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**Molecular imprinting of small, poorly  
functionalised organic compounds**

A thesis  
submitted in partial fulfilment  
of the requirements for the degree  
of  
**Master of Science in Chemistry**  
at  
**The University of Waikato**  
by  
**Alona Swee Hua Kueh**



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**Hamilton, New Zealand**

2008

## Abstract

Molecularly imprinted polymers (MIPs) have been compared to natural antibodies in that they can specifically bind target compounds in a similar way that antibodies specifically bind to an antigen. The attraction of the MIPs technology is the ease of creating binding elements which are relatively cheap compared with the process of isolating natural antibodies. In this research monoterpenes, such as  $\alpha$ -terpineol, were chosen to be the model compounds for investigating the molecular imprinting of small, poorly functionalised organic compounds. The conventional non-covalent approach was mainly used to synthesise these MIPs, but the sacrificial-spacer semi-covalent approach was also investigated. A less widely used method, porogen-imprinting – a variant of non-covalent imprinting – was adapted for  $\alpha$ -terpineol. The latter novel terpene MIP appeared to specifically bind  $\alpha$ -terpineol, by hydrogen bonding, so the polymer was characterised in detail. The main parameters which were altered for preparing non-covalent MIPs included the template ( $\alpha$ -terpineol, (-)-menthol or *trans*-terpin); the functional monomer (methacrylic acid, 2-hydroxyethyl methacrylate, bilirubin and phenol [for the semi-covalent MIP]); the cross-linking monomer (ethylene glycol dimethacrylate, divinylbenzene and trimethylolpropane trimethacrylate); and also the polymerisation method (block or precipitation polymerisation). The binding specificity and cross-reactivity for all the polymers were tested using a liquid batch-binding setup. The batch-binding setup required the detection of analyte that was not bound in order to calculate by difference the fraction of analyte bound to the polymer. Initially the terpenes were to be detected by a colorimetric method; however attempts to make the method sensitive and reliable were not successful. In comparison, gas chromatography was more reliable for the detection of terpenes and was used for the experiments presented in this thesis.

$^1\text{H-NMR}$  studies of the interaction between  $\alpha$ -terpineol and acetic acid (as a non-polymerisable analogue of methacrylic acid) were investigated as a basis for understanding the binding to the carboxyl functional group moiety employed in many of the non-covalent MIPs that were made. The interaction between (-)-menthol and phenol was also investigated because the phenol moiety was employed in the semi-covalent MIP.

Only selected MIPs, which appeared to specifically bind the template, were physically characterised. This included optimising the batch-binding parameters, scanning electron microscopy imaging, surface area and pore radius analysis and in some cases Fourier transform-infrared spectroscopy of the polymers.

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**List of Abbreviations**

|  |          |
|--|----------|
| Azobis(isobutyronitrile)               | AIBN     |
| Divinylbenzene                         | DVB      |
| Ethylene glycol dimethacrylate         | EDMA     |
| Fourier transform-infrared             | FT-IR    |
| Gas chromatography                     | GC       |
| 2-Hydroxyethyl methacrylate            | HEMA     |
| High performance liquid chromatography | HPLC     |
| Methacrylic acid                       | MAA      |
| Mol L <sup>-1</sup>                    | <i>M</i> |
| Parts per million                      | ppm      |
| Pounds per square inch                 | PSI      |
| Quartz crystal microbalance            | QCM      |
| Relative centrifugal force             | rcf      |
| Ring-opening metathesis polymerisation | ROMP     |
| Self-assembled monolayer               | SAM      |
| Scanning electron microscopy           | SEM      |
| Solid phase extraction                 | SPE      |
| Standard deviation                     | Stdev    |
| Tetrahydrofuran                        | THF      |
| Trimethylolpropane trimethacrylate     | TRIM     |
| Ultraviolet/visible                    | UV/Vis   |
| Volume per volume                      | v/v      |

# **Chapter One**

## **Introduction**

## 1 Synthetic polymers

Synthetic polymers are high molecular weight macromolecules composed of a number of repeating units covalently linked together. Polymers are formed by reacting monomers, which are the smallest repeating structural unit of a polymer. The classification of the two main types of polymers are based on their method of synthesis [1]. Polymers can be classed as:

- Step-growth polymers or
- Chain-growth polymers.

The step-growth polymerisation mechanism is characterised by step-wise coupling of monomers to form dimers, trimers and higher order species. Each coupling reaction is independent of each other. The monomers are rapidly consumed while the polymer weight increases slowly [2]. Condensation polymers are formed by step-growth polymerisation; they can be obtained from chemically bifunctional linear or cyclic monomers (which split open to form a linear bifunctional molecule). In most cases as the two functional groups couple there is a loss of a small molecule, such as water, hence the name condensation. For example the multipurpose material *nylon* can be synthesised from two linear bifunctional monomers, hexanedioic acid reacting with 1,6-hexanediamine (called *nylon 66* because both monomers contain six carbons; Figure 1.1).

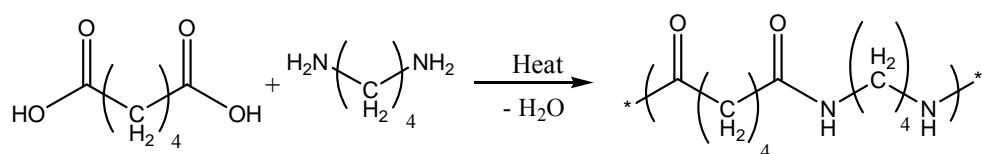


Figure 1.1 Schematic for the synthesis of *nylon 66*

Phenol-formaldehyde resins such as *Bakelite*<sup>TM</sup> are also a product of condensation polymerisation. They have been used in adhesives, coatings and the moulding industry and represent the oldest commercially available synthetic polymer [1]. These resins are formed when phenolics and formaldehyde react together to form an extensive three-dimensional network (Figure 1.2).

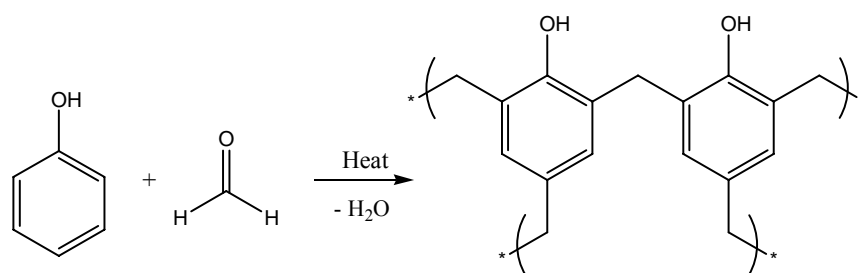
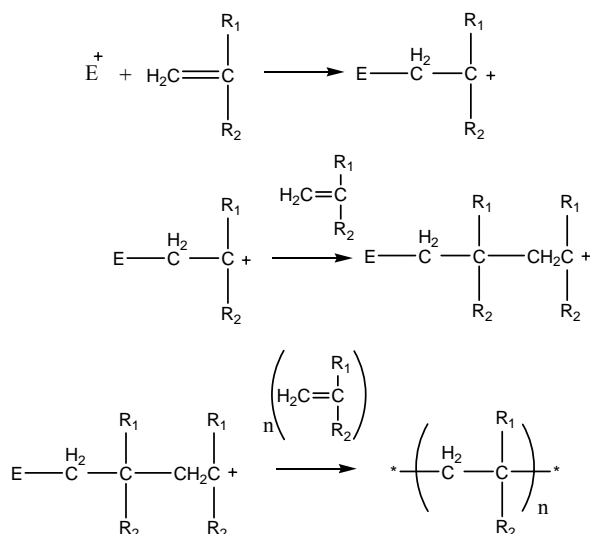
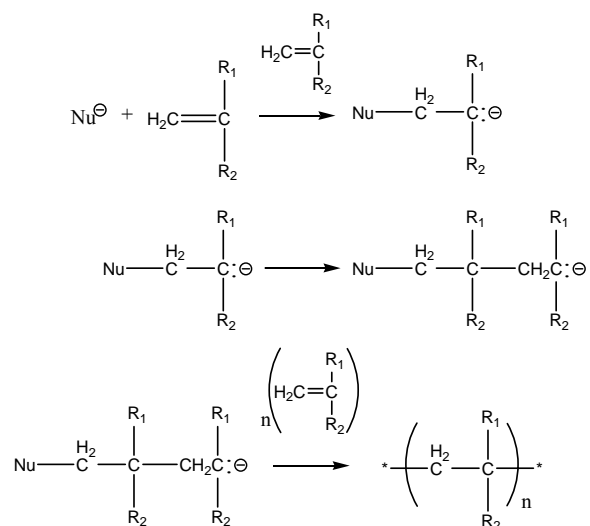


Figure 1.2 Schematic for the synthesis of *Bakelite*

Chain-growth polymerisation is characterised by vinyl-containing monomers successively linking onto the end of a growing polymer chain to form high molecular weight polymers with low monomer conversions [2]. Free radical polymerisation is a well known type of chain-growth polymerisation. Other types include cationic and anionic polymerisation. Cationic and anionic polymerisations are initiated by an electrophile or nucleophile, respectively. The monomers for cationic polymerisation must contain an electron donating group to stabilise the cationic intermediate that is formed after initiation; conversely a monomer for anionic polymerisation must contain electron withdrawing groups to stabilise the anionic intermediate (Figure 1.3).



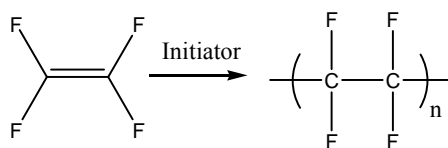
(a) Schematic of cationic polymerisation by an electrophile ( $\text{E}^+$ ), R = electron donating group. Polyisobutylene ( $\text{R}_1$  and  $\text{R}_2 = -\text{CH}_3$ ) is formed by cationic polymerisation and has been used as adhesives, sealants and as a moisture barrier [2].



(b) Schematic of anionic polymerisation by a nucleophile ( $\text{Nu}^-$ ), R = electron withdrawing group. Poly(methyl  $\alpha$ -cyanoacrylate) ( $\text{R}_1 = -\text{CN}$  and  $\text{R}_2 = -\text{COOCH}_3$ ), commonly known as *superglue*, is formed by anionic polymerisation [1].

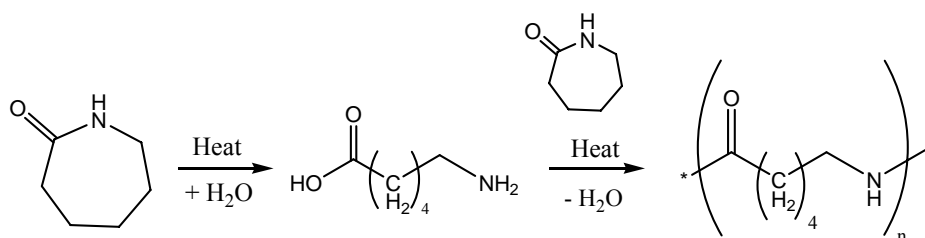
**Figure 1.3 Schematic of cationic (a) and anionic (b) polymerisation**

Radical polymerisations are initiated by free radical species; these are highly reactive and readily undergo homolytic scission with practically any monomer containing a vinyl group. Polytetrafluoroethylene (PTFE) commonly known as *Teflon<sup>TM</sup>* is synthesised by radical polymerisation from monomeric units of tetrafluoroethylene and the resulting non-reactive polymer has found many applications from the home kitchen to the laboratory (Figure 1.4).



**Figure 1.4 Schematic for the synthesis of polytetrafluoroethylene by radical polymerisation**

Ring-opening polymerisation is another type of polymerisation process which may be considered as either a step-growth or chain-growth polymerisation. For example *nylon 6* can be synthesised by ring-opening step-growth polymerisation from a cyclic six carbon monomer, *caprolactam*, Figure 1.5.



**Figure 1.5 Schematic for the synthesis of *nylon 6* by ring-opening polymerisation**

Cyclic vinyl monomers can also participate in ring-opening polymerisation; this is called ring-opening metathesis polymerisation (ROMP) and it often yields high molecular weight polymers in a mixture with some low molecular weight oligomers [2]. ROMP and radical polymerisation will be discussed more in Section 1.3.

Polymer research is an active area as there are a many different types of monomers and variables involved in polymer synthesis. Polymers prepared from the same monomers but using different polymerisation reactions may not have the same properties because different polymerisation processes can lead to differences in molecular weight, end groups, stereochemistry and chain-branching [2]. Therefore polymerisation can be used to create new types of materials that will meet industry demands. In some instances synthetic polymers have replaced natural resources that have restricted availability, such as wood, because polymers can be tailor-made to meet specifications. Polymers could have properties which may not be identified in a natural resource. For example plastic bags are strong and water-resistant compared to paper bags which lose mechanical strength when

wet. The chemical stability of many synthetic polymers is desirable when the product is being used; however their persistence has become an environmental problem because many synthetic polymers do not degrade fast enough. A branch of polymer research that is being undertaken by some research groups involves tailor making synthetic polymers which will degrade more readily when the object is discarded.

## **1.1 Molecularly imprinted polymers**

Molecularly imprinted polymers (MIPs) are cross-linked, synthetic polymers with a porous three-dimensional structure. MIPs have been of interest because of the scientific challenge of creating synthetic macromolecules able to recognise a specific compound or a class of compounds. There is demand for cheap and easy to produce materials that can echo the role of natural recognition systems such antibodies, enzymes or cell-surface receptors. MIPs are theoretically reusable; regeneration is possible by extracting the analyte prior to reuse.

Although they have great potential, MIPs are still not as sensitive as natural systems. However the technique is attractive because it may allow the development of synthetic recognition elements for target analytes which do not allow the synthesis of a natural recognition element. MIPs are tailor-made to specifically bind the target analyte by uptake into active cavities which are complementary in “size, shape and electron density” to the analyte [3]. In many reports, a reference polymer is generated to evaluate the amount of non-specific binding to the bulk polymer structure. The reference polymer and MIP are made under the same conditions with similar polymer recipes; the key difference is the MIP recipe contains the target analyte acting as the template to create a molecular imprint of the compound in the polymer structure whereas the template is omitted from the reference polymer recipe.

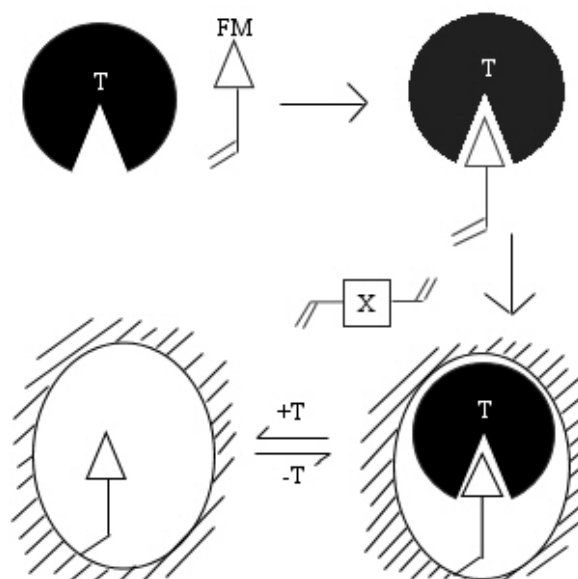


Figure 1.6 Schematic of molecular imprinting<sup>1</sup>

In general, creating a MIP first involves the template molecules interacting with the functional monomer through non-covalent interactions or covalent bonds (Figure 1.6). After thermal or photo-initiation, the vinyl group in the functional monomer polymerises with the cross-linking monomer; this process creates the molecular imprint by trapping the template-functional monomer unit in the polymer structure. After extracting the template from the polymer, the analyte can rebind specifically to the MIP through the active cavities or it can also non-specifically bind to the polymer by interacting with other sites that form the rest of the polymer structure. The extent of non-specific binding is evaluated from the reference polymer because the polymer structure does not contain imprinted cavities. The difference in the uptake of analyte between the MIP and the reference polymer is called specific binding. Attention should be paid to the definition of selectivity and specificity; MIPs are called “selective” if they had been tested against a range of compounds and found to only bind a certain one. In contrast, MIPs are called “specific” if they had been tested against a single analyte and were found to bind more compared to its reference polymer.

<sup>1</sup> Where “T” represents the template, “FM” is the functional monomer and “X” is the cross-linking monomer.

## 1.2 Basic composition of molecularly imprinted polymers

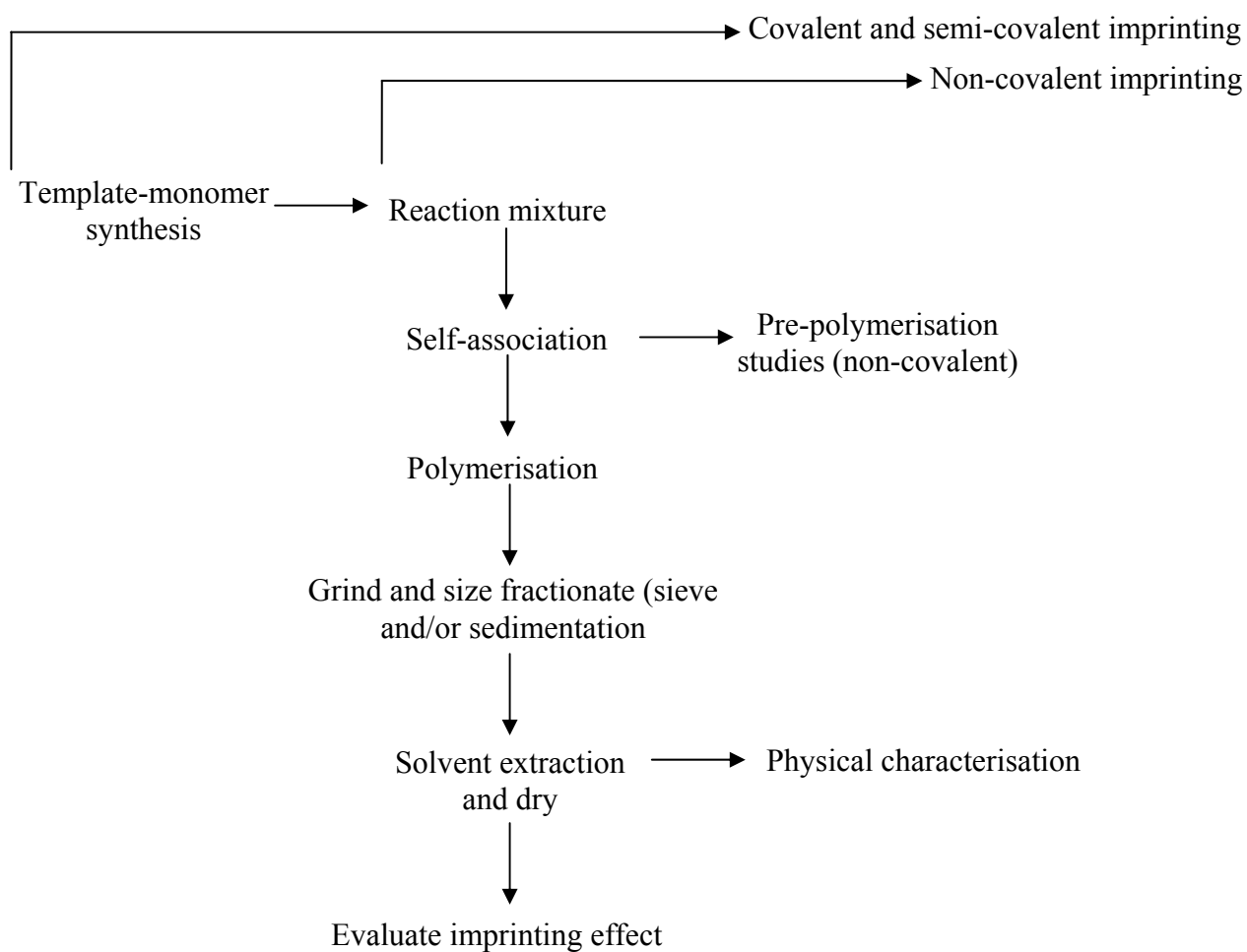
The mixture for an imprinted polymer contains a template (the target analyte), functional monomer and cross-linking monomer (or functionalised cross-linking monomer), porogen and initiator. In general the mole ratio of the template to cross-linking monomer is 1:20, the ratio depends on the type of cross-linking monomer used and the degree of structural rigidity required for the polymer [4]. Since the cross-linking monomer is present in greater amounts it may affect the binding properties of a MIP and may contribute to non-specific binding of the analyte. Along with having a similar composition as the MIP, the reference polymer is also made in the same format and subjected to the same treatments as the MIP. A comparison of the performance of the MIP and the reference polymer indicates whether the imprinted polymer has memory for the template.

Three different types of imprinting can be distinguished based on the type of interaction between the template and functional monomer during the synthetic process and rebinding events.

1. In covalent imprinting the template and functional monomer are covalently bound together and incorporated as a unit into the polymer. This approach is only useful if the covalent bond is reversible; it must form rapidly but it must also be weak enough to allow easy extraction of the template to leave behind a polymer with imprinted cavities.
2. Non-covalent imprinting is the most versatile of the approaches. The template and functional monomer form a complex through non-covalent intermolecular interactions which can be rapidly formed and easily disrupted. The drawback to the non-covalent approach is the random nature of complex formation which may lead to different orientations of the two species and therefore different types of imprinted cavities.
3. Semi-covalent imprinting merges the two previous approaches. The imprinted cavities are formed from a template which is covalently bound to a functional monomer; this minimises the types of template-monomer orientations incorporated into the MIP. The chosen covalent bond should be easily cleaved but should not

form easily under the rebinding conditions. The resulting MIP rebinds to the analyte through intermolecular interactions.

These three approaches will be discussed in more detail in Section 1.6 to Section 1.8. A summary of the overall process of forming a MIP is illustrated in Figure 1.7.



**Figure 1.7** The overall process of forming a MIP

### 1.2.1 The template and the importance of template extraction

The template molecule ideally should contain at least one functional group through which it can interact with the functional monomer as well a distinctive three-dimensional structure. The type of functional group controls the imprinting approach that can be utilised. Not all templates will readily form a covalent bond with a functional monomer that is easily cleaved. On the other hand the number of functional groups affects the affinity of the template for the MIP. Increasing the number of interactions between the template and functional monomer may increase the affinity with which the MIP rebinds the template [5]. However it also increases the non-specific binding of the template to the polymer. For all three MIP approaches listed in Section 1.2, the cross-linking monomer forms a large proportion of the polymer and therefore can potentially form sites able to non-specifically bind the template.

For a non-covalent MIP the mole ratio of template to functional monomer is usually 1:4; this is often specific to the template and functional monomer that is used because the template may have more than one functional group and thus can interact with more than one functional monomer moiety in the active sites. In some cases a slight excess of functional monomer is added to ensure complex formation. This means that some of the functional monomer will not be located within imprinted cavities; these sites can non-specifically rebind the template. A few examples of non-covalent interactions which have been commonly used include hydrogen bond, electrostatic, hydrophobic and  $\pi$ - $\pi$  electron interactions.

In a covalent MIP the template forms stoichiometric bonds with the functional monomer. Covalent bonds that have been used include the boronic ester, Schiff base and carbonate bonds; the use of these bonds in MIPs will be described in more detail in Section 1.6 and Section 1.8.

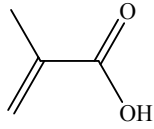
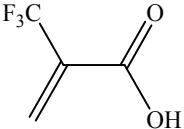
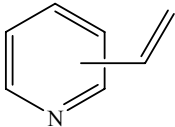
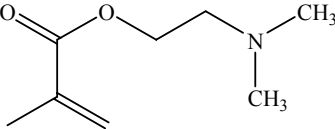
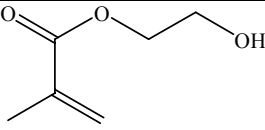
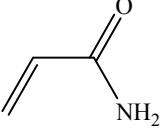
Removal of the template after polymerisation is necessary to reveal the imprinted cavities; if residual template remains these can leak out while performing tests on the polymers. This is called *template bleed* and it is a problem if the MIPs are used for analytical chemistry applications. This has been circumvented by using a method called analogue imprinting; a structural analogue of the target compound is used as the template. This method only works if the target analyte and its analogue can be detected as separate species (using

chromatography or spectroscopy) and has enabled MIPs to be used in solid-phase extraction (Section 1.5).

### **1.2.2 The functional monomer**

The functional monomer contains at least a vinyl group and another functional group, through which it can interact with the template. Non-covalent imprinting requires the selection of an appropriate functional monomer that will form strong intermolecular interactions with the template. Commonly used functional monomers can be classified into three main categories (Table 1.1). The acidic functional monomers are useful for templates that can accept a proton; alternatively basic functional monomers will form better interactions with templates that can donate protons. For uncharged templates, a neutral functional monomer may help increase their interaction [5]. To help in the selection process the strength of association between the template and functional monomer can be studied by spectroscopy, as the spectra changes are related to the change in the interaction between the template and functional monomer. FT-IR, <sup>1</sup>H-NMR or UV/Vis (depending on whether the template has a chromophore or not) have been used in the past [6].

**Table 1.1 Commonly used functional monomers for non-covalent imprinting**

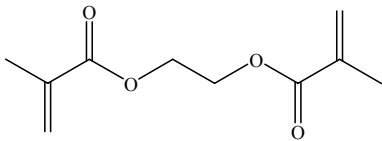
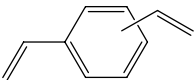
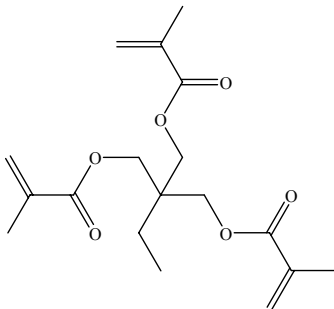
| Classification | Structure  | Functional monomer                           |
|----------------|--|--|
| Acidic         |     | Methacrylic acid (MAA)                       |
|                |    | Trifluoromethacrylic acid (TFMAA)            |
| Basic          |     | <i>ortho- or para-</i> Vinyl pyridine        |
|                |    | <i>N, N-</i> Dimethylaminoethyl methacrylate |
| Neutral        |    | 2-Hydroxyethyl methacrylate (HEMA)           |
|                |  | Acrylamide                                   |

The selection of functional monomers for covalent and semi-covalent imprinting is more restricted because of the antagonistic requirements of polymerisation and template extraction. The functional monomer must form a covalent bond with the template that is stable under polymerisation conditions. However the extraction of the template requires that the bond is readily cleaved once the polymer has been formed. In the case of covalent imprinting the template and functional group in the imprinted cavity must readily re-form the covalent bond to be useful. The boronate bond is commonly used in covalent imprinting of templates containing a diol functional group because it is readily formed and broken compared to other covalent bonds [7]. Monomers that can form amides and ester bonds with a template have been used for semi-covalent imprinting but they do not readily hydrolyse and thus generate a low number of active sites [7]. The design of the neighbouring groups of the functional monomer may increase the kinetics of bond breaking and bond formation, this could widen the range of templates that may be covalently imprinted [8].

### 1.2.3 The cross-linking monomer

MIPs are solid and porous because the multiple vinyl groups in the cross-linking monomer can co-polymerise with the functional monomer and thus interconnect two radical centres from different polymer chains. Their porous nature allows the template molecules to diffuse into and from the imprinted cavities; their solid state allows the MIP to maintain structural integrity of the imprinted cavities. The degree of cross-linking determines the rigidity of the polymer and may affect the selectivity of a MIP [9]. A selection of commonly used cross-linking monomers can be found in Table 1.2.

**Table 1.2 Commonly used cross-linking monomers**

| Structure   | Functional monomer                        |
|---|---|
|  | Ethylene glycol dimethacrylate (EDMA)     |
|  | Divinylbenzene (DVB)                      |
|  | Trimethylolpropane trimethacrylate (TRIM) |

An alternative method of creating MIPs is to use a single type of monomer that has the characteristics of both a functional monomer and a cross-linking monomer. An example of a dual-role monomer reported which has been used to create a MIP was *N,O*-bismethacryloyl ethanolamine (NOBE). This contains a functional group which can interact with the template and two vinyl groups for cross-linking polymer chains. The name *OMNiMIPs* (one monomer molecularly imprinted polymers) was coined by Sibrian and Spivak [10] to describe MIPs generated from a single functionalised cross-linking monomer. The functional amide group is covalently bound to the polymer backbone at two points and therefore would restrict its conformational freedom. This minimises the number of possible interactions between the template and the functionalised cavities and therefore increases the selectivity of the MIP. Another example of single monomer type MIPs, although not cross-linked) are poly(pyrroles) [11]. These may be the interface between polymers and electronics because the highly conjugated  $\pi$ -electron system may transduce the recognition event taking place in the MIP.

#### **1.2.4 The porogen**

The porogen plays an important role in polymer mixture as it dissolves the polymerisation components to form a single phase mixture; commonly used solvents include chloroform and acetonitrile [12]. The range of suitable porogens for a particular MIP system is limited by the type of interaction between the template and functional monomer because the strength of interaction is affected by the environment [12]. In some cases the template may be immiscible in the chosen organic porogen; however the addition of the monomers may increase the solubility by forming a soluble complex, thus the monomers behave like a surfactant [13]. Biphasic polymerisation systems have been reported where the polymerisation mixture is soluble in only one phase and not the other; the purpose of using an insoluble phase is to limit the extent of polymerisation so that small polymer particles can be formed. A more detailed discussion about different types of MIP morphologies can be found in Section 1.4.1.

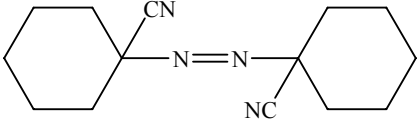
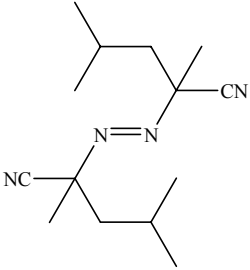
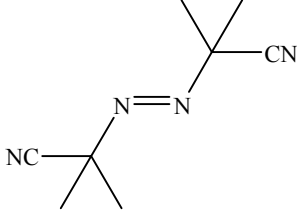
The porogen also plays a role in forming a porous polymer by solvating the template and monomers during the polymerisation process thus acting as a space-filler. It is eventually removed, just like the template, to create channels within the polymer which increases the accessibility of the imprinted cavities. The dimensions of the pores are determined by the porogen solubility phase [14]. Porogens with a low solubility phase poorly solvate the growing polymer and therefore separate from the polymer early on in the polymerisation process and form larger pores. The separation of the solvent from the polymer prevents the diffusion of the reactants to that site, effectively halting polymerisation and promoting polymer precipitation. Conversely porogens with high solubility phase form smaller pores because the polymer stays in solution for longer, thus polymerisation can continue for longer to yield a more dense structure.

### **1.2.5 The initiator**

The initiator starts the polymerisation process by providing a source of free radicals. These can be generated by thermal or photolytic decomposition of azobis(nitriles) or peroxides; azobis(nitriles) are more commonly used in molecular imprinting (Table 1.3). Alternatively free radicals can also be produced by the redox reaction between an oxidant and a reductant or by high energy radiation ( $\gamma$ -rays). The temperature of initiation can affect the strength of the complex formed by the reactants before polymerisation. This depends on the interactions between the template and monomer. Electrostatic interactions are stronger at lower temperatures whereas the strength of a covalent bond is not affected by moderate temperatures. In cases of using chiral templates, MIPs prepared at lower temperatures showed better separation of the template from its enantiomer. An example is the L-phenylalanine anilide MIP prepared at different temperatures ranging from 0 °C to 60 °C; this was accomplished by using different initiators suited for the temperature of polymerisation (either azobis(isobutyronitrile) or azobis(dimethylvaleronitrile) [15]. The azo bond decomposed when exposed to UV irradiation or elevated temperatures to form radical nitrile species and N<sub>2</sub>. The radical nitrile reacted with the vinyl group on nearby monomers to yield a new radical centre that led to propagation.

It is important that the temperature of initiation is lower than the boiling point of the porogen; this is discussed more in Section 1.4.2. The polymerisation reaction is exothermic and generates heat which increases the temperature of the reaction [16]; therefore initiating the reaction at lower temperatures allows the temperature of the system to be better controlled because it is easier to regulate the temperature. The polymerisation temperature of an (-)-ephedrine MIP, consisting of a HEMA-EDMA co-polymer, appeared to be related to the initiation temperature, lower polymerisation temperatures were recorded if the initiation temperature was lower.

**Table 1.3 Initiators commonly used for molecular imprinting**

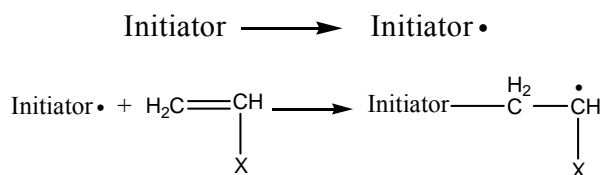
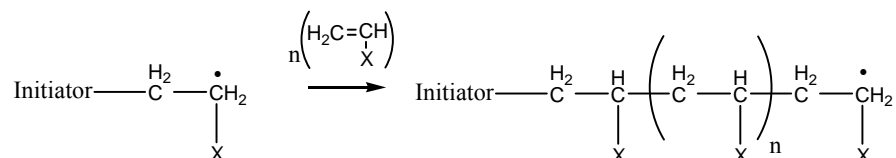
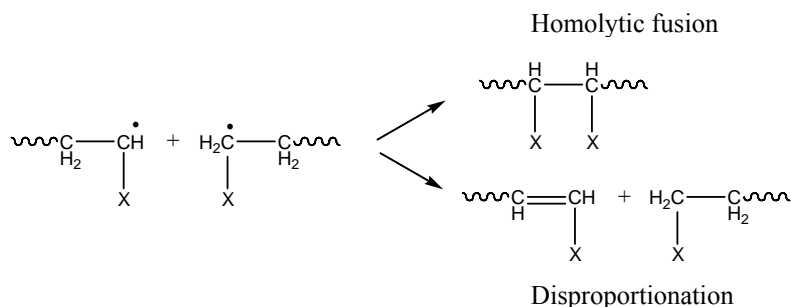
| Initiator name                               | Initiator structure  | Initiation temperature (°C) [17] and [18] |
|--|--|---|
| 1,1'-azobis(cyclohexanecarbonitrile) (ABCHC) |  | 88  |
| 2,2'-azobis(dimethylvaleronitrile) (ABDV)    |  | 50  |
| 2,2'-azobis(isobutyronitrile) (AIBN)         |  | 65  |

### **1.3 MIP polymerisation methods**

MIPs have been mainly synthesised by free radical polymerisation as this is a very quick and easy process to carry out. So far there have not been any MIPs generated by condensation polymerisation; it is possible that in this case the template permanently incorporates into the polymer backbone and thus would not be extracted, not forming cavities that reflect the shape and functional group orientation of the analyte. Ring-opening metathesis polymerisation has been used to create MIPs; it has been found to provide better thermodynamic control over the polymerisation process. However this method may not be suitable for all types of imprinting because the ROMP catalyst may affect the template-monomer complex [19].

#### **1.3.1 Free radical polymerisation**

MIPs and their corresponding reference polymers are commonly generated by free radical polymerisation. A schematic of the three elementary steps of radical polymerisation can be seen in Figure 1.8.

*Initiation**Propagation**Termination***Figure 1.8 Schematic of free radical polymerisation**

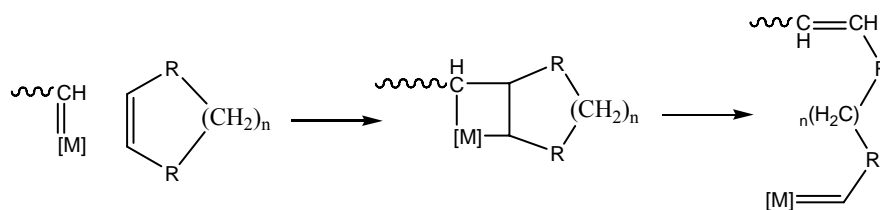
The first elementary step is the thermal or photolytic decomposition of the initiator which yields reactive free radicals. The free radicals of the initiators are more likely to react with the monomers (present in larger amounts) rather than reacting with themselves; this leads to propagation of the polymer chain, the second elementary step. The free radicals trigger the homolytic scission of double bonds (a feature of monomers) and thus generate new radical centres which can react with other double bonds. This results in a self propagating chain reaction that lengthens the polymer chain. The solvent and concentration of reactants used for polymerisation can control the length of the polymer chain (also known as the degree of polymerisation, d.p.) because viscosity determines how easy it is for a reactive radical to encounter another radical centre on the polymer [2]. Chain reactions are fast and the polymer chain increases in mass rapidly. The long polymer chains aggregate together and then precipitate out of solution when large

enough. MIPs are also insoluble because cross-linking monomers are used; these interconnect polymer chains to create a large three-dimensional network. MIPs formed by radical polymerisation have a porous structure because of cross-linking; the pore sizes and distribution are heterogeneous because each radical encounter forming the polymer depends on random nature of diffusion. The porosity and rigidity of the polymer can be controlled by changing the type and amount of cross-linker and solvent.

With time the likelihood of the third elementary step, termination, increases because the concentration of radicals is high enough. Termination can occur through recombination of two reactive polymer chains to form neutral products. This can occur by homolytic fusion of the radical centres or disproportionation (when one polymer chain forms a saturated product while the other forms an unsaturated product). There is a fourth elementary step which causes deviations in the kinetics of radical polymerisation [2], these are the chain transfer reactions. In this process a radical centre can be transferred onto any species in the reaction vessel, such as a monomer or another polymer chain, and often results in broad polymer molecular weight distributions. If a radical centre from a growing polymer chain transfers onto a monomer, chain growth terminates and a new radical monomer is formed which can participate in more reactions; if the radical centre is transferred onto another polymer chain this can increase the degree of cross-linking. Radical centres can also be transferred onto a modifier, such as thiol containing compound, to control polymer chain length. Chain transfer reactions can also prevent polymerisation if the radical centre is transferred onto an inhibitor; this is the mechanism through which alkylated phenols stabilise neat monomer solutions [2].

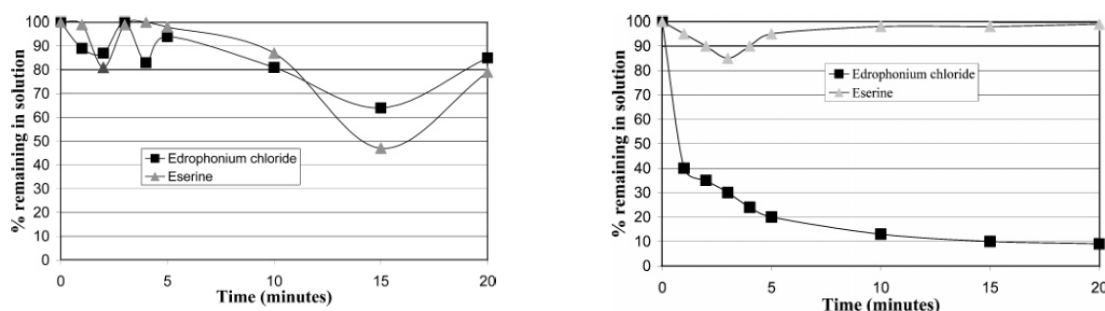
### **1.3.2 Ring-opening metathesis polymerisation**

MIPs can also be generated by ring-opening metathesis polymerisation (ROMP); in contrast to radical polymerisation the positions of the double bonds are rearranged rather than saturated and hence the polymers are called “living polymers” because they still contain polymerisable vinyl groups [2]; Figure 1.9 shows a schematic of one of the propagation steps.



**Figure 1.9** Schematic for a propagation step of ring-opening metathesis polymerisation

Polymerisation is initiated by using transition metal catalysts such as ruthenium complexes [20]. The rate determining step appears to be related to metal carbene reactivity rather than the ring strain in the cycloalkene [2]. ROMP MIPs appeared to form imprinted sites which were more selective for the target molecule compared to MIPs formed by radical polymerisation. Figure 1.10 illustrates that in a competitive assay, where the MIP was exposed to two competing analytes, edrophonium chloride ROMP MIPs were more selective for edrophonium chloride because very little remained in the supernatant in comparison to the competing analyte, eserine [21]. In contrast, MIPs prepared by radical polymerisation were less selective because at certain times the template molecule was bound to a greater extent compared to eserine but at other times the extent of uptake was reversed (Figure 1.10, left graph).



**Figure 1.10** Comparison of the specificity of edrophonium chloride MIPs prepared by radical polymerisation (left) and ROMP (right)<sup>2</sup>

<sup>2</sup> Reprinted with permission from *Macromolecules*, 2006, **39**: p. 7859-7862. Copyright 2006, American Chemical Society.

## 1.4 The physical nature of molecularly imprinted polymers

MIPs can exhibit different morphologies and topologies on the macro-level depending on the format chosen; this is often seen using an electron microscope. At the submicro-level the structure of the pores and cavities are more difficult to visualise but surface area studies, for example N<sub>2</sub> adsorption, can be used to evaluate the porous nature of the MIPs. Understanding the porosity of the MIP is important because diffusion controls the availability of the imprinted cavities, which are embedded within the polymer structure, to the analyte. This also allows solvents to permeate the structure; depending on the solvation properties of the solvent this may result in the polymer swelling or contracting which may affect its subsequent performance. MIPs are also more robust compared to biological receptors because of the chemical inertness of the polymeric structure; this will be discussed in more detail in Section 1.4.3.

### 1.4.1 Molecularly imprinted polymer formats

There are several different physical formats of MIP polymers and often the type chosen is based on the final application. The most widely used MIP format for research purposes is the block polymer, which initially is a solid cast of the vessel in which the polymerisation was carried out in. Prior to testing, the block polymers were ground (mechanically or manually) and sieved to collect particles of a desired size range. The small particles make solvent extraction of the template from the MIP more efficient because the template molecules trapped deep within the block polymer are exposed. This also makes it easier for the test analyte to diffuse to the imprinted cavities when testing the binding capacity of the MIP particles. Using ground MIP particles as the stationary phase of an HPLC column may lead to an undesired build-up of back pressure in the system due to the inevitable presence of very small sized particles, called fines [22]. The amount of fines can be minimised after sieving; this is done by the process called *sedimentation*, which involves numerous cycles of thoroughly stirring the particles in a solvent (such as acetone) and discarding the supernatant (which contains the fines) after waiting for a specified time. Although the synthesis of the polymer is fast, grinding and size fractionation is time-consuming. An

alternative block MIP format is the in situ synthesis of polymer within a chromatography column [23]; another approach has been to create thin films within a column which removes the problem of column back pressure [24]. A drawback to the block format is that it cannot be synthesised in batches larger than 100 g [25] because of the exothermic nature of polymerisation. A maximum temperature of 190 °C was reported to have been reached within a polymerisation solution that was thermally initiated at 80 °C [16]. Therefore the synthesis of block polymer format is not suitable to scale up for industrial applications because it would be more difficult to thermally regulate the system. Preparation of spherical MIP particles allows better dissipation of the heat generated by the radical reactions because the reaction mixture is more dilute. Another advantage of making MIP beads is that it overcomes the time consuming process of grinding and size fractionating. There are a few variations to preparation of MIP beads (Table 1.4). The basic principle behind forming polymer beads is to have a polymerisation mixture that is dilute enough to minimise the encounter event between radical centres on different polymer chains; this limits the size of the polymer network to small spheres.

**Table 1.4 Different methods to create MIP beads**

| <b>Parent method to form spherical polymers</b> | <b>Variants of the parent method</b>                        |
|---|---|
| Suspension polymerisation                       | Aqueous [25] or fluorocarbon suspension polymerisation [26] |
| Dispersion polymerisation                       | Precipitation polymerisation [27]                           |
| Two-step swelling polymerisation                | Core-shell polymerisation [28]                              |

For suspension polymerisation a mixture of monomers, template and porogen is added to a dispersion solvent to form an emulsion. The dispersion solvent can be an aqueous or an organic fluorocarbon solvent. Non-polar fluorocarbons have been used to develop non-covalent MIP beads by suspension polymerisation because they are chemically inert and therefore do not compete with the template-monomer intermolecular interactions. Fluorocarbons, for example perfluoro(methyl cyclohexane), are expensive but they can be reused [26]. Polymerisation is induced by adding an initiator that is soluble in the monomer-containing phase but not in the dispersion phase. A surfactant may be added to stabilise the monomers in the solvent, in some cases special polymeric

surfactants have been synthesised to suit the polymerisation system [26]. The dispersion solvent may interfere with the interaction that occurs between the template and the functional monomer which would not be suitable for molecular imprinting. Aqueous suspension polymerisation is only feasible for template and functional monomer interactions which are strong, such as covalent bonds, because non-covalent interactions can be disrupted by solvation of molecules. Therefore aqueous suspension polymerisation is limited to covalent or semi-covalent imprinting.

MIP beads prepared by suspension polymerisation are generally poly-disperse micron sized spheres [27]. Spherical MIPs can also be prepared by dispersion polymerisation in which a dilute homogeneous mixture of the template, monomers and initiator are dispersed in a porogen [29]. The point when a growing polymer network precipitates out of solution is determined by the solubility of the polymer within the polymerisation solvent [17]. A polymeric surfactant may be added to stabilise the growing polymer globules, this may control the final dimensions of the beads and result in a more uniform population of spheres. However addition of a stabiliser may interfere with the template-functional monomer interactions, it may incorporate into the polymer, or contribute to non-specific binding. Thus an alternative method, which omits the stabiliser, called precipitation polymerisation would be more suitable for molecular imprinting. Precipitation polymerisation beads may be mono-disperse depending on the solvent and polymerisation conditions [27].

Two-step swelling polymerisation can generally produce mono-disperse polymer beads [27]. Seed polymer beads (the core) are first synthesised, generally using emulsion polymerisation; this method is similar to suspension polymerisation except a surfactant is used to disperse monomers. Before initiating the second emulsion polymerisation step, the seed particles are equilibrated with a solution containing the monomers that have been chosen to form a polymer shell around the core. This has been used to form cholesterol MIP beads that have magnetic properties; magnetite was included in the core polymer which made it easier to collect the beads because they sedimented in a magnetic field and the MIP performed similarly to a non-magnetic version of the MIP [28].

A drawback to the use of small particles is the increased surface area for non-specific binding. The extent of non-specific binding can be evaluated by

comparing the extent the reference polymer binds to an analyte compared to the corresponding MIP. In all cases the template must be extracted from the MIP and the polymer dried prior to testing in the chosen test solvent.

#### **1.4.2 Effect of solvent on MIP properties**

The nature of the pores and active cavities within a MIP are greatly affected by the type of solvent they are exposed to. The synthesis of MIPs commonly occurs in the liquid phase in the presence of a porogen; the temperature of initiation is generally below the boiling points of the reactants. The dimensions of the pores within the polymer reflect the polymerisation conditions, mainly the temperature and porogen utilised. The temperature affects the vapour pressure of the reactants and thus the composition of the reaction solution. If the temperature was above the boiling point of the porogen it would not be effective at forming pores. The porogen must be liquid to solvate the growing polymer chain; this keeps the polymer in solution and allows the polymer chain to reach a size large enough for precipitation to then take place.

Once the polymers have had the template extracted the imprinted cavities are available for rebinding the target molecule. The strength of interaction between the template and the functional groups in the active cavities is affected by the solvent used in the test system. For example polar solvents can enhance interaction between hydrophobic moieties [12]. Different solvents can solvate the polymer network to different extents and may cause swelling or shrinkage of the polymer. A solvent must be chosen which does not cause shrinkage of the pores; otherwise the shrunken MIP may trap the template molecules and therefore block the imprinted cavities from participating in the process of selective binding.  $N_2$  adsorption and the Brunauer-Emmett-Teller (BET) model of gas adsorption can be used to evaluate the surface area of the MIPs and the Barrett-Joyner-Halenda (BJH) model or mercury intrusion porosimeter can be used to evaluate the porous nature of the MIP.

In practice, template extraction is not 100% efficient and often residual template remains in the polymer, trapped deeply within the polymer network. If a rebinding solvent is used which promotes swelling, the residual template may

leach out of the polymer during a test. This *template bleed* can impair the use of MIPs for analytical purposes.

After template extraction MIPs are dried; in the dry state the polymer structure will not be solvated and there may be shrinkage. The pores and imprinted cavities will also be smaller; therefore the diffusion of the test analyte to the cavities within the MIP will be sterically hindered. Even if the analyte molecule managed to diffuse through the polymer they may not bind specifically to the imprinted cavities because they are size excluded. However the MIPs are often tested in the liquid phase and the nature of the pores can change according to the types of solvent they are re-exposed to. Polar organic solvents such as acetonitrile promote polymer swelling whereas polymers developed or tested in less polar solvents such as cyclohexane swell less [30]. The flexible nature of polymers in different solvents may weaken the polymer network and decrease their life-span. This may be restrictive in some applications such as HPLC (when the mobile phase is changed to allow analyte separation) as polymer expansion/contraction may affect the pressure in the chromatography system. Testing polymers in the dry state may require the selection of a cross-linker and porogen combination which create a MIP that will not swell too much to ensure that the polymer has the correct structure for testing in the dry state.

The cross-linker plays a role in the rigidity of the polymer network and also in the degree of polymer solvation because in most cases it is the dominant species in the polymer. Aromatic cross-linkers such as divinylbenzene (DVB) are hydrophobic and chemically resistant [19] and form rigid co-polymers; however they are less commonly used in comparison to MIPs based on ethylene glycol dimethacrylate (EDMA) because aromatic analytes are often chosen for imprinting. These can non-specifically bind with DVB by  $\pi$ - $\pi$  interactions. EDMA MIPs interact better with most rebinding solvents [17] because of the electronegative oxygen atoms, however these can also form sites that non-specifically bind the template.

### 1.4.3 Stability of MIPs

The choice of monomers will dictate the stability of the resulting MIP. For instance theophylline MAA/EDMA non-covalent MIP was subjected to high

temperatures, up to 200 °C, that would often denature proteinaceous biological receptors and the MIPs still showed a greater affinity for the analyte in comparison to the reference polymer [31]. Thermal stability is a characteristic of cross-linked polymers [2].

The theophylline MIP (which consisted of a co-polymer of MAA and EDMA) was also subjected to acidic and alkaline conditions (up to 10 *M* HCl and 25% NH<sub>3</sub>, respectively) and this did not change its ability to take up the analyte when compared to a reference test of the MIP in water. However not all MIPs will have the same stability. A cholesterol EDMA-based semi-covalent MIP was hydrolysed in the presence of 1 *M* NaOH in methanol which resulted in the formation of carboxyl groups in the polymer backbone; this increased the non-specific binding sites on the polymer [32]. Therefore the composition of the polymer must be chosen to withstand the polymerisation conditions, the conditions required to remove the template and also the conditions used to test rebinding.

## 1.5 General applications of molecularly imprinted polymers

MIPs are selective solid surfaces that can theoretically be used as substitutes for proteins such as antibodies and cell-surface receptors; the imprinted cavities may also be chemically reactive and therefore the MIP may function like an enzyme. Catalytic MIPs have been prepared by imprinting an analogue of the transition state of a reaction because the transition state itself is unstable and is likely to decompose before the polymerisation is complete. The efficiency of catalytic MIPs is not as good as natural enzymes since they are finely tuned biomolecules which have evolved high catalytic activity in their natural medium; only modest rate enhancements have been achieved with catalytic MIPs [33].

Many variables are involved in polymerisation and assessing MIPs, therefore extensive optimisation studies may be required to achieve the efficiency of a natural enzyme. For polymerisation these include types of monomers, ratio of monomers, temperature and solvent; for assessing MIPs these include the rebinding solvent, concentration of polymer (if working with particles), concentration of test analyte, temperature and contact time between the MIP and the analyte. However compared to proteins, MIPs are more stable in extreme

temperatures and organic solvents. Therefore MIPs may be used in applications in which enzymes tend to degrade, such as in organic synthesis.

Natural antibodies have been used in enzyme-linked immunosorbent assays (ELISA); an enzyme-linked optical change (fluorescence or colorimetric) occurs when the target analyte is bound by the antibody. However, because proteins are easily denatured by organic solvents, this limits the scope of analytes which can be determined through this method. Therefore MIPs could be developed as substitutes for natural receptors. MIPs specific for atrazine and epinephrine could be used as a substitute for antibodies in an immunoassay [34]. A drawback is that often MIPs do not perform well in the presence of water which can be found in biological or environmental samples. The polarity of water can interfere with interactions between the target analyte and the imprinted sites. This would have a greater effect if specificity relied on non-covalent interactions such as hydrogen bonds but would be less of a problem for covalent MIPs (if the formation of the reversible covalent bond was not disrupted by the presence of water). MIPs that work in aqueous systems have employed stronger non-covalent interactions such as metal-ion chelation; an interesting application of this is to remove heavy metals, such as iron (III), from blood plasma. Iron (III) was allowed to complex with *N*-methacryloylamido-(L)-glutamic acid (functional monomer), and then HEMA and EDMA were added before polymerisation was initiated. Rebinding tests in human plasma showed that the MIP selectively bound iron (III); this could lead to MIP based extracorporeal iron removal from patients suffering from iron overdose or accumulation in the blood [35]. MIP sensors have been created which selectively re-bound glucose from aqueous carbonate buffer; glucose formed a complex with the copper (II) complex which had been polymerised into the polymer structure [36].

Receptor type MIPs, to a lesser extent catalytic MIPs, can be incorporated into a biomimetic sensor [37]. These mimic the role of a biological receptor/enzyme in a biosensor [38]. The binding event can be coupled to piezoelectric, optical or electrochemical transduction methods. An interesting detection method uses the “gate effect” observed in some MIPs; the binding of the template to the polymer causes the polymer structure to change which allows the passage of analytes through the polymer [37]. A theophylline MIP coupled to an electrochemical cell had an increased anodic current compared to the non-

imprinted polymer when it re-bound theophylline; this suggested that binding of the template increased the permeability of the MIP [39]. It was also shown by atomic force spectroscopy (AFS) that the surface of the MIP became rougher in the presence of theophylline which supported the idea that the polymer changed configuration and became more permeable.

The recognition capacity of MIPs can also be used as separation matrices. MIPs have been prepared for trapping biological and environmental analytes by solid phase extraction (SPE). Analogue imprinting plays an important role in the success of creating a MIP-SPE because template extraction is seldom 100% efficient. Theoretically the MIP can recognise the target analyte because it has a similar shape and functional group orientation to the template. It was observed by GC that template leaked from a MIP created using an analogue of sameridine. However the MIP could still be used to pre-concentrate the target analyte, sameridine, because their retention times did not overlap [40]. MIPs have better selectivity compared to common sorbents like alkyl bonded silica, therefore co-extraction of undesired compounds from a complex environmental or biological sample is minimised. MIPs can be used to separate a pair of enantiomers; this is influenced by the shape of the imprint, the spatial distribution of the functional groups and the number of interactions between the template and functional monomer. Enantiomeric pairs may be resolved because they exhibit multiple points of interaction with the functional monomer, in other cases enantiomers could be resolved even if they only had one point of interaction [3]. MIPs are also cheaper and easier to produce in comparison to immunosorbents; they are also more stable and have a higher load capacity. However site heterogeneity and low mass transfer in some MIPs may limit the performance of the polymers in certain applications [41].

## **1.6 Covalent imprinting**

Covalent imprinting involves coupling the template molecule to the functional monomer through a reversible covalent bond. The template-monomer is radically co-polymerised with a cross-linking monomer and thus the template is initially incorporated into the MIP structure. The MIP is only useful if the template is removed from the polymer structure (often using harsh conditions and

solvent extraction) to reveal cavities which are complementary in size and functional group orientation. The number of imprinted sites is fixed by the amount of template-monomer used. Thus the amount of non-specific binding should theoretically be reduced in comparison to a MIP prepared by the non-covalent method because there is not an excess of reactive functional groups incorporated into the polymer (see Section 1.7). These imprinted sites can selectively rebind the template molecule or may show *cross-reactivity* if the sites bind a template analogue. The recognition event in covalent imprinting is governed, to a lesser extent, by diffusion of the analyte through the MIP. The speed of covalent bond re-formation plays a greater role in the recognition process; although the interaction between the analyte and the functionalised cavities is stronger and therefore more stable, ideal for specificity or selectivity, these bonds do not form rapidly. This would be a drawback in sensor applications when a rapid response is usually required, but is less of a drawback in applications which allow time for equilibrium to be set up such as batch-binding experiments. The range of templates which can be covalently imprinted is limited by the type of chemical functional groups in the template; at this stage only templates which can form a reversible covalent bond with the functional monomer are useful. The boronate ester bond is well known for the covalent imprinting of mono-alcohols [42] or diols (such as carbohydrates); these form the strongest reversible functional group interaction and is most readily used to create synthetic molecular receptors [43]. *p*-Nitrophenyl- $\alpha$ -D-mannopyranoside was covalently imprinted using a boronate ester bond; the MIP was able to chromatographically distinguish the enantiomeric L-form from the D-form illustrating the selectivity of MIPs [44]. Schiff bases have been used to functionalise co-polymers of styrene/diisopropenylbenzene and methacrylamide [45]; however this bond does not equilibrate fast enough to be used for rapid chromatography [8]. Acetals/ketals have also been used to form covalent MIPs but these bonds also form slowly and therefore have not been as widely utilised [8]. Ester bonds do not readily hydrolyse [7] which would create a low number of binding sites in a MIP. Neighbouring groups may affect the rate of bond formation and could be a way of making covalent MIPs more suitable for applications which require rapid response. This has been reported for the boronate ester group; the presence of an appropriately positioned nitrogen group from a boronate ester group accelerated

the rate of trans-esterification [46]. The restrictions of covalent imprinting reduce the versatility of this imprinting approach; a more widely utilised approach, non-covalent imprinting, will be covered in the next section.

## 1.7 Non-covalent imprinting

The non-covalent imprinting approach utilises the concept of self-association in the MIP pre-polymerisation mixture; this refers to the mixture of template, monomers and initiator in a porogen prior to the initiation of polymerisation. Figure 1.6 (Section 1.1) illustrates a schematic of the non-covalent approach. The functional monomer organises around the template, this is dictated by the functional groups present on the template. The functional monomer can adopt numerous conformations around the template (affected by the presence of cross-linking monomer, initiator and porogen) which can be polymerised into the polymer structure. The self-association takes place through the formation of non-covalent interactions between the template and the functional monomer such as hydrogen bonding, electrostatic, Van der Waals and  $\pi$ - $\pi$  interactions. The choice of porogen can affect the strength of interaction; for example a hydrogen bond would be enhanced in aprotic solvents such as chloroform but in protic solvents, like alcohols, the strength of the hydrogen bond interaction would become weaker because the solvent can solvate the template and functional monomer and compete with the template/functional monomer interactions. The functional monomer is typically added in excess to the amount of template (often a ratio of 1 mole template to 4 moles functional monomer) to ensure that all the template molecules form imprinted cavities. The template-functional monomer complex is frozen into the MIP structure and the cavities are revealed by solvent extraction. The non-covalent imprinting approach has been widely accepted by MIP researchers because a larger range of template molecules can theoretically be imprinted by this method. This does not mean all organic molecules can be imprinted successfully. MIPs are often prepared in organic solvents therefore the template must be soluble in the solvent of choice. In some cases the solubility limit of the template may be too low in the chosen organic porogen. The addition of functional monomer may increase the solubility limit: ethynylestradiol is sparingly soluble in toluene but in the presence of

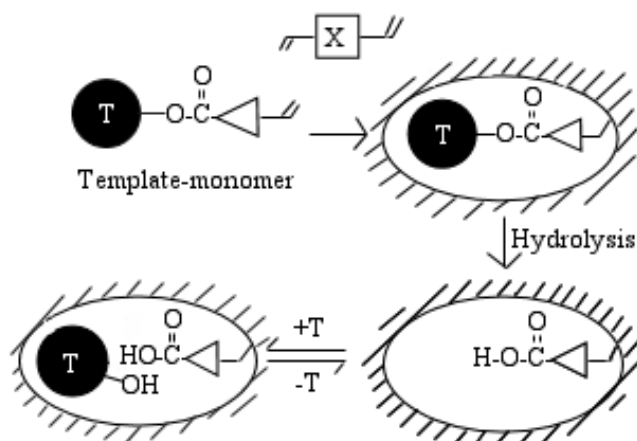
4-vinylphenol the solubility limit was exceeded by 10 times [13]. For non-covalent imprinting, the template ideally has structural rigidity. This would help to minimise the number of different template conformations which the functional monomer can complex with in the pre-polymerisation mixture; the same applies to the functional monomer. This would reduce the range of binding sites with different energies in the MIP and therefore result in more defined recognition because there would be more sites which fit the most abundant (and stable) conformation of the template. Binding a template into sites which have a better fit does not lose as much rotational entropy; this has been linked with a MIP with more selectivity and a higher affinity for the template [5].

The presence of more chemical functional groups on the template molecule may enhance the interaction with the appropriate functional monomer. Increasing the strength of association between the template and functional monomer influences the quality of the imprint; a more stable complex is likely to maintain its structural integrity during the polymerisation process so that imprinted cavities can be made from them. This can be affected by the polarity of the porogen and also the type and amount of cross-linking monomer [5, 17]. For liquid compounds with less chemical functionality, shape recognition may play a larger role in the binding process. Poorly functionalised aromatic solvents (such as xylene) may be imprinted by porogen-imprinting [47], it is likely that shape selectivity and also  $\pi$ - $\pi$  interactions with the aromatic cross-linker (DVB) played a role in the specific binding process. The strength of intermolecular interaction can be studied by spectroscopic methods, discussed more in Section 2.4.

## 1.8 Semi-covalent imprinting

Semi-covalent imprinting merges features of covalent and non-covalent imprinting. Just like covalent imprinting the template-functional monomer must be chemically synthesised and characterised prior to being used in the polymerisation step. There is no excess of functional monomer, the interaction between the template and functional monomer is stoichiometric and therefore decreases the amount of non-specific binding. The sites are more uniform, template bleed is less likely and template rebinding is not subjected to kinetic restrictions of covalent bond formation; diffusion remains as a limiting factor in

the uptake process [48]. Covalent bonds such as ester or amide have been used with this approach ; these are stronger and therefore do not spontaneously form once they have been hydrolysed. Thus for the rebinding step, weak intermolecular interactions between the analyte and the MIP are utilised (Figure 1.11).



**Figure 1.11 Schematic of semi-covalent imprinting using an ester bond<sup>3</sup>**

The template-monomer is often specifically synthesised. This is then mixed with a cross-linking monomer in a porogen and then thermally or photo-initiated. Once polymerisation is complete the covalent bond coupling the analyte and functional monomer is cleaved by hydrolysis to reveal imprinted cavities. The functional group moiety in the cavities can then rebind the template or its analogue. However upon rebinding, there may be steric crowding within the imprinted cavity because of the differences in covalent bond lengths and non-covalent interactions [8]. A variation of the semi-covalent technique is the use of a sacrificial spacer functional group, such as a carbonate or carbamate, to link the template and functional monomer. This approach has been used for a variety of different templates like steroids (such as cholesterol [49]), pharmaceutical chemicals (for example nortriptyline [50]) and environmental toxins (such as 2,3,7,8-tetrachlorodibenzodioxin [51]). The carbonyl group is a commonly used sacrificial spacer group and can be introduced as a carbonate (Figure 1.12),

<sup>3</sup> Where “T” represents the template and “X” is the cross-linking monomer.

carbamate or urea. Alternative spacer groups that have been used include dimethyl silyl ether [52] and dimethyl silyl ester groups [53]. The spacer group minimises steric hindrance because after it has been removed by hydrolysis there is space for the template to interact at the optimal bond distances [8].

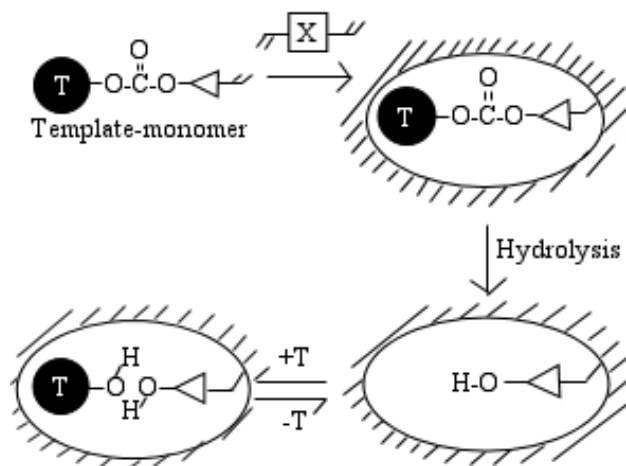


Figure 1.12 Schematic of the sacrificial spacer approach<sup>4</sup>

The performance of semi-covalent MIPs varies depending on the template and the monomers chosen. A non-covalent and semi-covalent MIP for 4-nitrophenol was compared [54]; it was found that the non-covalent MIP (a copolymer of MAA and EDMA) was more selective than the semi-covalent version (a polymer consisting of MAA, EDMA and styrene) but the semi-covalent MIP appeared to have a greater capacity. The semi-covalent MIP contained styrene which could interact through  $\pi$ - $\pi$  interactions with the phenyl ring in the template. The researchers thought that the poorer selectivity of the semi-covalent MIP was due to only a single point interaction in the cavities and not using the sacrificial spacer approach.

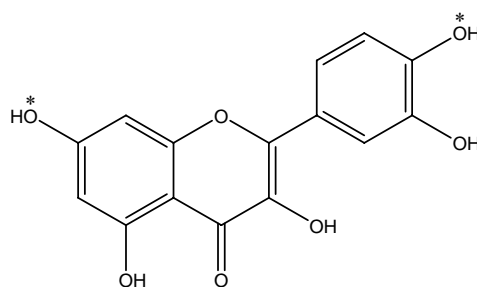
The decision of which of the three main imprinting approaches to use lies in the nature of the template, the monomers chosen and also any requirements of the final application. All three approaches have their merits but the trend at the moment leans towards using non-covalent and semi-covalent imprinting because of their versatility; the suitability of the approach is best evaluated empirically by synthesising and testing the MIPs.

<sup>4</sup> Where "T" represents the template and "X" is the cross-linking monomer.

## 1.9 Molecular recognition of small organic compounds

There are a vast number of reports of MIPs which can recognise small organic compounds. Many of these compounds are of biological or environmental interest (such as propofol – anaesthetic [55]; soman – war gas [56]; 2,4,5-trichlorophenoxyacetic acid – herbicide [57]). Molecular imprinting is often used as an alternative method of recognition because of its selectivity, robustness, ease of production and lower production costs. There are reports of imprinting larger templates such as proteins, viruses and bacteria; however their size limits diffusion through a typical MIP matrix. Surface imprinting the shape of a macromolecule or organism is one way of overcoming the diffusion problems [58] but this topic is out of the scope of this thesis which will only focus on imprinting small organic compounds.

A small organic compound found which has been studied is quercetin. It has a keto, ether and five hydroxyl groups and two aromatic rings which make it a candidate for non-covalent imprinting because there are many potential sites for interacting with functional monomers. The presence of multiple sites of interactions can potentially result in a MIP specific for the template. However, quercetin MIPs used to extract the compound from ginkgo leaves [59] showed that the quercetin non-covalent MIP had a high affinity for quercetin but it also could bind structural analogues which could fit into the imprinted cavities. It was shown that in acetone it only interacted through two of the hydroxyl groups (Figure 1.13, starred \*) with a pyridine moiety [60].



**Figure 1.13** Structure of quercetin

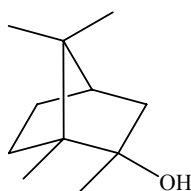
This may have been the case for other quercetin MIPs created using other polymerisation mixtures, because there is a degree of cross-reactivity towards structural analogues. However the interactions occurring in each system should

be studied individually because the mechanism of specificity can change in the presence of different monomer (functional and cross-linking) and porogen polarities. These can modify the types of interactions at the active sites and also affect their accessibility by changing the porosity of the MIP.

Other small organic compounds which have been extensively investigated were triazines herbicides [61]. They contain a number of functional groups which can interact in different combinations with the functional monomer as well and may result in a multi-point binding site. This contributes to site heterogeneity which is inherent to the non-covalent imprinting approach. It is possible to achieve better site heterogeneity in a MIP by using semi-covalent imprinting because the template is covalently attached to the monomer and thus reduces the number of possible orientations between the template and functional monomer. A semi-covalent MIP for cholesterol [62] was calculated to have a single dissociation constant (shown by a linear Scatchard plot) which meant there was a more homogeneous population of imprinted sites. Chemical modification (acylation) of the hydroxyl groups in the sites decreased the MIP affinity for cholesterol and therefore decreased its uptake; this was evidence that the formation of a hydrogen bond was involved in the specific recognition of cholesterol by the MIP. In contrast to the semi-covalent cholesterol MIP, a non-covalent theophylline MIP [63] had a non-linear Scatchard plot from which multiple dissociation constants were calculated, this implied that there were a number of different binding sites in the MIPs. Site heterogeneity in a non-covalent MIP may also be reduced (but not eliminated) by the presence of a ring structure in the template (such as those found in triazines) because the molecule will be more rigid. A rigid structure reduces the number of rotational conformations, thus decreasing the number of different template-functional monomer complexes formed during polymerisation and thus decrease site heterogeneity.

A ring structure in the template also allows an evaluation of the effect of molecular size on the recognition event because changing the side groups will not change the core shape too much. In some cases MIPs may show cross-reactivity to other analogues of the template. In these cases it is likely that shape recognition dominates recognition over the presence of functional group and

functional group orientation. These binding properties can be modulated by altering the rebinding conditions such as solvent, pH and temperature. Shape recognition is more likely to operate in MIPs imprinted against poorly functionalised organic compounds. They are likely to contain fewer functional groups in the cavities (which cause steric crowding), thus structural analogues may readily fit into the cavities. From studies probing shape selectivity of MIPs, the cavities of non-covalent MIPs of  $\alpha$ -methylbenzylamine or its derivatives were shown to exclude probe compounds that were bulkier than the template. It is likely that steric hindrance excluded bulky compounds from MIPs created with a smaller template [64]. Probing MIPs with  $\alpha$ -methylbenzylamine derivatives smaller than the template showed that they may enter the cavities but they did not form optimal contacts with the internal surface of the cavity. Thus the MIP showed lower selectivity for smaller compounds and this was termed “non-optimal spatial fit”, suggesting that there was a limit to how structurally similar an analyte must be compared to the template. For this reason analogue imprinting may be only useful for a certain number molecules from a chemical group. 2-Methylisoborneol has a rigid molecular structure and only a single hydroxyl group (Figure 1.14). A range of mono-alcohols were probed against the 2-methylisoborneol MIP but there was very little specific uptake [65]. A ketone was probed but only showed a small degree of selective uptake. This showed that in this case molecular structure screened against the uptake of template analogues because none of the mono-alcohols tested showed specific uptake but the ketone did.



**Figure 1.14** Structure of 2-methylisoborneol

Other poorly functionalised organic compounds which have been successfully imprinted include 4-nitrophenol and *N*-heterocycles such as pyridine. A sacrificial spacer, semi-covalent pyridine MIP was made from phenyldimethylsilylmethacrylate co-polymerised with DVB; after hydrolysis

methacrylic acid moieties were revealed which could hydrogen bond with the nitrogen on pyridine [53]. The phenyl group appeared to generate cavities which selectively excluded nitrogen containing aromatic compounds; the amount bound depended on the size of the molecule. Pyridine (one aromatic ring) was bound the most whereas acridine (three aromatic rings) was bound the least. The percentage of quinoline (two aromatic rings) taken up was in between the previous two.

As with other more complex analytes, many factors must be considered to create a successful MIP for a poorly functionalised organic compound. There is no universal method of successfully imprinting such compounds and therefore polymer development may be time consuming. However the multitude of factors in polymer synthesis is an indispensable tool because different combinations can be trialled to develop a tailor-made recognition element which will specifically bind to the target analyte.

### 1.9.1 Aim of current research

The goal of this research is to develop a molecularly imprinted polymer (MIP) against small, poorly functionalised organic compounds. The model class of compounds chosen were monoterpenes. In this study  $\alpha$ -terpineol and (-)-menthol were chosen because of their low molecular mass and their poor chemical functionality (a single hydroxyl group in both and an alkene group in  $\alpha$ -terpineol; Figure 1.15).



Figure 1.15 Structure of terpenes that were imprinted

They both also possess a distinct molecular shape which, as discussed before, can contribute to the imprinting effect. *trans*-Terpin was also chosen as a template because it has a similar shape to both  $\alpha$ -terpineol and (-)-menthol.

However it contains an additional hydroxyl group which may help to form stronger interactions with the functional monomer. There have been several reports of terpene MIPs. A non-covalent (-)-menthol MIP coupled to a quartz crystal microbalance (QCM) was shown to be enantioselective because there was less response with (+)-menthol compared to (-)-menthol [66]. The recognition mechanism appeared to be dominated by hydrogen bond formation with the functional monomer (methacrylic acid) because structural analogues that did not have a hydroxyl group gave a poor response. The ROMP method was used to create a sacrificial spacer semi-covalent MIP for (-)-menthol [3]. It could distinguish menthol enantiomers even though there was only one functional group moiety in the active sites. (-)-Menthol has also been used as a model compound to study the feasibility of creating MIPs by  $\gamma$ -radiation instead of using initiators [67]. Those MIPs bound menthol specifically and showed poor cross-reactivity against camphor, which was expected because it has a different molecular structure and functional group. A menthone MIP created for stereoselective organic synthesis could reduce menthol to a pair of diastereomeric menthol products if the polymer was activated by pre-treatment with  $\text{LiAlH}_4$  [68]. If the menthone MIP was not activated by a reductant it could selectively bind (-)-menthol. Post-translational processing of MIPs has been used to impart size selectivity in MIPs to improve the uptake of small, poorly functionalised *N*-heterocycles [52]; this may be an alternative method to conventional molecular imprinting. The option to modify MIPs after polymerisation is analogous to the post-translational modification of proteins and increases the repertoire of techniques to create MIPs that are better suited for their final application.

Detection of terpenes using MIPs has also been done in the gas phase. A limonene non-covalent terpene MIP was reported to be specific for gaseous limonene. The researchers found that their MIP-QCM system was also sensitive to humidity [69]. The non-covalent limonene MIP polymerisation mixture only contained a small percentage of limonene which is a common imprinting strategy. In another example of gas phase detection by a MIP-QCM setup, poorly functionalised volatile organic compounds such as toluene or *p*-xylene were imprinted by increasing the amount of template relative to the functional monomer in the polymerisation mixture [47]. The researchers found that the

toluene MIP bound toluene preferably to xylene and the xylene MIP bound xylene preferably to toluene. This was based on a technique called “porogen-imprinting” [70] where the solvent acted as a space-filling molecule and thus the polymer formed pores which could recognise the size and shape of the solvent. This method of imprinting may be useful for imprinting poorly functionalised terpenes, which will be discussed in Chapter Three. However it has not been encountered in the literature for terpenes, therefore it has not been fully characterised as a method of creating MIPs which can specifically recognise a terpene. The chosen terpene would have to be liquid and miscible with the functional monomer, cross-linking monomer and initiator.  $\alpha$ -Terpineol fulfilled these requirements because it is liquid at room temperature and it is a polar molecule with a hydroxyl group. It is possible that the alkene group in  $\alpha$ -terpineol may co-polymerise with conventional monomers; it was reported that  $\alpha$ -terpineol could co-polymerise with methyl methacrylate or styrene [71, 72]. From the perspective of conventional molecular imprinting theory, the alkene group in  $\alpha$ -terpineol may permanently incorporate the molecule into the polymer structure. This would make it difficult to remove the template; therefore imprinted cavities may not be formed. Thus radical polymerisation may not be suitable for imprinting terpenes with more than one alkene group because they may form soluble linear polymers rather than insoluble cross-linked polymers. However it was reported that a polymerisable template may increase the recognition of the MIP by rebinding a cluster of templates rather than a single molecule [73]. 2-(2,4,5-Trichlorophenoxyacetoxy)-ethyl methacrylate, a polymerisable derivative of the template 2,4,5-trichlorophenoxyacetic acid, was polymerised with vinyl pyridine and ethylene glycol dimethacrylate. This MIP had a higher affinity for the template compared to a MIP prepared without the polymerisable template; these were based on comparing the affinity constants calculated from different adsorption isotherm models. If the binding capacity of the MIP was correlated to the number of high affinity sites then a MIP with fewer high affinity sites would bind to less analyte and vice versa. However the MIP created with the polymerisable template had a larger affinity constant but a smaller binding site density, therefore they suggested that the covalently incorporated template acted as nucleation sites which bound template clusters rather than single molecules.

There has not been a terpene MIP made to recognise  $\alpha$ -terpineol. To investigate the most appropriate way of creating a successful imprint of the molecule two approaches were attempted: non-covalent and porogen-imprinting. Simultaneously (-)-menthol (using non-covalent and semi-covalent approaches) and *trans*-terpin (using non-covalent approach) MIPs were made so that cross-reactivity studies could be carried out on the MIPs.

### **1.10 Determination of terpenes**

It is important to be able to reliably quantitate the amount of non-bound terpenes in an equilibrium batch-binding experiment. The amount of analyte bound by terpene MIPs depends on the intrinsic property of the MIP and also the conditions used to carry out the batch-binding experiment. The analyte binds specifically to imprinted cavities and often also non-specifically with the bulk polymer structure. Due to the nature of the batch-binding experiment it is easier to determine the amount of analyte remaining and then the amount of bound analyte is calculated by difference from the initial amount of analyte added; these will be discussed further in Chapter Three. If the analyte was added in excess, the amount remaining theoretically should be lower because they would be bound by the MIP. In order to distinguish this value from the initial amount added an accurate and precise method of analyte detection is required. Two methods of detection were available during this research, colorimetric and gas chromatography (GC). The advantage of the colorimetric method over the GC was that the analysis time on a spectrophotometer can be faster per sample and therefore the results would be available sooner. The original colorimetric method available in literature, to detect  $\alpha$ -terpineol or (-)-menthol, was qualitative [74, 75]. Since then, there have been attempts to develop a quantitative method using modifications of the reaction for various types of compounds [76-80]. However in an attempt to adapt the colorimetric reaction to quantitatively determine  $\alpha$ -terpineol or (-)-menthol, the colour development turned out to be continuous which made it too variable for quantitative work. Attempts to decrease the absorbance variability were not successful enough for the colorimetric method to be used as a means of quantifying terpenes. The stages of developing the

colorimetric reaction for either  $\alpha$ -terpineol or (-)-menthol can be found in the Appendix One.

The GC method had been developed within our organisation so only validation of the method with the terpenes of interest was required. This was done by running calibration standards of  $\alpha$ -terpineol, (-)-menthol, terpinolene and  $\alpha$ -terpinene in different solvents; details can be found in Appendix Two.

**Chapter Two**  
**Synthesis of Molecularly Imprinted Polymers**

## 2 Introduction

A number of polymerisation techniques can be used to create a MIP that will rebind terpenes, a class of poorly functionalised organic compounds. One of them is block polymerisation described in Chapter One. This was the method of choice due to its simplicity and versatility for studying the rebinding of template by small (less than 38  $\mu\text{m}$ ) and large (38-150  $\mu\text{m}$ ) MIP particles from the same batch of polymer. This is advantageous when trying to optimise a new MIP system as it reduces the variables when trying to find a particle size range that has a high ratio of specific binding to non-specific binding. Precipitation polymerisation was subsequently tried as it theoretically generates small, spherical shaped MIP beads from a homogenous, one phase system. Each technique has an extensive set of variables which can be changed in the development of a MIP that will show a high level of specific uptake of terpenes. Discrete variables were modified in the synthesis stage to find a suitable MIP composition (listed below). In future these recipes can be further modified by changing continuous variables such as temperature of initiation and template:functional monomer mole ratio. A range of different MIPs were prepared and tested for the ability to specifically rebind  $\alpha$ -terpineol or an analogue (such as terpinolene or (-)-menthol) by carrying out equilibrium batch-binding experiments. From these studies low performance MIPs were eliminated. Further tests were carried out on selected polymers that performed better by changing the variables used in the batch-binding experiments.

The following discrete variables in the synthesis stage were chosen for modification:

1. Type of template
2. Type of functional monomer
3. Type of cross-linking monomer
4. Type of porogen

The variables which were modified when conducting equilibrium batch-binding experiments included:

1. Type of solvent used for analyte rebinding
2. Polymer concentration
3. Analyte concentration
4. Type of analyte

The synthesis of the polymers is discussed below. This is followed by the characterisation of polymer morphology and topology by SEM along with  $^1\text{H-NMR}$  investigations of the pre-polymerisation complexes.

## 2.1 Nomenclature for block polymers

Block terpene MIPs and corresponding reference polymers were prepared in 13 x 100 mm glass culture tubes with *Teflon*-lined cap. Every pair of MIP and non-imprinted polymer (i.e. the reference polymer) made on different days were treated separately even if the composition was the same because factors such as the temperature of initiation and quantity of polymer made may have had an impact on the characteristics of the polymer. Each pair of polymers was given an individual code to distinguish it from the other polymers. The composition and associated code for each pair of polymers can be found in Table 2.1. A sand bath was used to thermally initiate polymerisation in order to minimise potential exposure to water vapour from a water bath.

**Table 2.1 List of terpene block polymers**

| Code name | Type of MIP                        | Composition   |
|-----------|------------------------------------|---|
| t4        | $\alpha$ -terpineol, non-covalent  | $\alpha$ -terpineol, MAA, EDMA, chloroform, AIBN            |
| t5        | $\alpha$ -terpineol, non-covalent  | $\alpha$ -terpineol, MAA, DVB, chloroform, AIBN             |
| t6        | $\alpha$ -terpineol, non-covalent  | $\alpha$ -terpineol, MAA, TRIM, chloroform, AIBN            |
| terpin    | <i>trans</i> -terpin, non-covalent | <i>trans</i> -terpin, MAA, EDMA, chloroform, AIBN           |
| m9        | (-)-menthol, non-covalent          | (-)-menthol, MAA, EDMA, chloroform, AIBN                    |
| t10       | $\alpha$ -terpineol, non-covalent  | $\alpha$ -terpineol, HEMA, EDMA, chloroform, AIBN           |
| m10       | (-)-menthol, semi-covalent         | (-)-menthyl 4-vinylphenyl carbonate, EDMA, chloroform, AIBN |
| t11       | $\alpha$ -terpineol, non-covalent  | $\alpha$ -terpineol, bilirubin, EDMA, chloroform, AIBN      |

### 2.1.1 Synthesis of block polymers

Block polymers were prepared in most cases with a mole ratio of 1:4:20 between the template:functional monomer:cross-linker. The concentration of porogen in the polymerisation mixture (*porogen loading*) was often 2 mL/g of monomer. All chemicals were used as received; the amounts used and porogen loading can be found in Appendix Four, Table A4.1. An example of the protocol used to prepare the block polymers follows.

The MIP and reference polymer were prepared simultaneously in glass culture tubes. In one tube the template was mixed with the functional monomer, cross-linking monomer, porogen and initiator. In a second tube, the template was omitted to synthesise the reference polymer. The polymerisation mixture was vortexed to mix the contents and then thermally initiated for at least 12 hours. When the sand bath was used to thermally initiate polymerisation, a temperature gradient was observed in the bath. The heat was greatest at the base of the sand bath. In contrast the heat would have been more evenly distributed in a water bath because of convection currents. However there was a risk of exposing the

polymerisation mixture to water vapour which may disrupt intermolecular interactions between the template and functional monomer; it may also cause the formation of two phases. Therefore the sand bath option was used more often. The temperature of initiation was set between 60-80 °C (governed by the initiator and boiling point of the reactants) but it has been shown that polymerisation reactions are exothermic and generate heat [16]. Therefore the temperature of polymerisation was likely to be much higher than the initiation temperature, however this was not measured. The quantity of polymer could also influence how well the heat dissipated through the mixture and the efficiency of thermal initiation. In a large mixture, agitation may be required to distribute the heat evenly. However for the current research the polymers were created in small batches. If more polymers were required multiple small batches were synthesised.

Polymerisation was stopped, only if the content of the vial appeared solid, by letting the polymers cool down in a fume hood and allowing the solvent to evaporate away. The template-monomer for the semi-covalent MIP was synthesised in our laboratory (synthetic protocol in Appendix Three).

It is to be noted that using bilirubin as a functional monomer was previously developed within our organisation; the use of it for imprinting terpenes was a further investigation of using this compound as a functional monomer.

### **2.1.2 Size fractionation and template extraction**

The glass tubes were crushed to retrieve the solid polymer block. The polymer was then ground using a mortar and pestle (or an electronic ball mill for harder polymers). They were then dry sieved (with a brush) or wet sieved (with ethanol or acetone) through ATM standard testing sieves. The particle range collected for each polymer can be found in Appendix Four, Table A4.1.

## 2.2 Nomenclature for precipitation polymers

Precipitation terpene MIPs and corresponding reference polymers were prepared in 13 x 100 mm glass culture tubes with *Teflon*-lined cap. Every pair of MIP and reference polymers made on different days was treated separately as discussed in Section 2.1. Every pair of polymers was given an individual code (details in Table 2.2).

**Table 2.2** List of terpene precipitation polymers

| Code name | Type of MIP                       | Composition  |
|-----------|-----------------------------------|--|
| t15       | $\alpha$ -terpineol, non-covalent | $\alpha$ -terpineol, MAA, EDMA, acetonitrile, AIBN |
| t16       | $\alpha$ -terpineol, non-covalent | $\alpha$ -terpineol, MAA, EDMA, acetonitrile, AIBN |
| m17       | (-)-menthol, non-covalent         | (-)-menthol, MAA, EDMA, acetonitrile, AIBN         |
| t18       | $\alpha$ -terpineol, non-covalent | $\alpha$ -terpineol, MAA, EDMA, AIBN               |
| t19       | $\alpha$ -terpineol, non-covalent | $\alpha$ -terpineol, EDMA, AIBN                    |
| t24       | $\alpha$ -terpineol, non-covalent | $\alpha$ -terpineol, MAA, EDMA, AIBN               |

### 2.2.1 Synthesis of precipitation polymers

The mole ratios of precipitation polymers prepared were more varied and the porogen loading was generally greater compared to block polymers (details in Appendix Four, Table A4.2). All chemicals were used as received. The protocol was similar to the preparation of block polymers. The MIP and reference polymer were prepared simultaneously in glass culture tubes. In one tube the template was mixed with the functional monomer, cross-linking monomer, porogen and initiator. In a second tube, the template was omitted to make the reference polymer. The contents of the polymerisation mixture were mixed by vortexing, and then the tubes were thermally initiated for at least 3 hours in a sand bath. A similar procedure was carried out for synthesising MIPs by porogen-imprinting (details in Appendix Four, Table A4.3). Polymerisation was stopped, only if the contents of the vial appeared solid, by letting the polymers cool down in the fume hood before extracting the template.

## **2.2.2 Template extraction**

Unlike the block polymers, precipitation polymers were easier to remove from the glass tubes because they were only aggregates of small particles. The polymers were loosened from the tube using a spatula and lightly ground using a mortar and pestle before template extraction. Then they were transferred into 50 mL plastic centrifuge tubes to extract the template. The solvent chosen must be miscible with the template and the previous wash solvent. Approximately 20 mL of solvent was used to remove the template by repeated wash cycles which consisted of sonication or shaking at 560 rpm on an orbital shaker for at least 30 minutes in fresh solvent. The polymer particles were then pelleted by centrifugation at 2000 or 2400 rpm for at least 30 minutes. The supernatant was discarded if clear. If it was not clear the suspension was centrifuged for longer or the supernatant was poured into a fresh 50 mL centrifuge tube and centrifuged. The polymer pellets were re-suspended in fresh wash solvent and the wash cycle was repeated (details in Appendix Four, Table A4.4).

Once the wash cycles were complete the polymers were allowed to air dry, or in some cases the polymers were dried briefly (1-4 hours) in an 80 °C laboratory oven (as temperature does not affect the polymer behaviour as discussed in Section 1.4.3).

## **2.3 Physical characterisation of polymers**

### **2.3.1 Visualisation of polymers using SEM**

Only selected polymers were visualised by scanning electron microscopy (SEM) using a Hitachi S-4100 Field Emission Scanning Electron Microscope with X-ray analyser. The SEM images of polymers which showed potential specific binding or cross-reactivity from the batch-binding experiments (Chapter Three) were visualised. The images have been compiled in Appendix Six and they will be referred to in the text of the batch-binding discussion.

### **2.3.2 Surface area and pore analysis**

N<sub>2</sub> gas adsorption was used to determine the surface area and pore radius using a TriStar 3000. 0.22-0.35 g of solid polymer particles were degassed at

150 °C for 24 hours under nitrogen. The multi-point pressure data was used to determine the surface area (using the Brunauer–Emmett–Teller (BET) model) and the pore radius (using the Barrett–Joyner–Halenda (BJH) model). Surface area and pore analysis were only carried out on two polymers (t5 and t24) which were observed to specifically bind  $\alpha$ -terpineol (Table 2.3).

**Table 2.3** The results of surface and pore analysis for t5 and t24

| Polymer name          | Average BET surface area (m <sup>2</sup> /g) | Average BJH pore radius (nm) |
|-----------------------|--|------------------------------|
| t24-MIP               | 432.29                                       | 7.80                         |
| t24-reference polymer | 17.20  | 10.00                        |
| t5-MIP                | 815.43                                       | 5.36                         |
| t5-reference polymer  | 828.64                                       | 5.42                         |

It should be noted that t24 and t18 have the same composition but were a different batch; they both had a similar binding capacity for  $\alpha$ -terpineol in hexane (Section 3.2, Figures 3.17 and 3.18). All results were an average of duplicate tests; t5 averages were calculated from the results of two different size fractions: <38  $\mu$ m and fines.

t5-MIP had a similar but slightly lower BET surface area and BJH pore radius compared to t5-reference polymer. In contrast, t24-MIP had approximately 25 times the surface area of t24-reference polymer but a smaller pore radius. This may be a reflection of the different solvent properties of  $\alpha$ -terpineol and cyclohexane.

### 2.3.3 Fourier transform-infrared spectroscopy of polymers

Fourier transform-infrared (FT-IR) spectroscopy can be used to identify functional groups in polymers [81]. The FT-IR spectra were acquired only for t18 and t24 to help determine if  $\alpha$ -terpineol was covalently incorporated into the polymer structure (discussion in Section 3.2). A small amount of dry polymer was mixed with dry KBr and pressed to create a KBr disc. A Digilab Scimitar FT-IR spectrometer was used to acquire the FT-IR spectrum of the solid polymers. The FT-IR spectra of the neat liquid reagents ( $\alpha$ -terpineol, MAA,

EDMA) were also acquired and have been presented in Appendix Seven as a reference.

### *Results*

The peaks present in the spectra in Figures 2.1 to 2.4 appeared to be very similar to each other. When comparing the polymer spectra with the spectrum of neat  $\alpha$ -terpineol (Appendix Seven, Figure A7.1), there appeared to be no  $\alpha$ -terpineol incorporated into the polymers. The dominant signals originated from MAA and EDMA (FT-IR of neat monomers in Appendix Seven, Figure A7.2 and Figure A7.3). Overall these results suggested that:

- a)  $\alpha$ -Terpineol did not incorporate into the polymer structure.
- b) The two batches of polymer, t18 and t24, contained very similar functional groups.

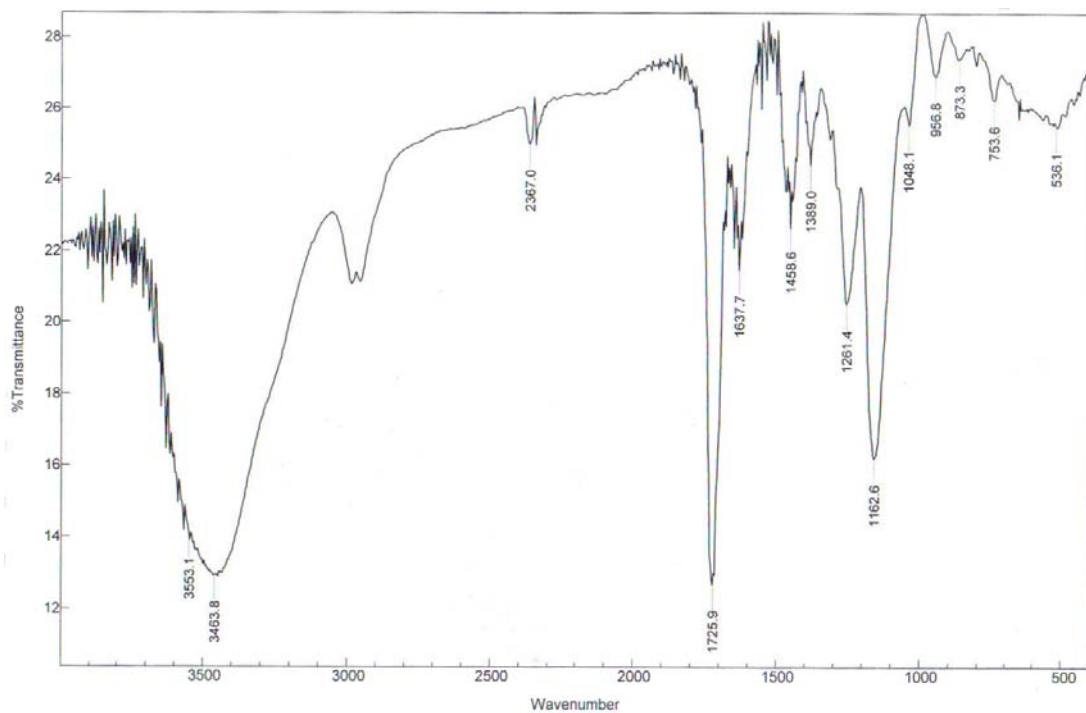


Figure 2.1 FT-IR of t18-MIP

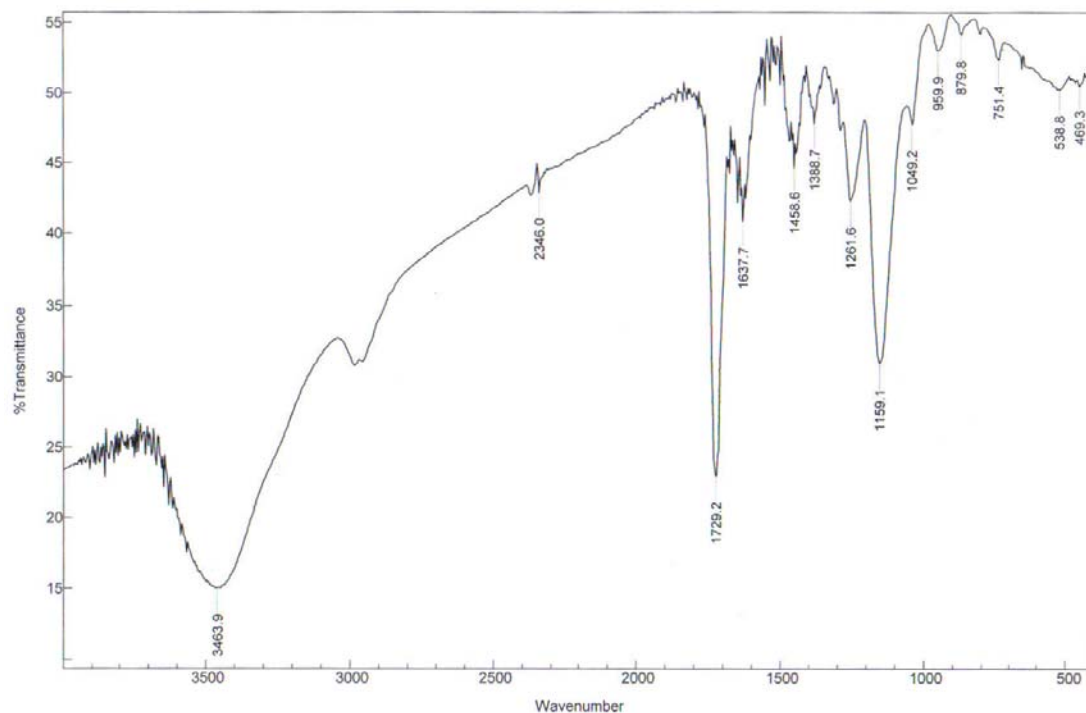


Figure 2.2 FT-IR of t18-reference polymer

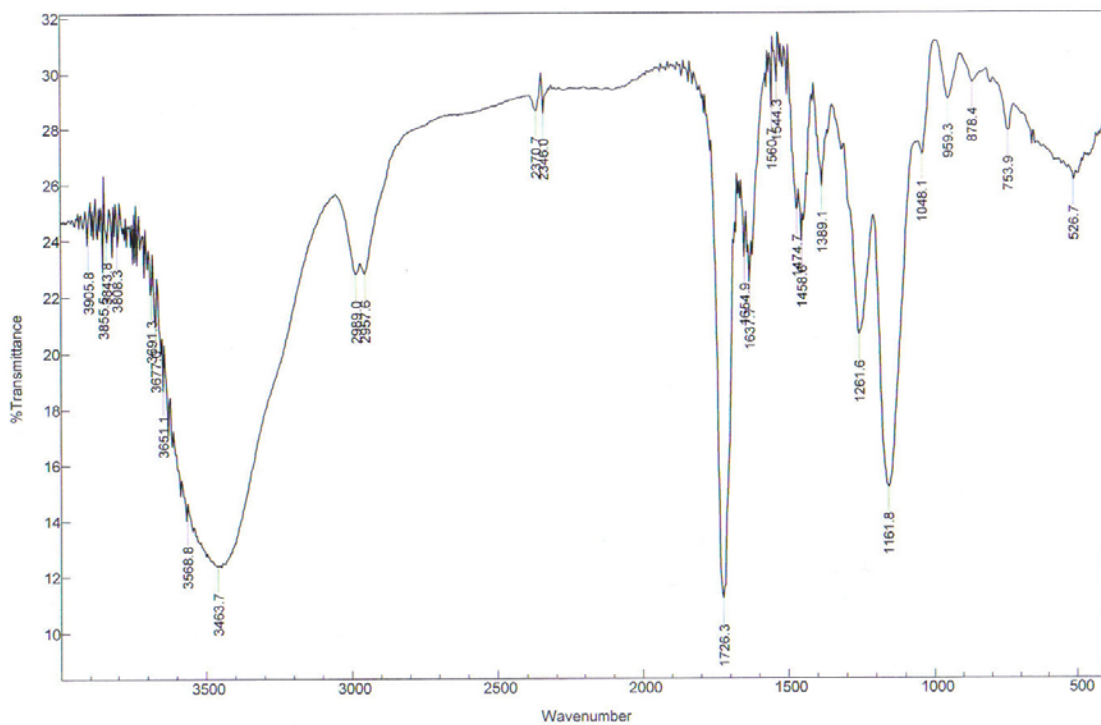


Figure 2.3 FT-IR of t24-MIP

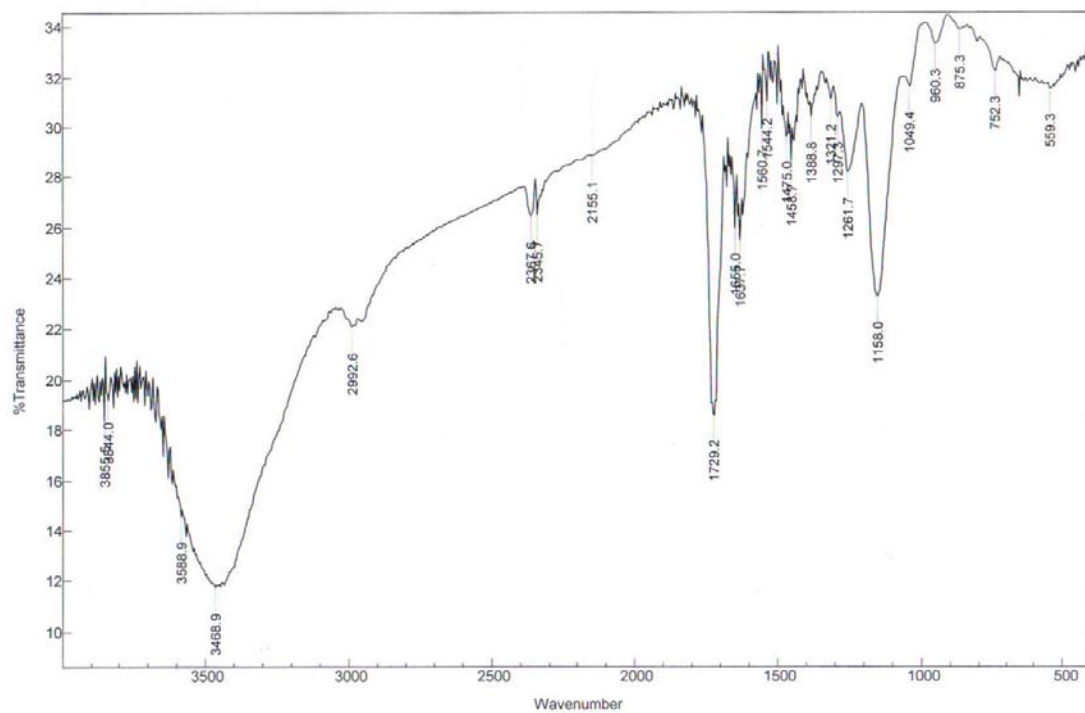


Figure 2.4 FT-IR of t24-reference polymer

## 2.4 Characterisation of pre-polymerisation complexes

In non-covalent imprinting the association of functional monomer around the template molecules forms complexes which are then frozen into a three-dimensional cross-linked polymer; theoretically these complexes give MIPs the property of being able to specifically rebind template molecules. Molecular interactions in the pre-polymerisation complexes can be studied by spectroscopy to evaluate the strength of interaction [82]. If the template has a chromophore any changes that occur in the UV/Vis spectra when the functional monomer is added indicates that there are changes in the surrounding environment which have redistributed the electrons in the template; these give rise to changes in the electronic transitions and therefore changes in the spectra. Alternatively the molecular interactions can be studied by  $^1\text{H-NMR}$  [4] because changes in the environment of the template give rise to changes in chemical shift, it is possible to identify the point on the polymer which interacts the most with the functional monomer because each proton yields a  $^1\text{H-NMR}$  signal. This method has been also used to screen template-functional monomer combinations and to select a pair which interact the strongest because those are more likely to form sites which bind more specifically [82].

### 2.4.1 $^1\text{H-NMR}$ analysis of pre-polymerisation complexes

A functional monomer analogue was used to probe the molecular interaction with  $\alpha$ -terpineol or (-)-menthol as it eliminated the chance of polymerisation taking place whilst the study was conducted.  $^1\text{H-NMR}$  was chosen because the two analytes did not have strong ultraviolet-active chromophores. A saturation isotherm was generated by carrying out  $^1\text{H-NMR}$  titration using a Bruker DRX400 FT-NMR spectrometer. A typical titration involved preparing 0.06 M stock solution of the template in deuterated chloroform ( $\text{CDCl}_3$ ). The solution containing the functional monomer analogue was prepared at the concentrations stated in Appendix Five and dissolved in 1 part  $\text{CDCl}_3$  and 1 part 0.06 M template solution; this ensured that the template concentration stayed constant during the titration. These solutions were stored in glass vials with *Teflon*-lined caps and then kept in a desiccating container in the refrigerator

when not in use. To begin the experiment, 250  $\mu\text{L}$  of the 0.06  $M$  template solution was added to 250  $\mu\text{L}$   $\text{CDCl}_3$  directly in an NMR tube and then mixed by inverting. The  $^1\text{H}$ -NMR spectrum was acquired (receiver gain set to “automatic”, number of scans: 16) before titrating the functional monomer analogue solution using the volumes specified in the “*volume added*” column in Appendix Five. After addition of the functional monomer analogue the tube was inverted to mix and then left at 303 K for 10-30 minutes which allowed the complexes to form. The  $^1\text{H}$ -NMR was acquired before proceeding with adding the next volume of functional monomer analogue solution. This method was used to investigate the complex formation between:

- $\alpha$ -Terpineol and acetic acid (to simulate the interaction between  $\alpha$ -terpineol and methacrylic acid in a non-covalent  $\alpha$ -terpineol MIP).
- (-)-Menthol and phenol (to simulate the interaction between (-)-menthol and vinylphenol in the semi-covalent (-)-menthol MIP).

All results were processed using MestReC software, version 4.9.9.9. Only chemical shifts ( $\delta$ ) belonging to protons which were likely to be influenced by complex formation were manually picked from the spectra. The difference in  $\delta$  ( $\Delta\delta$ ) was calculated using Equation 2.1 for each spectrum acquired.

$$\Delta\delta = (\delta \text{ for the proton of interest}) - (\text{smallest } \delta \text{ for that proton from all the spectra})$$

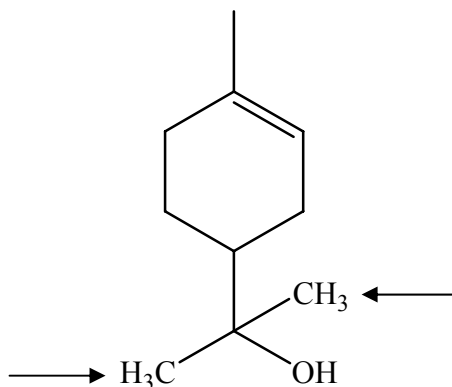
**Equation 2.1** Calculation of  $\Delta\delta$  for protons signals that shifted downfield as more functional monomer analogue was added

A plot of average  $\Delta\delta$  versus the concentration of functional monomer analogue gave the saturation isotherm.

## 2.4.2 Results and discussion

From the  $^1\text{H}$ -NMR titration study of  $\alpha$ -terpineol and acetic acid a comparison of the spectra suggested that the interaction between  $\alpha$ -terpineol and the carboxylic acid group of acetic acid was quite weak because the graph did not reach saturation even at very high concentrations of acetic acid (Figure 2.6). The

protons of interest were the methyl groups on  $\alpha$ -terpineol ( $\delta$  approximately 1.9 ppm, arrows in Figure 2.5)



**Figure 2.5** Protons which were monitored by  $^1\text{H-NMR}$  titration of  $\alpha$ -terpineol titrated with acetic acid

For this experiment, the  $\Delta\delta$  was calculated by subtracting  $\delta$  for the proton of interest from the largest  $\delta$  for that proton from all the spectra because the signals appeared to shift upfield as more acetic acid was added. The results of the first attempt (triangles, Figure 2.6) appeared to show that saturation was being approached but testing higher concentrations of acetic acid revealed that saturation was not achieved (solid circles, Figure 2.6). As the amount of acetic acid increased the change in  $\delta$  also increased. Since the maximum change in  $\delta$  could not be accurately defined by this NMR titration the dissociation constant was not calculated. These results suggested that the environment of the methyl groups became less electronegative as more functional monomer analogue was added. These results could also be interpreted in a different way; the interaction of the hydroxyl group with the carboxylate group may repel the methyl groups away from the hydroxyl group to minimise steric crowding and in this manner may enhance interaction. However, the changes in  $\delta$  were very small and more repetitions should be carried out in the future to confirm these findings; therefore it was too early to conclude the mechanism of interaction between  $\alpha$ -terpineol and acetic acid.

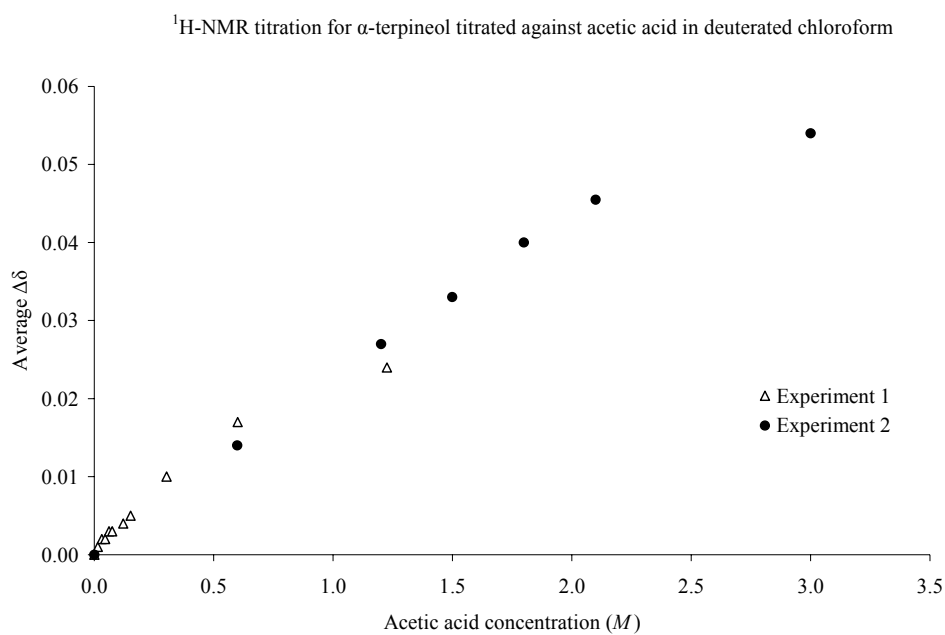


Figure 2.6 <sup>1</sup>H-NMR titration results for  $\alpha$ -terpineol titrated against acetic acid

The titration graph of (-)-menthol with phenol did reach a saturation point (Figure 2.8). The protons of interest were those of the methine group on (-)-menthol ( $\delta$  approximately 1.3 ppm, arrow in Figure 2.7).

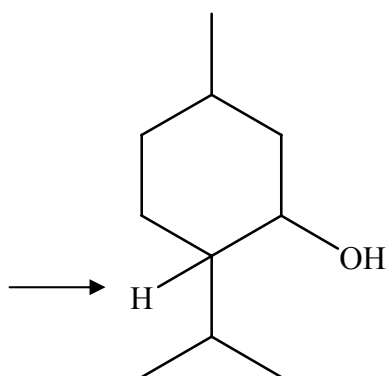
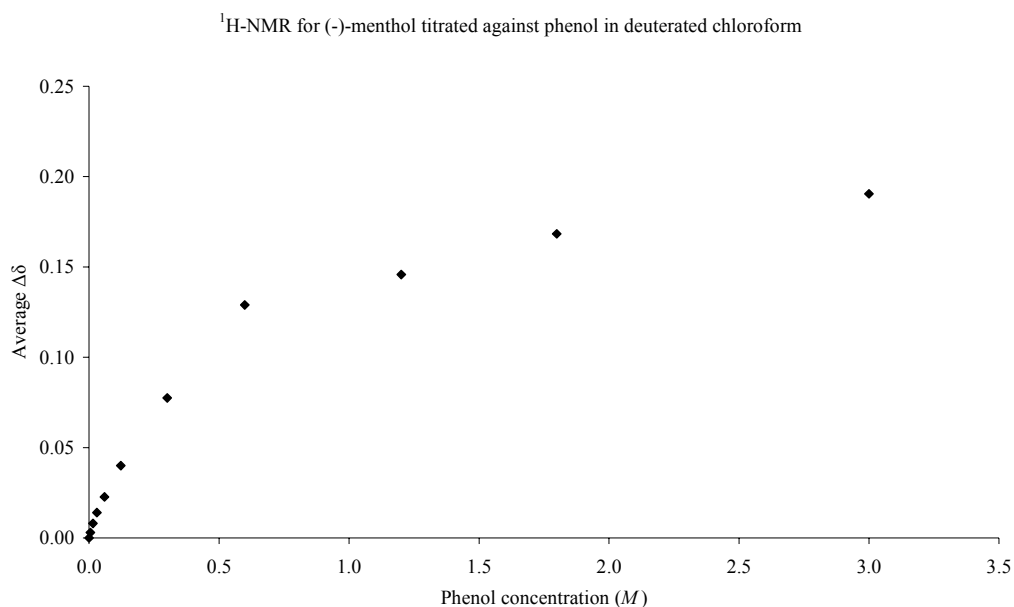


Figure 2.7 Protons which were monitored for the <sup>1</sup>H-NMR titration of (-)-menthol titrated with phenol

The graph line tended towards an asymptote of 0.20 (this was the  $\Delta\delta_{\max}$  for this experiment). The apparent dissociation constant ( $K_{d, \text{app}}$ ) for the (-)-menthol-phenol complex was estimated from  $\frac{1}{2}\Delta\delta_{\max}$  [83] which corresponded to approximately 0.3 M phenol in deuterated chloroform. Therefore there was some interaction (most likely hydrogen bonding) between (-)-menthol and the phenol group which may contribute to the rebinding mechanism in the menthol semi-covalent MIP (code name m10). In the future, tests that involve mixing (-)-menthol with other functional monomer analogues can be used to investigate the relative strength of the complexes.



**Figure 2.8** <sup>1</sup>H-NMR titration results for (-)-menthol titrated against phenol

*Summary of NMR titration studies*

The  $K_{d, \text{app}}$  for  $\alpha$ -terpineol and acetic acid could not be calculated but the results suggested that the two species do interact because there were changes in  $\delta$  as more acetic acid was added. (-)-Menthol and phenol also interact; the  $K_{d, \text{app}}$  was estimated to be approximately 0.3 M phenol in deuterated chloroform.

## **Chapter Three**

**Batch-binding studies to evaluate the capacity of MIPs to  
rebind the template or a structural analogue**

### 3 Introduction

A typical batch-binding test involves exposing the polymer to the analyte for long enough to allow the uptake of the analyte by the MIP and to a smaller extent by the reference polymer. On the molecular level, the analyte diffuses into the polymer particles and then specifically binds at high energy active sites such as the imprinted cavities; binding can also take place non-specifically in the bulk polymer structure. The amount of free analyte remaining in solution after binding has occurred is detected by a method appropriate to the compound. A range of polymers were screened using batch-binding studies to short-list polymers that showed specific uptake of the template. A description of the protocol for testing polymer particles follows.

#### *Preparation of polymer suspensions*

The polymer suspensions (10 mg/mL) were allowed to swell at least overnight before being used. Polymer suspensions were initially prepared in 20 mL scintillation vials, but later it was decided that 8 mL glass vials with *Teflon*-lined caps were compatible with more solvents. Therefore polymer suspensions in hexane, starting in Section 3.1 and all the following sections, were prepared in 8 mL vials and wrapped with parafilm to minimise the adsorption of water.

#### *Preparation of standards and test solutions*

All stock standards, test solutions and internal standards were prepared using glass equipment and were transferred into amber glass vessels with *Teflon*-lined caps and stored in the refrigerator when not in use. The method of preparing these solutions can be found in Appendix Eight.

#### *Testing polymer suspensions*

1.7 mL polypropylene micro-centrifuge tubes or 3 mL glass vials with *Teflon*-lined caps were used to carry out the batch-binding tests; selection depended on compatibility of solvent with polypropylene. If required the polymer suspensions were sonicated prior to testing if there were visible clumps to ensure homogeneity of the suspension. In the appropriate test vessel the polymer

suspension was mixed with test solution at a ratio of nine parts polymer suspension to one part test solution. The polymers were shaken at 560 rpm for 3 hours (block polymers) or half an hour (precipitation polymers); this was done at room temperature. The volumes used for the various tests can be found in Table 3.2.

For tests in micro-centrifuge tubes the samples were centrifuged at 14.1 rcf for 5 minutes for the 38-150  $\mu\text{m}$  particles. If the particles were  $<38 \mu\text{m}$  or precipitation polymers they were centrifuged for 10 minutes. Approximately 600  $\mu\text{L}$  of the supernatant was transferred into fresh micro-centrifuge tubes using air-displacement pipettes and re-centrifuged according to times specified above. Once the second centrifugation was complete, 450  $\mu\text{L}$  of that supernatant was transferred directly into a GC vial containing 450  $\mu\text{L}$  internal standard. The samples in GC vials were shaken by hand or for 5 minutes at 560 rpm on an orbital shaker then analysed on a GC fitted with a flame ionization detector (details Appendix Two). Samples which could not fit on the sample carousel were stored in the refrigerator.

For tests in glass vials the contents were poured into a fresh micro-centrifuge tube and put through the centrifugation process detailed above.

If the supernatant had visible polymer particles even after the second step they were re-centrifuged before transferring into the GC vials for analysis.

The peak areas were automatically or manually picked from the GC chromatograms using Millennium® software. Microsoft® Office Excel® was used for data analysis. Each peak area was corrected with respect to the internal standard peak area. The corrected peak area was then used to calculate the concentration of analyte using the equation of the daily calibration graph unless otherwise stated. The corrected concentration was used to calculate the bound fraction, expressed as B/T (bound by total). The B/T value represents the fraction of the target analyte taken up by the polymer. A B/T equal to one means the polymer bound all of the analyte that was spiked into the polymer suspension. Conversely a B/T equal to zero means that the polymer did not bind any of the analyte.

The B/T equation is given by Equation 3.1, where:

- $C_{ts}$  represents the concentration of analyte in the batch-binding samples without polymer ( $ts$  stands for “test solution”). It is the maximum concentration of analyte added. These samples undergo the same treatment as the batch-binding samples for the MIP and reference polymer, therefore any time-dependent loss of analyte or adsorption of analyte to equipment during batch-binding will be factored out without having to do a calculation to correct for the loss.
- $C_p$  represents the concentration of analyte detected in the supernatant and therefore it is the amount of analyte that did not bind to the polymer.

$$B/T = \frac{C_{ts} - C_p}{C_{ts}}$$

**Equation 3.1** Calculation to determine the amount of terpene not bound by polymer

### 3.1 Assessment of the specificity or cross-reactivity of MIPs

A number of variables can be modified to determine a batch-binding system that offers optimum specific binding of an analyte. The solvent and type of analyte were first investigated.

#### *Choosing a rebinding solvent*

Three solvents were chosen to assess the MIPs binding capacity. They each have a different characteristic and therefore affect the swelling of the polymer and the interaction between the polymer and the analyte (Table 3.1).

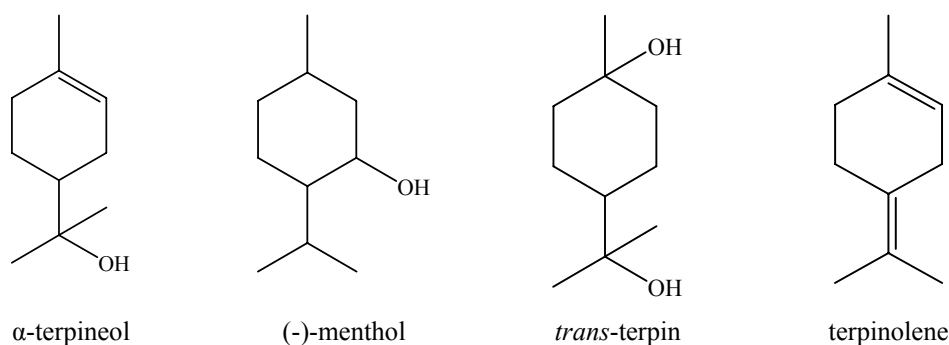
**Table 3.1** Solvents chosen to carry out equilibrium batch-binding experiments

| Solvent      | Solvent characteristic        |
|--------------|-------------------------------|
| Hexane       | Non-polar, no hydrogen bond   |
| Acetonitrile | Polar, weaker hydrogen bond   |
| Methanol     | Polar, stronger hydrogen bond |

Although it was not investigated in this research, changing the pH of a solvent by adding acid or base can affect the rebinding capacity of the MIP; the effect depends on the sensitivity of the functional group on the functional monomer to protonation or de-protonation. For example at acid pH, carboxylate groups found in methacrylate-based polymers will theoretically have a lower non-specific binding [63].

#### *Selecting analyte for polymer testing*

It is useful to test a MIP against a structural analogue in a variety of solvents. For the  $\alpha$ -terpineol, (-)-menthol or terpin MIPs, terpinolene was chosen as the structural analogue. Terpinolene has a similar molecular shape to the template but does not have a hydroxyl group (Figure 3.1), therefore it was used to probe if a polymer showed shape recognition.



**Figure 3.1** Structures of some terpenes

(-)-Menthol MIPs were also tested for cross-reactivity towards  $\alpha$ -terpineol. A polymer which binds the template analogue is said to show cross-reactivity. Testing against a template analogue can be useful when monitoring residual template. The supernatant is tested for template analogue and template bleed at the same time. Polymer specificity is tested by using the template as the probe analyte for batch-binding.

*Batch-binding protocol*

10 mg/mL polymer suspensions were allowed to equilibrate in the rebinding solvent at least overnight before use. Each polymer was tested in triplicate using the volumes specified in Table 3.2. The experiment detail and sample preparation was described in Section 3. The details of the standards, test solutions and internal standards that were prepared have been reported in Appendix Eight, Table A8.1. All the samples were analysed by GC-FID using the temperature program outlined in Appendix Two. All chromatograms were processed using Millennium® software, Microsoft® Office Excel® was used for data analysis.

**Table 3.2 Volumes used in the batch-binding experiments to test polymer cross-reactivity and specificity**

| <b>Solvent</b> | <b>Polymers tested</b>                                   | <b>Volume solvent (μL)</b> | <b>Volume 10 mg/mL polymer suspension (μL)</b> | <b>Test solution</b>         | <b>Volume test solution (μL)</b> |
|----------------|--|----------------------------|--|------------------------------|----------------------------------|
| Methanol       | t15, t16, m17  | 315                        | 315  | 1500 ppm terpinolene         | 70                               |
| Methanol       | t4, t5, t6, terpin, t8, m9, t10, t11, m10                | 472                        | 158  | 1500 ppm terpinolene         | 70                               |
| Acetonitrile   | t4, t5, t6, terpin, t8, m9, t10, t11, m10, t15, t16, m17 | 315                        | 315  | 1000 ppm terpinolene         | 70                               |
| Hexane         | t4, t5, t6, terpin, t8, m9, t10, t11, m10, t15, t16, m17 | 315                        | 315  | 1000 ppm terpinolene         | 70                               |
| Hexane         | t4, t5, t6, terpin, t8, m9, t10, t11, m10, t15, t16, m17 | 315                        | 315  | 1000 ppm $\alpha$ -terpineol | 70                               |
| Acetonitrile   | t4, t5, t6, terpin, t8, m9, t10, t11, m10, t15, t16, m17 | 315                        | 315  | 1000 ppm $\alpha$ -terpineol | 70                               |
| Methanol       | t4, t5, t6, terpin, t8, m9, t10, t11, m10, t15, t16, m17 | 315                        | 315  | 1000 ppm $\alpha$ -terpineol | 70                               |
| Hexane         | m9, m10, m17, t18, t19                                   | 315                        | 315  | 1000 ppm (-)-menthol         | 70                               |
| Acetonitrile   | m9, m10, m17, t18, t19                                   | 315                        | 315  | 1000 ppm (-)-menthol         | 70                               |
| Methanol       | m9, m10, m17, t18, t19                                   | 315                        | 315  | 1000 ppm (-)-menthol         | 70                               |

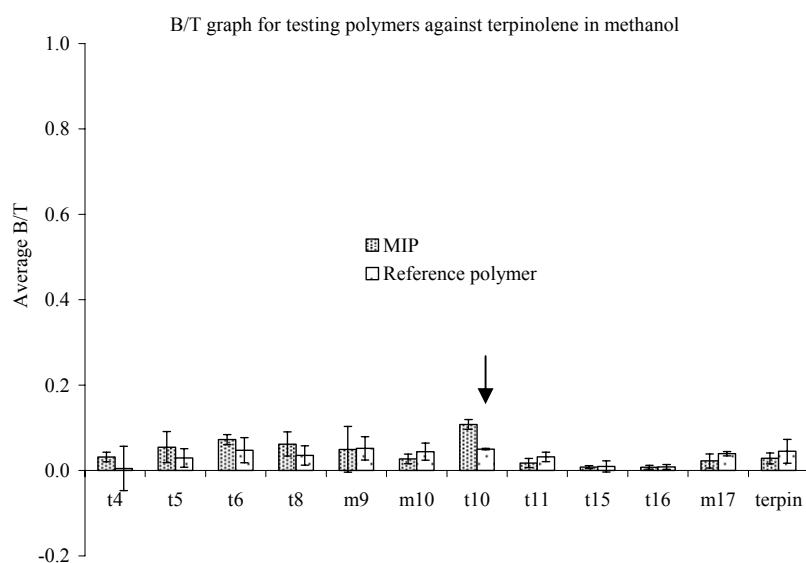
### 3.1.1 Results and discussion

#### *Efficiency of template extraction*

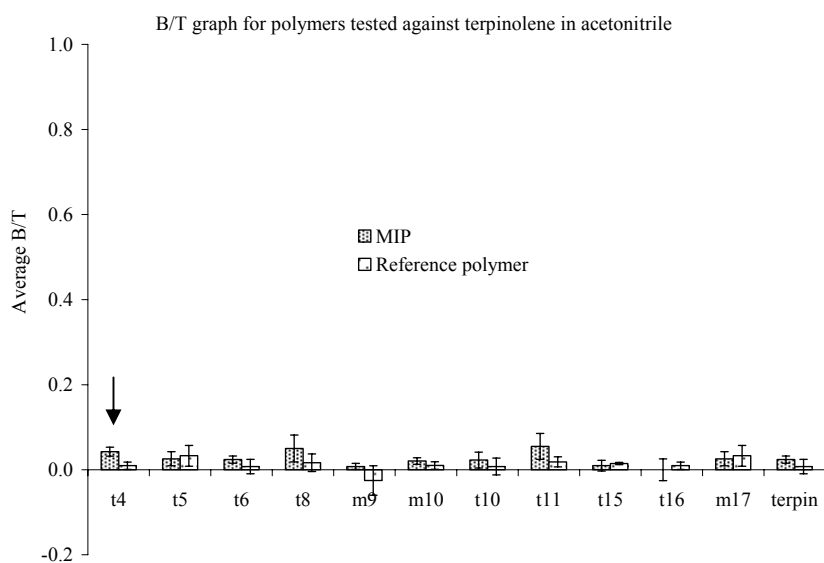
A number of polymers were synthesised and initially evaluated for the presence of any residual template in the rebinding solvent by analysing for template bleed in the polymer suspensions. Different solvents can expand or contract the polymer structure; any residual template could be released or trapped within the framework and therefore template bleed had to be assessed in the different solvents. The chromatograms of the samples from the cross-reactivity test could be evaluated for the presence of the template as well as analogues because the retention times of the compounds did not overlap. Since no peaks were seen at the retention time of the template in the different rebinding solvents it was inferred that template extraction was complete. This meant the imprinted cavities were available to bind probe compounds, such as terpinolene. The removal of template from the MIPs had to be inferred from cross-reactivity studies because either the washings were discarded or the washings were in a solvent, such as 1% glacial acetic acid in methanol, which was not suitable for GC analysis. For molecules with a chromophore the template molecule could be monitored by spectroscopy (such as using a UV/Vis spectrophotometer).

#### *Cross-reactivity of terpene MIPs, towards terpinolene, in different solvents*

The cross-reactivity tests were carried out in methanol, acetonitrile and hexane for most of the polymers. Cross-reactivity investigations were carried out with terpinolene first as it is a structural analogue of  $\alpha$ -terpineol, (-)-menthol and *trans*-terpin. Therefore terpinolene could also be used to assess if there was residual template on the polymers before doing further work. The following B/T graphs show that different solvent polarities affected the response of polymers towards terpinolene.



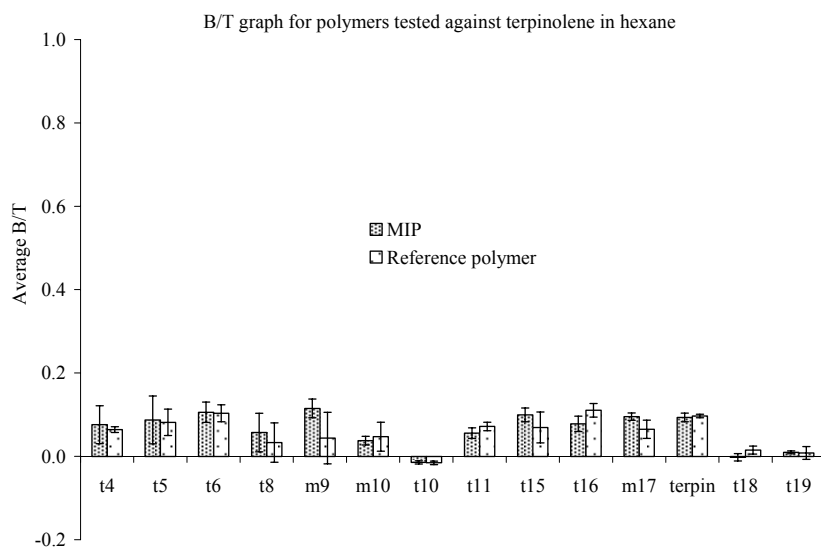
**Figure 3.2 Results of batch-binding in methanol; polymers tested against 150 ppm terpinolene<sup>5</sup>**



**Figure 3.3 Results of batch-binding in acetonitrile; polymers tested against 100 ppm terpinolene<sup>6</sup>**

<sup>5</sup> Average B/T calculated from triplicate samples for all except t4-MIP and t10-reference polymer which were based on duplicate samples. Error bars represent one standard deviation. No template bleed was observed in methanol.

<sup>6</sup> Average B/T calculated from triplicate samples for all polymers and error bars represent one standard deviation. No template bleed was observed in acetonitrile.



**Figure 3.4 Results of batch-binding in hexane; polymers tested against 100 ppm terpinolene<sup>7</sup>**

Figure 3.2 showed that out of twelve polymers tested only t10-MIP showed cross-reactivity in methanol (arrow, Figure 3.2). This implied that shape selectivity may be occurring because terpinolene does not contain any hydroxyl groups and thus it cannot hydrogen bond to the MIP. The errors for the other polymers were too large to be able to draw a conclusion without further testing. For instance t5, t6 and t8 may potentially show cross-reactivity if the batch-binding conditions were optimised. Evaporation at both the batch-binding and GC analysis stage may have contributed to the variability in the results because volatile solvents and solutes were used. Transferring organic solvents may have contributed to the negative B/T values for the reference polymer (for example, t4-reference polymer); solvent may have evaporated from the samples that did not contain polymer which gave the  $C_{ts}$  value. The relative amounts of analyte in the samples with and without polymer were important because of the way B/T values were calculated (Equation 3.1). Pipetting the polymer suspensions may have contributed to the data variability in all the batch-binding tests. There may have been a slightly different amount of polymer particles in the replicate tests. Although the polymer suspensions were continuously agitated whilst transferring an aliquot there may still have been a bias in the sampling method, as larger

<sup>7</sup> Average B/T calculated from triplicate samples for all polymers and error bars represent one standard deviation. No template bleed was observed in hexane.

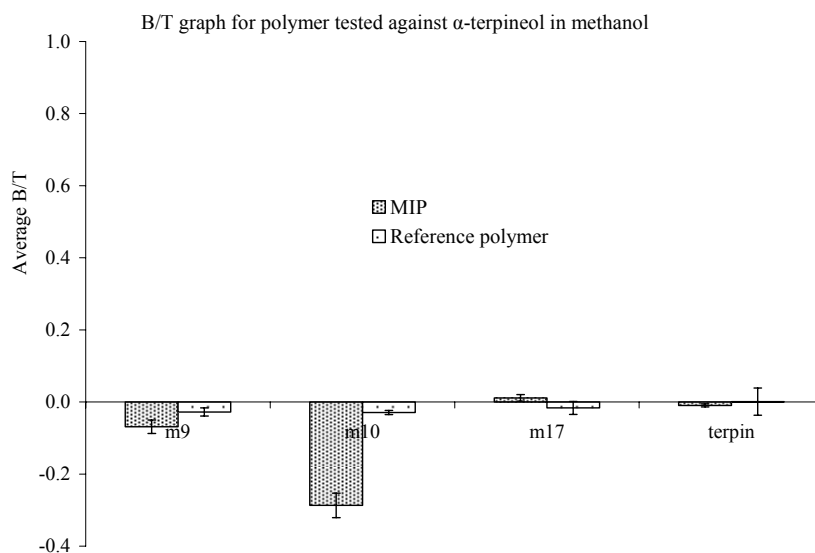
particles are heavier and will tend to sink whereas the smaller particles are lighter and will tend to float. This may introduce heterogeneity in the polymers test samples and may result in different binding capacities. However the method of testing a polymer suspension was not modified in this current research because all the polymers were generated as particles, which meant that either batch-binding or flow-through (such as HPLC) setups could be used. Since batch-binding tests did not require elaborate equipment used in a flow-through system or extensive method development, it was selected for its simplicity to be used as an initial way of assessing polymer particles. Batch-binding tests also provided enough time for the analyte to bind to the polymer and therefore avoided kinetic factors which may decrease binding [82]. Once a polymer is found that specifically binds a large fraction of the template then alternative polymer formats and test setups can be investigated.

The decision to use corrected concentration to present the results was made because it would correct for GC injection variability between days, and because the peak areas could be corrected by calculation with respect to the average peak area of the internal standard. The concentration calculated from the daily calibration graphs, based on corrected peak areas, accounted for any changes in the flame ionisation detector response to the standard solutions on a particular day. This normalised the results to the standard solutions.

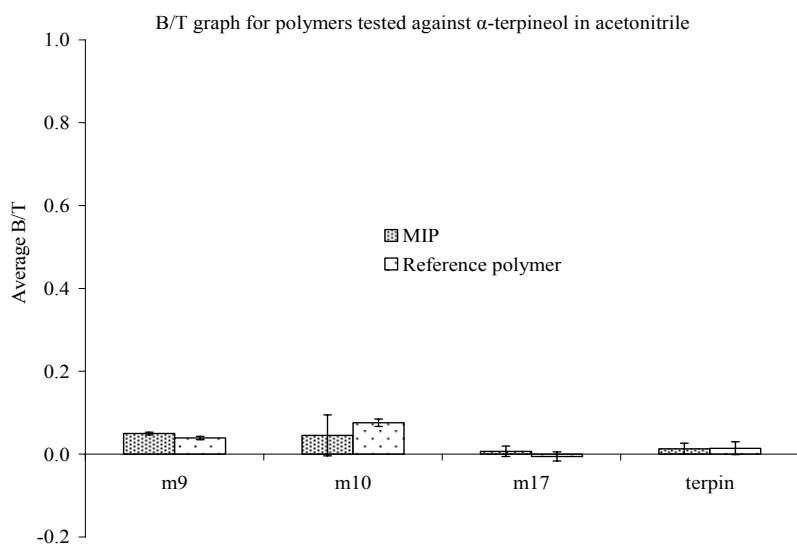
An interesting observation in Figure 3.2 was that t8 and t11 bound terpinolene to different extents in methanol even though their polymer composition was the same (Appendix Four, Table A4.1). This highlighted the variability of polymerisation and of the resulting polymers. The reactants may be the same but they may assemble differently depending on the conditions of polymerisation. In this case polymerisation temperature was most likely to contribute to polymer variability as radical polymerisation is exothermic; the heat generated by the reaction increased the temperature in the vessel. A sand bath has poor thermal control which may have given rise to polymers of different microstructures that may have affected the accessibility of the imprinted cavities.

Binding capacities can also be changed by using different rebinding solvents. Figure 3.3 showed that less terpinolene was bound by many of the polymers in acetonitrile. It was likely that acetonitrile, which has a lower

hydrogen bonding ability compared to methanol, was more effective at interfering with Van der Waals interactions (presumably the major type of interaction involved in the shape recognition of terpinolene by the polymers). Acetonitrile is somewhat basic and therefore could have competed for the acidic methacrylate groups in the functional cavities. However, t4-MIP in acetonitrile appeared to show cross-reactivity towards terpinolene because more was bound in comparison to the t4-reference polymer (arrow, Figure 3.3). In contrast, a non-polar solvent such as hexane appeared to enhance non-specific binding of terpinolene; the analyte was readily bound by most of the reference polymers (Figure 3.4). The negative t10 results reflected the limitations of using a second solution (*C<sub>ts</sub>*, the test solution) to compare how much analyte was taken up by the polymer. Another factor which may have affected the results was the use of different polymer concentrations and test solution concentrations; noted in Table 3.2. True comparisons of the cross-reactivity test in methanol can only be done if the batch-binding conditions are the same as for the other solvents. However, the purpose of the cross-reactivity test was to determine if template extraction was satisfactory; at the same time it illustrated the effect of solvent on the binding capacities of the polymers.

*Cross-reactivity of (-)-menthol and terpin MIPs, towards  $\alpha$ -terpineol, in different solvents*

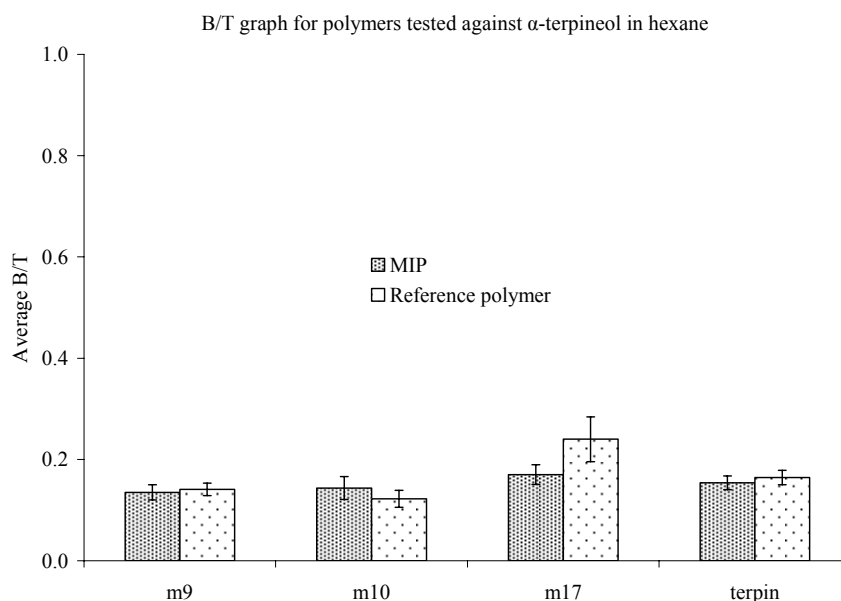
**Figure 3.5 Results of batch-binding in methanol; polymers tested against 100 ppm  $\alpha$ -terpineol<sup>8</sup>**



**Figure 3.6 Results of batch-binding in acetonitrile; polymers tested against 100 ppm  $\alpha$ -terpineol<sup>9</sup>**

<sup>8</sup> Average B/T calculated from triplicate samples for all polymers and error bars represent one standard deviation.

<sup>9</sup> Average B/T calculated from triplicate samples for all polymers and error bars represent one standard deviation.



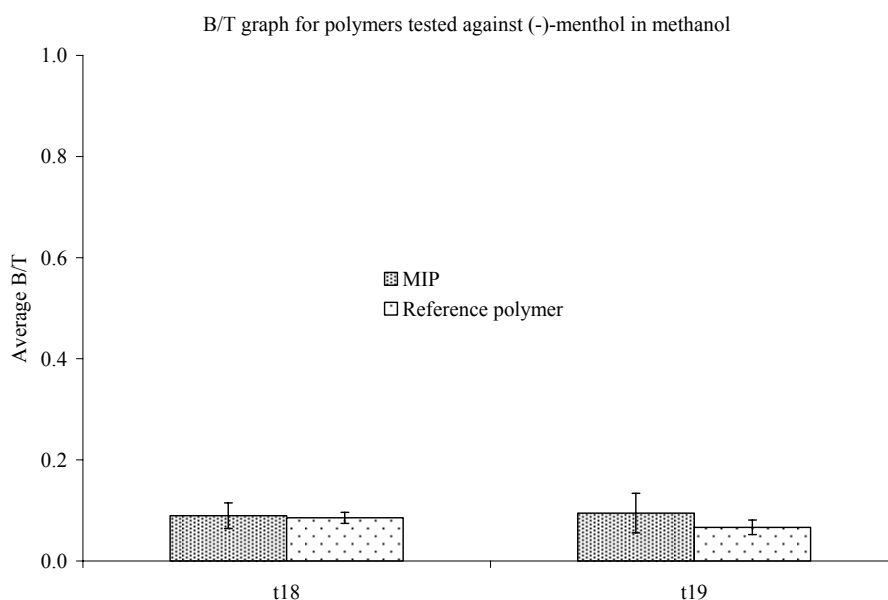
**Figure 3.7 Results of batch-binding in hexane; polymers tested against 100 ppm  $\alpha$ -terpineol<sup>10</sup>**

*trans*-Terpin,  $\alpha$ -terpineol and (-)-menthol have similar structures but vary in the number and position of hydroxyl groups. *trans*-Terpin has two tertiary hydroxyl groups,  $\alpha$ -terpineol has one tertiary hydroxyl and (-)-menthol has one secondary hydroxyl group (Figures 3.1). To investigate if terpin and (-)-menthol MIPs recognised  $\alpha$ -terpineol, cross-reactivity studies were carried out in methanol, acetonitrile and hexane (Figures 3.5, 3.6 and 3.7, respectively). In Figure 3.5, the negative B/T value for m9-MIP and m10-MIP in methanol reflected the difficulties of handling volatile organic such as solvent evaporation rather than template bleed; as terpinolene is an analogue of the template ((-)-menthol) and there was no residual template detected from the terpinolene cross-reactivity studies in methanol (Figure 3.2). Methanol can hydrogen bond with the functional groups on the polymer and weaken the affinity of the polymer towards  $\alpha$ -terpineol. Using a test solvent with less hydrogen bonding ability, like acetonitrile, can improve uptake; this was seen for m9 which showed cross-reactivity. Acetonitrile promoted non-specific uptake of  $\alpha$ -terpineol by m10

<sup>10</sup> Average B/T calculated from triplicate samples for all polymers and error bars represent one standard deviation.

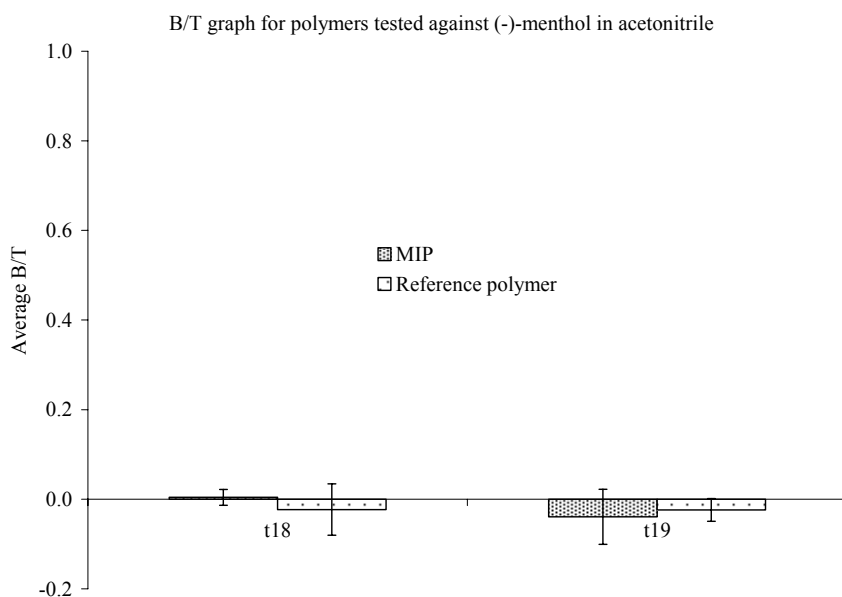
because both MIP and reference polymer had bound  $\alpha$ -terpineol to a similar extent. Terpin and m17 polymers bound very little  $\alpha$ -terpineol in acetonitrile. This could be related to the structure of the polymer. SEM images of m17 (Appendix Six, Figures A6.1 and A6.2) showed that it was very porous; this could indicate that compounds could easily access the imprinted cavities but they could also diffuse away easily. No SEM image was captured for the terpin polymers but based on the structure of *trans*-terpin the extra hydroxyl group would increase the electron density and the molecular size, it would occupy a larger volume and presumably form larger cavities which cannot effectively trap smaller structural analogues such as  $\alpha$ -terpineol. Hexane increased the affinity of  $\alpha$ -terpineol to the polymers (Figure 3.7) but there was no cross-reactivity for most of them (the MIP and reference polymers bound  $\alpha$ -terpineol to a similar extent). The slightly higher average B/T for m10-MIP may suggest it potentially can show cross-reactivity. The small difference may be enhanced by modifying the polymer concentration and testing at a constant  $\alpha$ -terpineol concentration (Section 3.2).

*Cross-reactivity of  $\alpha$ -terpineol MIPs, towards (-)-menthol, in different solvents*

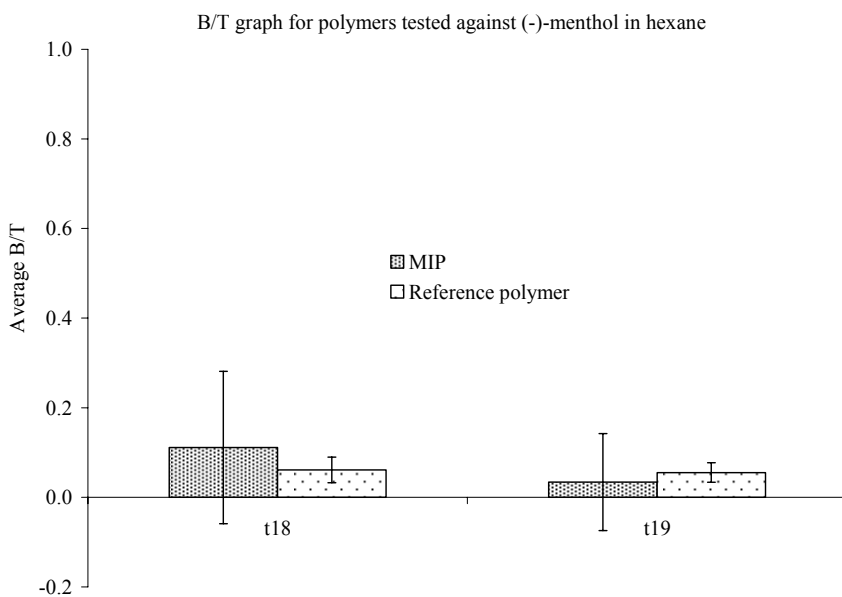


**Figure 3.8 Results of batch-binding in methanol; polymers tested against 100 ppm (-)-menthol<sup>11</sup>**

<sup>11</sup> Average B/T calculated from triplicate samples (based on uncorrected concentration) for both polymers and error bars represent one standard deviation.



**Figure 3.9 Results of batch-binding in acetonitrile; polymers tested against 100 ppm (-)-menthol<sup>12</sup>**



**Figure 3.10 Results of batch-binding in hexane; polymers tested against 100 ppm (-)-menthol<sup>13</sup>**

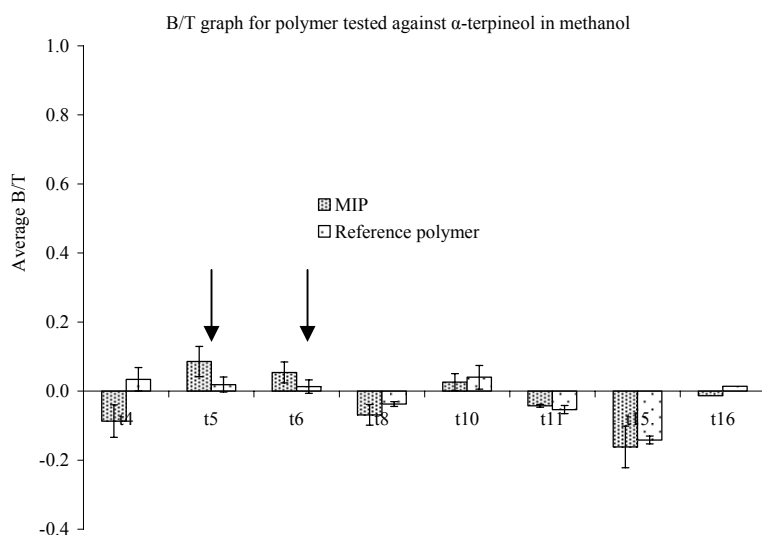
<sup>12</sup> Average B/T calculated from triplicate samples (based on uncorrected concentration) for both polymers and error bars represent one standard deviation.

<sup>13</sup> Average B/T calculated from triplicate samples (based on uncorrected concentration) for both polymers and error bars represent one standard deviation.

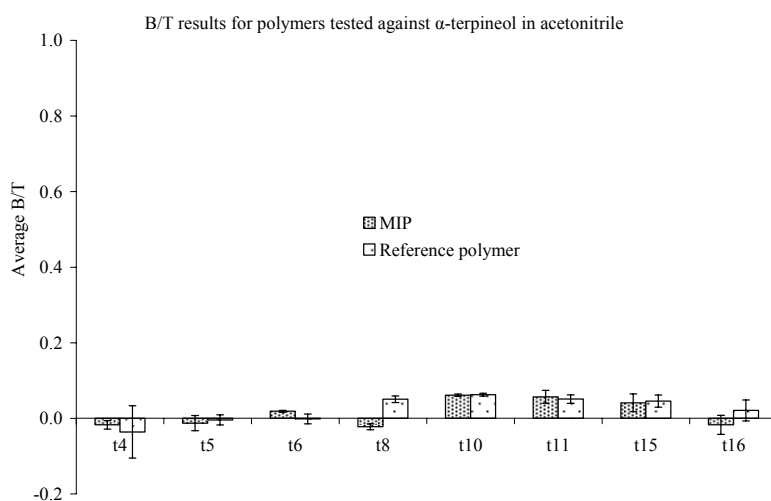
The chromatograms from the cross-reactivity studies for t18 and t19 showed that there was no residual  $\alpha$ -terpineol on the polymer; and since blank solvent rather than the internal standard was not added prior to GC analysis, the results for batch-binding with (-)-menthol (Figure 3.8, 3.9 and 3.10) were based on uncorrected concentration. This may have contributed to the variability of the results. However it can be summarised that the batch-binding results in acetonitrile showed very little binding to the polymers; therefore there was no cross-reactivity of the  $\alpha$ -terpineol MIPs towards (-)-menthol (Figure 3.9). In hexane the reference polymers bound (-)-menthol non-specifically; the performance of the MIPs in hexane was too variable and therefore it was too early to draw any conclusions about the cross-reactivity of t18 and t19 towards (-)-menthol in hexane. Methanol also promoted binding of (-)-menthol (Figure 3.8), although it was mainly non-specific uptake, because the MIP and reference polymer bound a similar fraction of (-)-menthol. Even though methanol theoretically could interfere with hydrogen bonding, the non-specific interactions which took place in t18 and t19 appeared to be strong enough to overcome any interference from the solvent.

*Specificity of  $\alpha$ -terpineol MIPs in different solvents*

The specificity of MIPs is also affected by the rebinding solvent as described next.



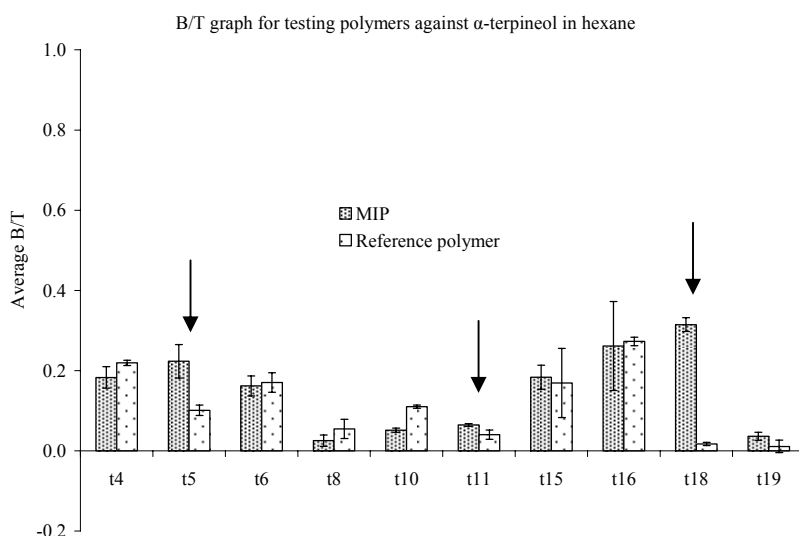
**Figure 3.11 Results of batch-binding in methanol; polymers tested against 100 ppm  $\alpha$ -terpineol<sup>14</sup>**



**Figure 3.12 Results of batch-binding in acetonitrile; polymers tested against 100 ppm  $\alpha$ -terpineol<sup>15</sup>**

<sup>14</sup> Average B/T calculated from triplicate samples for all polymers, except t8-reference polymer which was a result of duplicates; error bars represent one standard deviation.

<sup>15</sup> Average B/T calculated from triplicate samples for all polymers, except t10-reference polymer which was from duplicate samples; error bars represent one standard deviation.



**Figure 3.13 Results of batch-binding in hexane; polymers tested against 100 ppm  $\alpha$ -terpineol<sup>16</sup>**

Figure 3.11 showed that most of the polymers tested, except for t5 and t6 (arrows, Figure 3.11), did not specifically bind  $\alpha$ -terpineol in methanol. The negative B/T values for the MIP of t8, t11 and t15 once again indicated the difficulties of handling volatile organic solvents (with associated problems like solvent evaporation) rather than template bleed because the reference polymer also had a negative B/T value. The error bars for t5-MIP and t6-MIP overlapped with the B/T value for their reference polymer but the MIPs potentially could recognise  $\alpha$ -terpineol specifically because the average of the B/T value for the MIPs was higher than for the reference polymers. The negative B/T value for t4-MIP in methanol conflicted with the results of template extraction from the terpinolene cross-reactivity study which showed there was no residual  $\alpha$ -terpineol in the MIP; this may be an anomalous result due to the challenges of handling volatile organic solvents. This may also be the reason for the large error for t16-MIP in hexane.

Acetonitrile did not improve the extent of uptake of  $\alpha$ -terpineol by the polymers (Figure 3.12) probably because this solvent may interfere with hydrogen bonding (theoretically to a lesser extent compared with methanol) and Van der Waals interactions. Only t6-MIP showed the potential to take up  $\alpha$ -terpineol

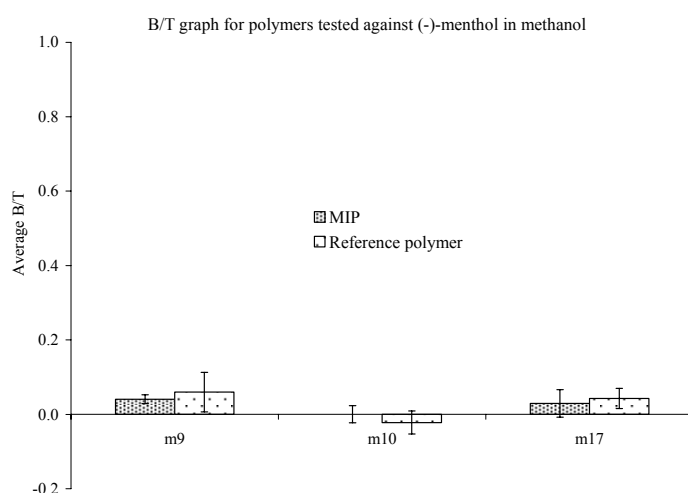
<sup>16</sup> Average B/T calculated from triplicate samples for all polymers, except t8 and t10-MIP which were from duplicate samples. Error bars represent one standard deviation.

specifically, but the error bars overlapped therefore a definite conclusion could not be drawn. The B/T values for MIPs t4, t5 and t16 were negative but like t8, t11 and t15 in methanol it was likely due to problems with handling volatile organic solvents rather than template bleed because their reference polymers also had negative B/T values. The negative B/T value for t8-MIP conflicted with the results of terpinolene cross-reactivity in acetonitrile (Figure 3.3); which indicated that there was no residual  $\alpha$ -terpineol in the MIP. Although solvent evaporation may have contributed to the negative B/T result, an alternative source of variability may be due to testing new polymer particles each time.

It was found that most polymers in hexane bound more  $\alpha$ -terpineol than in methanol or acetonitrile. Only t5-MIP, t18-MIP and t11-MIP specifically bound  $\alpha$ -terpineol in hexane (arrows, Figure 3.13). It was interesting to note the difference in binding capacities between t18-MIP and t19-MIP because the t18-MIP included functional monomer in the polymerisation mixture (methacrylic acid) but t19-MIP did not. This suggested that the functional monomer played a key role in the specific binding of  $\alpha$ -terpineol. The cross-reactivity data for t18-MIP (terpinolene and (-)-menthol in hexane; Figures 3.4 and 3.10, respectively) suggested that there may be a small degree of shape recognition, but the predominant method of recognition was most likely to be hydrogen bonding because more (-)-menthol was bound by t18; further investigation was required to confirm this. The recognition mechanism for t5-MIP was likely to be a combination of hydrogen bond and hydrophobic interactions. The hydrogen bond interaction was enhanced in a non-polar solvent like hexane; this can be seen by comparing the t5 results in methanol and in hexane (Figure 3.11 and 3.13, respectively). The polymerisation mixtures of t4, t5 and t6 were very similar; the only difference being the cross-linker. Ethylene glycol dimethacrylate was used in t4; t5 contained divinylbenzene and t6 contained trimethylolpropane trimethacrylate. The aromatic groups may have contributed to the better recognition of  $\alpha$ -terpineol by  $\pi$ - $\pi$  interaction. However, even polymers prepared with the same polymer recipe can perform differently; t8 and t11 (prepared using the same recipe, but different batches) showed different binding capacities which was likely to be due to differences in their microstructure; this was seen in their results of cross-reactivity test against terpinolene in methanol (Figure 3.2).

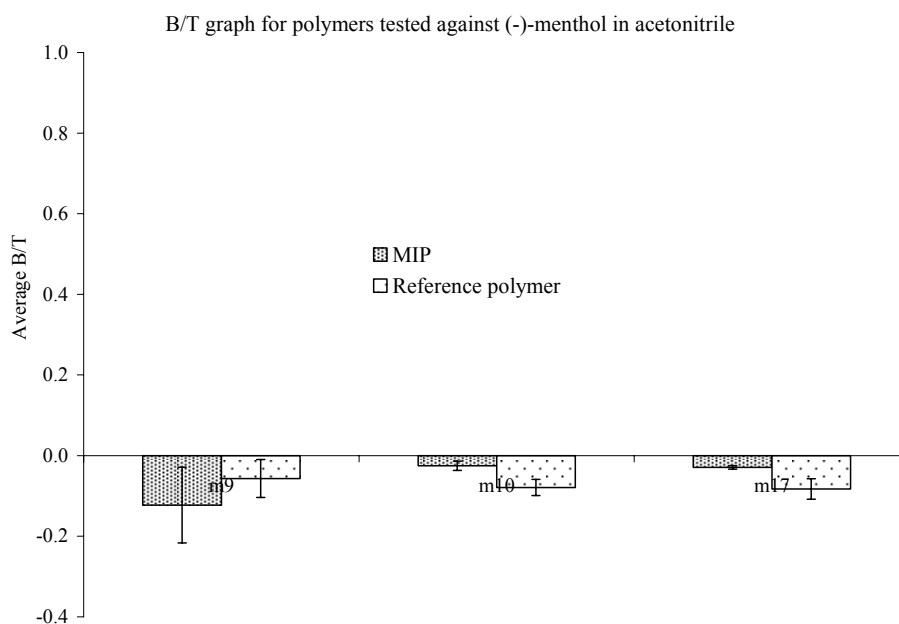
Polymers which appeared to have taken up  $\alpha$ -terpineol non-specifically in hexane included t4, t6, t8, t10 and possibly t15, as the reference polymer bound an equal or greater fraction of  $\alpha$ -terpineol in comparison to the MIP. The error bars for the MIP of t16 and t19 were large or overlapped with the error bars of the reference polymer, therefore no conclusion could be drawn about whether they specifically bound  $\alpha$ -terpineol; again the errors may reflect the challenges of using volatile organic solvents. It was interesting to note that t10 showed no specific uptake of  $\alpha$ -terpineol in hexane but the cross-reactivity studies (terpinolene in methanol, Figure 3.2) showed that t10-MIP could bind more terpinolene than the reference polymer. This may have reflected the effect of the solvent on the polymer structure. Polar organic solvents increase swelling and non-polar solvents do not swell the polymers as much [30]. Hexane may not effectively swell the polymer and therefore this may prevent  $\alpha$ -terpineol from accessing the imprinted cavities, a factor which appeared to hide the enhancement of hydrogen bonding usually seen in non-polar solvents. It was not possible to ascertain if t10 specifically bound  $\alpha$ -terpineol in methanol because of the large variability in the triplicate results.

#### *Specificity of (-)-menthol MIPs in different solvents*

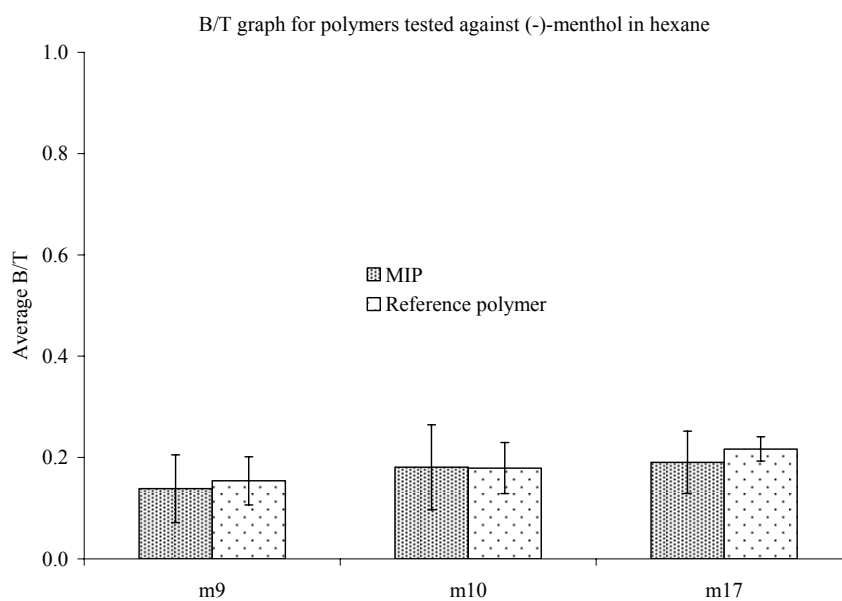


**Figure 3.14 Results of batch-binding in methanol; polymers tested against 100 ppm (-)-menthol<sup>17</sup>**

<sup>17</sup> Average B/T calculated from triplicate samples for all polymers and error bars represent one standard deviation.



**Figure 3.15 Results of batch-binding in acetonitrile, polymers tested against 100 ppm (-)-menthol<sup>18</sup>**



**Figure 3.16 Results of batch-binding in hexane; polymers tested against 100 ppm (-)-menthol<sup>19</sup>**

<sup>18</sup> Average B/T calculated from triplicate samples for all polymers (based on uncorrected concentration) and error bars represent one standard deviation.

<sup>19</sup> Average B/T calculated from triplicate samples for all polymers and error bars represent one standard deviation.

Figure 3.14 showed that (-)-menthol did not bind specifically to the (-)-menthol MIPs in methanol because their B/T values were similar to the B/T values for their reference polymers. The negative B/T values for the MIPs in acetonitrile (Figure 3.15) suggested there may have been solvent evaporation rather than template bleed because the reference polymers also had a negative B/T value. As seen in previous experiments, B/T values in hexane were usually higher than for the other solvents. Hexane enhanced the extent of (-)-menthol binding (Figure 3.16) but the variability of the data hid whether the MIPs specifically bound (-)-menthol. The terpin MIP was not tested for specificity against *trans*-terpin because the goal of this research was prepare a MIP to recognise small organic compounds with poor chemical functionality.

### *Summary*

From the cross-reactivity and specificity experiments hexane was the solvent which best promoted binding to the polymers because the B/T values were higher in most cases; therefore further studies were conducted in hexane only. In contrast, the B/T values were often lower in methanol and acetonitrile presumably because these solvents can disrupt intermolecular interactions, such as hydrogen bonds, between the analyte and polymer and weaken the affinity for each other. The polymers, t18 and t5, were chosen for further investigation because they both showed specific uptake of  $\alpha$ -terpineol in hexane (Figure 3.13). The polymers, t11 and t16, were also chosen for further testing because they showed potential to specifically bind  $\alpha$ -terpineol in hexane. The polymer m10 showed potential cross-reactivity towards  $\alpha$ -terpineol in hexane (Figure 3.7). Altering the batch-binding conditions may increase specific uptake.

## **3.2 Optimisation of the concentration of selected polymers used for batch-binding experiments in hexane**

Based on the results of the batch-binding experiments in Section 3.1 the polymers chosen for testing were t5, t18, t11, t16 and m10 because they either showed the potential to bind  $\alpha$ -terpineol specifically or showed potential cross-reactivity in hexane. To evaluate if the extent of binding could be improved in a batch-binding setup, the polymer concentration was varied while adding a

constant concentration of  $\alpha$ -terpineol. There are only a finite number of sites on the polymers which can specifically bind  $\alpha$ -terpineol. An excess amount of  $\alpha$ -terpineol could saturate these sites; non-specific binding may occur which can hide specific binding. Therefore varying the polymer concentration optimised the concentration at which the specific binding occurred; the  $\alpha$ -terpineol concentration was also decreased from previous tests so that the binding sites were not fully saturated at the lower polymer concentrations. Typically the polymer concentration which has a B/T of 0.5 is chosen to investigate the relationship between B/T and the test solution concentration (Section 3.3).

#### *Preparation of polymer suspension*

10 mg/mL stock polymer suspension in hexane was prepared in 8 mL glass vials with *Teflon*-lined caps and then allowed to swell at least overnight before use. Any visible polymer aggregates were broken by sonication; sometimes a glass stirring rod was used to crush the tighter aggregates. All batch-binding tests were carried out in triplicate using 3 mL glass vials with *Teflon*-lined caps.

#### *Preparation of standards and test solutions*

All stock standards, test solutions and internal standards were prepared using glass equipment (glass pipettes or volumetric flasks). They were transferred into amber glass vessels with *Teflon*-lined caps and stored in the refrigerator when not in use. The method of preparing these solutions can be found in Appendix Eight, Table A8.2.

#### *Batch-binding protocol*

The stock polymer suspension was diluted, using the volumes listed in Table 3.3, directly in 3 mL glass vials; the polymer suspensions were continuously stirred with a magnetic stirring bar to make the suspension as homogeneous as possible. Eppendorf® air-displacement pipettes were used and attached with wide-bore pipette tips.

**Table 3.3 Volumes used to dilute the stock polymer suspension**

| Final polymer concentration (mg/mL) | Volume of stock polymer suspension ( $\mu\text{L}$ ) | Volume of hexane ( $\mu\text{L}$ ) |
|-------------------------------------|--|------------------------------------|
| 0.00                                | 0.00   | 630.00                             |
| 2.25                                | 157.50   | 472.50                             |
| 4.50                                | 315.00   | 315.00                             |
| 6.75                                | 472.50   | 157.50                             |
| 9.00                                | 630.00   | 0.00                               |

The experiment was started by adding 70  $\mu\text{L}$  500 ppm  $\alpha$ -terpineol in hexane and then shaken at 320-400 rpm for 0.5 hours (precipitation polymers – t16 and t18) or 3 hours (block polymers – t5, t11 and m10) at room temperature. To assess how much  $\alpha$ -terpineol was not bound to the polymer, the samples were poured into labelled micro-centrifuge tubes and then the supernatant was collected by centrifuging for 5 minutes (38-150  $\mu\text{m}$  particles) or 10 minutes (<38  $\mu\text{m}$  and precipitation polymers) at 14.1 rcf. 450  $\mu\text{L}$  of the internal standard (50 ppm (-)-menthol in hexane) was added to all the GC vials. 450  $\mu\text{L}$  of the clear supernatant was transferred directly into the appropriately labelled GC vials, capped securely and then mixed by gently shaking the tray which held the samples. If the supernatant was not clear it was re-centrifuged before transferring into a GC vial. All the samples were analysed by GC-FID using the temperature program outlined in Appendix Two. All chromatograms were processed using Millennium® software, Microsoft® Office Excel® was used for data analysis.

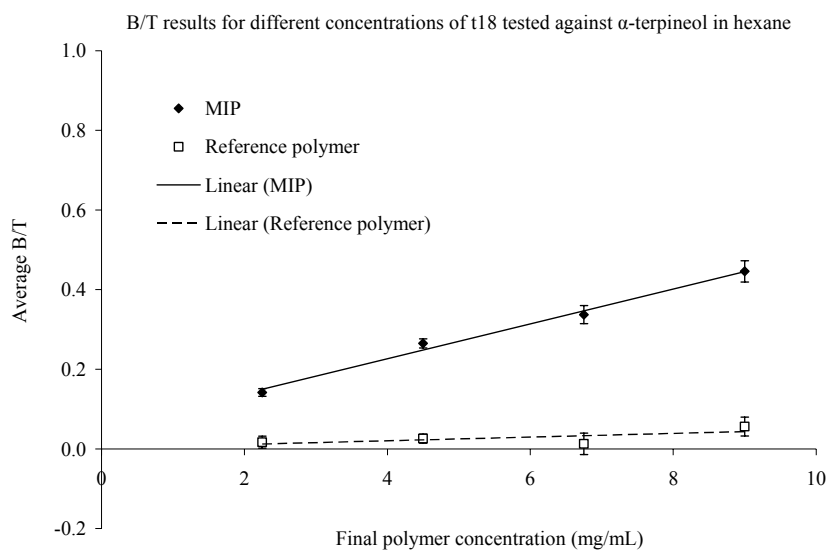
### 3.2.1 Results and discussion

The B/T value for t18 and t5 increased with polymer concentration which meant that the fraction of  $\alpha$ -terpineol bound by the MIPs increased with polymer concentration (shown later, Figure 3.17 and 3.19 respectively). However a B/T value of 0.5 was not achieved but the linearity of the graph line suggested that testing higher polymer concentrations may increase uptake. However, at a certain concentration the specific binding (i.e. the difference between the fraction bound by the MIP and the fraction bound by the reference polymer) may reach a maximum; this would occur when the MIP and reference polymer bound  $\alpha$ -terpineol to a similar extent. The specific binding by t5-MIP and t18-MIP also

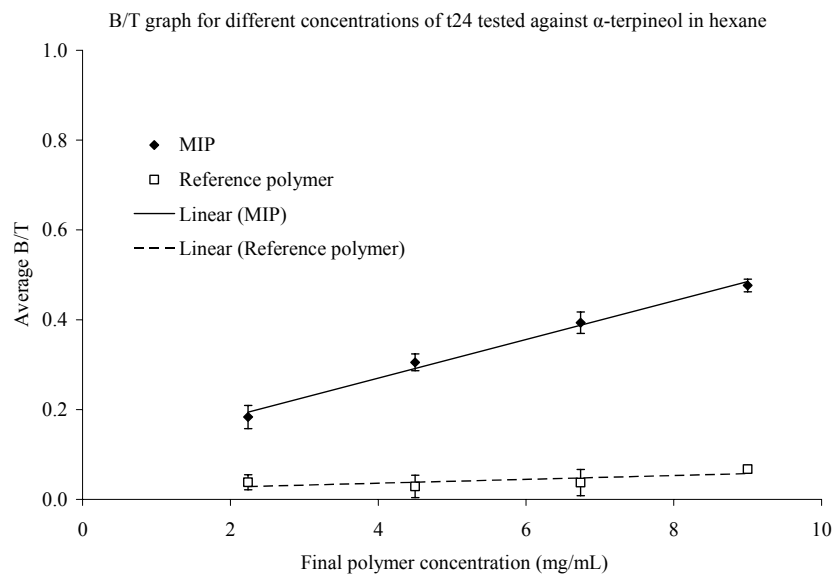
appeared to increase with polymer concentration. At the molecular level, as the polymer concentration increased, the sites of specific binding increased so more  $\alpha$ -terpineol could be taken up. However as there were more polymer particles, the total surface area also increased which meant the non-specific sites for binding increased too. This was seen for the reference polymer of t5 but the reference polymer for t18 had very little affinity for  $\alpha$ -terpineol over the range of polymer concentration tested. The surface topology of the MIP and reference polymer for t5 appeared similar (Appendix Six, Figures A6.5 and A6.6). As the polymer concentration increased, the degree of non-specific binding and physical trapping increased. Their surface areas were also of a similar magnitude (Table 2.3, Section 2.3.2). The surface topography of t18-MIP and t18-reference polymer visualised by SEM (Appendix Six, Figures A6.3 and A6.4) showed that they appeared very different; t18-MIP particles had an irregular shape and the surface had a dense appearance, in contrast t18-reference polymer was an aggregate of spherical beads. The average BET surface area for t18 was not measured because there was not enough polymer, instead the surface area analysis was done on t24 which was created using the same polymer recipe as t18. t24 had a similar polymer linearity result as t18 (Figure 3.18) and hence the surface area and pore radius results of t24 could be extrapolated to t18. The larger pores in t18-reference polymer may allow  $\alpha$ -terpineol to diffuse through the polymer easily and therefore it may not physically trap the analyte, which could explain why increasing the polymer concentration did not increase the fraction of  $\alpha$ -terpineol bound. In some aspects t18-reference polymer was not a “true” reference for t18-MIP because of the different BET surface areas, but in terms of molecular shape cyclohexane is a reference compound because it does not have the branch groups. Theoretically the reference polymer should have a similar morphology and surface area to the MIP because it represents a baseline extent of analyte uptake by that polymer structure. Once the baseline uptake can be determined, the extra analyte bound by the MIP is inferred to be taken up by the imprinted cavities. In the case of t18, the polymer was created using a liquid-template imprinting method which adapted the porogen-imprinting concept [70] for terpenes.

Liquid-template imprinting was devised to enhance the uptake of  $\alpha$ -terpineol by the MIP, which presumably would weakly interact with the

carboxyl group moiety in MAA because there is only one point through which it can form a hydrogen bond. Attempts to evaluate the strength of interaction by  $^1\text{H-NMR}$  titrations (Section 2.4) yielded ambiguous results. The liquid  $\alpha$ -terpineol functioned simultaneously as the solvent and the template and it formed the major component of the polymerisation mixture (Appendix Four, Table A4.3). Since  $\alpha$ -terpineol could be classified as a polar solvent and cyclohexane as non-polar solvent, the different solvent properties may be the main reason t18 MIP and reference polymers had different morphology, topology, surface areas and pore radii. A more polar solvent can solvate the growing polymer chain better than a non-polar solvent, hence the growing polymer chain does not undergo phase separation (i.e. precipitation of the polymer) until later. The solvent can keep the polymer in solution for longer and the final polymer has a more extensive network structure. The alkene group of  $\alpha$ -terpineol could be co-polymerised [71]; therefore the large amount of  $\alpha$ -terpineol in the polymerisation mixture of t18 may result in some of the terpene incorporating into the polymer structure. If some  $\alpha$ -terpineol was covalently incorporated into the polymer framework, then the incorporated  $\alpha$ -terpineol groups may act as nucleation sites during rebinding of  $\alpha$ -terpineol. The nucleation concept was reported for a 2,4,5-T MIP [73] (discussion in Section 1.9.1). However it could not be concluded that this was the mechanism operating for t18-MIP because there was no evidence for covalent incorporation of  $\alpha$ -terpineol into the polymer structure. The calculation of the yield of polymer matched only the total mass of functional monomer and cross-linking monomer and therefore suggested that the  $\alpha$ -terpineol was not covalently incorporated into the polymer structure. The FT-IR spectra of both the MIP and reference polymers (Section 2.3.3) were very similar; therefore the covalent incorporation of  $\alpha$ -terpineol into the MIP structure was not detected. The scent of  $\alpha$ -terpineol could also be detected by the nose after polymerisation was complete.



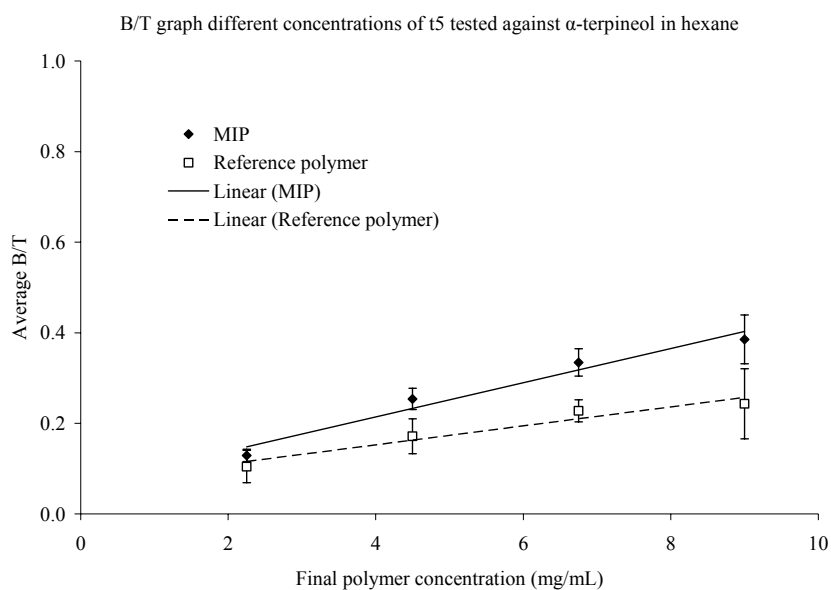
**Figure 3.17** Batch-binding results for testing t18, at different concentrations, against 50 ppm  $\alpha$ -terpineol in hexane<sup>20</sup>



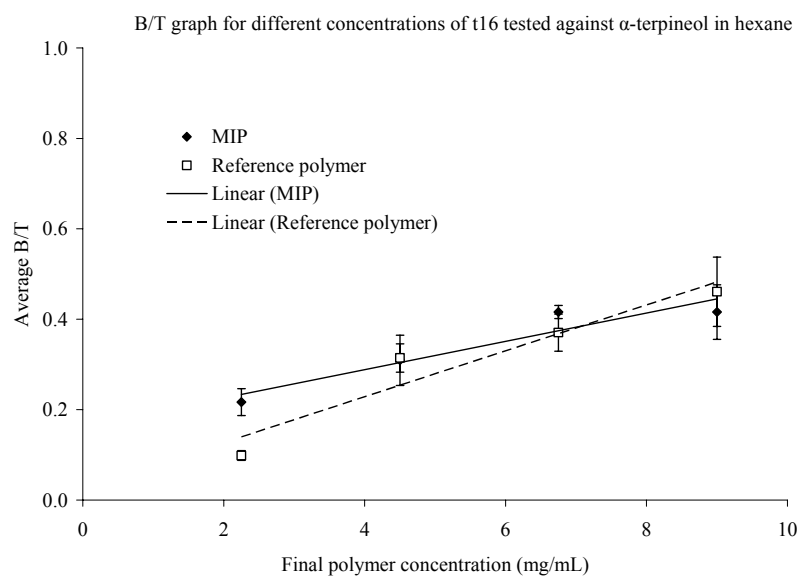
**Figure 3.18** Batch-binding results for testing t24, at different concentrations, against 50 ppm  $\alpha$ -terpineol in hexane<sup>21</sup>

<sup>20</sup> All results were calculated from triplicate samples. Error bars represent one standard deviation.

<sup>21</sup> All results were calculated from triplicate samples. Error bars represent one standard deviation.



**Figure 3.19** Batch-binding results for testing t5, at different concentrations, against 50 ppm  $\alpha$ -terpineol in hexane<sup>22</sup>

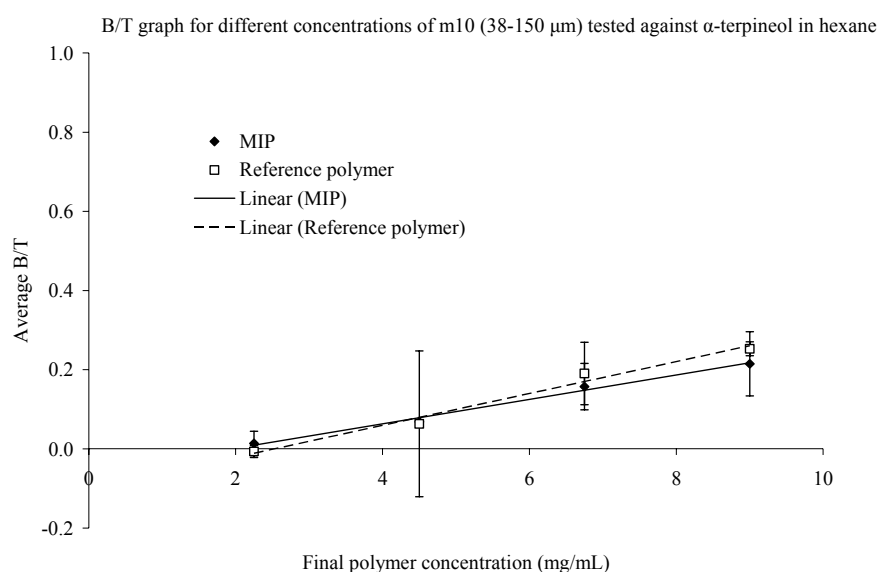


**Figure 3.20** Batch-binding results for testing t16, at different concentrations, against 50 ppm  $\alpha$ -terpineol in hexane<sup>23</sup>

<sup>22</sup> All results were calculated from triplicate samples. Error bars represent one standard deviation.

<sup>23</sup> All results were calculated from triplicate samples. Error bars represent one standard deviation.

t16 in hexane (tested against 50 ppm  $\alpha$ -terpineol) only specifically bound  $\alpha$ -terpineol at 2.25 mg/mL (Figure 3.20); this was a similar to the ratio of analyte to polymer concentration used in the specificity test which tested 4.5 mg/mL polymer against 100 ppm  $\alpha$ -terpineol (Figure 3.13). At higher concentrations of t16 the specific binding decreased because the amounts of  $\alpha$ -terpineol taken up by the MIP and reference polymer were similar.



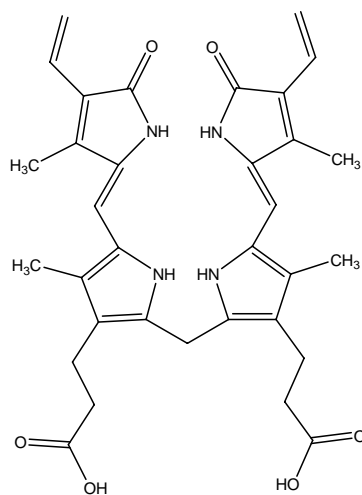
**Figure 3.21** Batch-binding results for testing m10 (38-150  $\mu$ m), at different concentrations, against 50 ppm  $\alpha$ -terpineol in hexane<sup>24</sup>

The polymer linearity result observed for m10 was too variable to decide whether it showed cross-reactivity over the polymer concentration range tested (Figure 3.21). It could be inferred that there was very little cross-reactivity because the B/T values for the MIP and reference polymer were close together. In comparison to the previous polymer linearity results, the B/T values for m10 were lower. This could be attributed to the different position of the hydroxyl group on  $\alpha$ -terpineol relative the hydroxyl group of (-)-menthol. The functional group (phenol moiety) in the imprinted cavity would be orientated to form hydrogen bonds with the secondary hydroxyl group of (-)-menthol because the MIP was created by the semi-covalent approach. The tertiary hydroxyl group on  $\alpha$ -terpineol would be sterically crowded by geminal dimethyl groups. Also it

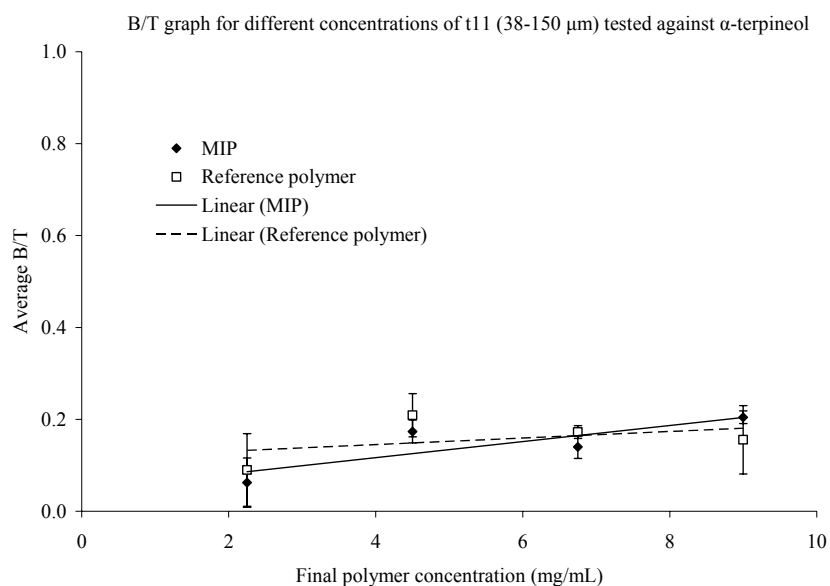
<sup>24</sup> All results were calculated from triplicate samples. Error bars represent one standard deviation.

would not be located at the optimal distance to form strong hydrogen bonds in the imprinted cavity.

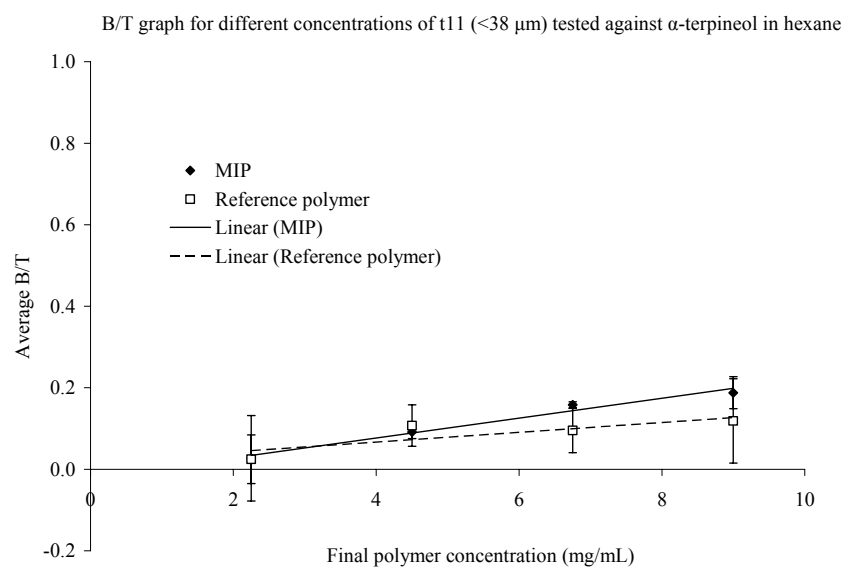
The results for t11 were also too variable to conclude if it bound  $\alpha$ -terpineol specifically over the polymer concentration range tested. Using different particle size range did not change the observed binding profile (Figure 3.23 and 3.24). It was noted that the B/T values were also lower compared to the polymer linearity results of t5, t16 and t18. The functional monomer in polymer t11, bilirubin, has many functional groups (Figure 3.22) and may form a number of different complexes with  $\alpha$ -terpineol. This could result in different types of binding sites with a large range of affinities; if there were more low-affinity sites, less  $\alpha$ -terpineol would bind to the polymer. This may be why lower B/T values were observed. Studies on the polymerisation complexes formed would be required to determine if the above hypothesis actually occurred. Alternatively, bilirubin may have created a polymer with a structure which may hinder the diffusion of  $\alpha$ -terpineol to the binding sites within the polymer particle. The SEM images for t11 were similar to t8 (Appendix Six, Figures A6.7 to A6.10).



**Figure 3.22** Structure of bilirubin



**Figure 3.23** Batch-binding results for testing t11 (38-150  $\mu\text{m}$ ), at different concentrations, against 50 ppm  $\alpha$ -terpineol in hexane<sup>25</sup>



**Figure 3.24** Batch-binding results for testing t11 (<38  $\mu\text{m}$ ), at different concentrations, against 50 ppm  $\alpha$ -terpineol in hexane<sup>26</sup>

<sup>25</sup> All results were calculated from triplicate samples. Error bars represent one standard deviation.

<sup>26</sup> All results were calculated from triplicate samples. Error bars represent one standard deviation.

*Summary*

The concept of using a liquid template, such as  $\alpha$ -terpineol, as the porogen was a novel adaptation of the porogen-imprinting effect; this method yielded a polymer (t18-MIP) which appeared to bind specifically to  $\alpha$ -terpineol in hexane compared to the reference polymer. The differences in the appearance of the polymers (t18-MIP and t18-reference polymer) may reflect a dominant factor contributing to the large difference in B/T values observed between them. The reference polymer was an aggregate of spherical particles; in contrast the MIP had irregularly shaped particles with a dense surface. Analysis revealed that they had different surface areas and t18-reference polymer had a slightly larger pore radius.

The polymer concentration range that was tested was not large enough to achieve a B/T of 0.5, the highest polymer concentration which approached this B/T value (9 mg/mL) was selected to investigate the effect of varying the test solution concentration on the B/T (Section 3.3). Further characterisation of t18 in hexane may help in the understanding of the mechanism responsible for the different binding properties between the MIP and reference polymer.

### **3.3 Optimisation of the concentration of $\alpha$ -terpineol used to test against t18 in hexane**

From the results of the previous section t18-MIP was observed to specifically adsorb  $\alpha$ -terpineol in hexane in comparison to t18-reference polymer. Varying the concentration of  $\alpha$ -terpineol, whilst keeping the polymer concentration constant at 9 mg/mL, may uncover an  $\alpha$ -terpineol concentration which increases the B/T value. It was expected that at higher concentrations of  $\alpha$ -terpineol, the B/T may be smaller because the fraction of  $\alpha$ -terpineol bound would presumably be less. At lower concentrations of  $\alpha$ -terpineol the B/T may be larger because the fraction of  $\alpha$ -terpineol bound presumably would be greater. Therefore, theoretically it is possible to achieve a B/T of 1, when all the analyte added is bound to the MIP, if a low enough concentration is tested.

*Preparation of polymer suspension*

10 mg/mL polymer suspension in hexane in 8 mL glass vials with *Teflon*-lined caps and then allowed to swell at least overnight before use. Any visible polymer aggregates were broken by sonication or a glass stirring rod was used to crush the tighter aggregates.

*Preparation of standards and test solutions*

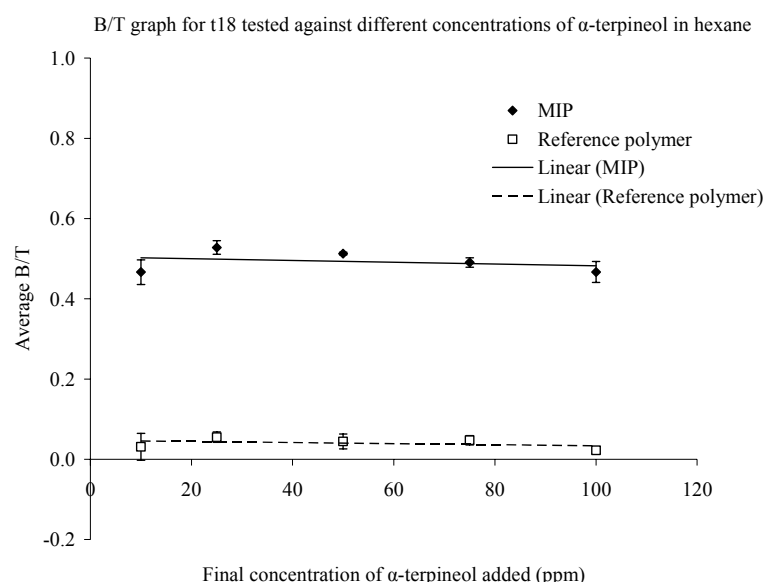
Unless specified otherwise, all stock standards, test solutions and internal standards were prepared using glass equipment (glass pipettes or volumetric flasks) and they were transferred into amber glass vessels with *Teflon*-lined caps (or clear glass vessels which were then wrapped in aluminium foil) and stored in the refrigerator when not in use. The method of preparation can be found in Appendix Eight, Table A8.3.

*Batch-binding protocol*

All batch-binding tests were carried out in 3 mL glass vials with *Teflon*-lined caps. Both polymers were tested in triplicate at each test solution concentration. 630  $\mu\text{L}$  of continuously stirred 10 mg/mL polymer suspension were transferred into 3 mL glass vials using Eppendorf® air-displacement pipettes attached with wide-bore pipette tips. 70  $\mu\text{L}$  of the appropriate concentration of  $\alpha$ -terpineol was added to labelled vials then they were shaken at 320 rpm for 0.5 hours at room temperature. To assess how much  $\alpha$ -terpineol was not bound to the polymer, the samples were poured into labelled micro-centrifuge tubes and then the supernatant was collected by centrifuging for 10 minutes at 14.1 rcf. 450  $\mu\text{L}$  of the clear supernatant was transferred directly into the labelled GC vials which contained 450  $\mu\text{L}$  internal standard ((-)-menthol in hexane). The vials were capped securely and then mixed by gently shaking the tray which held the samples. All the samples were analysed by GC-FID using the temperature program outlined in Appendix Two. All chromatograms were processed using Millennium® software, Microsoft® Office Excel® was used for data analysis.

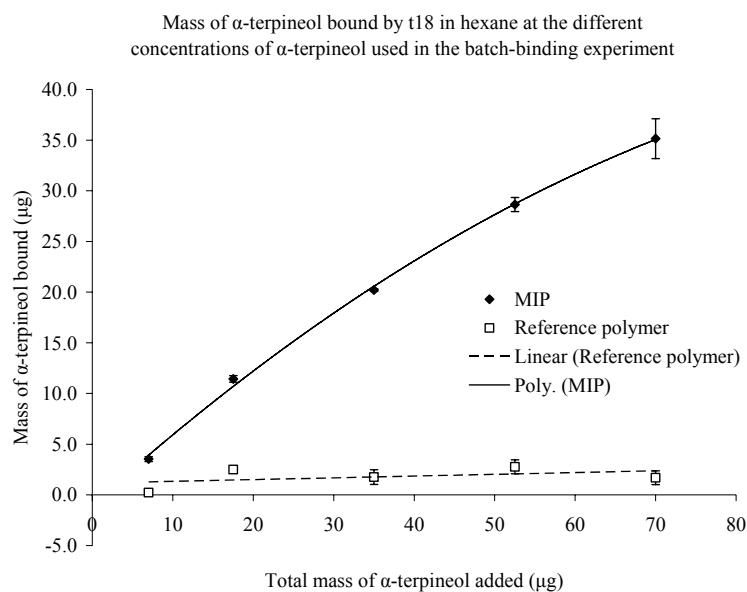
### 3.3.1 Results and discussion

It was observed that the MIP specifically bound more  $\alpha$ -terpineol compared to the reference polymer. In the test range of 10-100 ppm  $\alpha$ -terpineol, the B/T value for t18-MIP decreased slightly as the concentration of  $\alpha$ -terpineol increased (Figure 3.25). It was interesting to observe that the mass of  $\alpha$ -terpineol taken up by the polymer increased with a very slight curvature. This could mean that the concentration range tested may be near the saturation limit for t18-MIP (Figure 3.26; values were calculated from data in Figure 3.25). From this experiment the highest B/T values were around 0.5. Larger B/T values may not have been observed because the concentration range of  $\alpha$ -terpineol that was tested may have been too narrow and only showed part of the binding profile. The GC-FID which was available did not reliably detect  $\alpha$ -terpineol concentrations below 2.5 ppm. It would be worthwhile in the future to test t18 against lower concentrations of  $\alpha$ -terpineol in hexane to investigate if the B/T could be increased. It may also be possible to evaluate the lowest possible concentration of  $\alpha$ -terpineol that can be specifically bound by t18-MIP.



**Figure 3.25** Batch-binding results for testing t18 against different concentrations of  $\alpha$ -terpineol in hexane<sup>27</sup>

<sup>27</sup> All averages were calculated from triplicate samples, except the *C<sub>ts</sub>* solution for 25 and 75 ppm were calculated from duplicate samples. Error bars represent one standard deviation.



**Figure 3.26** Mass of  $\alpha$ -terpineol bound by t18 in hexane<sup>28</sup>

### Summary

The B/T value for 9 mg/mL t18 decreased with the concentration of  $\alpha$ -terpineol when tested between 10-100 ppm  $\alpha$ -terpineol in hexane. This was not a big decrease, therefore 50 ppm  $\alpha$ -terpineol was chosen for testing the kinetics of  $\alpha$ -terpineol binding the polymer (Section 3.4).

## 3.4 Optimisation of the time $\alpha$ -terpineol was exposed to t18 in hexane

The length of time that a polymer is exposed to the test analyte can affect the extent of analyte uptake. A large B/T value may arise from non-specific binding or physical trapping of the analyte by the sieve-like nature of cross-linked polymers. In these cases the kinetics of analyte diffusion to and from the imprinted cavities of the polymer will be the major factor determining the extent of binding. A polymer structure with small pores would sterically hinder free diffusion of analytes and therefore more time may be required to bind a larger amount. Specific binding of analyte by a MIP may also contribute to a large B/T

<sup>28</sup> All averages were calculated from triplicate samples, except the  $C_{ts}$  solution for 17.5 and 52.5  $\mu\text{g}$  total mass of  $\alpha$ -terpineol added which were calculated from duplicate samples. Error bars represent one standard deviation.

value; this is affected by both the kinetics of diffusion and the kinetics of the analyte forming an intermolecular interaction with the functional groups in the imprinted cavities. Since the MIPs which have been studied rebind analyte through non-covalent interactions, the speed of bond formation was not considered to be a limiting factor. The effect of exposure time on the extent  $\alpha$ -terpineol was taken up by t18 was investigated using the polymer concentration and  $\alpha$ -terpineol concentration determined from the previous two experiments (Section 3.2 and 3.3).

#### *Preparation of polymer suspension*

5 to 7 mL of 5 mg/mL polymer suspensions in hexane were prepared in 8 mL glass vials with *Teflon*-lined caps and then allowed to swell at least overnight before use. Any visible polymer aggregates were broken by sonication or a glass stirring rod. All batch-binding tests which were 5 minutes and under were carried out directly in micro-centrifuge tubes, batch-binding tests which were over 5 minutes were carried out in 3 mL glass vials with *Teflon*-lined caps. Each polymer was tested in triplicate for each exposure time.

#### *Preparation of standards and test solutions*

Unless specified otherwise, all stock standards, test solutions and internal standards were prepared using glass equipment (glass pipettes or volumetric flasks) and they were transferred into amber glass vessels with *Teflon*-lined caps (or clear glass vessels which were then wrapped in aluminium foil) and stored in the refrigerator when not in use. The method of preparing these solutions can be found in Appendix Eight, Table A8.4.

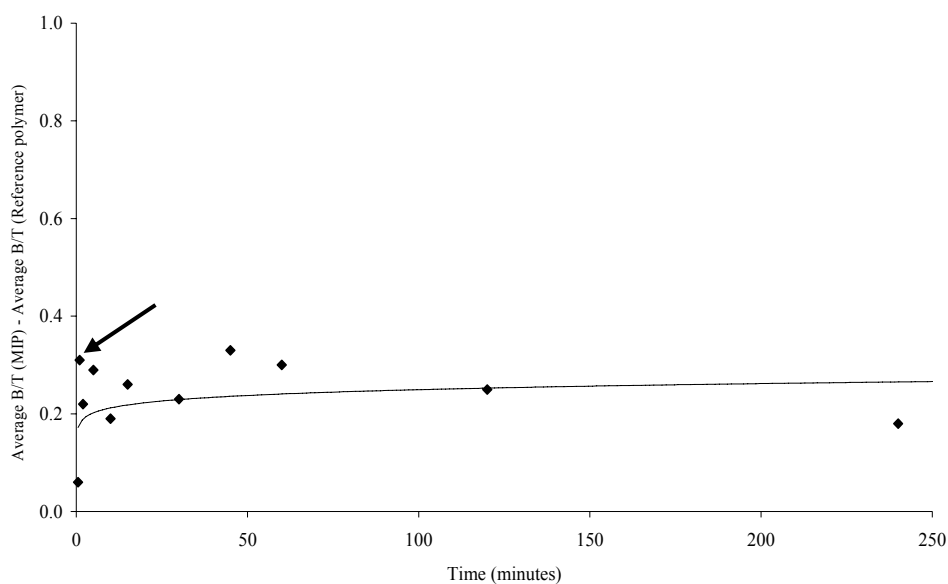
#### *Batch-binding protocol*

630  $\mu$ L of continuously stirred 10 mg/mL polymer suspension was transferred using Eppendorf® air-displacement pipettes attached with wide-bore pipette tips; this was mixed with 70  $\mu$ L of 500 ppm  $\alpha$ -terpineol in hexane. For more than 1 minute exposure times, the samples were shaken at 320 rpm for the appropriate time at room temperature. The 0.5 minute test samples were vortexed for half a minute at room temperature. To determine how much  $\alpha$ -terpineol was

not bound to the polymer, supernatant was collected. For samples in glass, the contents were poured into labelled micro-centrifuge tubes and then centrifuged for 10 minutes at 14.1 rcf. For samples already in micro-centrifuge tubes, they were centrifuged as they were for 10 minutes at 14.1 rcf. 600  $\mu\text{L}$  of supernatant was transferred into a fresh plastic micro-centrifuge and this was centrifuged for 10 minutes at 14.1 rcf to clean up the supernatant. 450  $\mu\text{L}$  of the internal standard (50 ppm (-)-menthol in hexane) was added to all the GC vials; then 450  $\mu\text{L}$  of the clear supernatant was transferred directly into the labelled GC vials, capped securely and then mixed by gently shaking the tray which held the samples. All the samples were analysed by GC-FID using the temperature program in Appendix Two. All chromatograms were processed using Millennium® software, Microsoft® Office Excel® was used for data analysis.

### 3.4.1 Results and discussion

The specific binding by t18-MIP appeared to reach a maximum after 1 minute (arrow, Figure 3.27). However oscillation in the specific binding was observed at shorter times; which appeared to stop after 60 minutes. This may reflect the dynamic nature of the analyte binding to the polymer. Non-covalent bonds are not permanent and may be disrupted by molecular motion when shaking the samples. It also represented the variability in assessing specific binding at short exposure times; at the molecular level  $\alpha$ -terpineol may bind to different extents for each replicate sample because they were a sub-sample of the stock suspension and thus may have a slightly different particle size distribution.

Specific binding of  $\alpha$ -terpineol by t18 in hexane as a function of time

**Figure 3.27** The effect of exposure time on the specificity of t18-MIP; tested against 50 ppm  $\alpha$ -terpineol in hexane

### 3.5 Further investigation of MIP cross-reactivity

From the previous results t18-MIP appeared to enhance the uptake of  $\alpha$ -terpineol in hexane. To probe if shape selectivity was involved in the recognition mechanism a range of terpinolene concentrations in hexane were exposed to the polymer, in a similar setup as described in Section 3.3. Although the cross-reactivity studies (Figure 3.4 in Section 3.1.1) suggested that very little terpinolene was bound by the MIP, investigating a range of terpinolene concentrations would determine if this was a concentration-dependent result or if the MIP intrinsically did not recognise terpinolene in hexane.

#### *Polymer preparation*

In 8 mL glass vials, 10 mL of 10 mg/mL t18 polymer suspensions were prepared in hexane. They were allowed to swell at least overnight before use. Prior to testing the suspensions, the polymer aggregates were minimised by sonication or by crushing with a glass rod.

*Preparation of standards and test solutions*

Unless specified otherwise, all stock standards, test solutions and internal standards were prepared using glass equipment (glass pipettes or volumetric flasks) and they were transferred into amber glass vessels with *Teflon*-lined caps (or clear glass vessels which were then wrapped in aluminium foil) and stored in the refrigerator when not in use. The method of preparing these solutions can be found in Appendix Eight, Table A8.5.

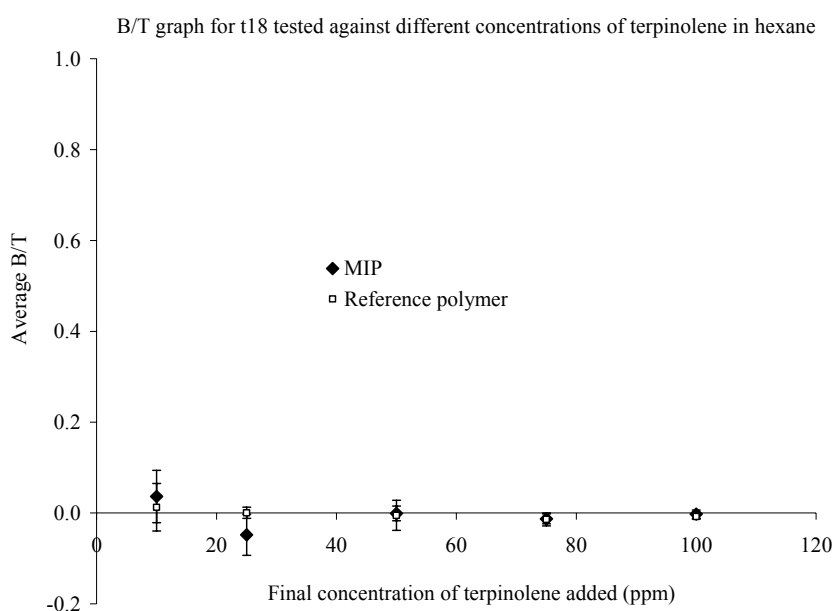
*Batch-binding protocol*

630  $\mu\text{L}$  of continuously stirred 10 mg/mL polymer suspension was pipetted (air-displacement pipette, wide-bore tip) into labeled 3 mL glass vials and mixed with 70  $\mu\text{L}$  of the appropriate terpinolene test solution. All MIPs, reference polymers and test solutions were tested in triplicate against each terpinolene concentration. The addition of the test solution to every set of 9 samples (i.e. one terpinolene concentration) was staggered by 10 minutes. All samples were shaken at 320 rpm for 0.5 hour at room temperature on an orbital shaker. At the end of 0.5 hour, the supernatants were prepared for GC analysis by pouring the sample into labelled micro-centrifuge tubes. All tubes were then centrifuged at 14.1 rcf for 10 minutes, and then 600  $\mu\text{L}$  of the supernatant was transferred into a fresh micro-centrifuge tube. This second supernatant was centrifuged for 14.1 rcf, 10 minutes. 450  $\mu\text{L}$  of the second supernatant was transferred into a labelled GC vial already containing 450  $\mu\text{L}$  of 50ppm menthol in hexane (internal standard). Samples were analysed on GC-FID using the temperature program outlined in Appendix Two. All chromatograms were processed using Millennium® software, Microsoft® Office Excel® was used for data analysis.

**3.5.1 Results and discussion**

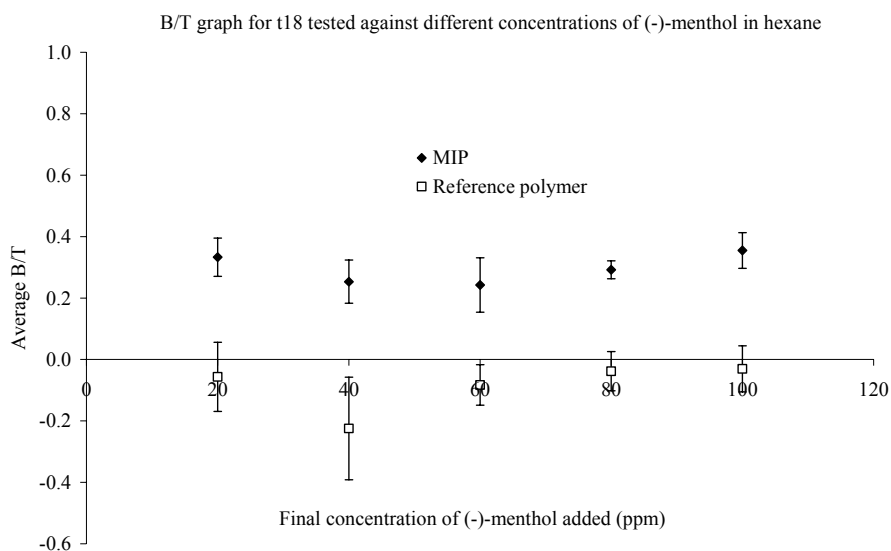
As shown in Figure 3.28, t18-MIP showed no cross-reactivity towards terpinolene in hexane even though the surface area of t18-MIP was much larger than t18-reference polymer (Section 2.3.2). This suggested that surface area alone did not dictate the binding capacity of the polymer. Testing different concentrations of (-)-menthol showed that t18-MIP showed cross-reactivity towards (-)-menthol (Figure 3.29; since  $\alpha$ -terpineol (internal standard) calibration

solutions were not run at the same time as these samples, only corrected peak areas could be shown). This suggested that t18 may specifically bind  $\alpha$ -terpineol through hydrogen bonding. Hydrophobic interactions and shape recognition presumably play a minor role because t18-MIP did not take up terpinolene which is a hydrophobic compound. A potential future experiment, which could help to clarify the mechanism of recognition by t18-MIP, may involve testing alcohols with different structures. This would probe the extent that shape recognition influences analyte uptake by this polymer.



**Figure 3.28** Batch-binding results for testing t18 against different concentrations of terpinolene in hexane<sup>29</sup>

<sup>29</sup> Average B/T calculated from triplicate samples; except for when 50 ppm (both MIP and reference polymer), 25 ppm (MIP) and 10 ppm (MIP) terpinolene was added, these averages were based on duplicate results. Error bars represent one standard deviation.



**Figure 3.29** Batch-binding results for testing t18 against different concentrations of (-)-menthol in hexane<sup>30</sup>

### 3.6 Final discussion and conclusion

A study of different terpene MIPs using widely accepted methods of imprinting (non-covalent and semi-covalent imprinting) yielded MIPs which showed poor specificity towards their template analyte. An  $\alpha$ -terpineol MIP was synthesised by precipitation polymerisation which appeared to specifically bind to  $\alpha$ -terpineol in hexane; the synthetic protocol involved adapting a method that has not been widely applied in the field of molecular imprinting, called porogen-imprinting. This method has never been previously reported for a terpene. The porogen-imprinting method, coupled with precipitation polymerisation, appeared to enhance the specific uptake of  $\alpha$ -terpineol by t18-MIP. It did not enhance the uptake of a hydrophobic structural analogue (terpinolene). In contrast, t18-MIP bound a polar structural analogue (such as (-)-menthol); this supported the idea that hydrogen bonding may play a dominant role in the recognition mechanism. The cross-reactivity of t18-MIP towards (-)-menthol and the poor cross-reactivity towards terpinolene suggested that the polymer did not recognise the shape of the compounds (which was the recognition mechanism proposed for porogen-

<sup>30</sup> Average B/T values were calculated from triplicate samples (based on corrected peak areas). Error bars represent one standard deviation.

imprinting of aromatic solvents [70]) but instead bound the analytes through their single hydroxyl group. Therefore at present, it can be concluded that  $\alpha$ -terpineol specifically bound to a MIP prepared by porogen-imprinting through hydrogen bonding. However further cross-reactivity studies using other alcohols may clarify how exclusively the MIP binds to  $\alpha$ -terpineol. The encouraging results shown by the porogen-imprinted  $\alpha$ -terpineol MIP suggested that porogen-imprinting could potentially be used to imprint other liquid terpenes.

The kinetics experiment showed that the maximum amount of  $\alpha$ -terpineol bound by t18-MIP was achieved in approximately one minute; this rapid uptake suggested that diffusion was not a limiting factor in the uptake mechanism and therefore suggested suitability for MIP applications that require rapid detection or separation of the analyte from a sample.

The precipitation polymerisation method was reproducible for porogen-imprinting; a second batch of t18 (called t24) was found to specifically bind  $\alpha$ -terpineol to a similar extent to t18 when both were tested in hexane. In contrast when block MIPs prepared by non-covalent imprinting were re-made they had a different binding capacity from one another (as observed for t8 and t11). Possibly relevant is that the exothermic nature of radical polymerisation elevated the temperature in the reaction vessel. The final temperature and rate of increase was not easily controlled and therefore may have given rise to differences in the microstructure of the polymers which could have influenced their performance.

$\alpha$ -Terpineol also bound specifically to t5-MIP, but further studies are needed to evaluate the effect of  $\alpha$ -terpineol concentration on the specificity of binding and also the kinetics of specific binding.

In conclusion, the most encouraging finding in this research project was that the porogen-imprinting method could be applied to other liquid compounds such as terpenes, when in the past this method was only reported for conventional aromatic organic solvents such as benzene derivatives. This is a major outcome from this research which should be further studied and developed.

## **Appendix One**

### **Colorimetric detection of terpenes**

## A1 Introduction

The reaction of an aromatic aldehyde with secondary or tertiary alcohols exposed to acid is called the Komarowsky reaction [84]. The Komarowsky reaction has been used to determine a wide range of alcohols; however primary alcohols showed no interference with the reaction [76]. The reaction has been used by others in a qualitative spray format to visualise terpenoid esters on thin layer chromatography (TLC) plates [85]. The reaction has also been modified previously by others to create a quantitative liquid assay for some alcohols including sugar alcohols [78], higher alcohols (fusel oils like isobutanol and isopentanol) in spirits and alcoholic drinks [86, 87], and monoterpenes from grapes [77].

The qualitative detection of the monoterpenes,  $\alpha$ -terpineol and (-)-menthol, has been described previously [74, 75]. A minor goal of this thesis was to develop an assay, based on one of the reactions reported, which could be accurate and precise enough for the quantitative determination of  $\alpha$ -terpineol and (-)-menthol by modifying various parameters including the type of acid, the amount of acid, the composition of the diluent and fixing the time interval when each step of the reaction was to be carried out. However the colorimetric method was too variable and attempts to quantify terpene uptake by MIPs was not successful in most cases; therefore the work conducted on the colorimetric reaction will only be presented here in the Appendix. Gas chromatography subsequently became the primary method of quantitating the terpenes as presented in the results section.

### A1.1 Validation of the colorimetric method found in the literature

A test for  $\alpha$ -terpineol and (-)-menthol had been described previously, the original protocol will be outlined first and then the modifications which were carried out will be described.

### **A1.1.1 Validation of colorimetric method found in the literature for detecting $\alpha$ -terpineol**

*Caution: Concentrated acids are corrosives and must be handled with safety protective gear in a fume hood.*

The original procedure [75] required 15 mL of  $\alpha$ -terpineol sample to be mixed with 5 mL ethanol and then 30 mL of 1% vanillin in concentrated hydrochloric acid. This was shaken for a minute and then heated at 60 °C for 25 minutes; a bluish-green colour developed as a positive result. However these volumes were too large and not suitable for analysing many samples. The first attempt was to test using smaller volumes. The original procedure was modified so that 2 mL of aqueous 25% ethanol was mixed with 50  $\mu$ L neat  $\alpha$ -terpineol and then 3 mL of 1% vanillin in concentrated hydrochloric acid (yellow coloured solution) was added prior to heating at 60 °C for 30 minutes; a blue-grey colour developed.

### **A1.1.2 Validation of the colorimetric method found in the literature for detecting (-)-menthol**

The original procedure [74] was tested against a 2 mg/mL (-)-menthol solution in methanol. In a glass test tube, 1 mL of the 2 mg/mL (-)-menthol solution was mixed with 5 mL of reagent (which consisted of 0.5% *p*-dimethylaminobenzaldehyde dissolved in 62% aqueous sulfuric acid). The reaction was observed for approximately 10 minutes at room temperature; the solution started off with a faint yellow colour and then turned pinky-orange. The original procedure required the mixture to be heated in boiling water for 2 minutes; therefore the pinky-orange mixture was capped with a CaCl<sub>2</sub> drying tube and then placed in a beaker of hot water. The reaction mixture turned blood red to indicate a positive test result because when the 2 mg/mL (-)-menthol solution was replaced by a blank methanol solution the mixture remained yellow after the heating step.

### **A1.1.3 Discussion**

The reaction volumes in the original protocol were too large for rapid throughput of samples. Therefore the reaction was scaled down and various other factors of the original protocol were changed, such as type of acid, type of solvent and type of terpene. The following sections in Appendix One will be focused on the vanillin reaction because, during the research period, more time was spent on it than the *p*-dimethylaminobenzaldehyde reaction before the colorimetric method approach was abandoned as it did not perform to our criteria.

### **A1.2 Altering the type of acid and the order of adding the reagents for the vanillin reaction**

In an attempt to remove the heating step from the original protocol it was found that substituting concentrated hydrochloric acid with concentrated sulfuric acid provided a more exothermic reaction which brought about a rapid colour change without an external heat source. If concentrated phosphoric acid was used as the acid there was no visual colour change. Concentrated sulfuric acid (98%) is a very strong acid, much stronger than concentrated hydrochloric acid (35%). In contrast to the other two acids, concentrated phosphoric acid (85%) is much weaker; the first  $pK_a$  of phosphoric acid is 2.16 [88]. An external heat source for colour development was needed when concentrated hydrochloric acid was used, therefore only concentrated sulfuric acid was used for future experiments.

A lot of heat was generated upon mixing sulfuric acid with the vanillin solution; therefore the second solution had to be added drop-wise to prevent bubbling of the mixture and solvent loss. This limited the speed with which the two solutions could be combined. In a concentrated acidic solution the vanillin will most likely be protonated. This may increase the reactivity of vanillin and may promote dimerisation with another vanillin molecule or bring about other side reactions, such as reaction with solvent molecules or with ions (like sulfate or bisulfate). Therefore a complex population of product species may be formed. The order of adding the vanillin and concentrated sulfuric acid reagents appeared to play a role in the colour developed by the mixed reagent. Adding the acid

drop-wise to the vanillin solution gave a more stable starting colour (yellow); but this only happened when an equal volume of the acid and vanillin solution were mixed together (details in Section A1.3).

### A1.3 Altering the strength of sulfuric acid for the vanillin colorimetric reaction

The volume of concentrated sulfuric acid used was found to affect the starting colour of the reagent, along with the stability as mentioned in Section A1.2. The reagent used was prepared by mixing 200  $\mu\text{L}$  2 mg/mL vanillin in methanol with different volumes of concentrated sulfuric acid (Table A1.1).

**Table A1.1 The effect of different volumes of concentrated sulfuric acid on the colorimetric reagent**

| Volume concentrated sulfuric acid ( $\mu\text{L}$ ) | Reagent colour                    |
|---|-----------------------------------|
| 200   | Yellow, stable                    |
| 100   | Yellow, then grey after 1.5 hours |

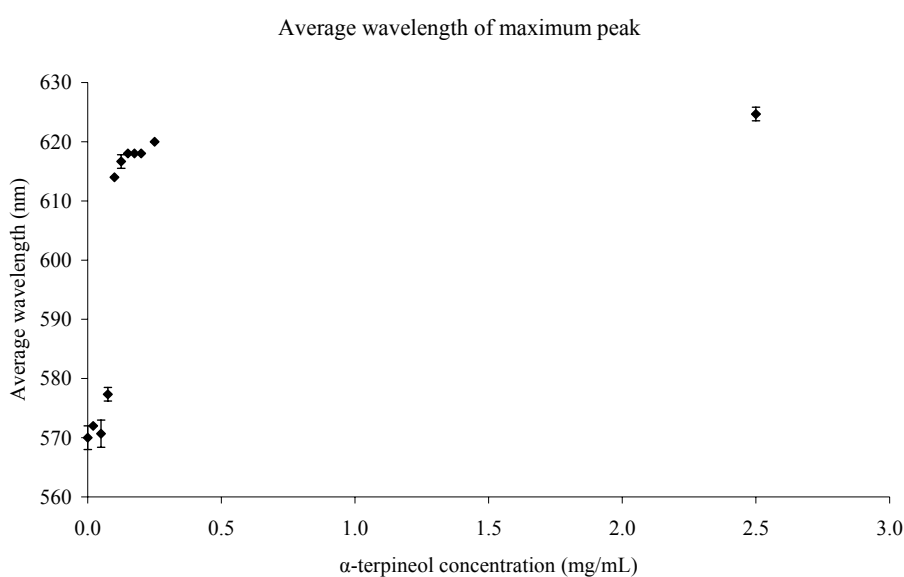
When different volumes of concentrated sulfuric acid were mixed with vanillin and then tested by adding  $\alpha$ -terpineol, different final colours were seen. At room temperature, 200  $\mu\text{L}$  2 mg/mL vanillin in methanol was mixed with different volumes of concentrated sulfuric acid (Table A1.2) and then 20  $\mu\text{L}$  1% (v/v)  $\alpha$ -terpineol [which was dissolved in a 25% ethanol solution in water] was added. The colours observed after mixing are listed in Table A1.2.

**Table A1.2 The effect of different volumes of concentrated sulfuric acid on the colorimetric reaction in the presence of  $\alpha$ -terpineol**

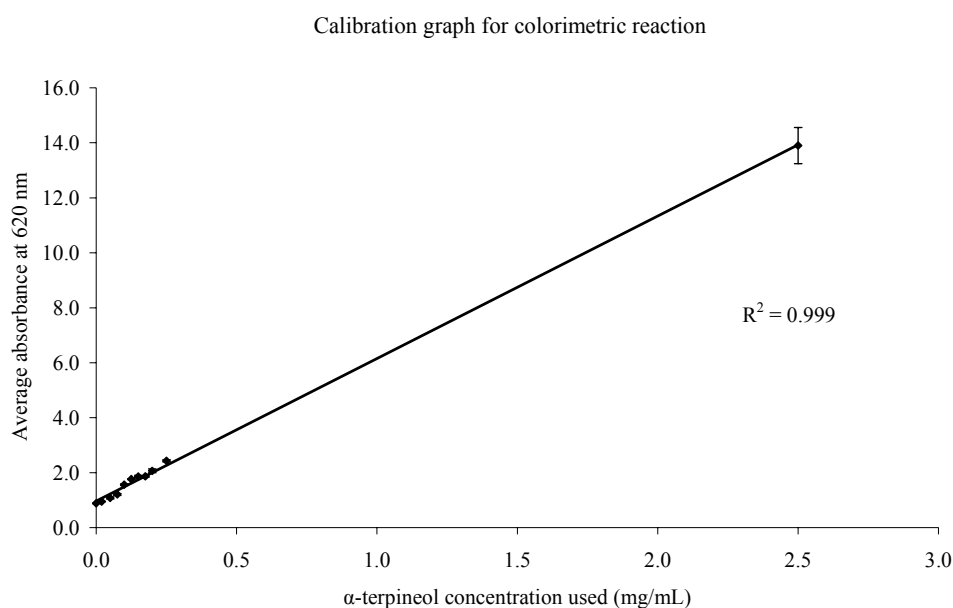
| Volume concentrated sulfuric acid ( $\mu\text{L}$ ) | Colour development |            |
|---|--------------------|------------|
|   | Initial            | Final      |
| 200   | dark red           | blue-red   |
| 100   | yellow             | green      |
| 75  | light green        | dark green |
| 50  | colourless         | faint pink |

Reagent consisting of 200  $\mu\text{L}$  2 mg/mL vanillin in methanol with 100  $\mu\text{L}$  concentrated sulfuric acid yielded a colour change that was obvious and also used

less acid and therefore was selected for further development. Calibration graphs were generated for a reagent consisting of 2:1 (v/v) 2 mg/mL vanillin in methanol: concentrated sulfuric acid. 0.72 mL of the reagent was mixed with 0.08 mL of  $\alpha$ -terpineol in ethanol. Each concentration of  $\alpha$ -terpineol was tested in triplicate. The colours ranged from purple at low  $\alpha$ -terpineol concentrations to blue at higher  $\alpha$ -terpineol concentrations; the colours may have been different from expected because water was not added to the mixture. The wavelength of the maximum spectral peak shifted over the  $\alpha$ -terpineol concentration range (Figure A1.1); the mixtures were more blue (greater absorbance around 620 nm) if there was more  $\alpha$ -terpineol present. The colours of the reaction solutions were too intense for measuring on a spectrophotometer, therefore methanol was used to dilute the reaction mixture by 1:10 or 1:100 before acquiring the spectra; the diluted solutions were transparent sky blue. These dilutions were accounted for when reporting the absorbance at 620 nm. Lower concentrations of  $\alpha$ -terpineol were tested but there was no observable colour change, therefore it was necessary to use a diluent when testing higher concentrations of  $\alpha$ -terpineol to allow spectral analysis. A plot of the average absorbance at 620 nm for each concentration of  $\alpha$ -terpineol yielded a linear absorbance versus concentration graph in the  $\alpha$ -terpineol concentration range of 0 to 2.5 mg/mL (Figure A1.2).



**Figure A1.1 Wavelength of maximum peak in spectra**



**Figure A1.2 Calibration graph for  $\alpha$ -terpineol tested against 2:1 vanillin:H<sub>2</sub>SO<sub>4</sub> reagent<sup>31</sup>**

For an undiluted reaction mixture which tested 0.025 mg/mL  $\alpha$ -terpineol, it was observed that the maximum wavelength of absorbance was red-shifted (bathochromic) from approximately 560 nm to 620 nm after 22 hours. This suggested that the reaction products were not stable and therefore investigations were carried out in an attempt to stabilise the absorbance.

#### **A1.4 Altering the solvent used to dissolve vanillin**

The changing absorbance intensity suggested that the reaction did not go to completion. Based on the reactants it was possible (though it is likely only a minor occurrence) that the colorimetric reaction may be forming an acetal product because aldehyde and alcohol are present together in an acidic solution [89]. Acetal formation is a reversible equilibrium reaction and readily decomposes which could be the reason why the absorbance intensity changed with time. As time progressed different species may have formed. An alcohol had been used as a solvent for  $\alpha$ -terpineol but this may be another species that could compete with  $\alpha$ -terpineol in the reaction. Different solvents were investigated to find a possible alternative for alcohols, however many of the solvents available were reactive

<sup>31</sup> Error bars represent one standard deviation (only visible for 2.5 mg/mL samples).

with concentrated acid. As expected many of the alternative solvents produced reagents which were not usable as part of a colorimetric test for  $\alpha$ -terpineol (Table A1.3). A stock of 2 mg/mL vanillin was prepared in each of the test solvents and then two parts of this was mixed with one part concentrated sulfuric acid; this was an exothermic reaction because a lot of heat was generated and each solvent gave a different reagent starting colour (Table A1.3). Each reagent was tested against 2 mg/mL  $\alpha$ -terpineol in the solvent (except for testing propylene glycol,  $\alpha$ -terpineol was dissolved in methanol) by mixing nine parts reagent to one part  $\alpha$ -terpineol solution.

**Table A1.3 Colours observed for reagent and tests carried out in different solvents**

| Solvent           | Colour                          |                             |                     |
|-------------------|---------------------------------|-----------------------------|---------------------|
|                   | Reagent                         | $\alpha$ -Terpineol added   | Blank solvent added |
| Propylene glycol  | Yellow to dark purple instantly | Dark purple                 | Dark purple         |
| Acetonitrile      | Yellow, two phases              | Not tested                  | Not tested          |
| Acetone           | Brown                           | Black                       | Black               |
| Ethyl acetate     | Yellow (hot), green (cool)      | Red to dark purple to green | Green               |
| Dimethylformamide | Yellow                          | Faint yellow                | Faint yellow        |
| Cyclohexane       | Vanillin did not dissolve       | Not tested                  | Not tested          |

A colour change was observed when  $\alpha$ -terpineol was tested with the ethyl acetate reagent. However the ester could have hydrolysed to form the alcohol and carboxylic acid in acidic conditions; this could increase the number of different species in the colorimetric reaction and may explain why there were so many colours observed upon addition of  $\alpha$ -terpineol. It was observed that vanillin dissolved in most solvents, except for cyclohexane. Also the vanillin solution reacted with the acid because a colour change was seen before  $\alpha$ -terpineol was added.

Propylene glycol created a viscous reagent and it was not easy to mix. It was likely that the diol reacted with the vanillin in the presence of acid and the colour formed was too intense to visually observe any other spectral changes when  $\alpha$ -terpineol or methanol was added. When acetonitrile was used as a solvent to dissolve vanillin subsequent addition of acid resulted in a highly exothermic reaction; the heat generated appeared to boil off the solvent because it was observed that the volume decreased and after a few hours the reagent was viscous.

It was possible that acetonitrile mixed with acid generated other toxic species; therefore acetonitrile was not tested again with such high volume ratios of acid. In acid, dimethylformamide could be protonated on the oxygen; the positive charge could be delocalized onto the nitrogen which would be inductively stabilised by the geminal methyl groups (Figure A1.3). Therefore dimethylformamide was potentially contributing another species to the colorimetric reaction which may bring about unknown side reactions that prevented colour change when  $\alpha$ -terpineol was added.

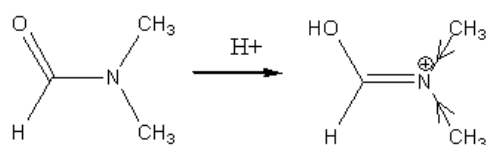


Figure A1.3 Effect of protonating dimethylformamide

### A1.5 Testing the vanillin colorimetric reaction on (-)-menthol

With some alterations, the vanillin colorimetric reaction could be used to detect (-)-menthol. After considering that the solvent may interfere with the colorimetric reaction the vanillin was dissolved directly in concentrated sulfuric acid. A 2 mg/mL vanillin solution in concentrated sulfuric acid was yellow; 40  $\mu$ L of this reagent was tested in triplicate against 40  $\mu$ L (-)-menthol dissolved in methanol at different concentrations. 720  $\mu$ L 2:1:1 concentrated sulfuric acid:water:ethanol (v/v/v; i.e. volume per volume per volume) was added immediately and then the mixture was vortexed. Solvent was added here to decrease the intense absorbance of the reaction mixture so that the visible spectra could be measured on a spectrophotometer. The spectra were acquired at 1, 19 and 24 hours after adding the diluent on the UV1601 spectrophotometer. Baseline was performed between 400-800 nm using 2:1:1 concentrated sulfuric acid:water:ethanol (v/v/v) as the reference solution. The results showed that over time the variability in absorbance (CV% in Table A1.4) decreased for the triplicate tests; however there may have been some solvent evaporation which may influence the absorbance readings because the samples were left in the

curvettes and not sealed tightly. The absorbance increased after 19 hours storage, but after 24 hours moisture in the air may have quenched the absorbance (the effect of water on the colorimetric reaction has been outlined in Section A1.6). Overall the data were too variable amongst the triplicate tests and the calibration graph was only approximately linear (Figure A1.4) so it was deemed to be unsuitable for quantifying the uptake of menthol by MIPs. The diluent [2:1:1 concentrated sulfuric acid:water:ethanol (v/v/v)] may influence the variability because it had been observed that solvent affected colour development (Section A1.4). The next section describes the effect of changing the diluent on the colorimetric reaction.

Table A1.4 Absorbance data for (-)-menthol calibration graph<sup>32</sup>

| (-)-Menthol concentration used<br>(mg/mL) | Time after dilution (hours) |                    |                   |         |                    |      |         |                    |       |
|---|-----------------------------|--------------------|-------------------|---------|--------------------|------|---------|--------------------|-------|
|   | 1                           |                    |                   | 19      |                    |      | 24      |                    |       |
|   | Average                     | Standard deviation | CV% <sup>33</sup> | Average | Standard deviation | CV%  | Average | Standard deviation | CV%   |
| 0.0                                       | 0.006                       | 0.002              | 29.8              | -0.017  | 0.001              | -5.9 | -0.005  | 0.001              | -10.8 |
| 0.2                                       | 0.114                       | 0.048              | 41.7              | 0.124   | 0.029              | 23.7 | 0.118   | 0.024              | 20.7  |
| 0.4                                       | 0.251                       | 0.072              | 28.9              | 0.272   | 0.042              | 15.5 | 0.249   | 0.034              | 13.7  |
| 0.6                                       | 0.442                       | 0.091              | 20.5              | 0.427   | 0.051              | 11.9 | 0.384   | 0.042              | 10.9  |
| 0.8                                       | 0.377                       | 0.117              | 31.2              | 0.475   | 0.078              | 16.4 | 0.437   | 0.064              | 14.5  |

<sup>32</sup> Absorbance reported = Absorbance ( $\lambda_{530nm}$ ) - Absorbance ( $\lambda_{800nm}$ ).

<sup>33</sup> CV% (percentage coefficient variability) = (standard deviation/average)\*100.

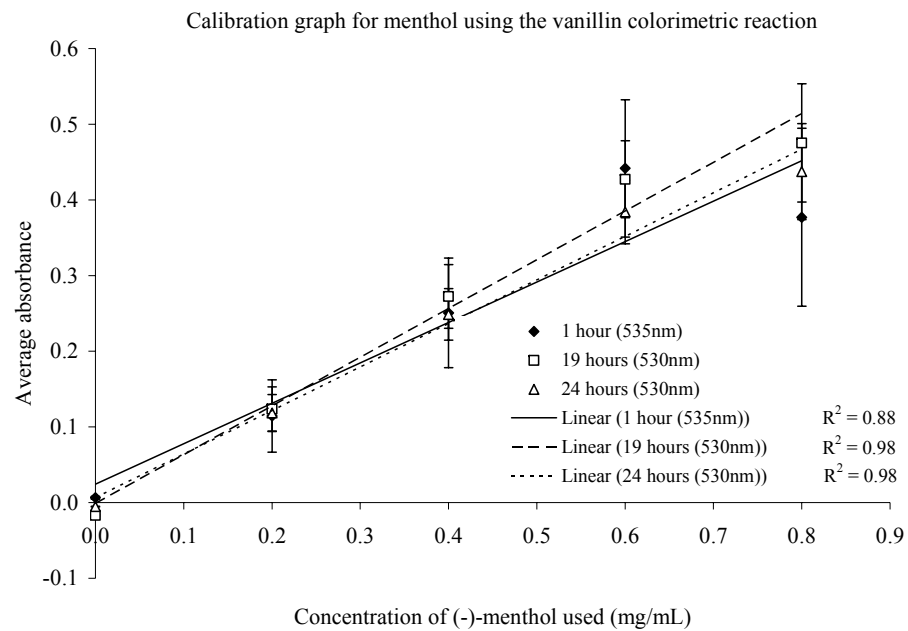


Figure A1.4 Calibration graph for menthol colorimetric reaction

### A1.6 Altering the diluent solution

The reaction mixtures containing vanillin reagent mixed with  $\alpha$ -terpineol or (-)-menthol were too intense to acquire a useful spectrum on a spectrophotometer. Therefore a diluent solution was required to decrease the intensity of the reaction mixture. It was seen that the solvent influenced the colorimetric reaction (Section A1.4) therefore different diluents were chosen for comparison. This included methanol, 2:1:1 concentrated H<sub>2</sub>SO<sub>4</sub>:water:ethanol (v/v/v) and different percentages of concentrated sulfuric acid in water. They resulted in different final colours (Table A1.5).

**Table A1.5 Effect of solvent on colour development**

| Solvent  | Colour                |                      |
|--|-----------------------|----------------------|
|  | Before adding solvent | After adding solvent |
| Methanol <sup>34</sup>   | Dark purple           | Sky blue             |
| 2:1:1 Concentrated H <sub>2</sub> SO <sub>4</sub> : water: ethanol (v/v/v) <sup>35</sup> | Red                   | Pink                 |
| Different percentages of H <sub>2</sub> SO <sub>4</sub> in water <sup>35</sup>           | Red                   | Colourless to pink   |

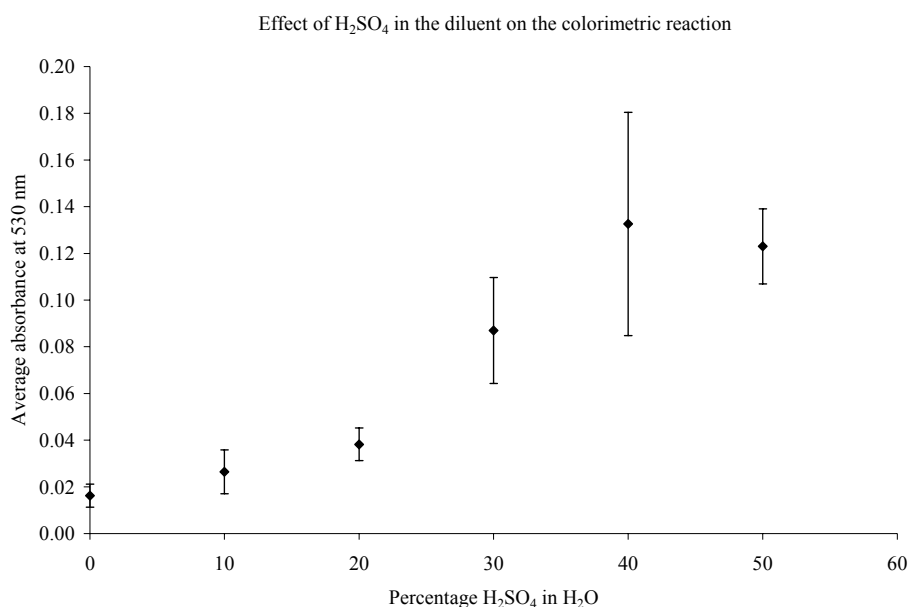
For methanol as a diluent, two parts  $\alpha$ -terpineol solution dissolved in methanol was mixed with one part 2 mg/mL vanillin in concentrated sulfuric acid. A dark purple solution developed instantly upon mixing. Diluting this solution by 1:10 with methanol resulted in a transparent sky blue solution. This colour was similar to the colour obtained in the experiments described in Section A1.3, although the reaction was prepared in a different manner the final diluted colours were similar; the acidity of the sulfuric acid presumably was strong enough to bring about a similar reaction.

2:1:1 concentrated H<sub>2</sub>SO<sub>4</sub>: water: ethanol (v/v/v) had been used as a diluent (Section A1.5) but it had many components to it and their relative importance was unknown. Two aspects were investigated, the importance of the percentage of sulfuric acid (0, 10, 20, 30, 40, 50% H<sub>2</sub>SO<sub>4</sub> in water H<sub>2</sub>O) and ethanol (this was omitted to assess the requirement for ethanol). 40  $\mu$ L of 0.6 mg/mL (-)-menthol in methanol was mixed with 40  $\mu$ L of 2 mg/mL vanillin dissolved in concentrated sulfuric acid. This was mixed with 720  $\mu$ L of a

<sup>34</sup> Tested  $\alpha$ -terpineol.

<sup>35</sup> Tested (-)-menthol.

H<sub>2</sub>SO<sub>4</sub>:H<sub>2</sub>O diluent and then the spectra were measured after waiting for 1 hour; the difference in absorbance at 530 and 800 nm was reported. Each diluent was tested with five replicates. Omitting ethanol did not appear to affect the colour because when either 2:1:1 concentrated H<sub>2</sub>SO<sub>4</sub>: water: ethanol (v/v/v) or 50% H<sub>2</sub>SO<sub>4</sub> in H<sub>2</sub>O was used to dilute the reaction mixtures, a pink colour was developed in both cases. Decreasing the amount of sulfuric acid in the diluent was observed to decrease the intensity of absorbance (Figure A1.5); if water was added the colour disappeared.



**Figure A1.5** Effect of different percentages of sulfuric acid in water on absorbance

The variability of the five replicates was large and it could be related to the time when each component was added. This was investigated using 2:1:1 concentrated H<sub>2</sub>SO<sub>4</sub>: water: ethanol (v/v/v) as the diluent because calibration graphs were available for it, as the menthol calibration for 50% H<sub>2</sub>SO<sub>4</sub> in water had not been carried out.

### A1.7 Fixing the time allowed for colour development

It was observed that the colorimetric reaction colour developed with time (Section A1.3). To investigate how the absorbance changed with time, numerous

spectra of one colorimetric reaction solution were acquired over 15 minutes (Figure A1.6). In a micro-centrifuge tube 400  $\mu\text{L}$  0.1 mg/mL (-)-menthol in methanol was mixed with 400  $\mu\text{L}$  2 mg/mL vanillin in concentrated sulfuric acid. The mixture was shaken at room temperature and a red colour developed straight away. The reaction solution was immediately transferred into a plastic disposable cuvette and multiple spectra were scanned between 400-800 nm on a UV1601 spectrophotometer. (Baseline performed with methanol as the reference). The spectrophotometer was set up to scan the same cuvette at one minute intervals for a total of 15 minutes.

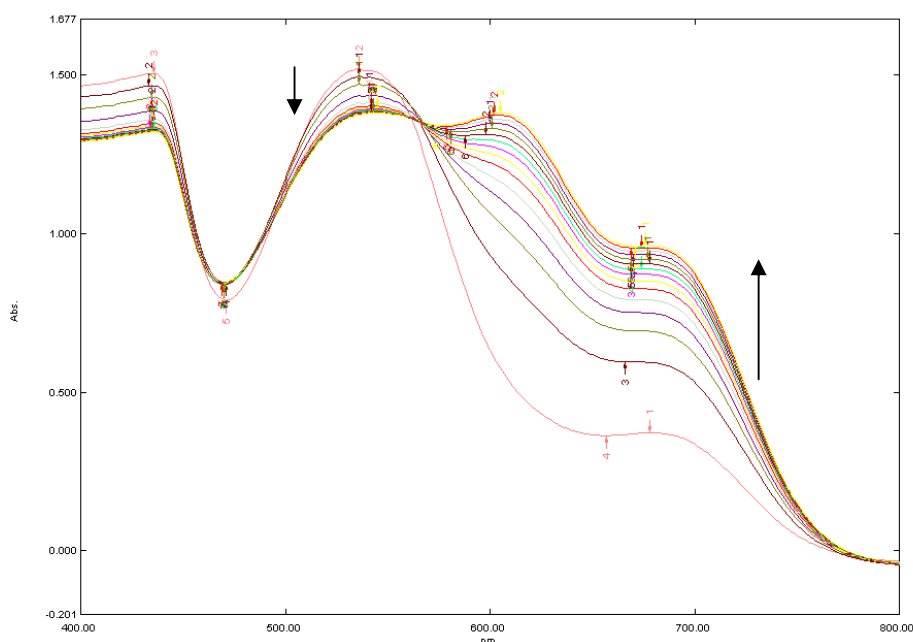


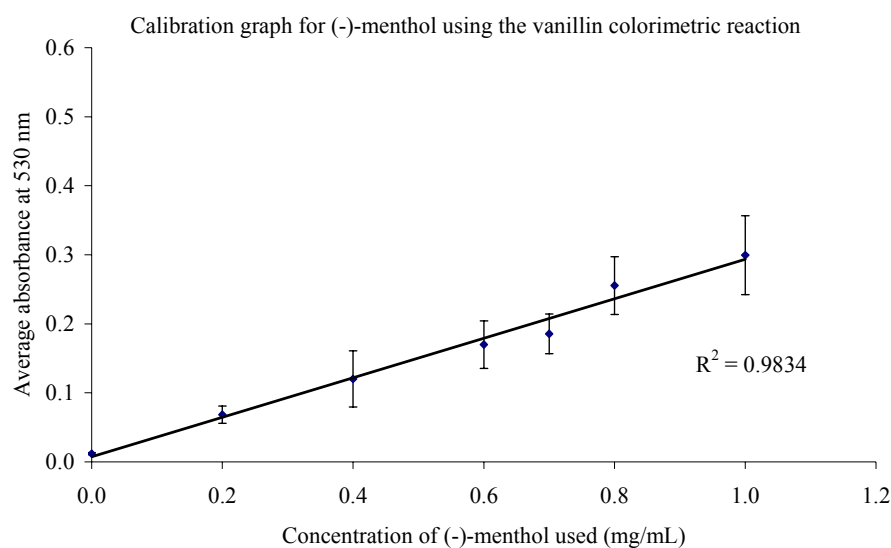
Figure A1.6 Changes in the colorimetric spectra over 15 minutes<sup>36</sup>

The spectra (Figure A1.6) were overlaid and showed that with time the species absorbing at 560 nm decreased slightly while the species absorbing at 620 nm increased the most within 15 minutes.

Since the absorbance profile for the (-)-menthol colorimetric reaction changed quickly with time it was thought necessary to fix the time when each solution was added to the colorimetric reaction. Each (-)-menthol concentration was tested in five replicates; the testing of each (-)-menthol concentration was staggered by approximately 10 minutes so that only five samples were being dealt with for each step. 40  $\mu\text{L}$  (-)-menthol solution (0, 0.2, 0.4, 0.6, 0.8, 1 mg/mL in

<sup>36</sup> Arrows indicated the changes in spectra with increasing time.

methanol) was mixed with 40  $\mu\text{L}$  2 mg/mL vanillin in concentrated sulfuric acid and then after 5 minutes 720  $\mu\text{L}$  2:1:1 concentrated  $\text{H}_2\text{SO}_4$ : water: ethanol (v/v/v) was shaken with the coloured mixture. The colour was allowed to develop for 1 hour before acquiring the spectra on the UV1601 spectrophotometer (performed baseline, between 400-800 nm, with 1:10 methanol in diluent as the reference solution). The difference in absorbance at 530 nm and 800 nm (Table A1.6) was plotted in the calibration graph (Figure A1.7).



**Figure A1.7 Calibration graph for (-)-menthol using the vanillin colorimetric reaction**

A linear calibration graph was obtained for the vanillin colorimetric reaction when tested against (-)-menthol. Fixing the time when each step of the colorimetric reaction was carried appeared to decrease the variability of the absorbance (compare CV% of the 1 hour readings, Table A1.4 and Table A1.6.); however it was still too large to be used to quantitate the terpenes from MIP batch-binding experiments (Figure A1.7).

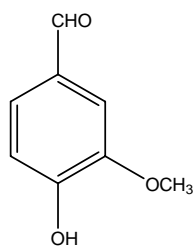
**Table A1.6 Data for (-)-menthol calibration graph**

| (-)-Menthol concentration (mg/mL) | Average Absorbance | Standard deviation | CV%  |
|-----------------------------------|--------------------|--------------------|------|
| 0.0                               | 0.012              | 0.001              | 12.6 |
| 0.2                               | 0.068              | 0.012              | 18.2 |
| 0.4                               | 0.120              | 0.041              | 33.8 |
| 0.6                               | 0.170              | 0.034              | 20.3 |
| 0.7                               | 0.185              | 0.029              | 15.5 |
| 0.8                               | 0.255              | 0.042              | 16.4 |
| 1.0                               | 0.299              | 0.057              | 19.1 |

### A1.8 An attempt to elucidate structure of the coloured species

The structure of the coloured species may help determine a method of stabilising the reaction and stop the colours from changing. However, an attempt to elucidate the structure of the coloured species by  $^1\text{H-NMR}$  spectroscopy did not yield useful structural data. 400  $\mu\text{L}$  of 1.2 mg/mL (-)-menthol in deuterated methanol was mixed with 400  $\mu\text{L}$  of 2 mg/mL vanillin in concentrated sulfuric acid. A dark purple coloured solution developed immediately which turned dark blue overnight. The proton on the hydroxyl group (approximately  $\delta = 11$  ppm) from the acid gave a large broad peak which swamped out the intensity of the other signals. A pre-saturation method was applied to remove the intensity of the hydroxyl signal and the resulting spectra were less dominated by the broad peak at  $\delta = 11$  ppm (Figure A1.9). Aromatic signals were seen in the region of 8-9 ppm; these would have arisen from the aryl protons from vanillin (or its derivative). The signals in the region of 2-3 ppm arose from alkyl protons that originated from (-)-menthol (Figure A1.10); in the  $^1\text{H-NMR}$  spectrum of vanillin alone in sulfuric acid (not shown) there were no peaks observed in this region as expected from the structure of vanillin (Figure A1.8). The peak pattern between 2-3 ppm (Figure A1.9) was different to the peak pattern in (-)-menthol alone; therefore this spectrum showed that (-)-menthol reacted in the acidified vanillin solution. These signals may represent (-)-menthol that had degraded in the acid solution and (-)-menthol that had reacted with vanillin; it is not possible, without further studies, to identify the product of the reaction. The singlet at approximately 5 ppm (inset, Figure A1.9) may have arisen from the methoxy group on vanillin.

Variably deuterated methanol (solvent) and variably deuterated dimethyl ethers may have contributed to the signals seen to the right of the methoxy singlet (inset, Figure A1.9). In spite of the intense colour, the amount of coloured species may have been too low to be detected by  $^1\text{H-NMR}$  spectroscopy.



**Figure A1.8** Structure of vanillin

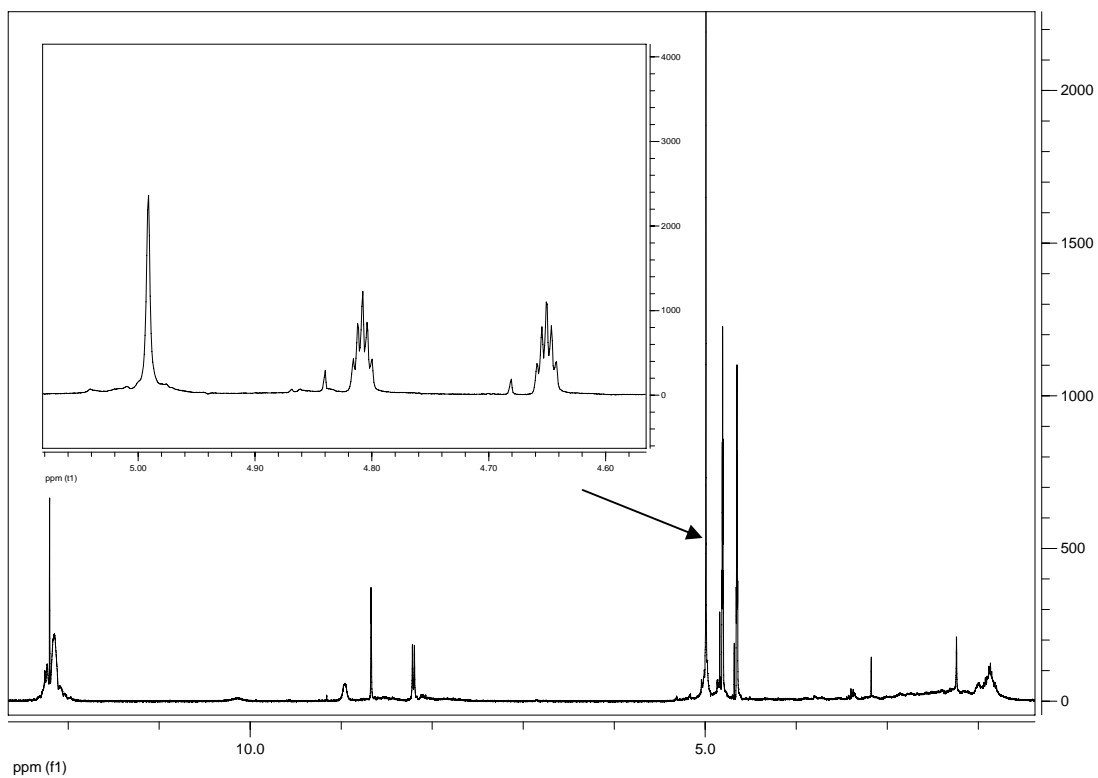


Figure A1.9  $^1\text{H-NMR}$  spectrum of (-)-menthol tested by the vanillin colorimetric reaction

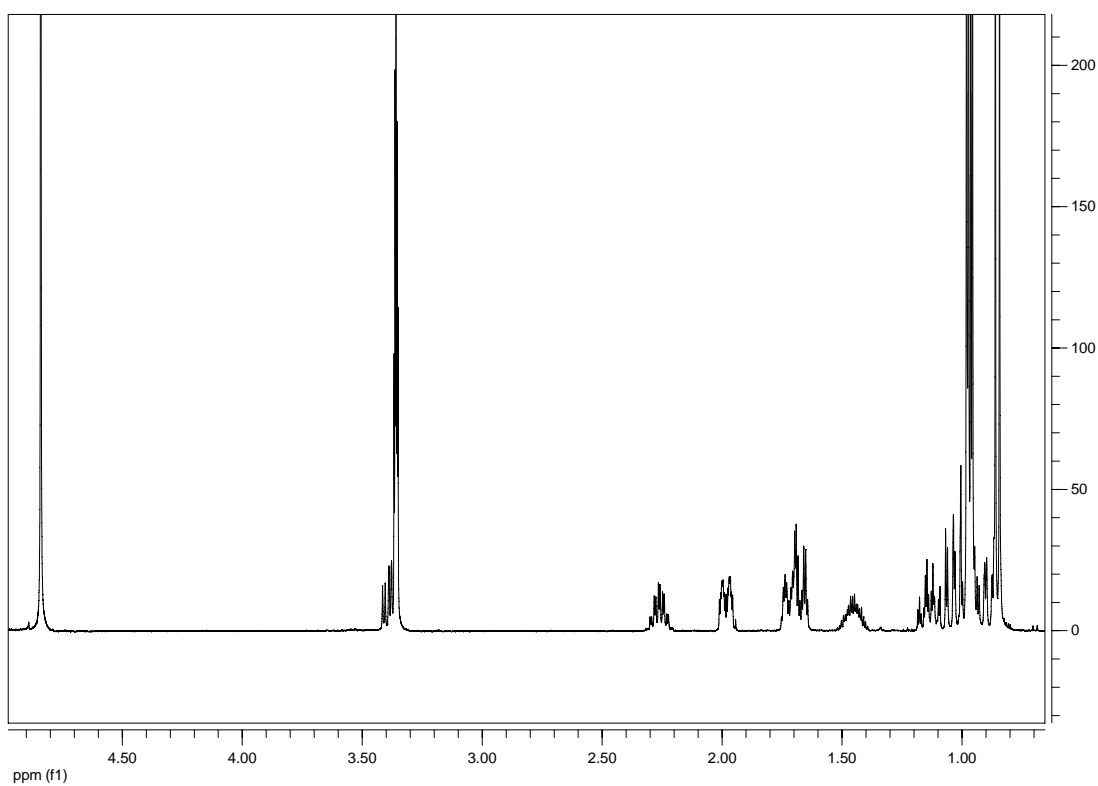


Figure A1.10  $^1\text{H-NMR}$  spectrum of (-)-menthol alone, in deuterated methanol

### **A1.9 Final discussion and conclusion**

The vanillin colorimetric reaction yielded vivid colour changes in the presence of  $\alpha$ -terpineol or (-)-menthol in methanol. Sulfuric acid appeared to be the only acid which could drive the reaction to produce a coloured solution without exposure to external heat; this was probably because of its strong acidity. The reaction between sulfuric acid and an alcohol generated a lot of heat; the absence of proper temperature control meant that there was no absolute consistency in how the samples were treated and thus may have also contributed to the absorbance variability. Although the absorbance values were shown to be concentration-dependent in a linear manner, the variability was too large for this method to be used to detect the binding of terpenes to MIPs. Considerable further development may have been necessary for achieving better reproducibility, however for time limit reasons colorimetry was abandoned in favour of gas chromatographic analysis.

**Appendix Two**  
**Gas chromatography method**

## A2 Gas chromatography method validation

The quantitation of terpenes was carried out using a gas chromatograph (Varian® 3400 with Varian® auto-sampler) coupled to a flame ionisation detector and fitted with a Supelco® SPB™1 capillary column (30 m x 0.2 mm column, 0.2 µm film thickness). The gas flow rates are listed in Table A2.1. The column was held at 60 °C for 1 minute and temperature ramped at 4 °C minute<sup>-1</sup> to 122 °C (0 minute hold time) and then ramped at 50 °C minute<sup>-1</sup> to a final temperature of 250 °C (1 minute hold time). Injector temperature set at 250 °C and detector set at 300 °C. Solvent plug volume was set at 1 µL and injection volume was 1 µL.

**Table A2.1 Gas flow rates for GC-FID**

| Gas             | Flow rates    |
|-----------------|---------------|
| H <sub>2</sub>  | 30 mL/minute  |
| Air             | 300 mL/minute |
| N <sub>2</sub>  | 30 mL/minute  |
| Split flow      | 20 mL/minute  |
| Helium pressure | 29 PSI        |

The GC method was validated for detecting  $\alpha$ -terpineol, terpinolene, (-)-menthol and  $\alpha$ -terpinene by running standard solutions (both single component and mixed component standard solutions) to check their retention times and the FID response to the analytes in hexane, methanol or 50% acetonitrile in methanol. The retention times were similar for both single component and mixed component standard solutions; the times were also similar in different solvents (Table A2.2).

**Table A2.2 Retention times for test terpenes in different solvents<sup>37</sup>**

| Component           | Retention times (minutes $\pm$ 1%) |
|---------------------|------------------------------------|
| $\alpha$ -terpinene | 8.5                                |
| terpinolene         | 10.8                               |
| (-)-menthol         | 13.5                               |
| $\alpha$ -terpineol | 14.0                               |

<sup>37</sup> Retentions times were for analytes in hexane, methanol or 50% acetonitrile in methanol.

Acetonitrile alone was not a suitable solvent because repeat injections of standard solutions were variable; to overcome this acetonitrile was mixed with methanol at different percentages (25, 50 and 75% acetonitrile in methanol). However it was found that 50% acetonitrile in methanol decreased variability more consistently than the other solvent mixtures (Table A2.3), therefore any tests in acetonitrile was diluted with an equal volume of methanol before GC analysis. The calibration graphs of  $\alpha$ -terpineol, terpinolene, (-)-menthol and  $\alpha$ -terpinene were linear in the concentration range of 10-100 ppm in hexane, methanol and 50% acetonitrile in methanol. This range covered the concentrations which were used to test the polymers and also could detect the amount of terpene that remained unbound to the polymers.

**Table A2.3 Average peak area for mixed component standards in different solvents analysed on GC-FID**

| Theoretical (-)-menthol<br>concentration (ppm) | 25% acetonitrile in methanol |        |     | 50% acetonitrile in methanol |         |     | 75% acetonitrile in methanol |        |      | 100% acetonitrile |        |      |
|--|------------------------------|--------|-----|------------------------------|---------|-----|------------------------------|--------|------|-------------------|--------|------|
|  | Average                      | Stdev  | CV% | Avg                          | Stdev   | CV% | Average                      | Stdev  | CV%  | Average           | Stdev  | CV%  |
| 5  | x                            | x      | x   | 1906.5                       | 87.0    | 4.6 | x                            | x      | x    | x                 | x      | x    |
| 10   | 3796.7                       | 162.4  | 4.3 | x                            | x       | x   | 4849.7                       | 295.8  | 6.1  | 5054.7            | 377.1  | 7.5  |
| 25   | x                            | x      | x   | 12351.0                      | 151.3   | 1.2 | x                            | x      | x    | 13149.0           | 485.2  | 3.7  |
| 50   | 26074.0                      | 522.9  | 2.0 | 26585.0                      | 663.3   | 2.5 | 31675.3                      | 157.6  | 0.5  | 29977.3           | 1826.2 | 6.1  |
| 80   | x                            | x      | x   | x                            | x       | x   | x                            | x      | x    | 47408.7           | 4877.3 | 10.3 |
| 100  | 50793.3                      | 2696.3 | 5.3 | x                            | x       | x   | 51728.0                      | 8009.5 | 15.5 | 63814.0           | 5465.9 | 8.6  |
| 125  | x                            | x      | x   | 391138.5                     | 8104.2  | 2.1 | x                            | x      | x    | x                 | x      | x    |
| 200  | x                            | x      | x   | x                            | x       | x   | x                            | x      | x    | 140053.0          | 4712.6 | 3.4  |
| 250  | 137191.7                     | 9556.1 | 7.0 | 768386.0                     | 72512.4 | 9.4 | 153809.3                     | 893.5  | 0.6  | x                 | x      | x    |
| 500  | 304062.0                     | 8890.5 | 2.9 | x                            | x       | x   | 313166.7                     | 2083.2 | 0.7  | x                 | x      | x    |

| Theoretical $\alpha$ -terpineol<br>concentration (ppm) | 25% acetonitrile in methanol |        |      | Acetonitrile |         |     | 75% acetonitrile in methanol |        |      | 100% acetonitrile |        |     |
|--|------------------------------|--------|------|--------------|---------|-----|------------------------------|--------|------|-------------------|--------|-----|
|  | Average                      | Stdev  | CV%  | Avg          | Stdev   | CV% | Average                      | Stdev  | CV%  | Average           | Stdev  | CV% |
| 5  | x                            | x      | x    | 1575.0       | 5.7     | 0.4 | x                            | x      | x    | x                 | x      | x   |
| 10   | 3128.3                       | 459.0  | 14.7 | x            | x       | x   | 4254.0                       | 585.3  | 13.8 | 4568.7            | 420.5  | 9.2 |
| 25   | x                            | x      | x    | 11377.5      | 120.9   | 1.1 | x                            | x      | x    | 12436.7           | 446.1  | 3.6 |
| 50   | 25195.7                      | 489.0  | 1.9  | 25328.0      | 705.7   | 2.8 | 30479.0                      | 207.5  | 0.7  | 28645.3           | 1709.4 | 6.0 |
| 80   | x                            | x      | x    | x            | x       | x   | x                            | x      | x    | 45534.0           | 4509.5 | 9.9 |
| 100  | 49132.3                      | 3306.5 | 6.7  | x            | x       | x   | 49587.0                      | 7684.6 | 15.5 | 61295.7           | 4827.7 | 7.9 |
| 125  | x                            | x      | x    | 351413.0     | 3597.8  | 1.0 | x                            | x      | x    | x                 | x      | x   |
| 200  | x                            | x      | x    | x            | x       | x   | x                            | x      | x    | 133278.7          | 4247.3 | 3.2 |
| 250  | 131963.0                     | 8678.0 | 6.6  | 673353.0     | 64271.8 | 9.5 | 147623.3                     | 335.8  | 0.2  | x                 | x      | x   |
| 500  | 289782.7                     | 7367.4 | 2.5  | x            | x       | x   | 297503.7                     | 566.7  | 0.2  | x                 | x      | x   |

**Appendix Three**  
**Synthesis and characterisation of the**  
**semi-covalent monomer**

## A3 Synthesis and characterisation of the template-monomer for the semi-covalent MIP

### A3.1 Synthesis and characterisation of menthyl chloroformate

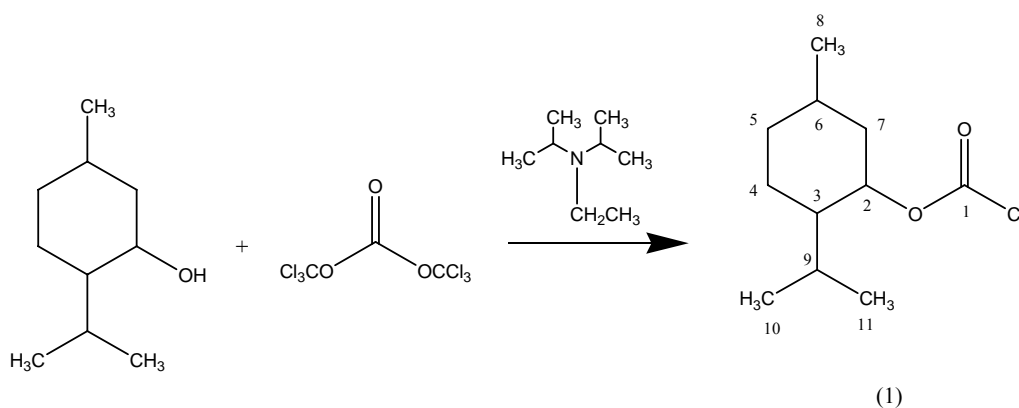


Figure A3.1 Synthesis of menthyl chloroformate

*CAUTION: triphosgene can form the hazardous gas, phosgene, and must always be handled in a fume hood with protective safety goggles and safety gloves.*

All chemicals were used as received. In a 3 neck flask, 0.962 g triphosgene (0.962 g) was dissolved in toluene (25 mL) under  $N_2$  while kept cool. (-)-Menthol (1.25 g) and diisopropylethylamine (Hünig's base; 1.3 mL) were dissolved in toluene (10 mL). This mixture was added drop-wise over 10 minutes. The reaction was monitored against the reactants by TLC using an eluant of dichloromethane/cyclohexane (2:5 v/v) and allowed to proceed until finished (24 hours). After the reaction was completed the product mixture was vacuum-dried to remove the solvent. The cream coloured solid was characterised by NMR spectroscopy (Figure A3.2).  $C^{13}$ -NMR ( $CDCl_3$ )  $\delta$  (ppm)<sup>38</sup>: 16.6, (C-8); 20.8 and 22.1 (C-10/C-11); 23.8 (C-4); 26.7 (C-9); 31.8 (C-6); 34.1 (C-5); 40.5 (C-7); 47.2 (C-3); 84.4 (C-2); 150.2 (C-1). Signals at 54.1, 42.2, 18.9, 17.7, and 12.4 ppm may indicate impurity from salt by-products and diisopropylethylamine. The crude product (1) was used without purification for the synthesis of the menthyl 4-vinylphenyl carbonate (Section A3.3).

<sup>38</sup> Assignments were aided by ChemDraw Ultra NMR prediction tool and NMR data in [90].

The attempt to synthesise  $\alpha$ -terpinyl chloroformate was not successful because the carbonyl peak was not present. Therefore only (-)-menthyl chloroformate was used to synthesise the 4-vinylphenyl carbonate to be used as the template-monomer for semi-covalent imprinting of menthol.

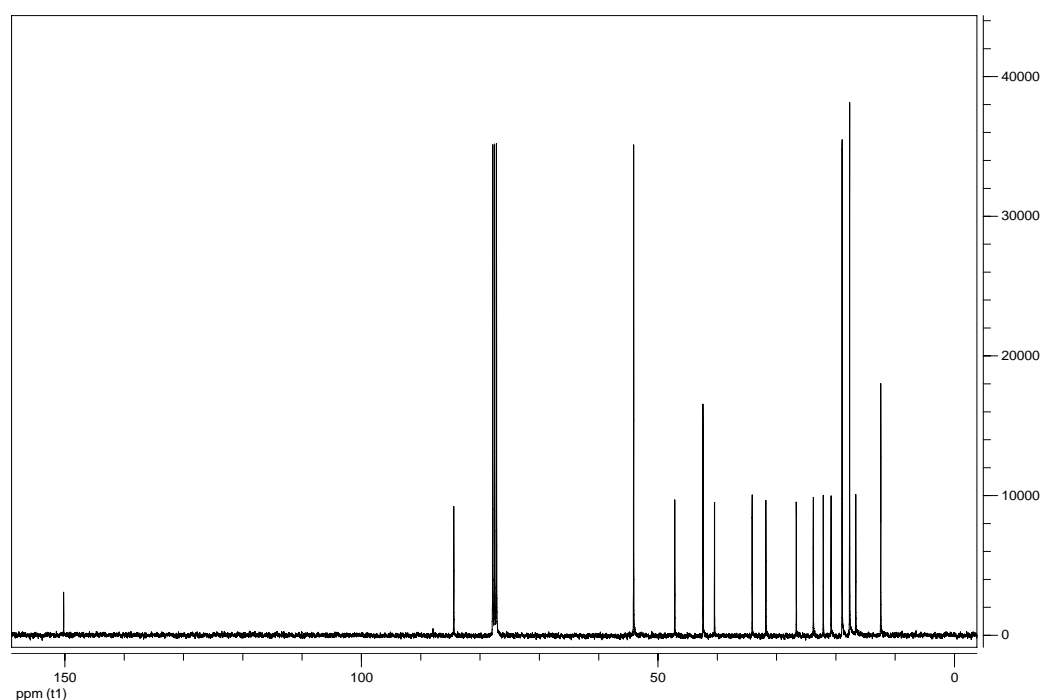


Figure A3.2  $^{13}\text{C}$ -NMR for menthyl chloroformate<sup>39</sup>

### A3.2 Synthesis of 4-vinylphenol

4-Vinylphenol is not stable, therefore it was synthesised fresh from *p*-acetoxystyrene and potassium hydroxide. The protocol used was adapted from the method in [55]. *p*-Acetoxystyrene (10.5 mL) was pipetted into a round bottom flask, sitting in an ice bath, and the reaction was started by adding drop-wise  $0.156 \text{ g mL}^{-1}$  potassium hydroxide in water (88 mL) with continuous stirring over 10 minutes. The reaction was checked by running TLC against *p*-acetoxystyrene dissolved in chloroform (eluant: cyclohexane/dichloromethane; 1:5 v/v) and checking the plates under an ultraviolet lamp. It was found that the reaction was not complete after 4 hours of stirring, therefore more potassium hydroxide (10-20

<sup>39</sup> 30 degree pulse, 303 K, 400 MHz NMR.

pellets) was added and the mixture was allowed to stir overnight. Once no *p*-acetoxystyrene was detected by UV/TLC the mixture was acidified by stirring in solid CO<sub>2</sub> (dry ice) until the pH reached 8, this was monitored by testing a small drop on JT Baker pHIX strips (pH 0-14). The product was crystallised from boiling hexane before use; no characterisation was carried out because the synthetic protocol had been used routinely in the past.

### A3.3 Synthesis and characterisation of menthyl 4-vinylphenyl carbonate

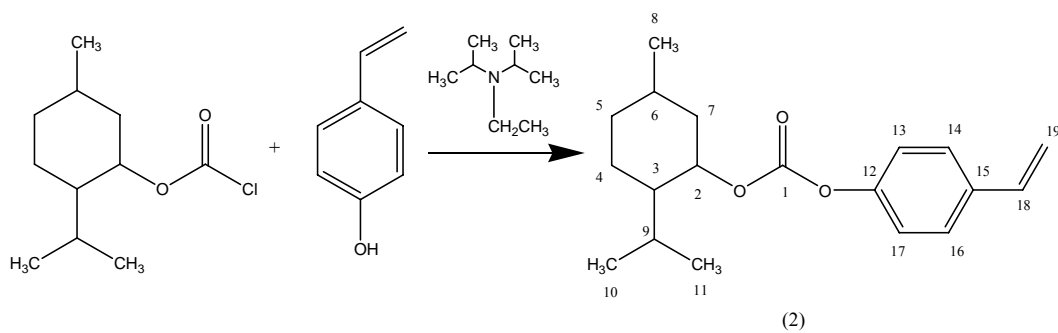


Figure A3.3 Synthesis of menthyl 4-vinylphenyl carbonate

4-Vinylphenol (1.2 g) in toluene was mixed with diisopropylethylamine (3.2 mL), then menthyl chloroformate was added drop-wise over 30 minutes under nitrogen atmosphere while the reaction was maintained at 0 °C. The reaction was allowed to proceed until considered complete by comparing the reaction mixture with the starting material using TLC (eluant: dichloromethane/hexane; 2:5 v/v). The crude product was purified using a silica column (eluant: dichloromethane/hexane; 3:10 v/v). The resulting colourless oil was characterised by NMR spectroscopy (Figure A3.3). <sup>13</sup>C-NMR (CDCl<sub>3</sub>) δ (ppm)<sup>40</sup>: 16.7 (C-8); 21.0 and 22.2 (C-10/C11); 23.7 (C-4); 34.4 (C-5); 41.0 (C-7); 47.4 (C-3); 79.8 (C-2); 114.3 (C-19); 121.4 (C-13, C-17); 127.4 (C-14, C-16); 135.6 (C-15); 136.1 (C-18); 151.1 (C-12); 153.5 (C-1). Signals at 30.6 and 31.7 ppm may indicate impurity in the product, signals at 128.5 and 129.3 may have arisen from the presence of a small amount of other isomers of vinylphenol. The monomer was subsequently used to prepare the semi-covalent MIP.

<sup>40</sup> Assignments were aided by ChemDraw Ultra NMR prediction tool and NMR data in [91].

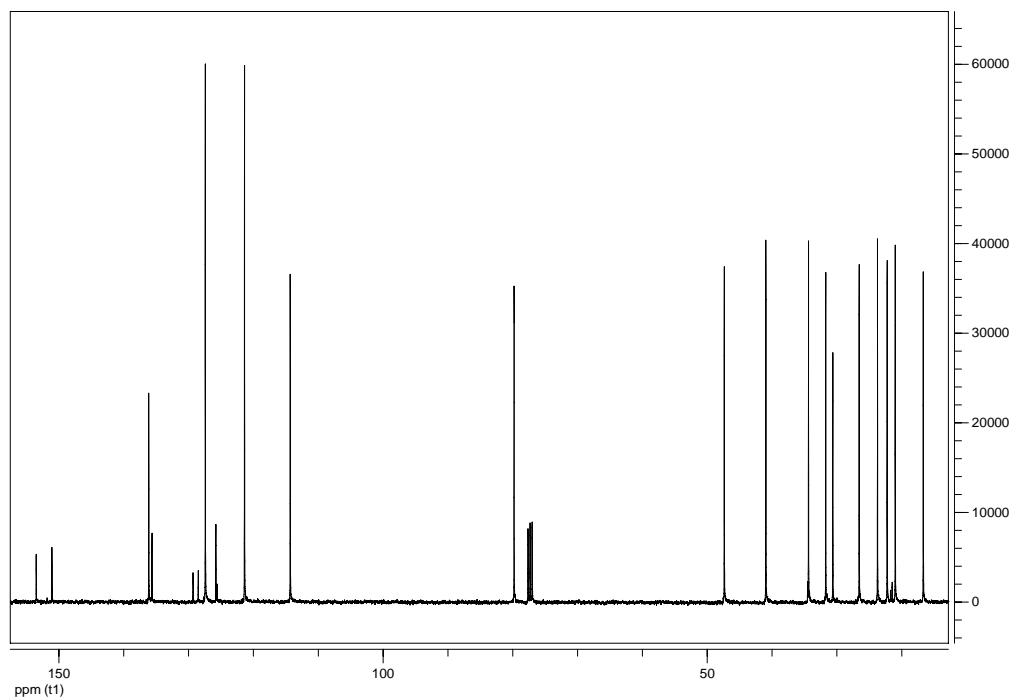


Figure A3.4  $^{13}\text{C}$ -NMR spectra of menthyl 4-vinylphenyl carbonate<sup>41</sup>

<sup>41</sup> 70 degree pulse, 303 K, 400 MHz NMR.

**Appendix Four**  
**Polymer composition**

## A4 Polymer composition

**Table A4.1 Composition of block polymers**

*All were polymerised for approximately 24 hours. Template was extracted by soxhlet extraction in ethanol for approximately 24 hours<sup>‡</sup>.*

| Code name                         | Cross-linker (X) | Template (T)        | Functional monomer (FM) | Temperature of initiation (°C) | Particle size range collected (µm) | Amount T (mol) | Amount FM (mol) | Amount X (mol) | Mole ratio T | Mole ratio FM | Mole ratio X | AIBN # | Porogen loading § |
|-----------------------------------|------------------|---------------------|-------------------------|--------------------------------|------------------------------------|----------------|-----------------|----------------|--------------|---------------|--------------|--------|-------------------|
| t4-MIP <sup>*</sup>               | EDMA             | $\alpha$ -terpineol | MAA                     | 60                             | fines,<38 and 8-38                 | 0.00010        | 0.00041         | 0.00196        | 1            | 4.1           | 19.6         | 1      | 2.1               |
| t4-reference polymer <sup>*</sup> | EDMA             | x                   | MAA                     | 60                             | fines,<38 and 8-38                 | x              | 0.00042         | 0.00196        | x            | 4.2           | 19.6         | 1      | 2.1               |
| t5-MIP <sup>*</sup>               | DVB              | $\alpha$ -terpineol | MAA                     | 60                             | fines,<38 and 8-38                 | 0.00016        | 0.00063         | 0.00303        | 1            | 3.9           | 19           | 1      | 2.5               |
| t5-reference polymer <sup>*</sup> | DVB              | x                   | MAA                     | 60                             | fines,<38 and 8-38                 | x              | 0.00064         | 0.00302        | x            | 4             | 18.9         | 1      | 2.1               |
| t6-MIP <sup>*</sup>               | TRIM             | $\alpha$ -terpineol | MAA                     | 60                             | fines,<38 and 8-38                 | 0.00006        | 0.00024         | 0.00114        | 1            | 4             | 19           | 1.3    | 2                 |
| t6-reference polymer <sup>*</sup> | TRIM             | x                   | MAA                     | 60                             | fines,<38 and 8-38                 | x              | 0.00025         | 0.00115        | x            | 4.2           | 19.2         | 1.3    | 2                 |

<sup>‡</sup> Mole ratio calculated relative to the template, for the reference polymer it is relative to the template in the corresponding MIP.

<sup>#</sup> Percentage of total monomer mass.

<sup>§</sup> mL/g of total monomer mass.

<sup>\*</sup> N<sub>2</sub> purge before polymerization and thermally initiated in a water bath. All others were not purged with N<sub>2</sub> and were thermally initiated in a sand bath.

**Table A4.1 (continued) Composition of block polymers**

All were polymerised for approximately 24 hours. Template was extracted by soxhlet extraction in ethanol for approximately 24 hours<sup>‡</sup>.

| Code name                          | Cross-linker (X) | Template (T)        | Functional monomer (FM)              | Temperature of initiation (°C) | Particle size range collected (µm) | Amount T (mol) | Amount FM (mol) | Amount X (mol) | Mole ratio T | Mole ratio FM | Mole ratio X | AIBN # | Porogen loading § |
|------------------------------------|------------------|---------------------|--------------------------------------|--------------------------------|------------------------------------|----------------|-----------------|----------------|--------------|---------------|--------------|--------|-------------------|
| terpin-MIP                         | EDMA             | $\alpha$ -terpineol | MAA                                  | 72                             | 38-150                             | 0.00010        | 0.00041         | 0.00198        | 1.0          | 4.1           | 19.8         | 1.2    | 2.3               |
| terpin-reference polymer           | EDMA             | x                   | MAA                                  | 72                             | 38-150                             | x              | 0.00041         | 0.00198        | x            | 4.1           | 19.8         | 1.2    | 2.3               |
| t11-MIP                            | EDMA             | $\alpha$ -terpineol | Bilirubin                            | 90                             | <38 and 38-150                     | 0.00014        | 0.00005         | 0.00313        | 1            | 0.4           | 22.4         | 3      | 4.6               |
| t11-reference polymer              | EDMA             | x                   | Bilirubin                            | 90                             | <38 and 38-150                     | x              | 0.00005         | 0.00313        | x            | 0.4           | 22.4         | 3      | 4.6               |
| m9-MIP                             | EDMA             | (-)-menthol         | MAA                                  | 72                             | <38 and 38-150                     | 0.00013        | 0.00071         | 0.00477        | 1            | 5.5           | 36.7         | 1      | 0.9               |
| m9-reference polymer               | EDMA             | x                   | MAA                                  | 72                             | <38 and 38-150                     | x              | 0.00071         | 0.00477        | x            | 5.5           | 36.7         | 1      | 0.9               |
| t8-MIP                             | EDMA             | $\alpha$ -terpineol | Bilirubin                            | not recorded                   | <38 and 38-150                     | 0.00012        | 0.00005         | 0.00313        | 1            | 0.4           | 26.1         | 3.1    | 4.6               |
| t8-reference polymer               | EDMA             | x                   | Bilirubin                            | not recorded                   | <38 and 38-150                     | x              | 0.00005         | 0.00313        | x            | 0.4           | 26.1         | 3.1    | 4.6               |
| t10-MIP                            | EDMA             | $\alpha$ -terpineol | HEMA                                 | not recorded                   | <38 and 38-150                     | 0.0001         | 0.00036         | 0.00313        | 1            | 3.6           | 31.3         | 3      | 4.5               |
| t10-reference polymer              | EDMA             | x                   | HEMA                                 | not recorded                   | <38 and 38-150                     | x              | 0.00036         | 0.00313        | x            | 3.6           | 31.3         | 3      | 4.5               |
| m10-MIP <sup>†</sup>               | EDMA             | $\alpha$ -terpineol | (-)-Menthyl 4-vinyl phenyl carbonate | not recorded                   | <38 and 38-150                     | x              | 0.00081         | 0.01622        | x            | 1             | 20           | 1.4    | 1.9               |
| m10-reference polymer <sup>†</sup> | EDMA             | x                   | 4-Vinylphenol                        | not recorded                   | <38 and 38-150                     | x              | 0.00081         | 0.01622        | x            | 1             | 20           | 1.4    | 1.9               |

<sup>‡</sup> Mole ratio calculated relative to the template, for the reference polymer it is relative to the template in the corresponding MIP; except for m10 it was calculated relative the functional monomer.

<sup>#</sup> Percentage of total monomer mass.

<sup>§</sup> mL/g of total monomer mass.

<sup>†</sup> Semi-covalent MIPs. All other polymers in this table were non-covalent MIPs.

**Table A4.2 Composition of precipitation polymers**

*All were thermally initiated in a sand bath. Template extraction details can be found in Table A4.4<sup>‡</sup>.*

| Code name             | Cross-linker (X) | Template (T)        | Functional monomer (FM) | Temperature of initiation (°C) | Approximate length of polymerisation time (hours) | Amount T (mol) | Amount FM (mol) | Amount X (mol) | Mole ratio T | Mole ratio FM | Mole ratio X | AIBN <sup>#</sup> | Porogen loading <sup>§</sup> |
|-----------------------|------------------|---------------------|-------------------------|--------------------------------|---|----------------|-----------------|----------------|--------------|---------------|--------------|-------------------|------------------------------|
| t15-MIP               | EDMA             | $\alpha$ -terpineol | MAA                     | 70                             | 3.25  | 0.00157        | 0.00101         | 0.00586        | 1            | 1.6           | 3.7          | 2.4               | 4                            |
| t15-reference polymer | EDMA             | x                   | MAA                     | 70                             | 3.25  | x              | 0.00101         | 0.00586        | x            | 1.6           | 3.7          | 2.4               | 4                            |
| t16-MIP               | EDMA             | $\alpha$ -terpineol | MAA                     | 60                             | 3   | 0.00008        | 0.00051         | 0.00293        | 1            | 6.4           | 36.6         | 2.4               | 8                            |
| t16-reference polymer | EDMA             | x                   | MAA                     | 60                             | 3   | x              | 0.00051         | 0.00293        | x            | 6.4           | 36.6         | 2.4               | 8                            |
| m17-MIP               | EDMA             | (-)-menthol         | MAA                     | 80                             | 17  | 0.00008        | 0.00051         | 0.00293        | 1            | 6.4           | 36.6         | 2.4               | 8                            |
| m17-reference polymer | EDMA             | x                   | MAA                     | 80                             | 17  | x              | 0.00051         | 0.00293        | x            | 6.4           | 36.6         | 2.4               | 8                            |

<sup>‡</sup> Mole ratio calculated relative to the template, for a reference polymer it is relative to the template in the corresponding MIP.

<sup>#</sup> Percentage of total monomer mass.

<sup>§</sup> mL/g of total monomer mass.

**Table A4.3 Composition of precipitation polymers prepared by porogen-imprinting**

*All were thermally initiated in a sand bath. Template extraction details can be found in Table A4.4.*

| Code name             | Cross-linker (X) | Porogen             | Functional monomer (FM) | Temperature of initiation (°C) | Approximate length of polymerisation time (hours) | Amount FM (mol) | Amount X (mol) | AIBN <sup>#</sup> | Porogen loading <sup>§</sup> |
|-----------------------|------------------|---------------------|-------------------------|--------------------------------|---|-----------------|----------------|-------------------|------------------------------|
| t18-MIP               | EDMA             | $\alpha$ -terpineol | MAA                     | 70                             | overnight   | 0.00101         | 0.00586        | 2.4               | 4                            |
| t18-reference polymer | EDMA             | cyclohexane         | MAA                     | 70                             | overnight   | 0.00101         | 0.00586        | 2.4               | 4                            |
| t19-MIP               | EDMA             | $\alpha$ -terpineol | x                       | 70                             | overnight   | x               | 0.00586        | 2.6               | 4.3                          |
| t19-reference polymer | EDMA             | cyclohexane         | x                       | 70                             | overnight   | x               | 0.00586        | 2.6               | 4.3                          |
| t24-MIP               | EDMA             | $\alpha$ -terpineol | MAA                     | 70                             | overnight   | 0.00101         | 0.00586        | 2.4               | 4                            |
| t24-reference polymer | EDMA             | cyclohexane         | MAA                     | 70                             | overnight   | 0.00101         | 0.00586        | 2.4               | 4                            |

<sup>#</sup> Percentage of total monomer mass.

<sup>§</sup> mL/g of total monomer mass.

Table A4.4 Template extraction protocol for precipitation polymers

| Solvent (used in the order listed below)  | t15              |                 | t16             |                 | m17             |                 | t18              |                 | t19             |                 | t24             |                 |
|---|------------------|-----------------|-----------------|-----------------|-----------------|-----------------|------------------|-----------------|-----------------|-----------------|-----------------|-----------------|
|   | MIP              | Reference       | MIP             | Reference       | MIP             | Reference       | MIP              | Reference       | MIP             | Reference       | MIP             | Reference       |
| Ethanol                                   | 0                | 0               | 0               | 0               | 0               | 0               | 0                | 3               | 0               | 3               | 0               | 3               |
| Acetonitrile                              | 0                | 0               | 0               | 0               | 1               | 1               | 0                | 0               | 0               | 0               | 0               | 0               |
| 1% glacial acetic acid in methanol        | 3                | 3               | 3               | 3               | 3               | 3               | 6                | 3               | 6               | 3               | 6               | 3               |
| Methanol                                  | 1                | 1               | 1               | 1               | 2               | 2               | 6                | 4               | 6               | 4               | 4               | 4               |
| Acetone                                   | 1                | 1               | 1               | 1               | 1               | 1               | 2                | 2               | 2               | 2               | 3               | 2               |
| <b>Other details</b>                      |                  |                 |                 |                 |                 |                 |                  |                 |                 |                 |                 |                 |
| Sonicate (minutes)                        | x                | x               | x               | x               | 5               | 5               | 15               | 15              | 15              | 15              | <5              | <5              |
| Shake (minutes, at approximately 560 rpm) | x                | x               | x               | x               | 25              | 25              | 15               | 15              | 15              | 15              | 30              | 30              |
| Centrifuge (minutes)                      | 30 <sup>42</sup> | 30 <sup>a</sup> | 30 <sup>a</sup> | 30 <sup>a</sup> | 30 <sup>a</sup> | 30 <sup>a</sup> | 30 <sup>43</sup> | 30 <sup>b</sup> | 30 <sup>b</sup> | 30 <sup>b</sup> | 30 <sup>b</sup> | 30 <sup>b</sup> |
| Oven (hours)                              | x                | x               | x               | x               | x               | x               | 4                | 1               | 4               | 1               | x               | x               |

<sup>42</sup> Centrifuged at 2000 rpm.<sup>43</sup> Centrifuged at 2400 rpm.

## **Appendix Five**

### **NMR titration**

## A5 <sup>1</sup>H-NMR titration

The *volume added* column (Table A5.1) shows the volume of 2.1 M acetic acid solution (containing 0.03 M  $\alpha$ -terpineol in CDCl<sub>3</sub>) titrated against 500  $\mu$ L 0.03 M  $\alpha$ -terpineol in CDCl<sub>3</sub> in an NMR tube. To investigate higher values of M/T (ratio of concentration of  $\alpha$ -terpineol to the concentration of acetic acid), volumes in the *volume added* column (Table A5.2) were used. These were the volumes of 5 M acetic acid solution (containing 0.03 M  $\alpha$ -terpineol in CDCl<sub>3</sub>) titrated against 500  $\mu$ L 0.03 M  $\alpha$ -terpineol in CDCl<sub>3</sub> in an NMR tube. Details of these experiments have been outlined in Section 2.4.

The *volume added* column (Table A5.3) shows the volume of 5 M phenol (containing 0.03 M (-)-menthol) added to 500  $\mu$ L 0.03 M (-)-menthol in CDCl<sub>3</sub> in an NMR tube to investigate the interactions between (-)-menthol and phenol; the experiment was outlined in Section 2.4.

**Table A5.1 Volumes of 2.1 M acetic acid added to 0.03 M  $\alpha$ -terpineol in CDCl<sub>3</sub> solution for the <sup>1</sup>H-NMR titration experiment**

| Experiment number | Volume added ( $\mu$ L) | Total volume added ( $\mu$ L) | Total volume in NMR tube ( $\mu$ L) | Concentration of $\alpha$ -terpineol (M) | Concentration of acetic acid (M) | M/T ratio |
|-------------------|-------------------------|-------------------------------|-------------------------------------|--|----------------------------------|-----------|
| 1                 | 0                       | 0                             | 500                                 | 0.03                                     | 0.00                             | 0.0       |
| 2                 | 3.5                     | 3.5                           | 503.5                               | 0.03                                     | 0.01                             | 0.5       |
| 3                 | 4                       | 7.5                           | 507.5                               | 0.03                                     | 0.03                             | 1.0       |
| 4                 | 3.5                     | 11                            | 511                                 | 0.03                                     | 0.05                             | 1.5       |
| 5                 | 4                       | 15                            | 515                                 | 0.03                                     | 0.06                             | 2.0       |
| 6                 | 3.5                     | 18.5                          | 518.5                               | 0.03                                     | 0.07                             | 2.5       |
| 7                 | 12                      | 30.5                          | 530.5                               | 0.03                                     | 0.12                             | 4.0       |
| 8                 | 8.5                     | 39                            | 539                                 | 0.03                                     | 0.15                             | 5.1       |
| 9                 | 53.5                    | 92.5                          | 592.5                               | 0.03                                     | 0.33                             | 10.9      |
| 10                | 116                     | 208.5                         | 708.5                               | 0.03                                     | 0.62                             | 20.6      |
| 11                | 500                     | 708.5                         | 1208.5                              | 0.03                                     | 1.23                             | 41.0      |

**Table A5.2 Volumes of 5 M acetic acid added to 0.03 M  $\alpha$ -terpineol in CDCl<sub>3</sub> solution for the <sup>1</sup>H-NMR titration experiment**

| Experiment number | Volume added ( $\mu$ L) | Total volume added ( $\mu$ L) | Total volume in NMR tube ( $\mu$ L) | Concentration of $\alpha$ -terpineol (M) | Concentration of acetic acid (M) | M/T ratio |
|-------------------|-------------------------|-------------------------------|-------------------------------------|--|----------------------------------|-----------|
| 1                 | 0                       | 0                             | 500                                 | 0.03                                     | 0.00                             | 0.0       |
| 2                 | 68                      | 68                            | 568                                 | 0.03                                     | 0.60                             | 20.0      |
| 3                 | 90                      | 158                           | 658                                 | 0.03                                     | 1.20                             | 40.0      |
| 4                 | 56                      | 214                           | 714                                 | 0.03                                     | 1.50                             | 50.0      |
| 5                 | 67                      | 281                           | 781                                 | 0.03                                     | 1.80                             | 60.0      |
| 6                 | 81                      | 362                           | 862                                 | 0.03                                     | 2.10                             | 70.0      |
| 7                 | 388                     | 750                           | 1250                                | 0.03                                     | 3.00                             | 100.0     |

**Table A5.3 Volumes of 5 M phenol added to 0.03 M (-)-menthol in CDCl<sub>3</sub> solution for the <sup>1</sup>H-NMR titration experiment**

| Experiment number | Volume added ( $\mu$ L) | Total volume added ( $\mu$ L) | Total volume in NMR tube ( $\mu$ L) | Concentration of (-)-menthol (M) | Concentration of phenol (M) | M/T ratio |
|-------------------|-------------------------|-------------------------------|-------------------------------------|----------------------------------|-----------------------------|-----------|
| 1                 | 0                       | 0                             | 500                                 | 0.03                             | 0.00                        | 0.0       |
| 2                 | 0.5                     | 0.5                           | 500.5                               | 0.03                             | 0.00                        | 0.2       |
| 3                 | 1                       | 1.5                           | 501.5                               | 0.03                             | 0.01                        | 0.5       |
| 4                 | 1.5                     | 3                             | 503                                 | 0.03                             | 0.03                        | 1.0       |
| 5                 | 3                       | 6                             | 506                                 | 0.03                             | 0.06                        | 2.0       |
| 6                 | 6.5                     | 12.5                          | 512.5                               | 0.03                             | 0.12                        | 4.1       |
| 7                 | 6                       | 18.5                          | 518.5                               | 0.03                             | 0.18                        | 5.9       |
| 8                 | 13.5                    | 32                            | 532                                 | 0.03                             | 0.30                        | 10.0      |
| 9                 | 36                      | 68                            | 568                                 | 0.03                             | 0.60                        | 20.0      |
| 10                | 90                      | 158                           | 658                                 | 0.03                             | 1.20                        | 40.0      |
| 11                | 123                     | 281                           | 781                                 | 0.03                             | 1.80                        | 60.0      |
| 12                | 469                     | 750                           | 1250                                | 0.03                             | 3.00                        | 100.0     |

**Appendix Six**

**SEM images**

## A6 SEM images of polymers

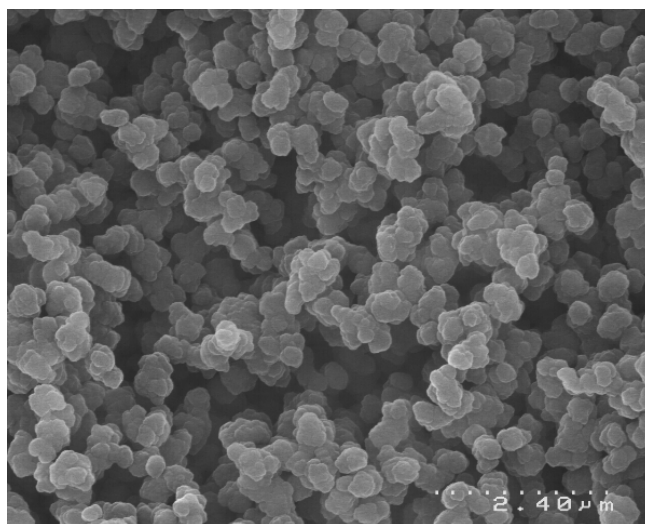


Figure A6.1 SEM image of m17-MIP

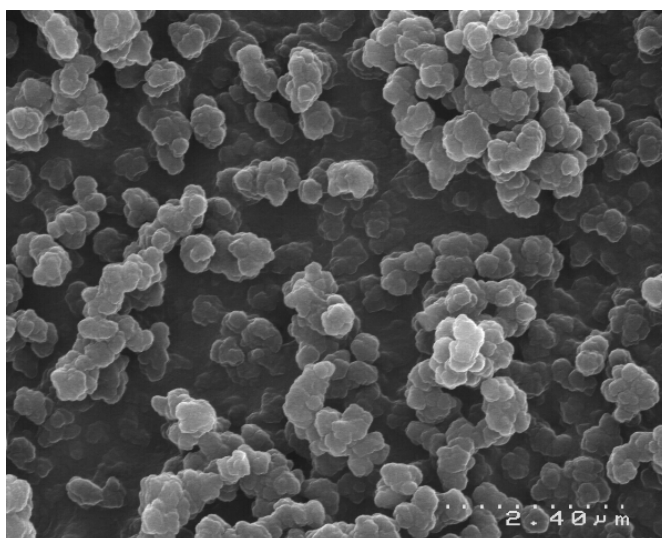
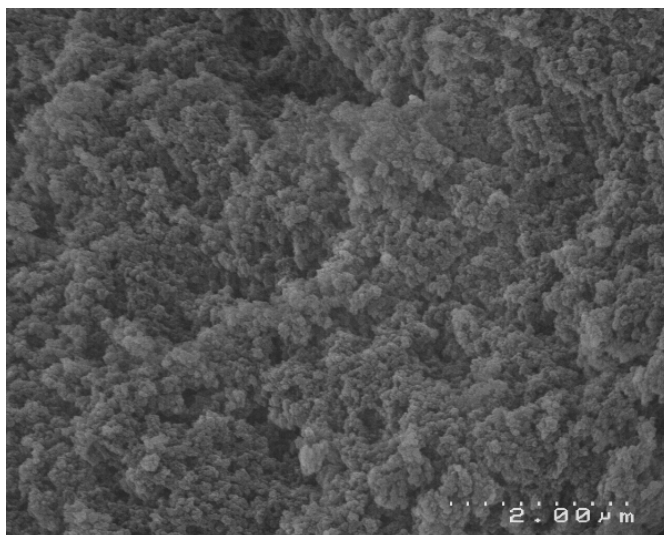
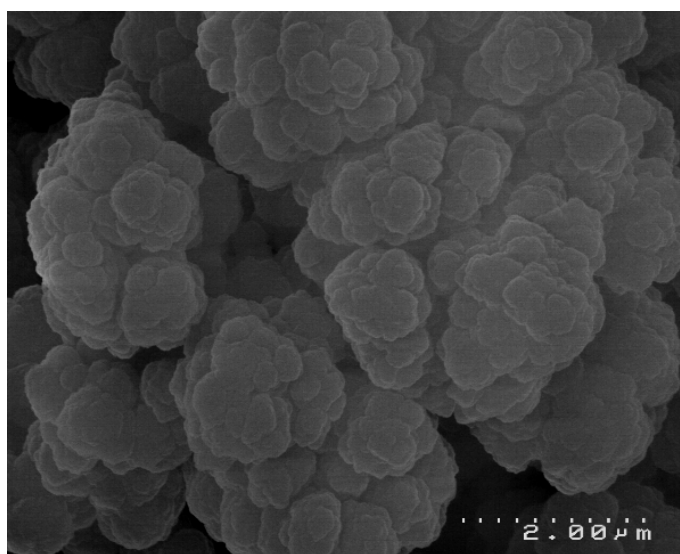


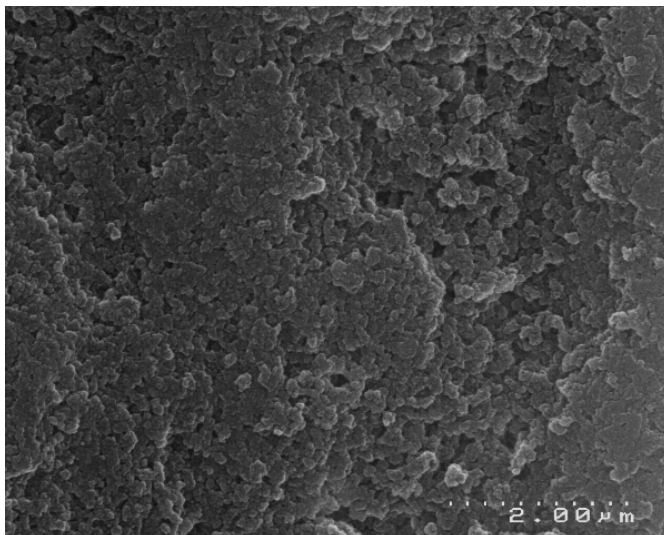
Figure A6.2 SEM image of m17-reference polymer



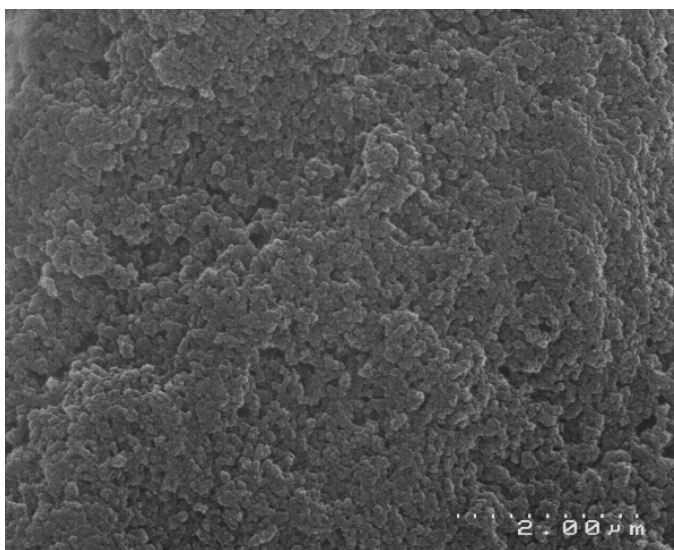
**Figure A6.3 SEM image of t18-MIP**



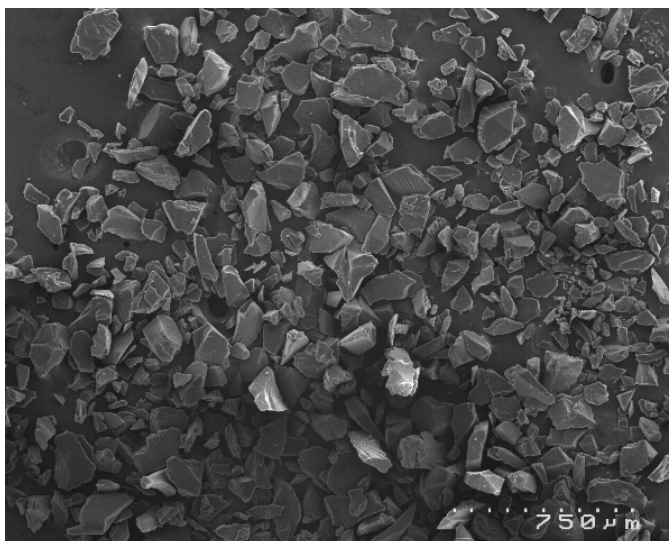
**Figure A6.4 SEM image of t18-reference polymer**



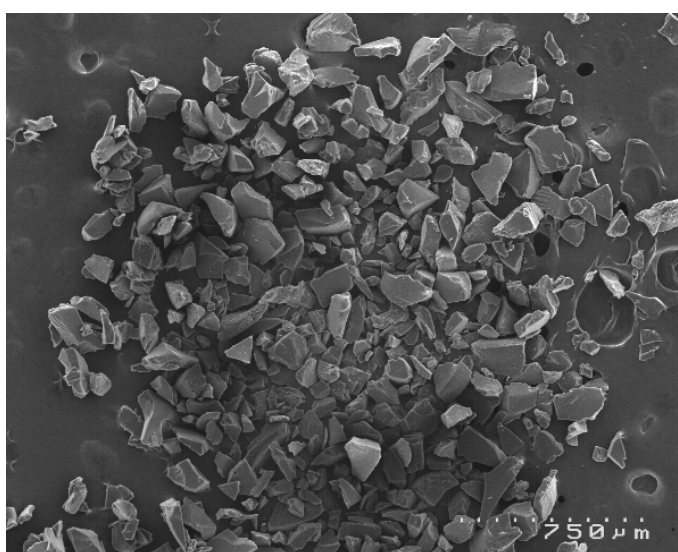
**Figure A6.5 SEM image of t5-MIP**



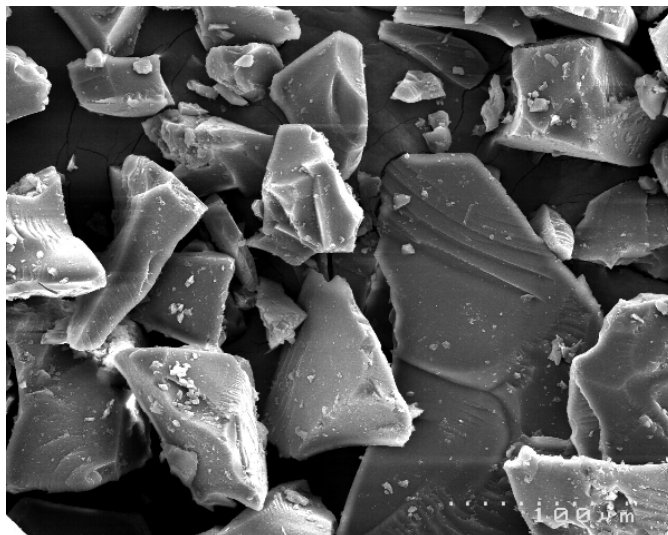
**Figure A6.6 SEM image of t5-reference polymer**



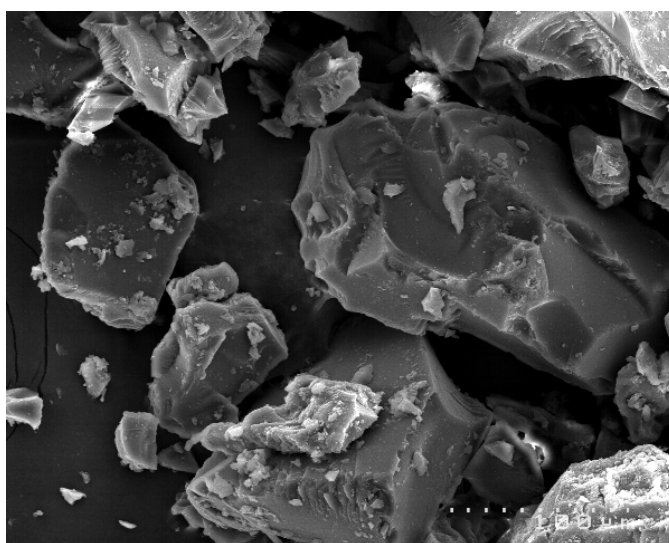
**Figure A6.7 SEM image of t8-MIP**



**Figure A6.8 SEM image of t8-reference polymer**



**Figure A6.9 SEM image of t11-MIP**



**Figure A6.10 SEM image of t11-reference polymer**

## **Appendix Seven**

### **FT-IR spectra**

## A7 FT-IR spectra

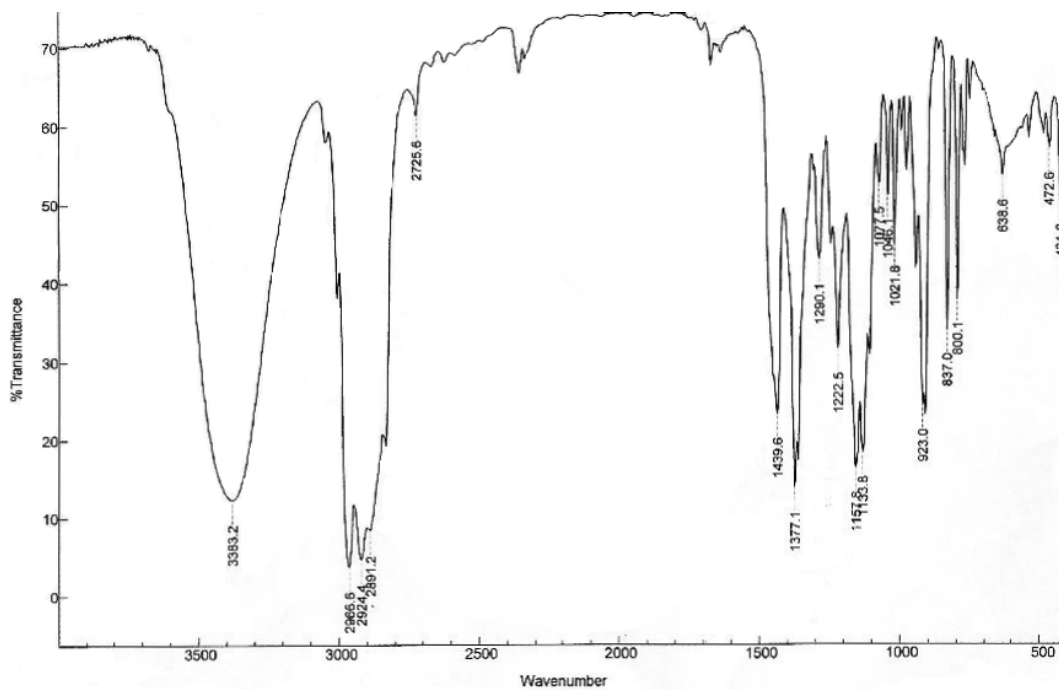


Figure A7.1 FT-IR of neat  $\alpha$ -terpineol

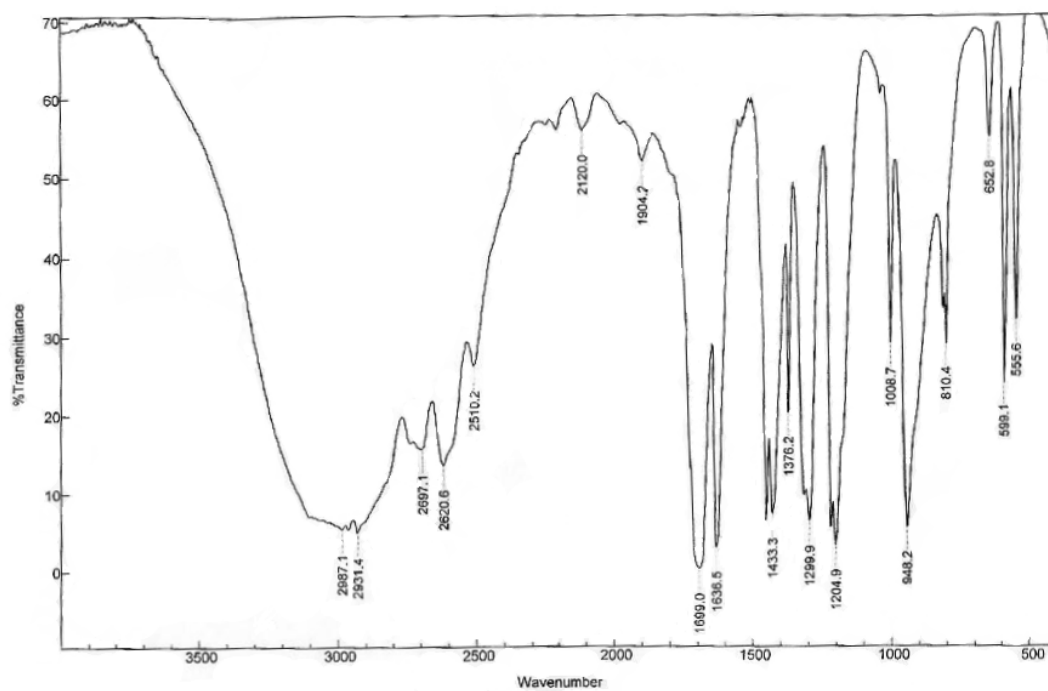


Figure A7.2 FT-IR of neat methacrylic acid

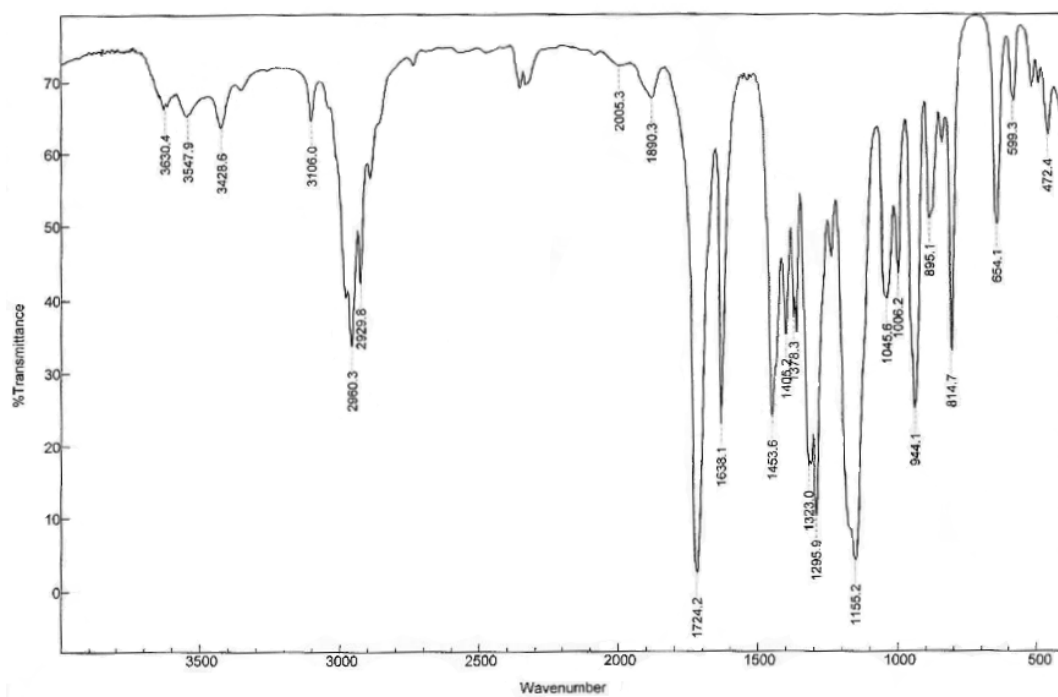


Figure A7.3 FT-IR of neat ethylene glycol dimethacrylate

## **Appendix Eight**

### **Preparation of standards and test solutions**

## A8 Details for preparing standards and test solutions

**Table A8.1** Details for preparing the standards and working solutions for Section 3.1

*Testing polymer cross-reactivity against terpinolene in methanol*

| Type of solutions       | Description   | Details on preparation   |
|-------------------------|---|--|
| Stock and test solution | 1500 ppm terpinolene in CH <sub>3</sub> OH                        | 30.20 mg terpinolene in 20 mL CH <sub>3</sub> OH   |
| Stock solution          | 1500 ppm $\alpha$ -terpinene in CH <sub>3</sub> OH                | 30.42 mg $\alpha$ -terpinene in 20 mL CH <sub>3</sub> OH   |
| Internal standard       | 150 ppm $\alpha$ -terpinene in CH <sub>3</sub> OH                 | 5 mL 1000 ppm $\alpha$ -terpinene; topped up to 50 mL with CH <sub>3</sub> OH  |
| Standards               | 150 ppm terpinolene and $\alpha$ -terpinene in CH <sub>3</sub> OH | 2 mL 1500 ppm terpinolene mixed with 2 mL 1500 ppm $\alpha$ -terpinene; topped up to 20 mL with CH <sub>3</sub> OH   |
|                         | 75 ppm terpinolene and $\alpha$ -terpinene in CH <sub>3</sub> OH  | 1 mL 1500 ppm terpinolene mixed with 1 mL 1500 ppm $\alpha$ -terpinene; topped up to 20 mL with CH <sub>3</sub> OH   |
|                         | 10 ppm terpinolene and $\alpha$ -terpinene in CH <sub>3</sub> OH  | 0.13 mL 1500 ppm terpinolene mixed with 0.13 mL 1500 ppm $\alpha$ -terpinene (plastic pipette tips used in both instances); topped up to 20 mL with CH <sub>3</sub> OH |

*Testing polymer cross-reactivity against terpinolene in acetonitrile*

| Type of solutions       | Description   | Details on preparation  |
|-------------------------|---|---|
| Stock and test solution | 1000 ppm terpinolene in CH <sub>3</sub> CN  | 20.31 mg terpinolene in 20mL CH <sub>3</sub> CN   |
| Stock solution          | 1000 ppm $\alpha$ -terpinene in CH <sub>3</sub> OH  | 20.30 mg $\alpha$ -terpinene in 20mL CH <sub>3</sub> OH   |
| Internal standard       | 100 ppm $\alpha$ -terpinene in CH <sub>3</sub> OH   | 5 mL 1000 ppm $\alpha$ -terpinene; topped up to 50 mL with CH <sub>3</sub> OH   |
| Standards               | 500 ppm terpinolene and $\alpha$ -terpinene in 1:1 CH <sub>3</sub> CN: CH <sub>3</sub> OH | 3.5 mL 1000 ppm terpinolene (CH <sub>3</sub> CN) mixed with 3.5 mL 1000 ppm $\alpha$ -terpinene (CH <sub>3</sub> OH)  |
|                         | 100 ppm terpinolene and $\alpha$ -terpinene in 1:1 CH <sub>3</sub> CN: CH <sub>3</sub> OH | 4 mL 500 ppm terpinolene and $\alpha$ -terpinene in 1:1 CH <sub>3</sub> CN: CH <sub>3</sub> OH; topped up to 20 mL with 1:1 CH <sub>3</sub> CN: CH <sub>3</sub> OH                      |
|                         | 50 ppm terpinolene and $\alpha$ -terpinene in 1:1 CH <sub>3</sub> CN: CH <sub>3</sub> OH  | 2 mL 500 ppm terpinolene and $\alpha$ -terpinene in 1:1 CH <sub>3</sub> CN: CH <sub>3</sub> OH; topped up to 20 mL with 1:1 CH <sub>3</sub> CN: CH <sub>3</sub> OH                      |
|                         | 10 ppm terpinolene and $\alpha$ -terpinene in 1:1 CH <sub>3</sub> CN: CH <sub>3</sub> OH  | 0.4 mL 500 ppm terpinolene and $\alpha$ -terpinene in 1:1 CH <sub>3</sub> CN: CH <sub>3</sub> OH (plastic tip used); topped up to 20 mL with 1:1 CH <sub>3</sub> CN: CH <sub>3</sub> OH |

Appendix Eight – Preparation of standards and test solutions

*Testing polymer cross-reactivity against terpinolene in hexane*

| Type of solutions       | Description   | Details on preparation   |
|-------------------------|---|--|
| Stock and test solution | 1000 ppm terpinolene in hexane                        | 20.10 mg terpinolene in 20 mL hexane   |
| Stock solution          | 1000 ppm $\alpha$ -terpinene in hexane                | 20.20 mg $\alpha$ -terpinene in 20 mL hexane   |
| Internal standard       | 100 ppm $\alpha$ -terpinene in hexane                 | 5 mL 1000 ppm $\alpha$ -terpinene; topped up to 50 mL with hexane                      |
| Standards               | 500 ppm terpinolene and $\alpha$ -terpinene in hexane | 4 mL 1000 ppm terpinolene mixed with 4 mL 1000 ppm $\alpha$ -terpinene, both in hexane |
|                         | 100 ppm terpinolene and $\alpha$ -terpinene in hexane | 4 mL 500 ppm terpinolene and $\alpha$ -terpinene; topped up to 20 mL with hexane       |
|                         | 50 ppm terpinolene and $\alpha$ -terpinene in hexane  | 2 mL 500 ppm terpinolene and $\alpha$ -terpinene; topped up to 20 mL with hexane       |
|                         | 10 ppm terpinolene and $\alpha$ -terpinene in hexane  | 2 mL 100 ppm terpinolene and $\alpha$ -terpinene; topped up to 20 mL with hexane       |

*Testing polymer specificity against  $\alpha$ -terpineol in methanol*

| Type of solutions       | Description   | Details on preparation   |
|-------------------------|---|--|
| Stock and test solution | 1000 ppm $\alpha$ -terpineol in CH <sub>3</sub> OH                | 20.14 mg $\alpha$ -terpineol in 20 mL CH <sub>3</sub> OH   |
| Stock solution          | 1000 ppm (-)-menthol in CH <sub>3</sub> OH                        | 20.12 mg (-)-menthol in 20 mL CH <sub>3</sub> OH   |
| Internal standard       | 100 ppm (-)-menthol in CH <sub>3</sub> OH                         | 5 mL 1000 ppm (-)- menthol; topped up to 50 mL with CH <sub>3</sub> OH   |
| Standards               | 500 ppm $\alpha$ -terpineol and (-)-menthol in CH <sub>3</sub> OH | 4 mL 1000 ppm menthol (CH <sub>3</sub> OH) mixed with 4 mL 1000 ppm $\alpha$ -terpineol (CH <sub>3</sub> OH)                 |
|                         | 100 ppm $\alpha$ -terpineol and (-)-menthol in CH <sub>3</sub> OH | 4 mL 500 ppm $\alpha$ -terpineol and (-)-menthol in hexane in CH <sub>3</sub> OH; topped up to 20 mL with CH <sub>3</sub> OH |
|                         | 50 ppm $\alpha$ -terpineol and (-)-menthol in CH <sub>3</sub> OH  | 2 mL 500 ppm $\alpha$ -terpineol and (-)-menthol in hexane in CH <sub>3</sub> OH; topped up to 20 mL with CH <sub>3</sub> OH |
|                         | 10 ppm $\alpha$ -terpineol and (-)-menthol in CH <sub>3</sub> OH  | 2 mL 100 ppm $\alpha$ -terpineol and (-)-menthol in hexane in CH <sub>3</sub> OH; topped up to 20 mL with CH <sub>3</sub> OH |

Appendix Eight – Preparation of standards and test solutions

*Testing polymer specificity against  $\alpha$ -terpineol in acetonitrile*

| Type of solution        | Description  | Details on preparation   |
|-------------------------|--|--|
| Stock and test solution | 1000 ppm $\alpha$ -terpineol in CH <sub>3</sub> CN                                       | 20.16 mg $\alpha$ -terpineol in 20 mL CH <sub>3</sub> CN   |
| Stock solution          | 1000 ppm (-)-menthol in CH <sub>3</sub> OH   | 20.09 mg (-)-menthol in 20 mL CH <sub>3</sub> OH   |
| Internal standard       | 100 ppm (-)-menthol in CH <sub>3</sub> OH  | 5 mL 1000 ppm (-)-menthol; topped up to 50 mL with CH <sub>3</sub> OH  |
| Standards               | 500 ppm $\alpha$ -terpineol and (-)-menthol in 1:1 CH <sub>3</sub> OH:CH <sub>3</sub> CN | 4 mL 1000 ppm (-)-menthol (CH <sub>3</sub> OH) mixed with 4 mL 1000 ppm $\alpha$ -terpineol (CH <sub>3</sub> CN)   |
|                         | 100 ppm $\alpha$ -terpineol and (-)-menthol in 1:1 CH <sub>3</sub> OH:CH <sub>3</sub> CN | 4 mL 500 ppm $\alpha$ -terpineol and (-)-menthol in hexane in 1:1 CH <sub>3</sub> OH:CH <sub>3</sub> CN; topped up to 20 mL with 1:1 CH <sub>3</sub> OH:CH <sub>3</sub> CN |
|                         | 50 ppm $\alpha$ -terpineol and (-)-menthol in 1:1 CH <sub>3</sub> OH:CH <sub>3</sub> CN  | 2 mL 500 ppm $\alpha$ -terpineol and (-)-menthol in hexane in 1:1 CH <sub>3</sub> OH:CH <sub>3</sub> CN; topped up to 20 mL with 1:1 CH <sub>3</sub> OH:CH <sub>3</sub> CN |
|                         | 10 ppm $\alpha$ -terpineol and (-)-menthol in 1:1 CH <sub>3</sub> OH:CH <sub>3</sub> CN  | 2 mL 100 ppm $\alpha$ -terpineol and (-)-menthol in hexane in 1:1 CH <sub>3</sub> OH:CH <sub>3</sub> CN; topped up to 20 mL with 1:1 CH <sub>3</sub> OH:CH <sub>3</sub> CN |

*Testing polymer specificity against  $\alpha$ -terpineol in hexane*

| Type of solution        | Description   | Details on preparation   |
|-------------------------|---|--|
| Stock and test solution | 1000 ppm $\alpha$ -terpineol in hexane                | 20.33 mg $\alpha$ -terpineol in 20 mL hexane   |
| Stock solution          | 1000 ppm (-)-menthol in hexane                        | 20.33 mg (-)-menthol in 20 mL hexane   |
| Internal standard       | 100 ppm (-)-menthol in hexane                         | 5 mL 1000 ppm menthol; topped up to 50 mL with hexane                                      |
| Standards               | 500 ppm $\alpha$ -terpineol and (-)-menthol in hexane | 4 mL 1000 ppm $\alpha$ -terpineol (hexane) mixed with 4 mL 1000 ppm menthol (hexane)       |
|                         | 100 ppm $\alpha$ -terpineol and (-)-menthol in hexane | 4 mL 500 ppm $\alpha$ -terpineol and (-)-menthol in hexane; topped up to 20 mL with hexane |
|                         | 50 ppm $\alpha$ -terpineol and (-)-menthol in hexane  | 2 mL 500 ppm $\alpha$ -terpineol and (-)-menthol in hexane; topped up to 20 mL with hexane |
|                         | 10 ppm $\alpha$ -terpineol and (-)-menthol in hexane  | 2 mL 100 ppm $\alpha$ -terpineol and (-)-menthol in hexane; topped up to 20 mL with hexane |

Table A8.2 Details for preparing the standards and working solutions for Section 3.2

| Type of solution  | Description   | Details on preparation   |
|-------------------|---|--|
| Stock solutions   | 1000 ppm (-)-menthol in hexane  | 20.27 mg (-)-menthol in 20 mL hexane   |
|                   | 1000 ppm $\alpha$ -terpineol in hexane  | 20.12 mg $\alpha$ -terpineol in 20 mL hexane   |
| Test solution     | 500 ppm $\alpha$ -terpineol in hexane   | 10 mL 1000 ppm $\alpha$ -terpineol; topped up to 20 mL with hexane                         |
| Internal standard | 50 ppm (-)-menthol in hexane  | 5 mL 1000 ppm (-)-menthol; topped up to 100 mL with hexane                                 |
| Standards         | 500 ppm $\alpha$ -terpineol and (-)-menthol in hexane (prepared in scintillation vial, discarded after use) | 4 mL 1000 ppm menthol (hexane) mixed with 4 mL 1000 ppm $\alpha$ -terpineol (hexane)       |
|                   | 100 ppm $\alpha$ -terpineol and (-)-menthol in hexane   | 4 mL 500 ppm $\alpha$ -terpineol and (-)-menthol in hexane; topped up to 20 mL with hexane |
|                   | 50 ppm $\alpha$ -terpineol and (-)-menthol in hexane  | 2 mL 500 ppm $\alpha$ -terpineol and (-)-menthol in hexane; topped up to 20 mL with hexane |
|                   | 10 ppm $\alpha$ -terpineol and (-)-menthol in hexane  | 2 mL 100ppm $\alpha$ -terpineol and (-)-menthol in hexane; topped up to 20 mL with hexane  |

**Table A8.3 Details for preparing the stock and working solutions for Section 3.3**

| Type of solution   | Description   | Method of preparation  |
|--------------------|---|--|
| Stock solutions    | 1000 ppm $\alpha$ -terpineol in hexane (also used as a test solution)                                       | 50.14 mg $\alpha$ -terpineol dissolved in 50 mL hexane   |
|                    | 1000 ppm (-)-menthol in hexane  | 50.10 mg (-)-menthol dissolved in 50 mL hexane   |
|                    | 500 ppm $\alpha$ -terpineol and (-)-menthol in hexane (prepared in scintillation vial, discarded after use) | 4 mL 1000 ppm $\alpha$ -terpineol mixed with 1000 ppm (-)-menthol, both in hexane                              |
| Test solutions     | 750 ppm $\alpha$ -terpineol in hexane   | 1.5 mL hexane mixed with 4.5 mL 1000ppm $\alpha$ -terpineol in hexane  |
|                    | 500 ppm $\alpha$ -terpineol in hexane   | 3 mL hexane mixed with 3 mL 1000 ppm $\alpha$ -terpineol in hexane   |
|                    | 250 ppm $\alpha$ -terpineol in hexane   | 4.5 mL hexane mixed with 1.5 mL 1000 ppm $\alpha$ -terpineol in hexane   |
|                    | 100 ppm $\alpha$ -terpineol in hexane   | 5.4 mL hexane mixed with 0.6 mL 1000 ppm $\alpha$ -terpineol in hexane (plastic tips used)                     |
| Internal standards | 100 ppm (-)-menthol in hexane   | 2 mL 1000 ppm (-)-menthol in hexane; topped up to 20 mL with hexane  |
|                    | 75 ppm (-)-menthol in hexane  | 1.5 mL 1000 ppm (-)-menthol in hexane; topped up to 20 mL with hexane  |
|                    | 50 ppm (-)-menthol in hexane  | 1 mL 1000 ppm (-)-menthol in hexane; topped up to 20 mL with hexane  |
|                    | 25 ppm (-)-menthol in hexane  | 0.5 mL 1000 ppm (-)-menthol in hexane; topped up to 20 mL with hexane  |
|                    | 10 ppm (-)-menthol in hexane  | 0.2 mL 1000 ppm (-)-menthol in hexane; topped up to 20 mL with hexane  |
| Standards          | 100 ppm $\alpha$ -terpineol and (-)-menthol in hexane   | 4 mL 500 ppm $\alpha$ -terpineol and (-)-menthol; topped up to 20 mL with hexane                               |
|                    | 50 ppm $\alpha$ -terpineol and (-)-menthol in hexane  | 2 mL 500 ppm $\alpha$ -terpineol and (-)-menthol; topped up to 20 mL with hexane                               |
|                    | 10 ppm $\alpha$ -terpineol and (-)-menthol in hexane  | 2 mL 100 ppm $\alpha$ -terpineol and (-)-menthol; topped up to 20 mL with hexane                               |
|                    | 5 ppm $\alpha$ -terpineol and (-)-menthol in hexane   | 2.5 mL hexane mixed with 2.5 mL 10ppm $\alpha$ -terpineol and (-)-menthol in hexane                            |
|                    | 2.5 ppm $\alpha$ -terpineol and (-)-menthol in hexane   | 0.5 mL hexane mixed with 0.5 mL 5 ppm $\alpha$ -terpineol and (-)-menthol in hexane (plastic pipette tip used) |

**Table A8.4 Details for preparing the stock and working solutions for Section 3.4**

| Type of solution   | Description   | Method of preparation  |
|--------------------|---|--|
| Test solutions     | 500 ppm $\alpha$ -terpineol in hexane                 | 10 mL 1000 ppm $\alpha$ -terpineol (used the stock made in Table A8.3); topped up to 20 mL with hexane |
| Internal standards | 50 ppm (-)-menthol in hexane                          | 5 mL 1000 ppm (-)-menthol (used the stock made in Table A8.3); topped up to 100 mL with hexane         |
| Standards          | 100 ppm $\alpha$ -terpineol and (-)-menthol in hexane | Used the standard solution made in Table A8.3  |
|                    | 50 ppm $\alpha$ -terpineol and (-)-menthol in hexane  | Used the standard solution made in Table A8.3  |
|                    | 10 ppm $\alpha$ -terpineol and (-)-menthol in hexane  | Used the standard solution made in Table A8.3  |

TableA8.5 Details for preparing the standards and working solutions for Section 3.5

*Testing different concentrations of terpinolene against t18*

| Type of solution   | Description   | Details on preparation  |
|--------------------|---|---|
| Stock solutions    | 1000 ppm terpinolene in hexane                        | 50.02 mg terpinolene in 50 mL hexane  |
|                    | 1000 ppm $\alpha$ -terpinene in hexane                | 50.16 mg $\alpha$ -terpinene in 50 mL hexane  |
| Test solutions     | 750 ppm terpinolene in hexane                         | 4.5 mL 1000 ppm terpinolene in hexane mixed with 1.5 mL hexane.                           |
|                    | 500 ppm terpinolene in hexane                         | 3 mL 1000 ppm terpinolene in hexane mixed with 3 mL hexane                                |
|                    | 250 ppm terpinolene in hexane                         | 1.5 mL 1000 ppm terpinolene in hexane mixed with 4.5 mL hexane                            |
|                    | 100 ppm terpinolene in hexane                         | 0.6 mL 1000 ppm terpinolene in hexane mixed with 5.4 mL hexane (plastic pipette tip used) |
| Internal standards | 100 ppm $\alpha$ -terpinene in hexane                 | 2 mL 1000 ppm $\alpha$ -terpinene; topped up to 20 mL with hexane                         |
|                    | 75 ppm $\alpha$ -terpinene in hexane                  | 1.5 mL 1000 ppm $\alpha$ -terpinene; topped up to 20 mL with hexane                       |
|                    | 50 ppm $\alpha$ -terpinene in hexane                  | 1 mL 1000 ppm $\alpha$ -terpinene; topped up to 20 mL with hexane                         |
|                    | 25 ppm $\alpha$ -terpinene in hexane                  | 0.5 mL 1000 ppm $\alpha$ -terpinene; topped up to 20 mL with hexane                       |
|                    | 10 ppm $\alpha$ -terpinene in hexane                  | 0.2 mL 1000 ppm $\alpha$ -terpinene; topped up to 20 mL with hexane                       |
| Standards          | 500 ppm terpinolene and $\alpha$ -terpinene in hexane | 4 mL 1000 ppm terpinolene mixed with 4 mL 1000 ppm $\alpha$ -terpinene, all in hexane     |
|                    | 100 ppm terpinolene and $\alpha$ -terpinene in hexane | 4 mL 500 ppm terpinolene and $\alpha$ -terpinene; topped up to 20 mL with hexane          |
|                    | 50 ppm terpinolene and $\alpha$ -terpinene in hexane  | 2 mL 500 ppm terpinolene and $\alpha$ -terpinene; topped up to 20 mL with hexane          |
|                    | 10 ppm terpinolene and $\alpha$ -terpinene in hexane  | 2 mL 100 ppm terpinolene and $\alpha$ -terpinene; topped up to 20 mL with hexane          |
|                    | 5 ppm terpinolene and $\alpha$ -terpinene in hexane   | 2.5 mL 10 ppm terpinolene and $\alpha$ -terpinene mixed with 2.5 mL hexane                |
|                    | 2.5 ppm terpinolene and $\alpha$ -terpinene in hexane | 2.5 mL 5ppm terpinolene and $\alpha$ -terpinene mixed with 2.5 mL hexane                  |

Appendix Eight – Preparation of standards and test solutions

*Testing different concentrations of (-)-menthol against t18*

| Type of Solution  | Description                           | Details on preparation   |
|-------------------|---------------------------------------|--|
| Stock solutions   | 1000 ppm (-)-menthol in hexane        | 20.07 mg (-)-menthol in 20 mL hexane                               |
|                   | 500 ppm $\alpha$ -terpineol in hexane | Used 500ppm $\alpha$ -terpineol made in Table A8.4                 |
| Test solutions    | 1000 ppm (-)-menthol in hexane        | Used undiluted 1000ppm (-)-menthol in hexane                       |
|                   | 800 ppm (-)-menthol in hexane         | 4 mL 1000 ppm (-)-menthol in hexane mixed with 1 mL hexane         |
|                   | 600 ppm (-)-menthol in hexane         | 3 mL 1000 ppm (-)-menthol in hexane mixed with 2 mL hexane         |
|                   | 400 ppm (-)-menthol in hexane         | 2 mL 1000 ppm (-)-menthol in hexane mixed with 3 mL hexane         |
|                   | 200 ppm (-)-menthol in hexane         | 1 mL 1000 ppm (-)-menthol in hexane mixed with 4 mL hexane         |
|                   | 0 ppm (-)-menthol                     | Hexane   |
| Internal standard | 100 ppm $\alpha$ -terpineol in hexane | 4 mL 500 ppm $\alpha$ -terpineol in hexane mixed with 16 mL hexane |
| Standards         | 100 ppm (-)-menthol in hexane         | 1 mL 1000ppm (-)-menthol in hexane mixed with 9 mL hexane          |
|                   | 50 ppm (-)-menthol in hexane          | 5 mL 100ppm (-)-menthol in hexane mixed with 5 mL hexane           |
|                   | 10 ppm (-)-menthol in hexane          | 1 mL 100ppm (-)-menthol in hexane mixed with 9 mL hexane           |

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