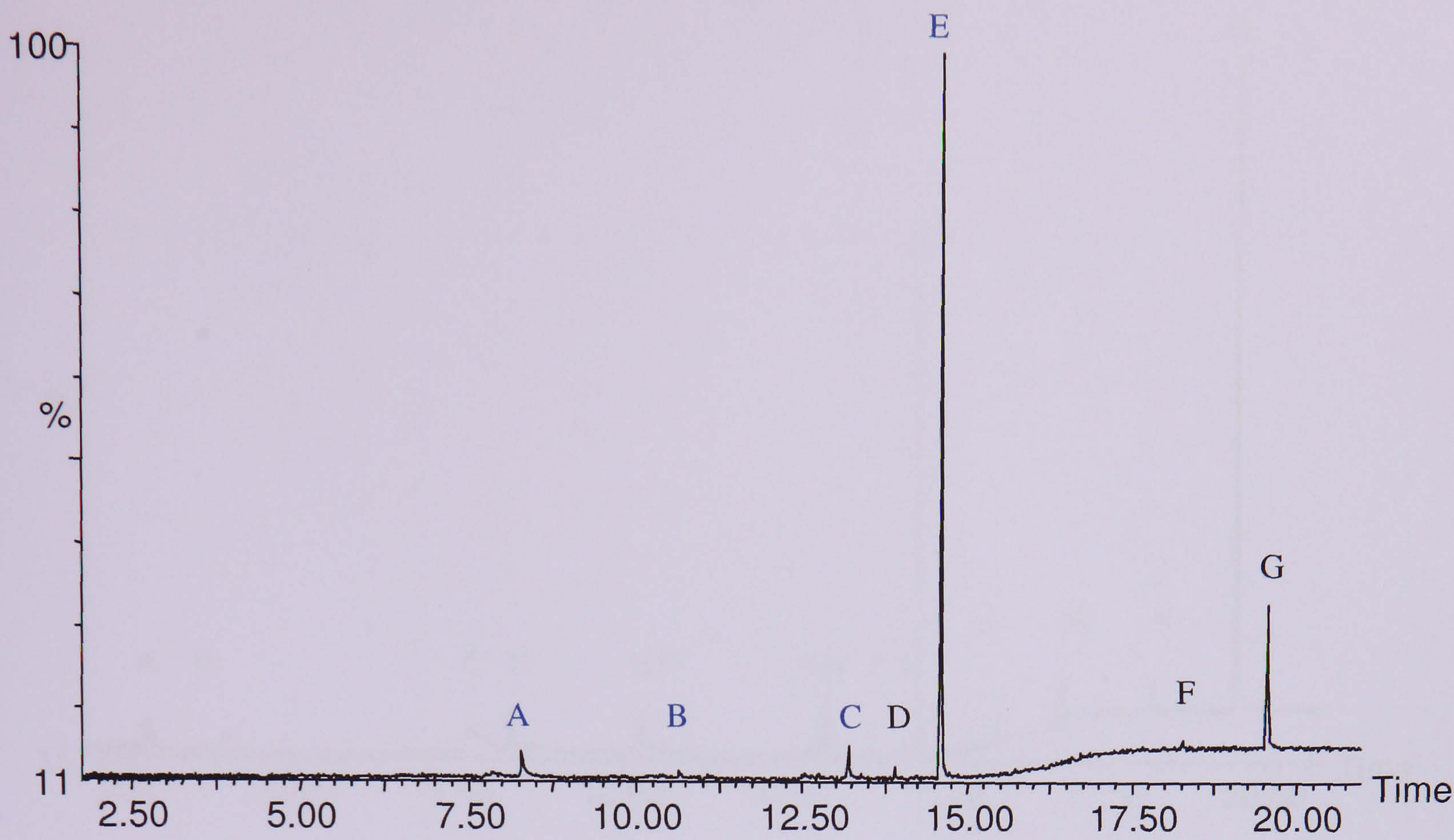


Figure 4.35 TD-GC-MS trace for gold wire after exposure to a headspace of hexane-1-thiol and thermally desorbed at 200°C.

Peak	Assignment
A	1-hexane-1-thiol
B	1-(isopropylsulfanyl)hexane
C	1,1'-thiodihexane
D	Unassigned
E	1,1'-dithiodihexane
F	Hexanedioic acid bis(2-ethyl hexyl) ester
G	Di octyl phthalate

Table 4.7 Assignment of peaks from gold wire exposed to a headspace of hexane-1-thiol and thermally desorbed at 200°C (Figure 4.35).

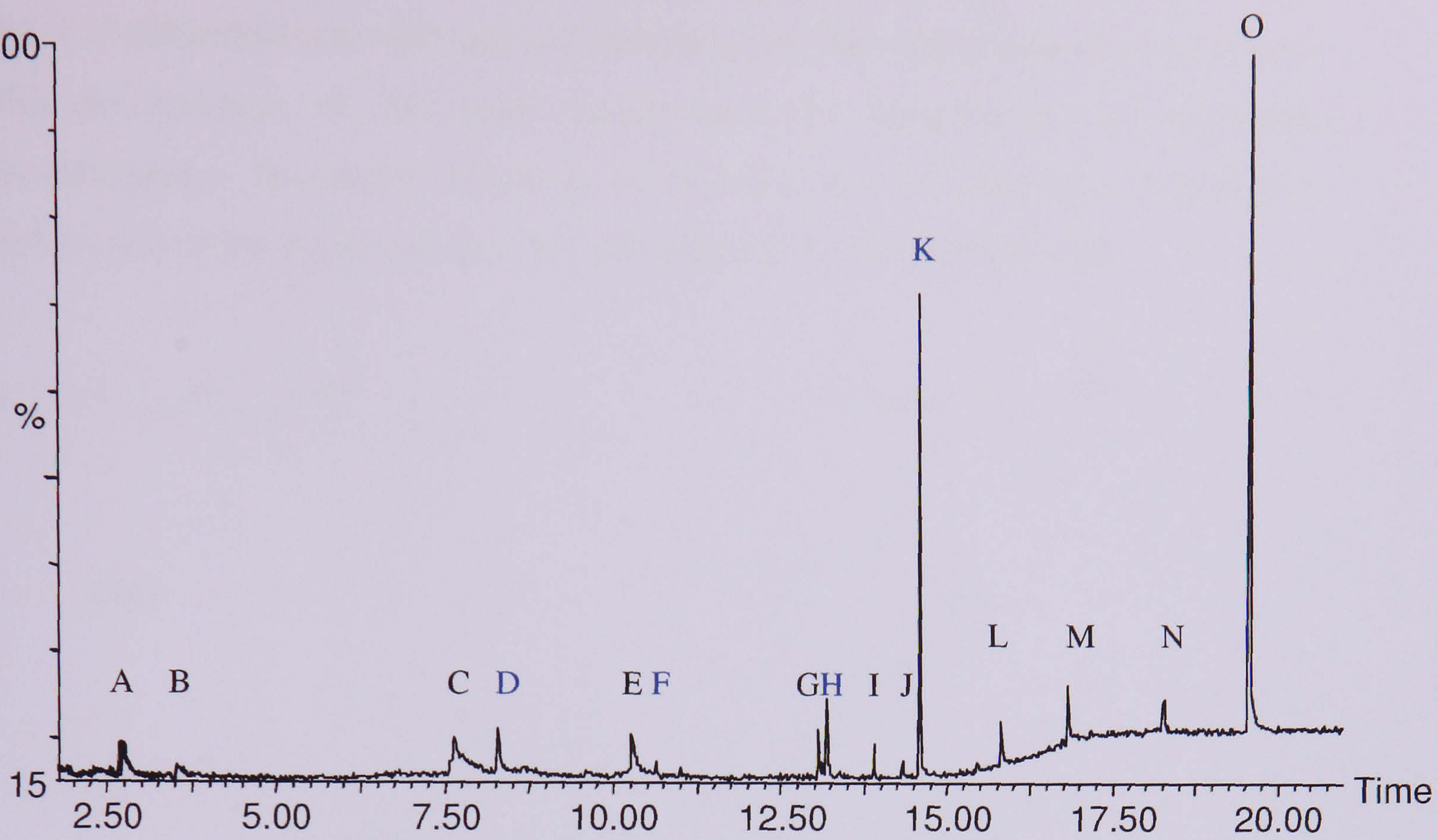
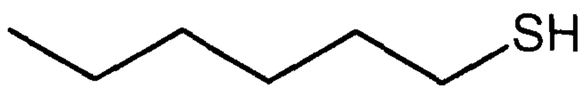


Figure 4.36 TD-GC-MS trace for gold wire after exposure to a headspace of hexane-1-thiol and thermally desorbed at 200°C.

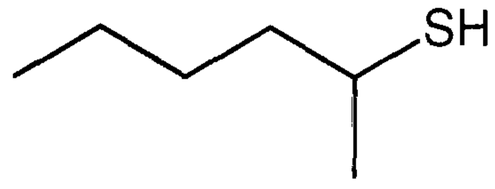
Peak	Assignment	Peak	Assignment
A	ethanediol	**H**	1,1'-thiodihexane
B	unassigned	**I**	unassigned
C	D-limonene	**J**	dichlorohexadecane
D	hexane-1-thiol	**K**	1,1'-dithiodihexane
E	cyclopentanol	**L**	hexadecanoic acid
F	hexane-1-thiol isomer	**M**	octadecanoic acid
G	chloroalkane	**N**	hexanedioic acid bis(2-ethyl hexyl) ester
		O	di octyl phthalate

Table 4.8 Assignment of peaks from gold wire exposed to a headspace of hexane-1-thiol and thermally desorbed at 300°C (Figure 4.36).

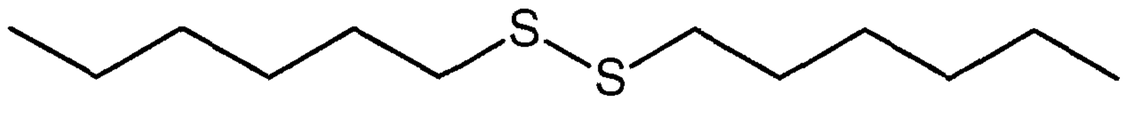
The standard of hexane-1-thiol (**35**) contained minor impurities of hexane-2-thiol (**36**), 1,1'-dithiodihexane (**37**) and 1,1'-thiodihexane (**38**) (chromatogram not shown). With the exception of (**36**) these species were also identified in the desorption chromatograms. The major difference is the relatively low intensity of hexane-1-thiol in each of the traces and the very intense peak for the disulfide (**37**).



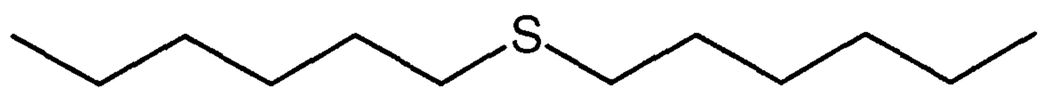
(35)



(36)



(37)



(38)

It is likely that this disulfide is formed during the high temperatures of the thermal desorption experiment. It could also form once adsorbed on the gold surface. This would be expected given the ease with which thiol compounds oxidise to the corresponding disulfide product. There are also a number of impurities present, particularly in the higher temperature experiment. Considerably fewer impurities were seen in the experiment at 200°C, which indicates that this is the more appropriate operating temperature for thermal desorption. The non-sulfur containing impurities detected are likely to have originated from the reagents and equipment used in the headspace adsorption experiment as a clean blank of the thermal desorber was obtained prior to the analysis. Hexane, which was used to dissolve the hexane-

1-thiol, was not present in the headspace, which indicates there is some selectivity of the gold for sulfur containing compounds.

4.6 Discussion and conclusions

Three methods have been investigated for selective isolation of thiols based on exploiting their reactivity. An important consideration in selecting a route for thiol trapping was the ability to concentrate the thiols. This led to a focus on solid-phase reagents. For the maleimide based and disulfide reagents, reactions were investigated in the solution-phase first before applying to the solid phase. Table 4.9 summarises the results from the experiments conducted.

Approach	**Phase**	**Sample**	**Comments**
Maleimide reagent	Solution	hexane-1-thiol	Trapping and oxidation reaction successful, elimination reaction requires optimisation.
	Solid	hexane-1-thiol	Analysis of trapping and oxidation reaction is inconclusive. The elimination step was partially successful.
	Solution	Buchu leaf oil	Not successful. Contradictory analytical evidence for final stage.
	Solid	Buchu leaf oil	Not successful.
	Solution	Standards of Buchu leaf oil thiol	Not successful. Contradictory analytical evidence.
Disulfide reagent	Solution	Mixture of thiols	Successful.
	Solid	3-mercapto-2-butanone	Successful.
Gold wire	Solid	Hexane-thiol	Successful, but mixed disulfides have potential to form.

Table 4.9 Comparison of methods for trapping thiols.

4.6.1 Maleimide reactions

The method development reactions in the solution-phase using hexane-1-thiol suggested that the reaction steps worked, although the reaction of the elimination did not go to completion and was hampered by a side reaction between the methylating reagents and the base, DBU. The first step, trapping with maleimide, was the most

consistent, confirming the common use of maleimide for isolating thiols. The reaction on the solid-phase silica reagent also produced the expected product; although again, the reaction solution also contained methylated-DBU indicating that the elimination procedure could certainly be improved. An alternative elimination reaction avoiding the oxidation step was attempted but was not successful under the conditions used. This could be developed further. The trapping reaction was also shown to be specific to thiols in the presence of alcohols.

The reaction with Buchu leaf oil containing the thiol, p-mentha-8-thiol-3-one, in solution, on the solid-phase and with the standard was less successful. The evidence suggests that there is an unexpected reaction at the first stage, which has not been resolved. This could be a feature of the structure of this thiol. Further work would be required (such as exact mass experiments) or two-dimensional NMR experiments to identify additional species.

The possibility of using solid-phase reagents for isolating thiols from food matrices has been demonstrated, although significant additional work would be required to be able to apply this to a flavour system. Further developments could be on finding a simpler procedure for eliminating the thiol from the resin. For example, by using a resin that is designed with a facile release linker, such as photo-cleavable reagents or under mild acid/base release conditions. This would maintain the benefits of a solid-phase reaction. It has also been mentioned that water compatibility is important but there are no commercially available maleimide-silica based reagent with flexible linkers so this would have to be synthesised, with the added complexity that this would entail. Macroporous resins also exist which are designed to be used with water.

4.6.2 Disulfide reagent

The experiments described in Section 4.4 suggest that the use of a disulfide reagent would be suitable for trapping of low molecule weight thiols, either in the solution-phase or using the covalent chromatography gel. Reactions in both phases meet some of the selection criteria, namely selectivity towards thiols and regeneration of the thiol for organoleptic analysis. The downside of releasing the thiol again for

analysis is the poor stability, plus all the usual difficulties for chromatographic analysis would apply such as the thiols being absorbed by the column. A way to overcome this would be to analyse the mixture before release by LC-MS to generate some structural information, using the characteristic ion at *m/z* 153 to identify the reacted species. This would only be possible for the solution-phase reaction. This could be combined with analysis of the thiols after displacement with the reducing agent for assessment of olfactory properties and further structural data (GC-MS in conjunction with GC-O). A disadvantage of using the solution-phase derivatising reagent is that this does not provide a way of isolating the thiols from the other components in the mixture. DTNB also has a limited pH range of solubility (6.8 – 8.2) which would limit the application with some food matrices.

A further disadvantage of the solution-phase reaction is that it does not provide a method for concentration of thiols, whilst the covalent chromatography method does have the potential for passing large amounts of food matrix through the column. When the reactive disulfide is bound to a solid support, a wider pH range can be used since the solubility is no longer an issue.

The recovery of the thiols was not measured and would need to be investigated. Other issues to explore are whether the reaction will take place with very dilute solutions of thiols. It is also possible that during analysis, the release of the displaced species would swamp the signals from the analyte thiols. More refined experiments would be required to take steps to eliminate oxygen which causes oxidation and to remove any metal ions which may be present in a more complicated sample. The experiments described above provide proof of principle. Further experimentation would be required to optimise the reaction conditions.

4.6.3 Adsorption onto a gold wire

The experiments described in Section 4.5 showed the potential of using a metal such as gold to selectively isolate thiol species. The major drawback of such a method is that the formation of disulfides could make the identification of the thiols originating from the sample more difficult. This is especially true if there are several thiols present which are likely to form mixed disulfides. If disulfides are detected it would not be known for certain if the disulfide originated from the sample or were formed

during the experiment. For this reason, no further experiments were conducted using these methods.

Further experiments which could have been conducted would be to observe if the gold was absorbing solely the sulfur containing species in the presence of a more complex matrix and thus truly being selective. It would also be illuminating to have tested the ability of the gold to absorb sulfur species from a solution containing trace thiol compounds, perhaps in the kind of adaptation of the equipment used in stir-bar absorption experiments (see Section 1.5.2.4). Other refinements would be to have a much greater surface areas of gold exposed, using finely divided particles. The latter method would be most compatible with a headspace experiment.

4.6.4 Conclusion

The traditional method using disulfide reagents was the most successful on the model compounds, and should be the simplest to apply to a food matrix without much further optimisation, and hence reach the goal of discovering new thiol compounds to be used in flavour applications.

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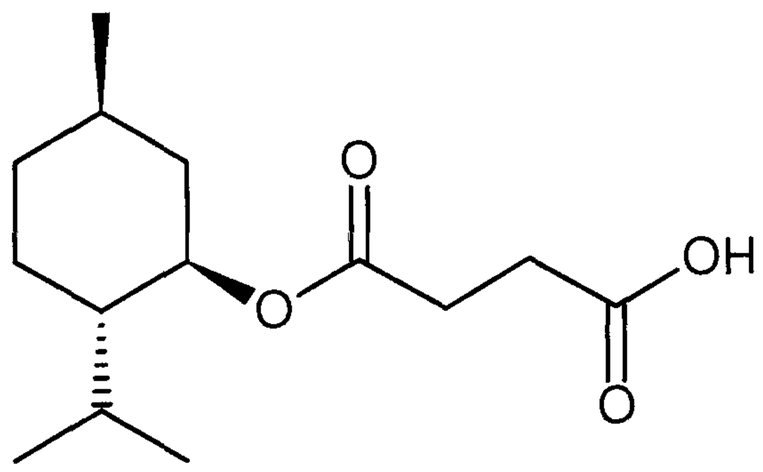
5

Identification of a Menthyl Ester in Nature

In Chapter 1, menthyl esters were introduced as compounds which are capable of generating a cooling sensation without the drawbacks of menthol. Disadvantages of menthol include a bitter taste, high volatility and a burning sensation when used in high concentration. The strong minty flavour is also a limitation for applications where other flavours are desired. Beverages are an attractive application area because cooling compounds, in general, deliver sensory perception best in polar systems. Ester derivatives of menthol have been the subject of several patents, covering their use in numerous fragrance and flavour applications (Bauer *et al.*, 1977; Dewis *et al.*, 2005; Grainger *et al.*, 2002; Jarboe, 1963; Mane and Ponge, 1998).

This chapter describes the identification of L-monomenthyl succinate (**1**) in two natural food sources: the berries and leaves of the plant *Lycium barbarum.* Preparative liquid chromatography was used for simplifying the complex plant extracts, with further separation and subsequent identification by nano-LC-ESI-MS/MS.



(1)

This short introduction discusses the criteria required for nature-identical status to be established; the choice of a suitable food source for identifying L-monomenthyl

succinate and the non-standard instrumentation that was used in this work including advanced chromatographic techniques. A comparison is made of the use of microwaves for extracting materials when compared to the traditional Soxhlet extraction technique. This work also provides a brief demonstration of the utility of microwaves in organic synthesis.

5.1 Establishing nature-identical status

It was mentioned in the Introduction that there are three categories of flavours: natural, nature-identical and artificial, as defined by the European Union, and in some other countries. (Many other countries, including the USA, have only natural and artificial categories). Natural flavours are extracted by some method from a natural source. Nature-identical flavourings are chemically identical to natural flavours but manufactured synthetically. Artificial flavours are synthesised flavours not found in nature. Nature-identical is preferable to artificial flavouring status for flavour companies for regulatory and consumer perception reasons. From a legislative point of view, most national states have lists of permitted artificial flavouring substances. Nature-identical substances do not have to be listed because they are presumed to be safe. Note that the category of nature-identical may change in the future, with current draft proposals from the EU to categorise these as artificial flavours because of lack of consumer understanding of the legal definitions. Today, the overwhelming regulatory trend is towards a positive list of all flavouring substances, that is, those substances not listed are prohibited. Flavouring substances are added to the list following a safety evaluation by qualified experts based on the conditions of intended use (Schrankel, 2004).

Because of the importance of establishing nature-identical status to the flavour industry, the industry association the International Organisation of the Flavour Industry (IOFI) has established a Working Group on Methods of Analysis (WGMA) to evaluate the validity of identifications in nature of flavouring substances. Recently, this working group published details on the criteria for accepting nature-identical status in order to avoid mistaken identification (Brevard *et al.*, 2006). These guidelines mean that that any particular substance must have its identity confirmed by at least two methods, for example, by comparison of chromatographic and spectroscopic data (which may include mass, IR and NMR spectra) with those of

an authentic sample. In the specific case of papers aiming to demonstrate the first discovery of a given compound in a natural source, the author(s) are required to provide full data obtained by their own measurements of both the unknown and the authentic sample (for example, full mass, IR, or NMR spectra, retention indices, etc.). The need for these criteria has partly arisen due the publication of misleading lists of natural products, containing compounds which could be due to artifact formation or because of contamination from a variety of sources (such as plasticisers from storage containers).

5.2 Identification of suitable food sources

The nature-identical status of a number of menthyl esters was first established by Hiserodt and co-workers (Hiserodt *et al.*, 2004). L-monomenthyl succinate was found in extracts of *Lycium barbarum* berries and *Mentha piperita* (peppermint leaves). L-monomenthyl glutarate and dimenthyl glutarate were found in the dried fruit of *Litchi chinesis* (lychee). These researchers utilised LC-MS/MS with APCI ionization for the identification of these compounds. One of the same authors also identified L-menthyl-L-lactate in nature in the essential oil *Mentha arvensis L* (Gassenmeier, 2006). The presence of L-monomenthyl succinate in fresh and dried mint leaves of *M. piperita* (peppermint) and *M. spicata* (spearmint) has also been demonstrated by use of LC-ESI-MS/MS and GC-MS (Marin and Schippa, 2006).

The identification of a suitable food source of L-monomenthyl succinate by Hiserodt *et al.* (2004) was based on previous identification (Kim *et al.*, 1997) of the precursors to this compound, namely menthol and succinic acid, in *Lycium chinense* P.Mill leaves. Consequently, these authors looked for and found L-monomenthyl succinate in the berries from the closely related plant, *L. barbarum.* They also established that this compound was not formed primarily as an artefact of the extraction process by spiking a flour extract with menthol and succinic acid and using the same extraction procedure as for the fruit. Although a small amount of L-monomenthyl succinate was found in the flour extract this was 270 times less than the level found in *L. barbarum* berries and 1700 times less than the level found in peppermint leaves. For the research described here, the leaves of *L. barbarum* were investigated for the presence of L-monomenthyl succinate as the previous work suggested a high probability of this compound's presence.

L. barbarum is a plant native to Asia and belongs to the family Solanaceae. It has a number of common names including Chinese Wolfberry, Chinese boxthorn, Chinese desert thorn, Duke of Argyll's Tea tree or Matrimony Vine. The berries are known commonly as goji berries in English (or gǒuqǐ in Chinese). This plant is a major Chinese tonic herb with a history of almost 2,000 years of medicinal use. Both the berries and the root are used and traditionally the plant is believed to promote long life (Chevallier, 1996). The fruits are commonly used in Asia to maintain good health, and have recently seen increasing popularity in the Western world because of their high nutrient richness and antioxidant qualities (Gross *et al.*, 2006). The dried leaves can be used to make tea and are also sold fresh as a leaf vegetable (Facciola, 1990). The plant (a) and dried berries (b) are shown in Figure 5.1.

(a) **(b)**

Figure 5.1 *Lycium barbarum* (a) plant and (b) dried berries.

5.3 Choice of instrumentation

The use of highly-sensitive characterisation techniques, such as mass spectrometry, is invariably required to establish nature-identical status because of the potentially low concentration of target molecules. Food sources are also complex so chromatography is normally used. For this work, two chromatographic systems were used to separate the components of plant material extracts. The preparative chromatography system separates the mixture and collects fraction for further analysis. This provides an effective method of removing interfering substances, and thereby concentrating the sample. The second separation makes use of a nanoscale HPLC system interfaced with an ESI-quadrupole-TOF mass analyser.

5.3.1 Preparative chromatography system.

The preparative chromatography equipment used was a two-dimensional protein fractionation platform (Proteome™ Lab PF 2D Protein Fractionation System) from Beckman Coutler. This system is designed to provide a completely automated method for separating complex mixtures of proteins for studies on protein expression in biological systems (proteomics). The method uses chromatofocusing (CF) to fractionate proteins in a first dimension based on their isoelectric point (pI), followed by nonporous silica RP-HPLC separation of the pI fractions to separate proteins further based on their hydrophobicity. A two-dimensional map of the protein content of the samples can be visualized based upon pI versus hydrophobicity. Differential display maps can be generated to highlight differences in protein expression, such as between normal and abnormal states.

Traditionally, two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) gels have been used to separate protein mixtures but it is becoming increasingly common to use two-dimensional chromatography as the separation method. Some of the advantages of liquid phase protein separations include flexibility, relative speed, ease of automation of sample handling, and hyphenation to mass spectrometry. For further details, a review of two-dimensional chromatography in conjunction with mass spectrometry for protein expression studies has been published (Nilsson and Davidsson, 2000). Examples which demonstrate the use of this particular system includes a study comparing drug-treated and untreated colon cancer cells (Yan *et al.*, 2003) and more recently, for differential phosphoprotein mapping in cancer cells (Pal *et al.*, 2006).

A schematic of the instrument is shown in Figure 5.2. For the normal mode of operation of this instrument, both columns are used to separate the protein mixture, with the fractions collected from the first chromatofocussing column monitored by pH and UV detectors. For this application, the first column was bypassed and only the reverse phase column was used, as shown by the areas highlighted in yellow in Figure 5.2. Another UV detector is used for monitoring the separation on the second column, set to 214 nm, the absorption maximum of the amide bond. Note that this setting cannot be changed on this instrument because it is designed for analysing

proteins. Fortunately, the ester bond of L-monomenthyl succinate also absorbs in this region. The prepared samples are collected from a 96-well plate and injected on to the RP column and the fractions collected at intervals set by the user. Only the fractions around the retention time of the standard need be collected.

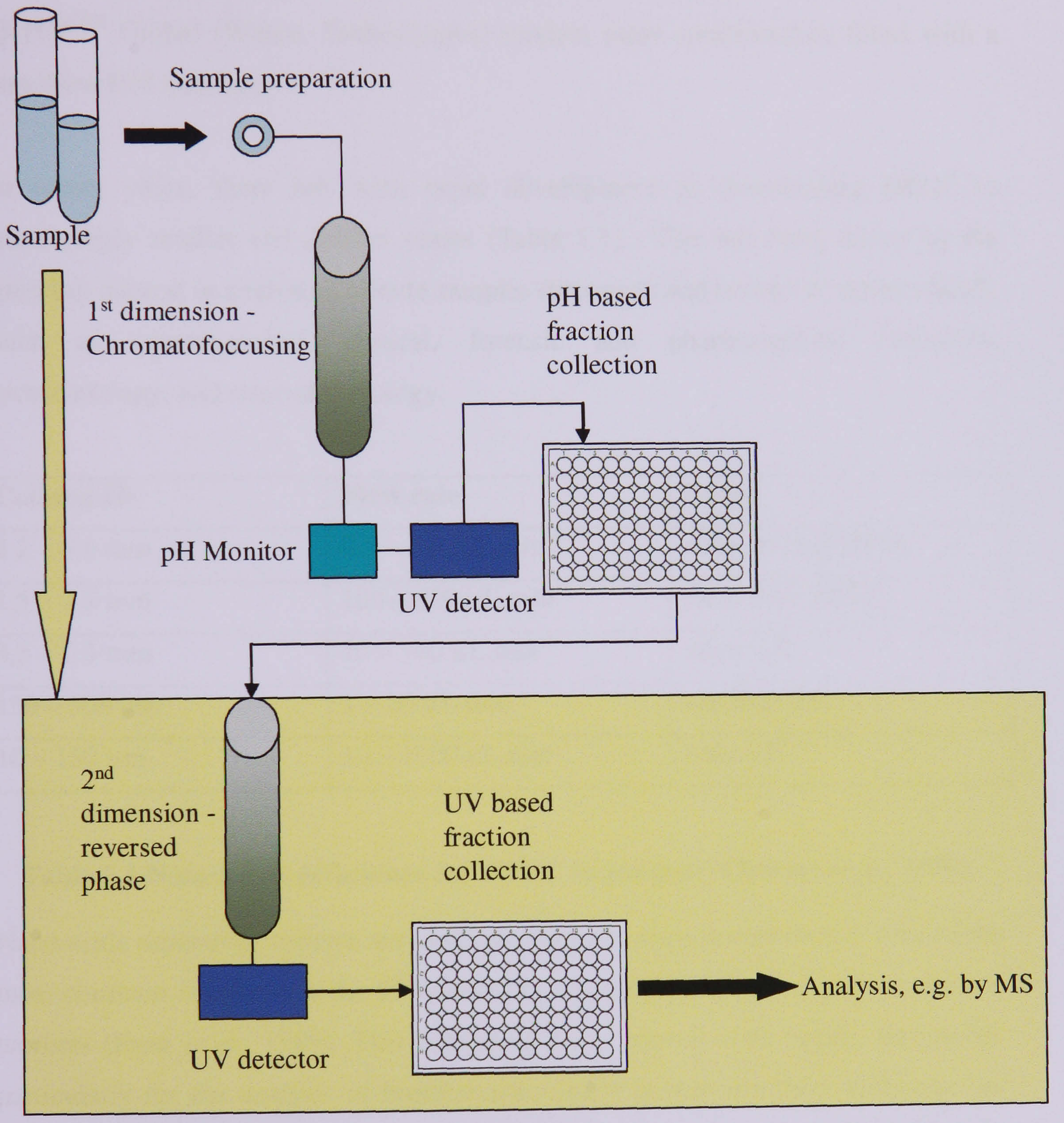


Figure 5.2 Schematic of the PF2D system.

(Only the parts of the system highlighted in yellow were used for the separation of components in plant extracts).

5.3.2 Nano-LC-ESI-MS/MS

A nano-capillary LC-ESI-MS-MS system was used to analyse fractions collected from the first separation. The system allows for the analysis of minimal samples amounts with high selectivity and sensitivity. Highly automated analysis is also possible. The system utilised consists of a capillary LC (CapLC^{TM}) interfaced with a Q-TOF^{TM} Global (Waters Technologies) tandem mass spectrometer, fitted with a nanoflow ESI interface.

In recent years, there has been rapid development in downscaling HPLC to increasingly smaller and smaller scales (Table 5.1). This has been driven by the growing interest in analysing minute samples (picomole and below) in various fields, such as environmental, clinical, forensic and pharmaceutical chemistry, biotechnology, and structural biology.

Column ID	**Flow rate**	**Name**
3.2 – 4.6 mm	0.5 – 2.0 mL.min^{-1}	conventional HPLC
1.5 – 3.2 mm	100 – 500 µL.min^{-1}	microbore HPLC
0.5 – 1.5 mm	10 – 100 µL.min^{-1}	micro LC
150 – 500 µm	1 – 10 µL.min^{-1}	capillary LC
10 – 150 µm	10 – 1000 nL.min^{-1}	nano LC

Table 5.1 Names and definitions for HPLC techniques (Chervet *et al.*, 1996).

Nano-scale separation systems are often interfaced to mass spectrometers. One of the most common interfaces is the ESI interface, originally introduced by Fenn and co-workers (Fenn *et al.*, 1989). This combination has proved to be hugely successful, particularly for the analysis of biomolecules and it is now the main technique for liquid chromatography coupled on-line to mass spectrometry. It is particularly powerful when ESI is miniaturised as a nanospray system. This requires low sample consumption, low flow rates (nL.min^{-1}), and there is improved ion transfer efficiency from source to detector when compared with conventional ESI sources. Reviews have been published on the developments in miniaturised electrospray interfaces (Gelpi, 2002; Niessen, 1998; Wood *et al.*, 2003) and nanoscale LC (Chervet *et al.*, 1996).

ESI is a soft ionisation technique and therefore usually generates little fragmentation. For more structural information, MS/MS is required. The Q-TOF™ mass spectrometer utilised for the work described below was a hybrid quadrupole time of flight mass spectrometer with MS/MS capability. A schematic of the instrument is shown in Figure 5.3. The quadrupole is operated as an ion guide in MS mode and as mass selection device in MS/MS mode. A reflectron time-of-flight (TOF) analyzer is placed orthogonally to the quadrupole and serves as a mass resolving device for both MS and MS/MS modes. A collision cell is located between the quadrupole and the TOF analyzer to induce fragmentation in MS/MS experiments. The final detector is a microchannel plate with high sensitivity. This combination of quadrupole and TOF mass spectrometer unites the high selectivity of a quadrupole with the high efficiency of the TOF analyser.

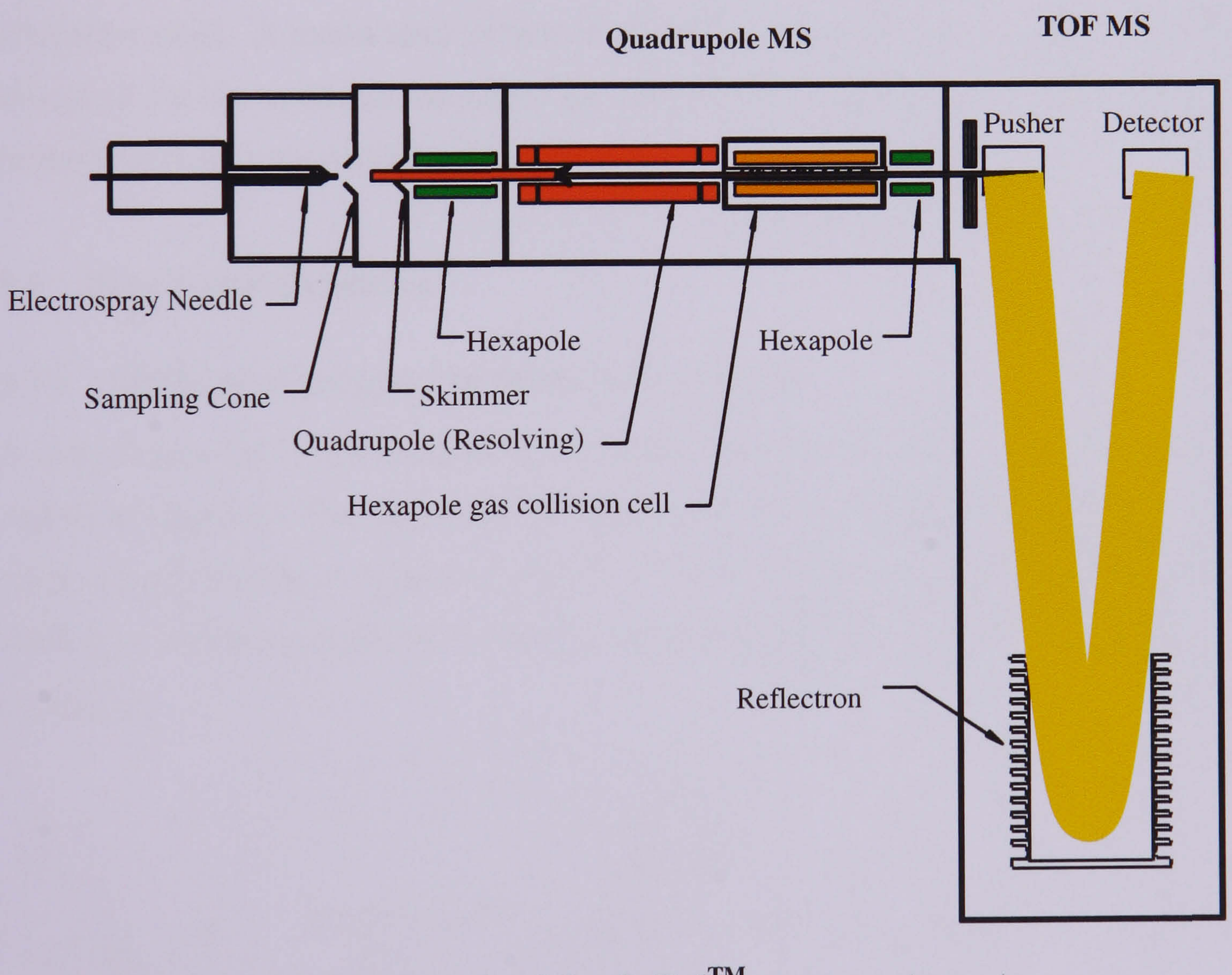


Figure 5.3 Schematic of the Q-Tof™ mass spectrometer

5.3.3 Microwave systems

Commercial microwaves are typically closed-vessel systems. They consist of a magnetron tube, an oven where the extraction vessels are set upon a turntable, and monitoring devices for controlling the temperature and pressure. Up to 12 extraction vessels (usually made of PTFE) can be irradiated simultaneously. One of the vessels is a reference vessel controlling heat and pressure. The pressure is measured by means of a water manometer and the temperature probe is a fibre optic with a phosphorous sensor, which allows temperature in the range of 20 - 200°C to be selected. Extraction conditions such as the percentage power input, the pressure and the temperature can be varied accordingly. Reviews comparing commercial microwave systems have been published (Erickson, 1998; Eskilsson and Bjorklund, 2000). Two different commercial microwave ovens were used in the synthesis and extraction steps. A microwave extraction system usually used for solvent extraction was used for the synthesis step and was found to be suitable for this purpose, as demonstrated by a successful synthesis.

5.4 Results and discussion

5.4.1 Synthesis of standard by microwave synthesis

It was found that L-monomenthyl succinate was not available from the usual chemical suppliers. This provided an opportunity for a simple exploration of the utility of microwaves in organic synthesis. Formation of esters from anhydrides and acids is a common synthetic reaction (Larock, 1989). The reaction is shown in Figure 5.4.

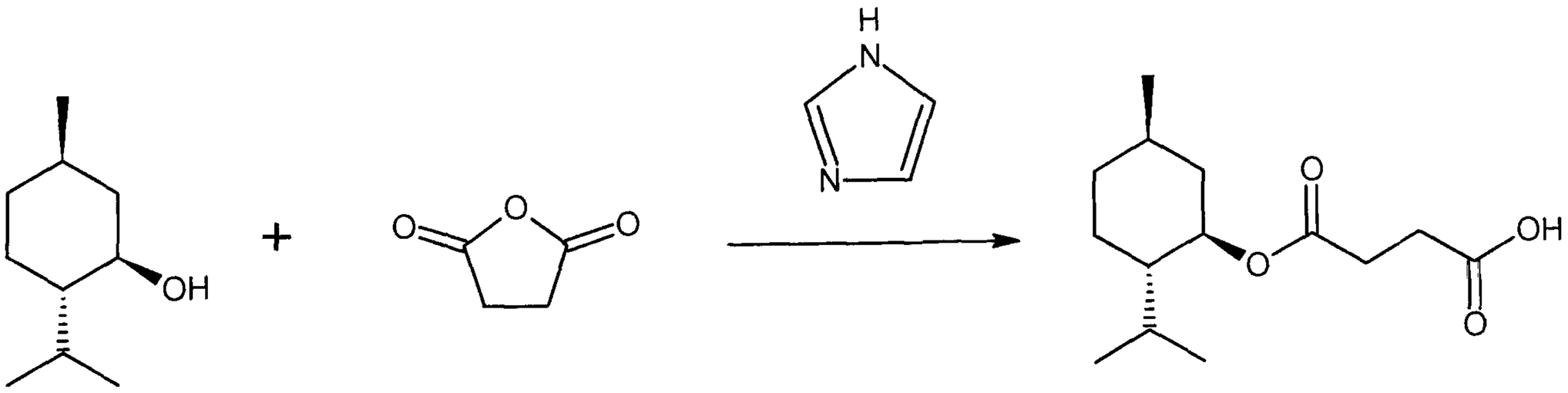


Figure 5.4 The formation of L-monomenthyl succinate by combination of menthol and succinic anhydride, catalysed by imidazole.

The reaction was facile when a microwave oven unit was utilised. It involved combining the solid starting materials, succinic anhydride and menthol with the catalyst imidazole in equimolar amounts. All ingredients were reacted as solids, with no solvent required, although care must be taken not to heat the sample too rapidly, which results in charring. A few minutes at low power was all that was required to generate the ester. (Method adapted from (Hirose *et al.*, 2003)). The more time-consuming process was the reaction work-up to purify the menthyl ester (see Materials and Methods, Section 2.3.2.1). The confirmation of the synthesis was provided by FTIR, ^{1}H and ^{13}C NMR and MS data. IR: 1724 cm^{-1} (ester C=O stretch) and 1705 cm^{-1} (acid C=O stretch); ^{1}H NMR (500 MHz, d6 DMSO) δ = 4.63 (td, 1H, J = 4.4 Hz, J = 10.9 Hz), 2.42 (s, 4H), 1.85 (m, 2H), 1.5 (m, 2H), 1.39 (m, 2H), 0.96 (m, 3H), 0.87 (d, 3H, J = 7 Hz), 0.86 (d, 3H, J = 6.4 Hz), 0.72 (d, 3H, J = 6.9 Hz); ^{13}C NMR (50 MHz, d6 DMSO) δ =173.5 (C), 171.5 (C), 74.7 (CH), 47.4 (CH), 41.2 (CH_2), 34.6 (CH_2), 31.7 (CH), 29.9 (CH_2), 29.4 (CH_2), 26.6 (CH), 23.8 (CH_2), 22.4 (CH_3), 21.2 (CH_3), 16.7 (CH_3); MS (ESI-ve) = 255.2 $[C_{14}H_{23}O_4]^-$, 99.0 $[C_4H_3O_3]^-$

5.4.2 Preparative chromatography

The standard of L-monomenthyl succinate was injected onto the RP-HPLC column of the fractionation system (20 mmoles on column). The resulting chromatogram for the standard is shown in Figure 5.5. It was found that the standard was poorly retained on the column and eluted in the first 2 minutes. Although the poor retention of L-monomenthyl succinate on the column was not ideal, it still allowed for simplification of the mixture by only collecting the fractions at the start of the elution. Figure 5.6 and Figure 5.7 illustrate that much of the sample (as monitored by the UV detector) was present at later retention times, and thus significant simplification of the sample is achieved by this first separation.

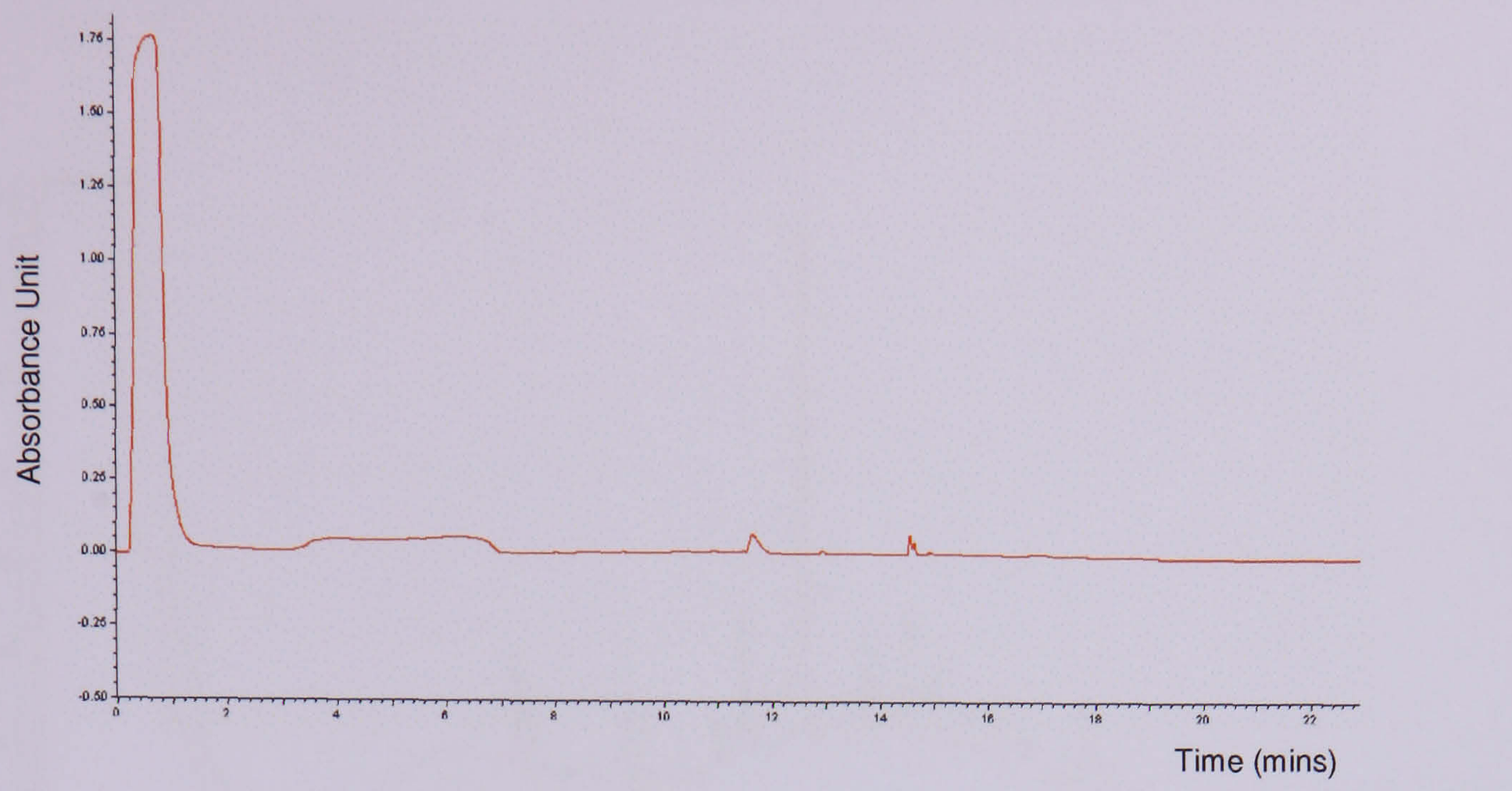


Figure 5.5 HPLC preparative chromatogram of standard of L-monomenthyl succinate.

The prepared extracts of *L. barbarum* dried fruit and dried leaves from Soxhlet and microwave extraction were injected on the column and the first six fractions were collected over a time period of three minutes in 30 second intervals. The chromatograms for the fruit extracted by Soxhlet and microwave are shown in Figure 5.6. The chromatograms for the leaves extracted by Soxhlet and microwave are shown in Figure 5.7. The two extraction techniques gave very similar elution profiles in both cases. This is evidence that the techniques are comparable for extraction.

The repeatability of the preparative chromatography was also investigated. A sample of the *L. barbarum* dried fruit preparation (Soxhlet extraction) was split into five fractions which were subsequently injected five times through the RP-HPLC system. The chromatograms are shown in Figure 5.8, indicating good repeatability between runs. Figure 5.9 shows the blank between runs. The fractions collected from the preparative stage were transferred to the sample tray for analysis by nano LC-MS/MS.

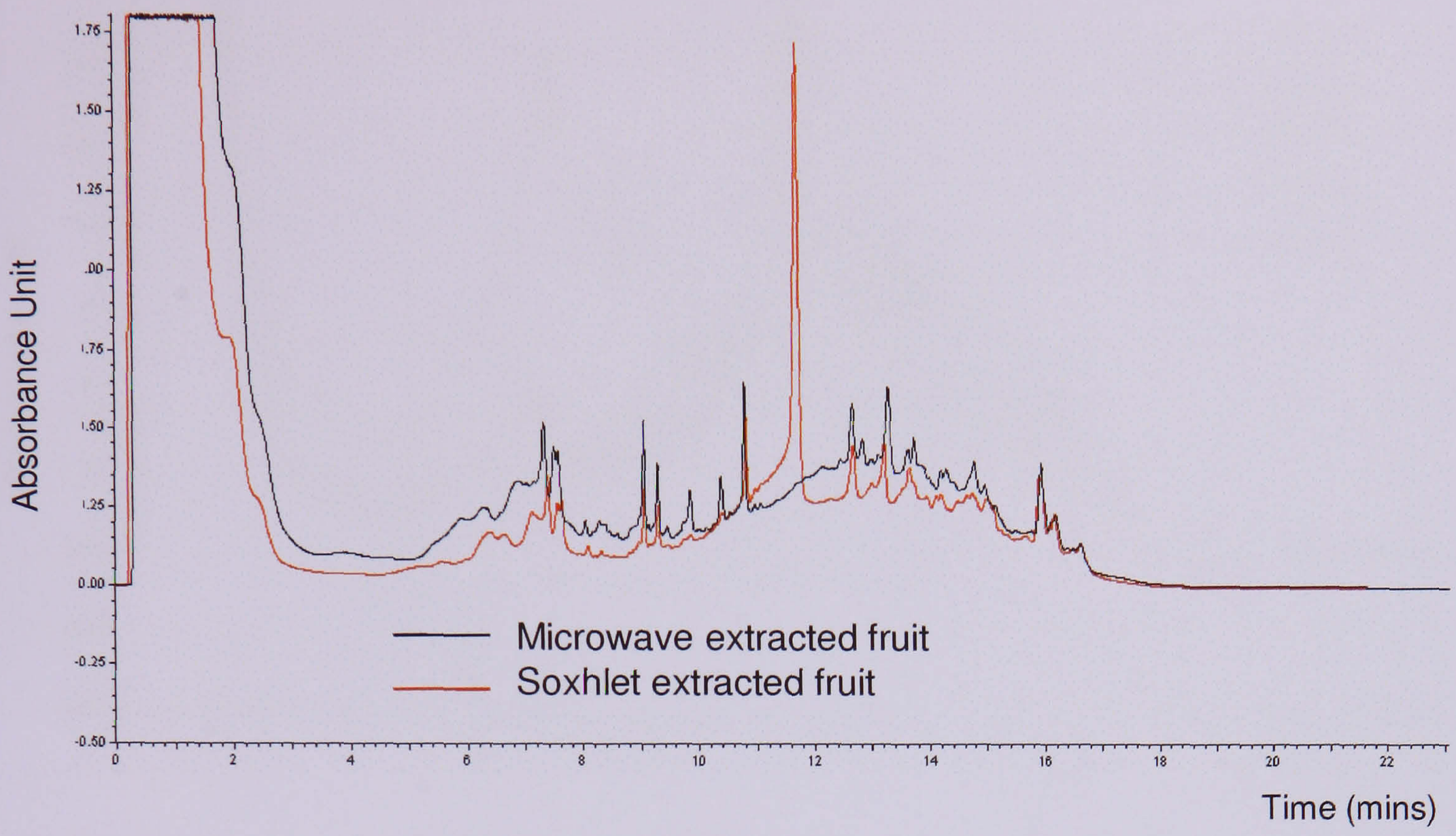


Figure 5.6 HPLC preparative chromatogram of *L. barbarum* dried fruit, comparison of microwave and Soxhlet extraction.

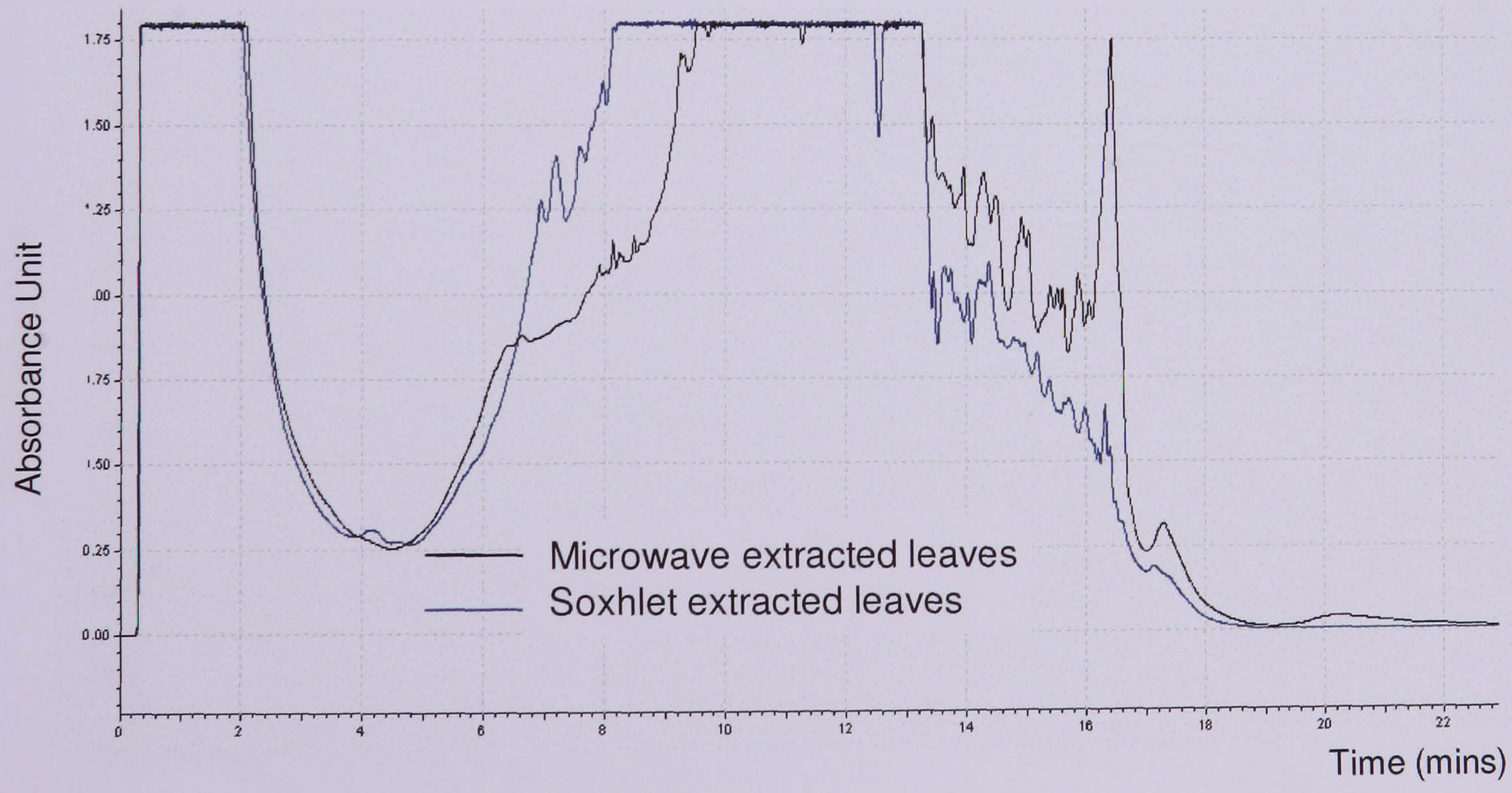


Figure 5.7 HPLC preparative chromatogram of *L. barbarum* leaf extracts, comparison of microwave and Soxhlet extraction

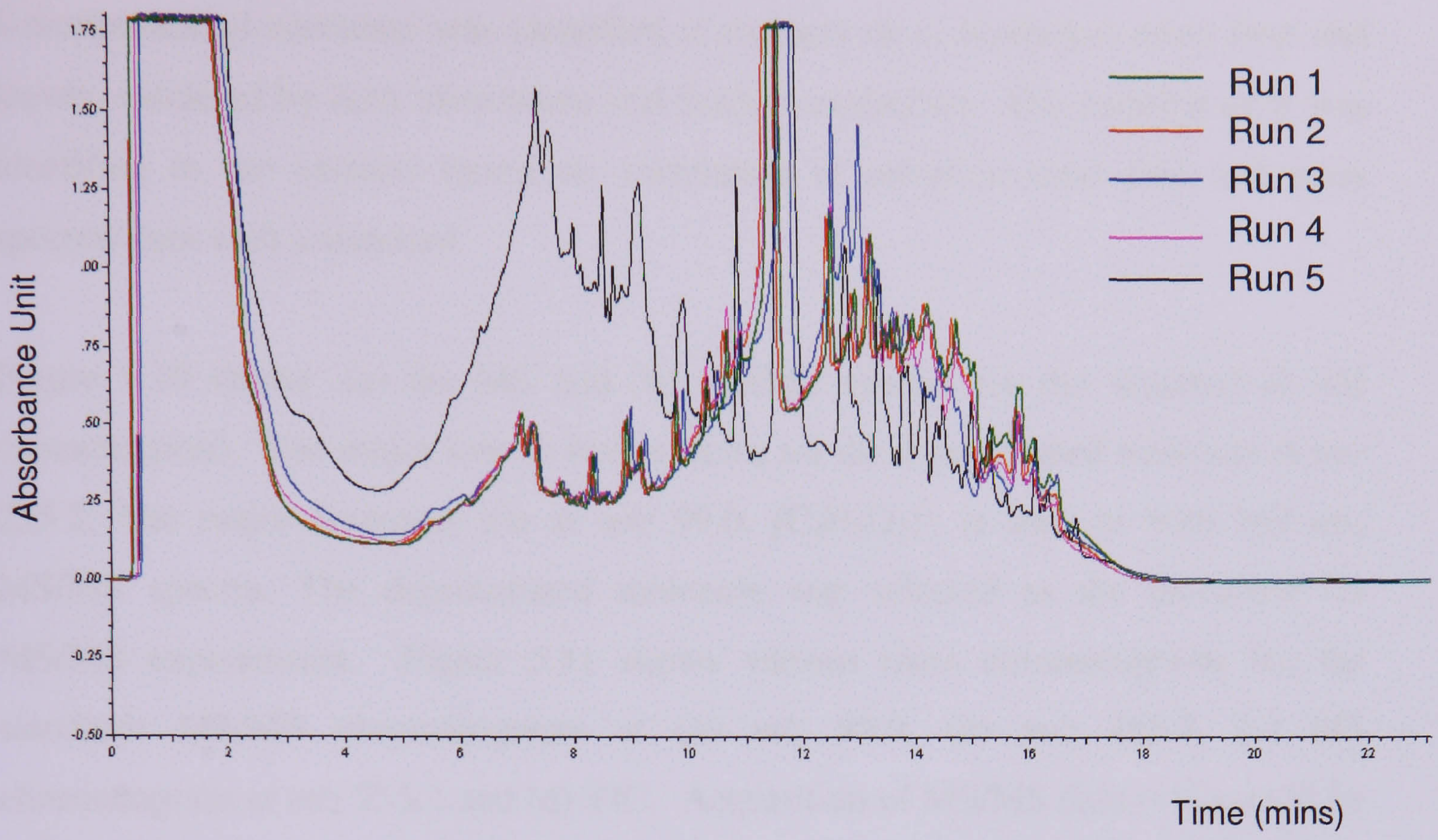


Figure 5.8 HPLC chromatogram of five-repeat analyses of fruit (Soxhlet extraction).

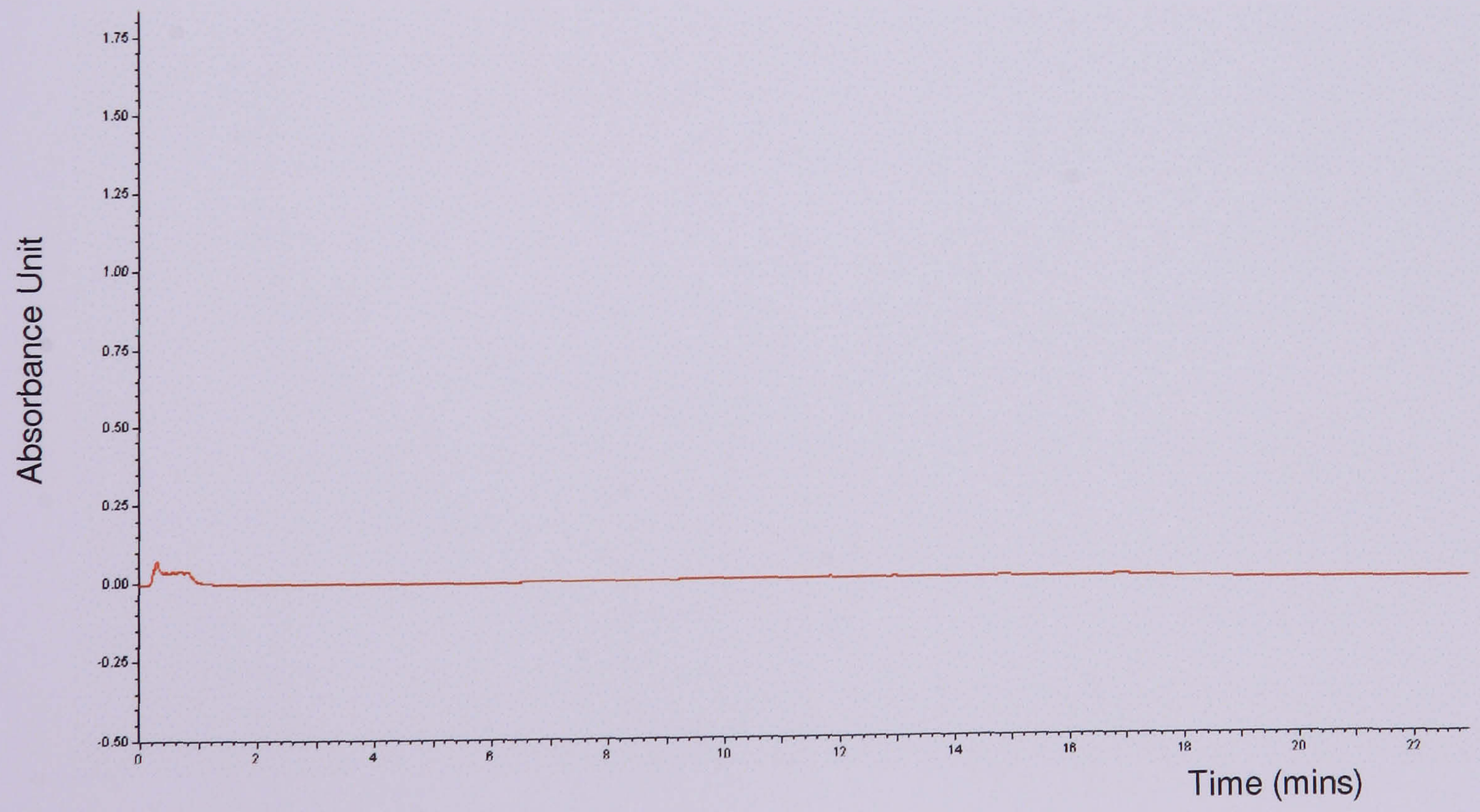


Figure 5.9 HPLC chromatogram of blank.

5.4.3 Nano-LC-ESI-MS/MS

L-monomenthyl succinate was identified in extracts of *L. barbarum* dried fruit and leaves, extracted by both microwave and Soxhlet extraction. The menthyl ester was identified in the extracts based on correlation of retention time data and mass spectral data with a standard.

Figure 5.10 shows: (a) the MS and (b) MS/MS spectra for the standard (2 nM concentration). The major ions in both spectra are the deprotonated molecule at *m/z* 255.2. The major fragment ion at *m/z* 99.0, $[C_4H_3O_3]^-$, is seen in both MS and MS/MS spectra. The deprotonated molecule was selected as the precursor for MS/MS experiments. Figure 5.11 shows various mass chromatograms for the standard: MS/MS chromatograms at (a) *m/z* 99.0, (b) *m/z* 255.2, (c) MS chromatogram at *m/z* 255.2 and (d) TIC. Acquisition of MS/MS data is triggered by detection of the ion at m/z 255.2 in the MS spectrum (data dependent acquisition), hence the truncated signal in Figure 5.11 (a) and (b).

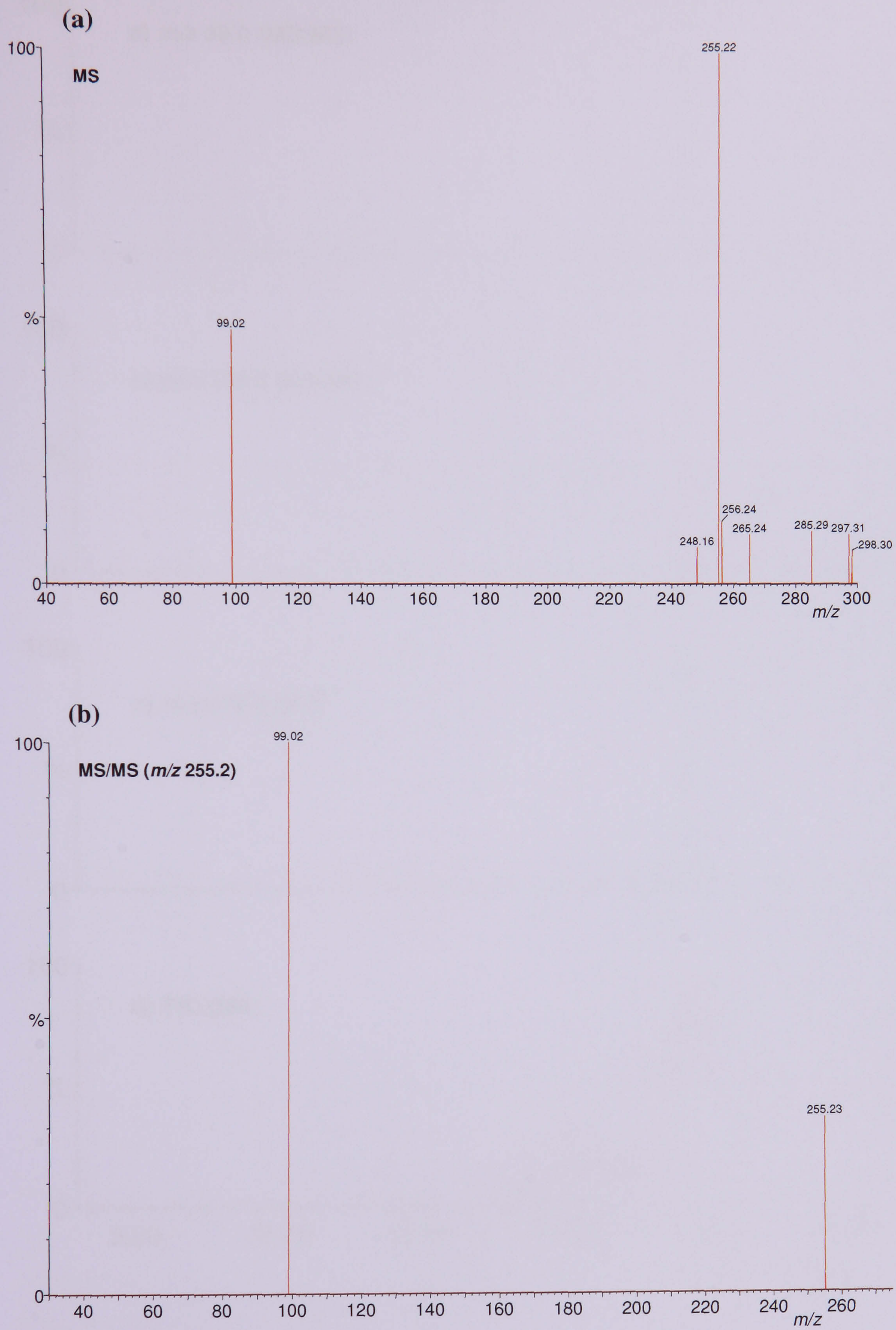


Figure 5.10 Mass spectra of the standard in (a) MS mode and (b) MS/MS mode.

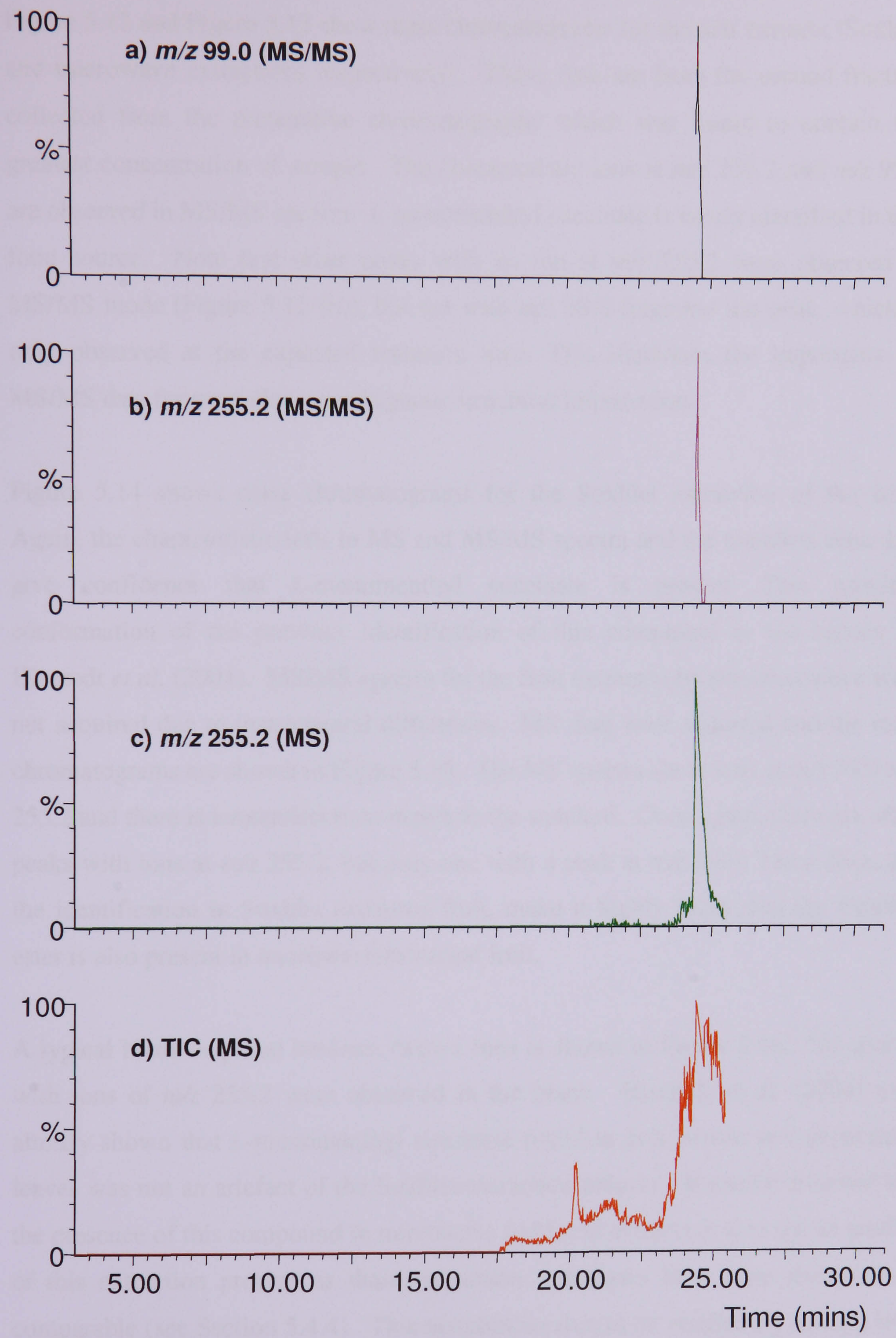


Figure 5.11 Mass chromatograms of the standard of L-monomenthyl succinate.

Figure 5.12 and Figure 5.13 show mass chromatograms for the leaf extracts (Soxhlet and microwave extractions respectively). These data are from the second fraction collected from the preparative chromatography which was found to contain the greatest concentration of sample. The characteristic ions at *m/z* 255.2 and *m/z* 99.0 are observed in MS/MS spectra. L-monomenthyl succinate is newly identified in this food source. Note that other peaks with an ion at *m/z* 255.2 were observed in MS/MS mode (Figure 5.12 (b)), but not with *m/z* 99.0 fragment ion peak, which is only observed at the expected retention time. This illustrates the importance of MS/MS data for providing unambiguous structural information.

Figure 5.14 shows mass chromatograms for the Soxhlet extraction of the fruit. Again, the characteristic ions in MS and MS/MS spectra and the retention time data give confidence that L-monomenthyl succinate is present. This provides conformation of the previous identification of this compound in the berries by Hiserodt *et al.* (2004). MS/MS spectra for the fruit extracted by the microwave were not acquired due to instrumental difficulties. MS data were acquired and the mass chromatograms are shown in Figure 5.15. The MS spectra show ions at *m/z* 99.0 and 255.2 and there is a retention time match to the standard. Once again, there are other peaks with ions at *m/z* 255.2, but only one with a peak at *m/z* 99.0. These data, and the identification in Soxhlet extracted fruit, make it highly likely that the menthyl ester is also present in microwave extracted fruit.

A typical blank acquired between extract runs is shown in Figure 5.16. No species with ions of *m/z* 255.2 were observed in the blank. Hiserodt *et al.* (2004) have already shown that L-monomenthyl succinate found in goji berries and peppermint leaves was not an artefact of the Soxhlet extraction process. It can be assumed that the presence of this compound in microwave produced extracts is also not an artefact of this extraction process as these extraction techniques have been shown to be comparable (see Section 5.4.4). This assumption should be verified by spiking flour with succinic acid and menthol in flour and using the same microwave extraction procedure. (This spiking protocol is described by Hiserodt *et al* (2004)).

In some samples, a shift in retention time was observed of up to 0.5 min. This is not unexpected when using nano LC which is sensitive to small variations in the flow

rate which in turn can shift the retention time. Retention time shifts can also be a result of matrix effects.

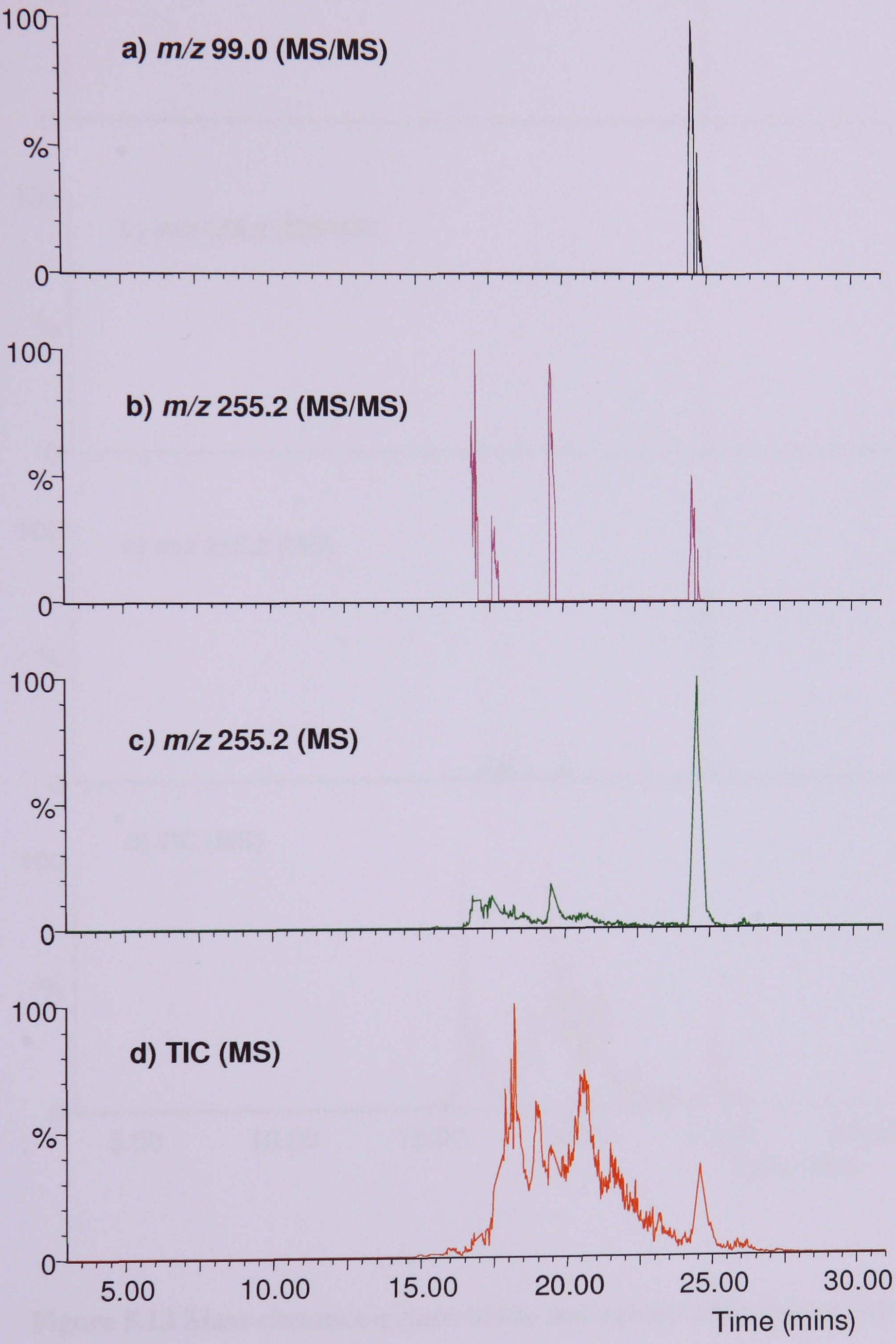


Figure 5.12 Mass chromatograms of the leaf extract (Soxhlet extracted).

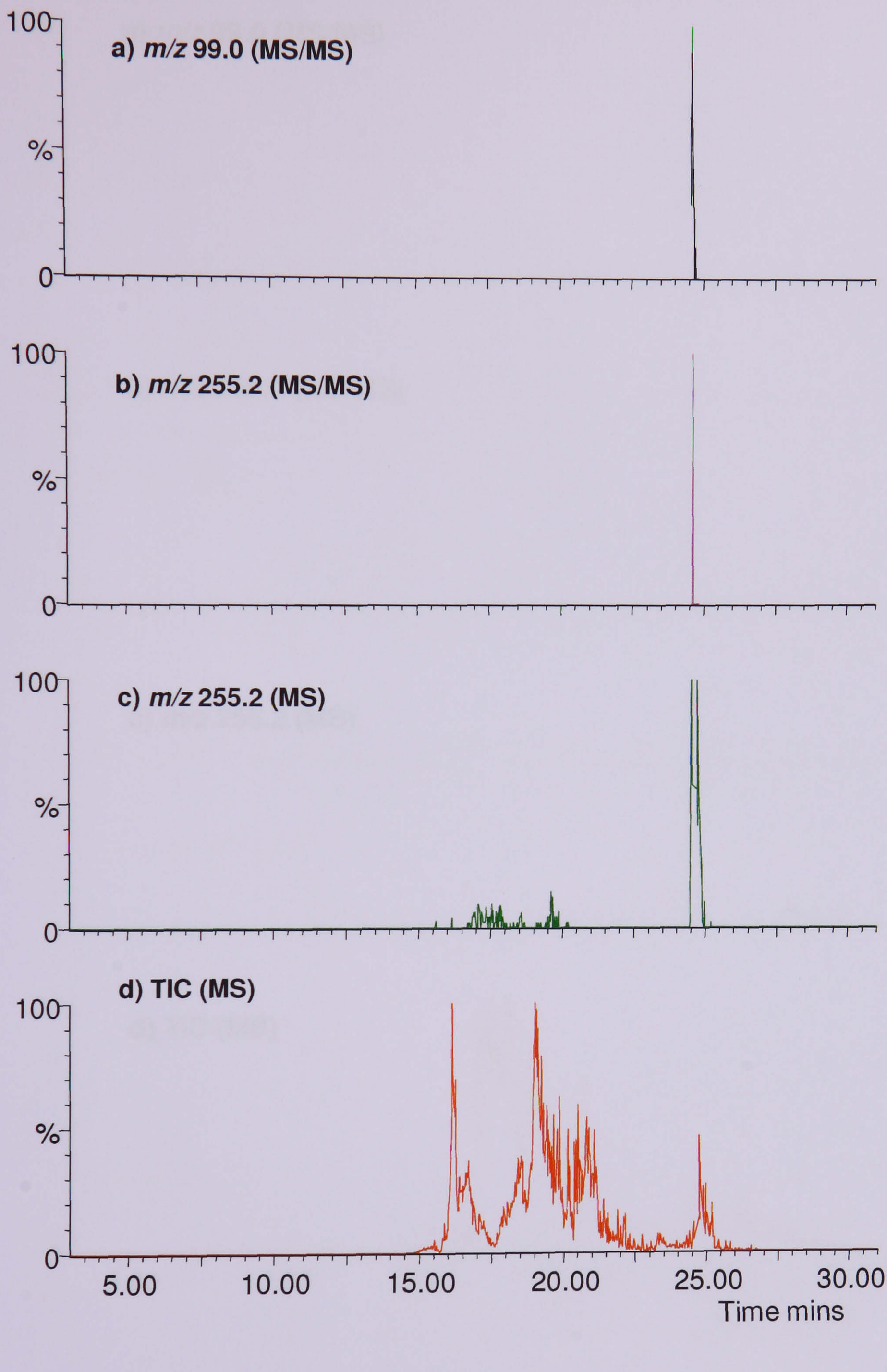


Figure 5.13 Mass chromatograms of the leaf extract (microwave extracted).

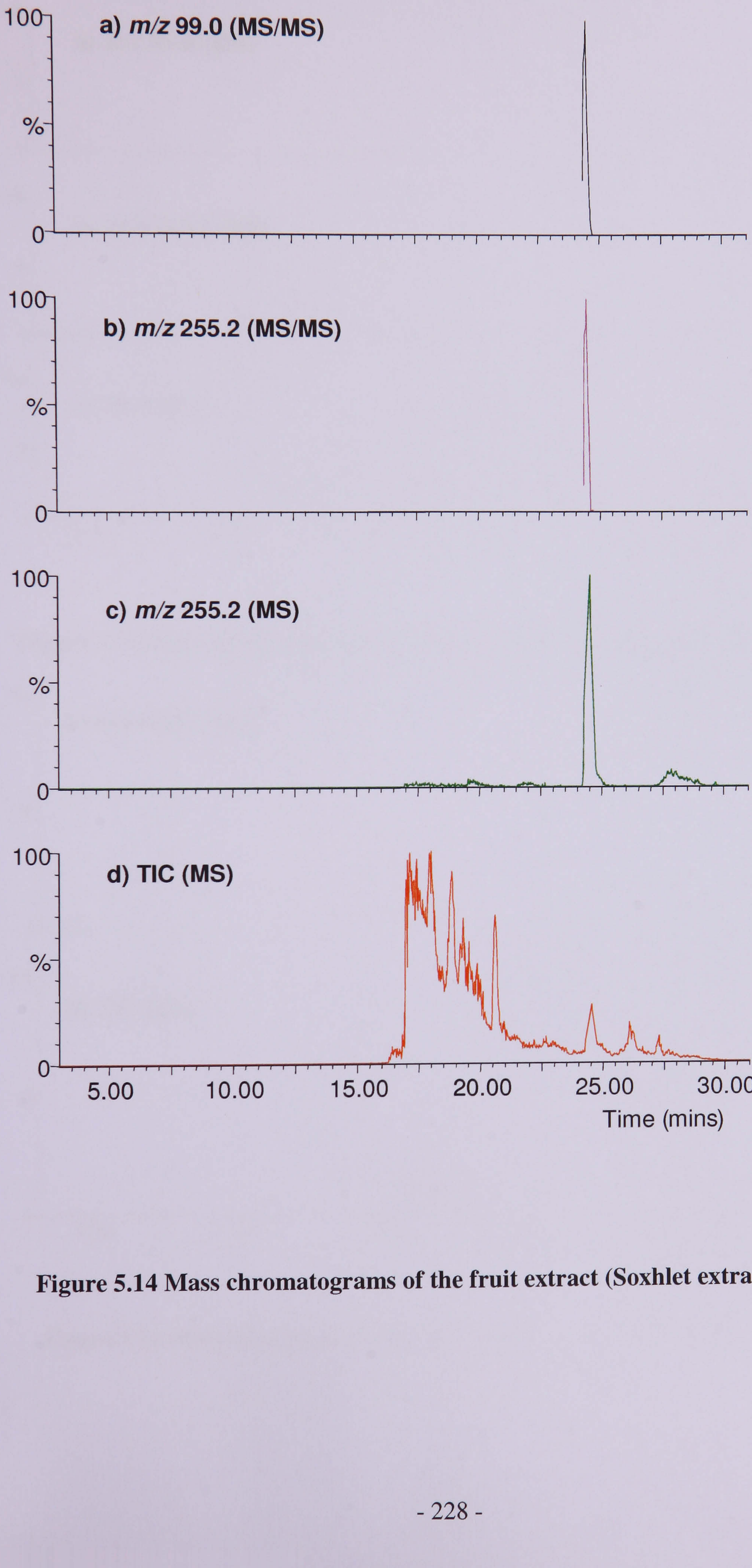


Figure 5.14 Mass chromatograms of the fruit extract (Soxhlet extracted).

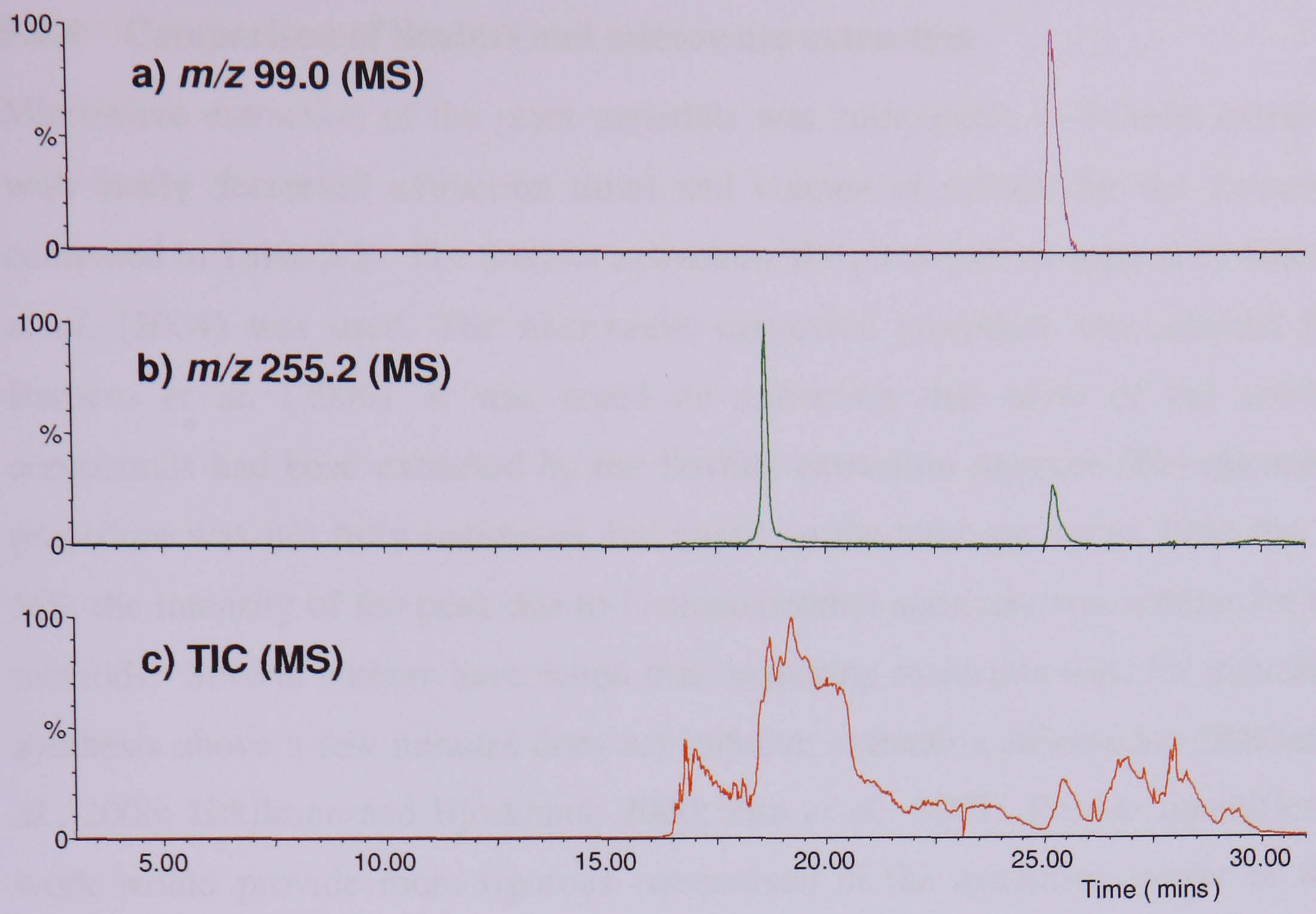


Figure 5.15 Mass chromatograms of the fruit extract (microwave extracted).

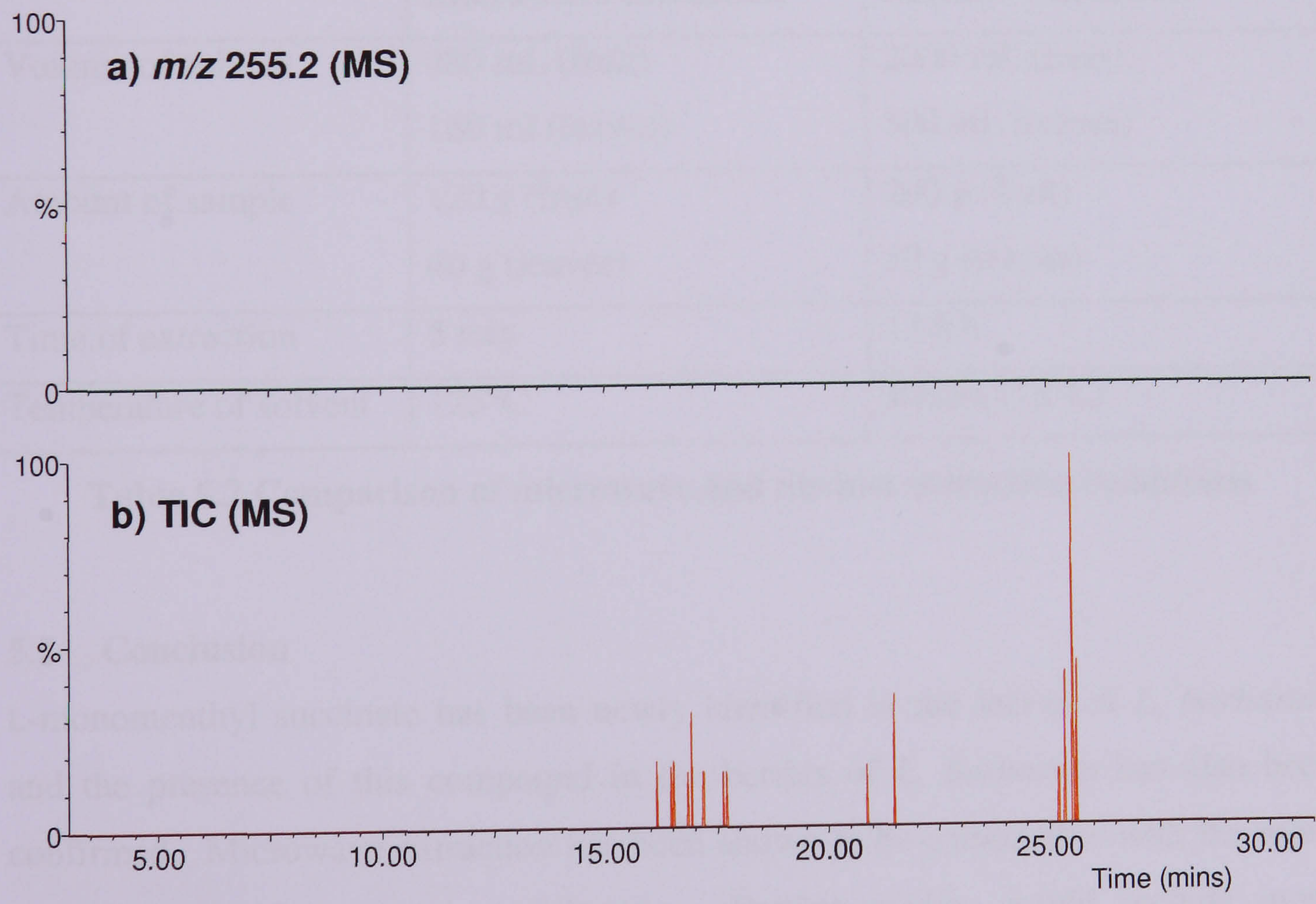


Figure 5.16 Mass chromatograms of the blanks a) *m/z* 255.2 and b) TIC.

5.4.4 Comparison of Soxhlet and microwave extraction

Microwave extraction of the plant materials was comparable to Soxhlet extraction with vastly decreased extraction times and volume of solvent for the former, as compared in Table 5.2. For Soxhlet extraction, the procedure as applied by Hiserodt *et al.* (2004) was used. The microwave extraction procedure was adapted from Barbero *et al.* (2006). It was noted on extraction that more of the coloured compounds had been extracted by the Soxhlet extraction protocol. The microwave procedure was not fully optimised, but based on the total ion count, from the LC-MS, the intensity of the peak due to L-monomenthyl succinate was similar for both methods. Several authors have found that increasing extraction time for microwave synthesis above a few minutes does not improve extraction efficiencies (Barbero *et al.*, 2006; Eskilsson and Bjorklund, 2000; Zhu *et al.*, 2007). Further quantification work would provide more rigorous comparison of the extraction power of these techniques.

	Microwave extraction	**Soxhlet extraction**
Volume of solvent	480 mL (fruit) 160 mL(leaves)	2000 mL (fruit) 500 mL (leaves)
Amount of sample	120 g (fruit) 40 g (leaves)	200 g (fruit) 50 g (leaves)
Time of extraction	5 min	13.5 h
Temperature of solvent	125°C	Reflux (78°C)

Table 5.2 Comparison of microwave and Soxhlet extraction conditions

5.5 Conclusion

L-monomenthyl succinate has been newly identified in the leaves of *L. barbarum* and the presence of this compound in the berries of *L. barbarum* has also been confirmed. Microwave extraction has been shown to be comparable with the more traditional Soxhlet extraction technique. Further studies would include more quantitative comparison of extraction techniques, optimisation of the extraction conditions, and verification that use of microwaves does not lead to artefact formation. Microwave extraction could be applied to other food samples to look for

nature-identical components. The simple use of microwaves in synthesis has also been shown.

The preparative HPLC system utilised here significantly simplified the plant extracts by only collecting fractions around the retention time window of the standard. Fractions could be further simplified by improving the chromatographic separation of the first dimension, and collecting 'tighter' fractions. The system was also shown to have good reproducibility. Highly concentrated samples could be obtained by repeated injection of extracts and collection of the same fractions, which can be combined and the solvent evaporated. This is extremely useful for trace analysis. The system could be utilised with a wide range of flavour systems. The power of MS/MS in unambiguous identification of trace level analytes has also been illustrated. The CapLC-QTof system could also be completely automated which maximises the efficient use of analysis time.

The main problems identified with this system were in precipitation of components in the extracts which caused blockage of the nanoscale columns, despite the presence of a pre-column. This could be overcome by improving the filtering procedure for samples. The next step for this work would be to quantify the level of analytes using standard addition, and to investigate the limits of detection of these experiments. Hiserodt *et al.* (2004) showed the concentration of L-monomenthyl succinate in the extracts was 0.8 ppm (equivalent to approximately 100 ppb in the dried fruit). Further exploration of this system would be to demonstrate the capability of detecting much lower levels of analytes than those expected with this system, in order to prove nature-identical status of food analytes.

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6

Final Discussion and Concluding Remarks

6.1 Summary

The work described in this thesis has focused on methods to isolate specific functional groups of fragrance and flavour compounds: aldehydes, thiols and a menthyl ester, from natural sources. Each of these families of compounds provides challenges to the analyst, in particular they can be:

- unstable during isolation,
- present in lower concentrations than the detection limits of instrumentation,
- part of a complex mixture.

The methods chosen for isolating and identifying carbonyl and thiol species were to transform them chemically to create a more stable species for analysis. For the isolation of carbonyl compounds, a trap has been built consisting of the derivatising reagent, PFBHA, coated onto the adsorbent, Tenax TA. The trap is light, portable and suitable to use for headspace trapping on field trips. Concentration of the analytes is achieved by use of dynamic sampling. The trap has been tested on two natural sources: a blue hyacinth plant and lemon oil. In both cases, aldehydes expected to be present were detected. The characteristics of the trap were explored in some detail in order to determine the parameters for operation.

For thiol isolation, different methods were investigated to overcome the primary issues of high instability and low concentration of analytes. Three methods were used:

- a solid-phase reagent based on maleimide;
- covalent chromatography with a disulfide based reagent and
- adsorption onto gold wire.

Each of these methods made use of an immobilised reagent to provide for simplified isolation of the components of interest and concentration of the sample. From the results presented, it was concluded that the more traditional disulfide reagent was the method with the most potential to be easily applied to isolation of compounds from a flavour matrix. The use of silica-maleimide beads was considered the most innovative method of those investigated. This route was pursued in the most detail, primarily because of the important driver for commercial research to generate ideas that are patentable. This is not only for the intellectual property rights, but also can be important to support the marketing of a product discovered by using the novel technology.

The use of chemical reactions to trap species has been shown to overcome the issues of stability and detectability, but this method has not been without its own challenges. Introducing extra steps can have the downside of increasing complexity. This was particularly evident for the protocol that was designed for trapping and release of thiols using silica-maleimide beads. The use of a one-stage process was more successful (as demonstrated by the reactions with PFBHA). The analysis of thiol compounds continues to present a technical challenge.

The menthyl ester, confirmed as nature identical in Chapter 5, did not have the problem of instability and hence derivatisation was not necessary to isolate this compound. The primary challenge was the complexity of the samples of fruit and leaf extracts. Two chromatographic separations were used to simplify significantly the natural source extracts. L-monomenthyl succinate has been newly identified in the leaves of the plant *Lycium barbarum* to a high confidence level, using tandem mass spectrometry (MS/MS) data and retention time data correlated with an external standard. This system could be applied to the analysis of other natural source extracts containing trace and ultra-trace level compounds.

The important role that advanced characterisation techniques have to play in the identification of components and in providing inventive methods of measurement, has also been demonstrated throughout this thesis. Examples include using:

- IR and Raman imaging to demonstrate the coating homogeneity of PFBHA on Tenax;
- an MS-Nose as a method of monitoring the breakthrough of individual analytes;
- a pseudo two dimensional chromatography system to simplify a complex natural extract;
- MS/MS to identify unambiguously a component in a new food source and combinations of powerful analytical techniques to generate complementary structural data, as demonstrated by the analysis of maleimide reactions.

6.2 Further work

Several experimental refinements were suggested in the discussion sections in individual chapters. This section looks at some of wider issues that can be drawn form this work.

The logical next step for the aldehyde trap described in Chapter 3 would be to use it in the field to sample headspaces in situ. It would also be interesting to investigate further some of the PFBHA-related components that were detected in the headspace of blue hyacinth and lemon oil to confirm their identity. One of the obvious trends in recent research in the analysis of volatiles is the increasing use of sorptive enrichment techniques such as solid-phase microextraction (SPME), stir-bar sorptive extraction (SBSE) and head-space sorptive extraction (HSSE) (introduced in Chapter 1). SPME fibres have already been used as a support for the derivatising reagent, pentafluorophenylhydrazine, which has been used to trap volatile aldehyde compounds (Stashenko *et al.*, 2000). SBSE also been used recently with PFBHA to isolate aldehydes from urine (Stopforth *et al.*, 2006). In that work, the derivatisation takes place in solution and the oxime products are extracted with the stir-bar with further derivatisation before analysis by thermal desorption-GC. Both of these sets of researchers used thermal desorption rather than the solvent desorption utilised in the work described in this thesis. The disadvantage of the 'one-shot' nature of

thermal desorption has already been discussed, particularly with samples that have been difficult to obtain, and this is one reason why these recent developments were not explored further in this case. To overcome the 'one-shot' limitation, several sets of sampling equipment would be required. The work of Stopforth *et al.* (2006) also illustrates the point that the use of PFBHA is not limited to gaseous reactions and could also be used with liquid extracts containing aldehydes. In theory, the reactive trap could be tailored to different analytes by changing the derivatising reagent on the adsorbent.

In Chapter 4, several literature methods for trapping thiols were described. One of the methods that was considered but not pursued was to use ionic liquids. Ionic liquids are low volatility solvents made of ions rather than molecules which can give distinct selectivity and reactivity when compared to conventional organic solvents. Ionic liquids have been used as novel 'green' solvents to replace traditional volatile organic solvents in organic synthesis, although claims of green chemistry need to be evaluated carefully as many as these liquids are toxic. Ionic liquids could be investigated for their ability to isolate compounds in liquid natural extracts.

The work described in Chapter 5 showed the utility of microwaves in both organic synthesis and extractions. The benefits include shorter reaction/extraction times and lower consumption of solvents. Use of microwaves as an efficient method for both syntheses and extraction would be an interesting area to pursue. Microwaves could even have been used to make ionic liquids, as has already been demonstrated (Varma and Namboodiri, 2001).

6.3 Future aspects for the flavour and fragrance industry.

Both flavour and fragrance industries continue to operate by using nature as one source of new molecules. Headspace isolation techniques described here will continue to be important. More publications can be expected that make use of the SPME, SBSE and HSSE, because of the attractive simplicity of solventless extraction and concentration. There is also an increasing trend for portable field sampling, because the composition of organic volatile emission can only be reliably characterised with plants in their natural habitats (with minimum disturbance to the

plant required). Ultimately, this requires not only rugged, portable trapping equipment but ideally also portable instrumentation for immediate analysis to be carried out.

As long as the flavour and fragrance industries are faced with the analysis of complex mixtures, chromatography will always be an important technique. Developments in fast GC and LC can help speed up analysis and the use of two-dimensional chromatography can also be used for simplification as was demonstrated in the work described in Chapter 5. GCxGC is also increasingly being used to separate complex mixtures. Although these techniques provide increased separation, they do increase the complexity of data which requires more detailed knowledge and the aid of chemometrics for interpretation.

Highly sensitive and selective analytical techniques, such as the nano-LC-ESI-MS/MS described in Chapter 5, are becoming increasingly important for providing the structural information required to identify unambiguously compounds in complex mixtures. LC-MS/MS is well suited for detecting polar and non-volatile compounds. These include taste active components which tend to have such properties, and are becoming interesting targets for research as more is known about the molecular recognition process of taste.

Finally, in the introduction, some of the present knowledge in understanding the molecular biology of olfaction and gustation was presented. Some potential areas of research immediately relevant to theses industries are:.

- Investigation of factors influencing the development of taste and odour preferences e.g. age, gender, previous experience, genetics
- Genetic influence on individual preferences e.g. in tastes and nutritional choices
- How odorants and tastants are detected and signal transmission to the brain.
- Investigation of factors influencing food choice over the human lifetime
- Relationship between odour perception, memory, and emotion
- Role of odours in feelings of health and well-being

- Factors influencing the development of taste and odour preferences e.g. age, gender, previous experience, genetics

It is likely that future research in the flavour and fragrance industry will make the most of discoveries in these areas.

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