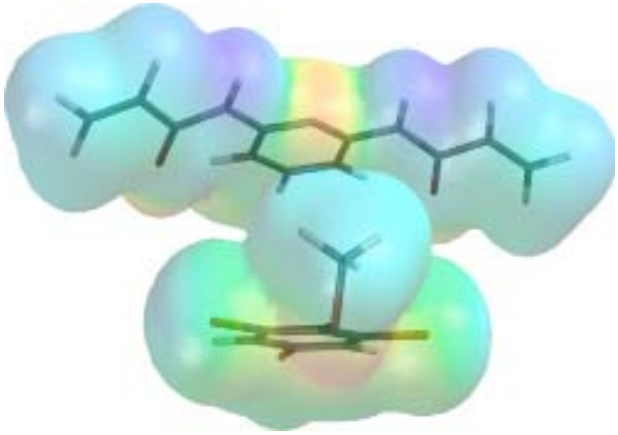
Selective detection of trichloroanisole and other flavour contaminants in wines using molecularly imprinted polymer technology.



FINAL REPORT to

GRAPE AND WINE RESEARCH & DEVELOPMENT CORPORATION

Project Number: **UN 98/1**

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Research Organisation: **The University of Newcastle**

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1. Abstract

Spoilage through organoleptic taint represents a significant source of financial loss to the wine industry. The musty or mouldy flavours associated with the presence of chlorinated aromatic compounds such as trichloroanisole (TCA) and trichlorophenol (TCP) in contaminated corks have particularly low thresholds that renders the wine unpalatable.

Molecular imprinting is an emerging technology that enables a target molecule to be selectively sorbed from a solution by trapping them in tailored cavities within a bulk polymer matrix. Research was undertaken to assess the viability of developing covalently and non-covalently imprinted polymers selective for TCA and TCP. The project aims to initiate future development of sensory devices to identify and quantify the levels of these contaminants corkwood and wine.

2. Executive Summary

Organoleptic taint is a serious economic concern to the wine industry, with losses estimated to exceed ten billion dollars annually worldwide. To date, no simple, cost effective method for the rapid evaluation of the levels of polychlorinated phenols and / or anisoles present either in corkwood or in wine has yet been developed. Current technologies are slow, infrastructure intensive and require highly skilled operators. This project was undertaken to investigate the feasibility of adapting new experimental technologies to address the issue of cork taint.

Molecular imprinting is a branch of supramolecular chemistry that has evolved rapidly over the past decade. The technology enables artificial and robust recognition sites for a specific target such as a molecule, protein, virus or bacterial surface to form in a polymer matrix. The cavity forms as a result of complimentary interactions between functional groups present on the monomer units that compose the bulk polymer structure and those and on the target species. Subsequent extraction of the entity from the resultant polymer leaves behind a well-defined cavity that contains binding points that interact uniquely with the target. The end result is a system that possesses highly specific recognition elements. It is this feature of molecular imprinting that has enabled it to be combined with existing technologies such as chemical separation and molecular sensing.

The aim of this study was to:

- Investigate the synthesis of a range of trichloroanisole (TCA) and trichlorophenol (TCP) based imprinted polymers using a combinatorial approach involving commercially available monomers, cross-linking agents and solvents.

- To investigate the applicability of molecular modelling techniques in monomer – template selection.

- To assess the specificity and binding characteristics of these materials under a range of conditions and to attempt to improve their overall performance.

- To attempt to develop a self-indicating detection system for trichloroanisole (TCA).

The first three objectives were carried out within the timeframe specified in the original project application (3 years). The latter was not pursued after initial investigations revealed that the proposed use of metal – ligand based recognition elements proved to be impractical. An examination of trichlorophenol based on covalent imprinting techniques was instead incorporated into the program.

Polymers were synthesised in a range of porogens (solvents) using both non-covalent and non-covalent imprinting techniques. The selectivity and binding capacity of each system was then assessed and compared in a range of solvents. Rebinding of the target molecule to the each MIP (assessed against a non-imprinted control) was found to be highest when conducted in the porogen used to form the polymer. Rebinding affinities fell significantly when the assessment was conducted in other solvents - particularly those with high polarities. This finding was consistent with previous literature reports.

TCA and TCP based MIPs prepared in dichloromethane (CH_2Cl_2) were found to exhibit particularly high target affinities. Competitive binding studies conducted on these systems (in CH_2Cl_2) also produced good results. The findings clearly demonstrate the presence of highly defined and specific cavities within the bulk polymer that promote good levels of target recognition, even in the presence of structurally similar analogues (e.g. TCA in the presence of TCP and *vice versa*). The results also suggest that different binding interactions exist for TCA and TCP, a finding that was in line with predictive modelling studies undertaken during the initial stages of the project.

Assessment of polymer performance in more polar solvent conditions (80% aqueous methanol) proved disappointing, with the selectivity levels collapsing to background (i.e. non-specific) levels. Preliminary studies are now underway to explore the use of emulsion and precipitation polymerisation techniques to develop TCA and TCP based MIPs capable of operating under aqueous conditions.

At the commencement of this project, conventional opinion dictated that only target species containing distinct and well-defined functional groups could be used as MIP targets. Our findings, together with a number of recently published findings have broadened this envelope to include less well-defined molecules such as polychlorinated arenes. It is our opinion that the results presented in this report demonstrate that MIP technology will provide a substantial avenue of future opportunity for addressing the

issue of TCA and TCP contamination in corkwood by incorporating MIP technology with existing sensory and analytical technologies.

3. Background

Bark cork has been traditionally used to stopper most wine[1]. Physical properties including compressibility, impermeability to air and liquids, chemical inertness[2] and cheap production costs enhance perceptions that the material is a superior closing agent, particularly for premium wines.

A significant drawback arising from the use of cork is the phenomenon known as *cork taint*, which arises when contaminant chlorinated arenes such as 2,4,6-trichloroanisole (TCA) and 2,3,4,6-tetrachloroanisole (TTCA) leech from the stopper into the wine[3]. The limit of TCA for humans is particularly low (~ 4 ppt). Consequently, wine containing even minute quantities of TCA develops a characteristic musty or mouldy taste and aroma that renders it undrinkable[4].

Estimates of the extent of the cork taint problem in the wine industry vary[4,5], however the figure is generally thought to be between 2 and 5% of all bottled wine. Globally, this represents a cost to the industry of approximately 10 billion dollars per annum. With modern wine making techniques continuing to refine the delicacy and complexity of wine bouquets, the intrusion of contaminants in wine has become increasingly apparent and consequently represents a greater threat to the industry than at any time in the past.

TCA contamination of corkwood is generally considered to arise as a consequence of poor quality control practices at the point of cork manufacture. As natural corkwood contains relatively low concentrations of simple phenols, it is believed that TCA contamination is introduced during post-harvest processing. Microbial action is thought to be the major source of TCA contamination, with chlorinated bleaching agents (such as sodium hypochlorite) used in the decontamination of corkwood, acting as a source of chlorine for microbial biosynthesis of TCA from both phenolic and non-phenolic precursors[3,6,]. Inadequate separation of the freshly sterilised corkwood from untreated stocks during storage leads to cross-contamination which perpetuates the problem[6].

TCA contamination has also been reported in other food processing industries such as coffee[7] and raisins[8]. In this case, the source of contamination is thought to arise from biotransformation (via methylation of the OH group) of 2,4,6-trichlorophenol – a material used to decontaminate wooden objects such as barrels, chests and other packaging materials[9].

Portugal is the dominant producer of corkwood in the world market, supplying approximately 50% of the total export market. This fact, coupled with increasing demands for cork and the very traditional nature of the corkwood industry has stymied attempts by external bodies to raise awareness of the cork taint issue at the point of production, and to bring about improvements in quality control[10].

Alternatives to cork seals such as screw caps and synthetic cork analogues have been developed, but met with only limited success. Consumer resistance to such innovations is strong, with market research indicating that the use of such devices is perceived to be associated with inferior quality wine[11]. This accounts for the reluctance of many winemakers – particularly those at the premium end of the market to such changes.

The need to develop a simple, rapid and cost effective method of detecting TCA contamination in corkwood has been earmarked by the wine industry for sometime[5]. Organoleptic evaluation is currently used to detect the presence of TCA, however, the method is unsatisfactory because it is extremely slow, laborious and requires the participation of highly trained staff[12]. Instrumental techniques such as gas chromatography (GC) and high performance liquid chromatography (HPLC)[13], while extremely sensitive, are impractical for large-scale evaluation because of their inherently high infrastructure costs and a reliance on skilled operators.

Like the agricultural and food manufacturing sectors, the wine industry is constantly seeking to implement technologies that promote improvements in safety, efficiency and quality control[14,15]. Molecular imprinting (MIP) is one such technology shows great promise as a basis for the development of low cost sensing devices.

Molecular recognition, the process of weak, selective and reversible binding between molecules, is the governing principle of many chemical processes that occur in biological systems. The mechanistic aspects of molecular recognition processes such as DNA replication, cellular ion transport, receptor-effector, enzyme-inhibitor and antibody-antigen interactions have been intensely studied and are now reasonably well understood[16].

In recent years, attention has turned towards utilising this behaviour in the production of synthetic enzyme-substrate mimics. MIP technology has emerged as a leading branch

of supramolecular chemistry, producing polymers containing *pre-organised* cavities that possess a high affinity for specific target species.

Conceptually, MIP technology has been postulated in scientific circles for some time. The production of working examples of the technique have however only recently been realised through advances in microelectronics and improved laboratory techniques[17]. Two principle approaches have been used to form imprinted polymers, namely reversible covalent binding and non-covalent interactions[18]. Although somewhat different conceptually, both follow the same preparation sequence of (i) pre-association, (ii) polymerisation, (iii) target removal / cavity formation (see Figure 3.1)[19].

In covalent imprinting, a formal association (in the form of a covalent bond) is established between complimentary groups on the target molecule and functionalised monomers units (e.g. carboxylic acid + alcohol → ester linkage) in the polymer system. Polymerisation is carried in a solvent in the presence of a crosslinking agent, which is added to promote structural rigidity in the final bulk polymer. The monomer – target unit becomes incorporated into the polymer structure during polymerisation, resulting in the formation of a cavity that houses the target molecule. The solvent molecules that are also trapped within the superstructure add porosity to the polymer, thereby enabling movement through the system.

Subsequent severing of the monomer-target linkage (usually by a hydrolysis reaction) removes the template leaving behind the cavity (or imprint) containing the monomer functional groups in a proximal relationship that is specific to those on the target compound. If the polymer is then placed in a solution containing a mixture of compounds that includes the original template, it will be selectively concentrated in the polymer cavities.

In non-covalent imprinting, pre-association between the functional groups on the target molecule and the functionalised monomer units is established through weaker interactions such as hydrogen bonding, complimentary electrostatic forces, or in the case of non-polar species, hydrophobic interactions such as Van der Waals forces. Polymerisation is followed by a grinding step, then by a washing step (with a polar solvent such as methanol) to remove the target from the resulting molecular cavity.

Research into the potential applications of MIP technology has diverged into three primary areas:

(i) *The development of tailor-made separation materials*[20]*:* The pharmaceutical industry has committed significant resources towards the development of imprint polymer technology. There is keen interest in a number of key development strategies including the development of:

- MIP-based chromatographic stationary phases to improve the separation of selected analytes in routine HPLC analysis.
- MIP based stationary phases for selective accumulation of target species from solution by solid phase extraction.
- Low cost MIP-based stationary phases for separation of racemic drug mixtures.

The final point is of particular importance, given that USFDA legislation governing the sale of pharmaceuticals sold as racemic mixtures dictates that a full assessment of the pharmokinetic and toxicological properties of each enantiomeric form of the drug must be undertaken separately before approval for general release can be granted[14].

(ii) *The development of catalytically active polymers to act as enzyme mimics*[21,22]. Scientific advancement, particularly in emerging fields such as biotechnology, is critically dependent upon catalysis. Translating the principles of enzyme-based catalysis to synthetic enzyme mimics offers the possibility of an improved understanding of enzyme-based catalysis through the ability to chemically modify functional groups at the active site. MIP based enzyme mimics exhibiting high thermochemical stability may in the future be used as catalysts for a range of industrially important chemical manufacturing processes.

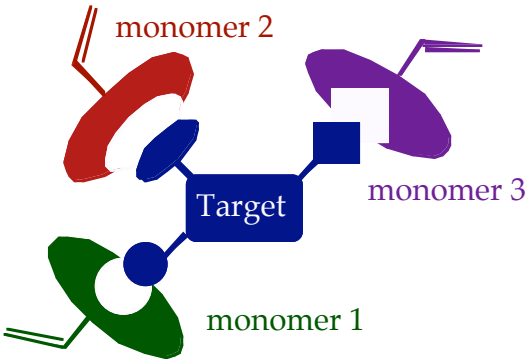
At present, the technology is at an embryonic stage of development, however, researchers have successfully demonstrated the feasibility of the system, with results from studies showing an ability to accelerate the rates of simple reactions (such as ester hydrolysis) using MIP systems tailored to the morphology of the reaction transition state[2324].

(iii) *The development of substrate-selective sensors for the detection of contaminants.* The merging of MIP technology with the field of biosensory detection and microelectronics has yielded a range of highly selective sensors. Detection methods employing technology such as potentiometry and optical surface ellipsometry have been successfully trialed[25]. A conductivity-based morphine sensor with a detection limit of 100 nM L^{-1} is one of a number of MIP-based systems recently reported in the literature[26].

Food and beverage industries have been quick to realise the potential of imprint technology. Modern food technology is comprised of a multitude of highly organised businesses aimed at supplying the demands of an ever more discerning public. This fact, coupled with the need to comply with strict national and international legislation governing the preparation and export of food products has placed increased pressure on the food industry to develop safer, more accurate and more rapid quality assurance measures.

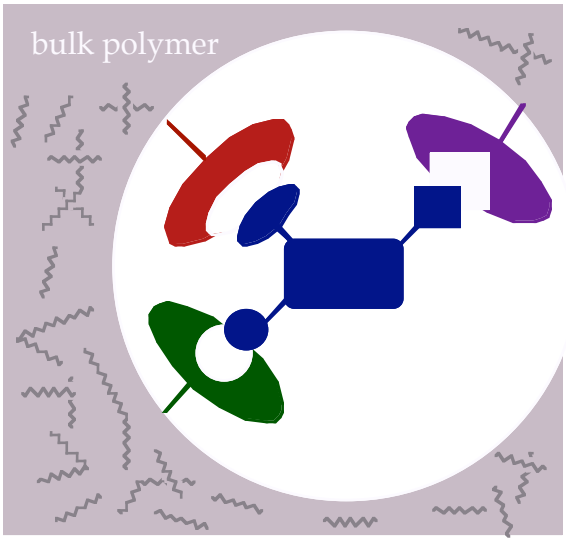
Analytical testing for contaminants such as microorganisms, toxins, antibiotic residues, and synthetic additives such as hormones is a key factor in any quality management system. To be compatible, such testing procedures must fit within the framework of existing "just in time" manufacturing practices[27]. Numerous rapid detection systems including enzyme-linked immunosorbent assays (ELISAs), flow cytometry and electrical impedance techniques are commercially available to test for the presence of selected pathogens[28]. In many cases however, these techniques suffer from a lack of robustness or require large amounts of capital funding to implement.

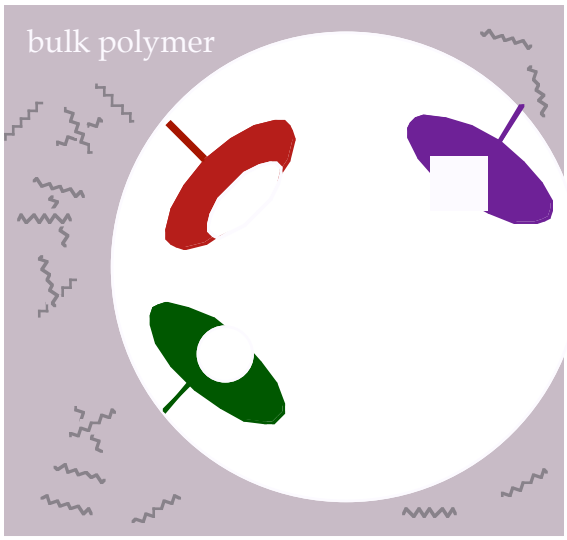
Figure 3.1: Overview of MIP Formation.



(i). Preassociation:
Monomer units containing complimentary functional groups to those found on the target molecule align in solution prior to bulk polymerisation.

(iii). Polymerisation
The associated monomer units combined with crosslinking agent in solution to form a solid polymer matrix.





(iii). Target Removal
Target molecule is removed from the bulk polymer by washing, leaving behind a vacant cavity with functional groups located in an idealised proximaal relationship to interact strongly with the target molecule.

As in the wine industry, the determination of specific chemical contaminants in food entails the use of specialised chromatographic techniques[29]. Continuing research has however demonstrated that imprint technology offers an efficient, low cost alternative to existing practices. Imprinted polymers designed to concentrate or detect specific target species from solution have been successfully developed. Examples include pharmaceutical agents such as clenbuterol[30] and nicotine[31]; food additives such as caffeine[32] and aspartame[33]; and herbicides such as atrazine[34] and 2,4-triichlorophenoxyacetic acid (2,4,5-T)[35] .

The principle aim of this project was to undertake a preliminary study to investigate the development of imprinted polymers to recognise TCA and TCP in solution. Although polychlorinated arenes represent a serious environmental problem, they have received little attention as MIP targets, primarily because of an absence of functional groups deemed suitable for non-covalent or covalent imprinting.

Although the targeting of TCA and TCP MIPs poses significant challenges, success would represent the first steps of a new an innovative approach to confronting the problem of cork taint in the wine industry. In the foreseeable future such technology could lead to the development of devices capable of;

- Detecting and quantifying the levels of phenolic contaminants in corkwood prior to bottling.

- Reducing or eliminating TCA and / or TCP contamination in wine through selective removal processes.

Such technology also has the potential to merge with existing analytical techniques (such as GCMS and HPLC) to significantly reduce current sampling times.

4. Project Aims and Performance Targets

Investigations were scheduled to be performed over a three year period, with the majority of the work being undertaken by Mr Lachlan Schwarz, a postgraduate candidate in the School of Environmental & Life Sciences at the University of Newcastle, Callaghan. The key features of the investigation as outlined in the original proposal were as follows:

- To trial a range of commercially available monomers and crosslinking agents to develop a non-covalent molecularly imprinted polymer system that would selectively bind to trichloroanisole.

- To assess the effectiveness of this pseudo enzyme-substrate system in a range of solvent matrices.

- To study and modify electrostatic interactions within the cavity of the polymer to development a self-indicating detection system upon occupation by the target (TCA).

- To assess the effectiveness of the modified imprinted polymer system in an aqueous environment

The timetable set to achieve key project objectives is shown in Table 4.1.

Table 4.1: Timetable of Key Project Objectives.

Objective	Time Frame
Completion of preliminary computer modelling studies of commercial monomers and cross-linking agents	0-6 months
Preparation of trial imprint polymers from commercial reagents	3-9 months will run concurrently with molecular modelling examination in a "feed-back-loop" scenario
Assessment of the selectivity of trial polymer systems by HPLC	9-12 months
Development of stage two MIP's; those generated via the combination of various ratios of the "high-ranking" single monomer MIP's generated as outlined above	12-18 months
Benchmarking of the new multi-monomer MIP's in close collaboration with Dr Eric Wilkes at Rosemount Estates	18-24 months
Custom synthesis of high performance MIP's and the development of a mixed MIP system that will allow the removal and detection of both TCA & TCP. Evaluation of these new systems.	24-36 months

Most elements set out in the original project outline were successfully addressed within the scheduled timeframe. A number of variations to the original proposal were however undertaken as a result of findings that were generated during the course of our investigations. These are detailed below.

(i) Extension of the scope of the original objectives to incorporate the imprinting of trichlorophenol.

The original aims of the project focused exclusively on the development of an imprint polymer system for trichloroanisole. The decision to incorporate a tandem development of a TCP based-MIP was undertaken for several reasons:

- *The existence of potential links between cork taint attributed to TCA and the incidence of TCP in corkwood.* Reports[36,37] published during the course of our research specified that TCP was present as a contaminant in a number of European and North American wines. In all cases, the compound was present in both the wine itself and the stopper. Like TCA, TCP contamination is a concern for wine producers, although the threshold for organoleptic taint is significantly higher (~350 ngL^{-1} *c.f.* ~2 ngL^{-1} for TCA)[37]. The development of a TCP MIP was therefore deemed to be complimentary to the overall goals of the project.

- *Structural advantages of TCP as a model system for TCA imprinting.* The absence of distinct functional groups on TCA capable of forming complimentary interactions with functionalised monomers during pre-association were a potential impediment to the formation of defined cavities in the final bulk polymer. By contrast, the acidic alcohol group (-OH) on TCP permitted a range of non-covalent and electrostatic interactions to be investigated. It was felt that the outcomes from these investigations would greatly aid the development of a TCA-based MIP.

- *Extension of applications for TCP-based MIPs to the assessment of water quality.* Chlorinated phenols (along with their anisole equivalents) are by-products of domestic and industrial water treatment processes and are known to affect the organoleptic qualities of drinking water[38,39,40]. The development of TCA and TCP based-MIPs capable of operating under aqueous conditions was therefore seen a complimentary adjunct to our investigations.

(ii) Abandonmant of an original objective to pursue the development of a self-indicating MIP for detection of TCA / TCP.

It was originally envisaged that the development of a colour based self-indicting MIP might be possible through judicious choice of the functional monomers used in to form the target cavity. Conformational changes occurring as a result of the target - molecule binding within the cavity (such as π-π stacking or metal-ligand interactions) were seen as potential trigger for colour production. Iinitial investigations towards the development a suitable metal – ligand donor system proved disappointing and was consequently not pursued.

This is not to say however that such an approach is not still feasible. There is ample literature evidence to suggest that systems employing visible light or other sectors of the electromagnetic spectrum can be successfully incorporated into MIP based detection systems[41]. Luminescence-based sensory devices that involve interactions between a target species and a metal ion have already been successfully developed[42,43]. These systems are particularly sensitive and represent an attractive approach, particularly where low level sensing is required.

(iii) Examination of covalent imprinting strategies for the development of a TCP based MIP.

Unlike TCA, TCP contains a free OH group that is capable of undergoing reaction with a range of functional groups, thereby presenting an opportunity to examine the development of a MIP system using covalent imprinting techniques. Covalent imprinting offers potentially greater selectivity because the existence of a formal linkage between the target and monomer during the polymerisation step ensures the formation of a highly defined cavity. It was considered that the approach might prove especially useful in developing a MIP system capable of functioning under aqueous conditions.

(iv) Deferment of trials to assess the performance of MIPs in a wine matrix.

Our original project proposal outlined a series of studies to evaluate the performance of TCA and TCP based imprinted polymers under field conditions. It was proposed that this work would be carried out with the cooperation of members of the wine industry (Mr Eric Wilkes – Rosemount Wines) and would assess the ability of the polymers to concentrate contaminants from a wine matrix.

Delays experienced during early phases of the project did not permit these studies to proceed within the original timeframe.

Our investigations found the performance of the MIPs to be highly solvent dependent. Polymers exhibited moderate levels of rebinding activity and good levels of target selectivity in non-polar solvents - particularly when the solvent used for evaluations was the same as that in which the polymer was originally prepared. The performance of TCA and TCP imprinted MIPs proved disappointing in aqueous media, with rebinding activity becoming indistinguishable from the levels exhibited by control polymers. The loss of selectivity is primarily attributed to the increased polarity of water, which dramatically affects the functioning recognition elements within the imprint cavities.

Emulsion and precipitation polymerisation (polymer synthesis under aqueous conditions) offers a potential solution to the selectivity issue, but is still largely at an experimental stage of development. A number of recent studies examining emulsion based imprinting show the technique to be a viable means of achieving selectivity under aqueous conditions[44,45,46]. The formation of TCA based MIPs in a mixture of methanol and water was investigated during the course of our work. While the results showed some promise, replication of data has proved problematic. As a result, it was felt that the testing of the present generation of MIPs in actual wine samples would offer no further insights into the factors governing selectivity within the polymer cavities.

Further investigations into the use of emulsion polymerisation are planned for the near future. Assessments involving wine will be undertaken pending the outcomes of these studies.

5. Methods.

A combination of techniques was employed during the course of the project. These are summarised below.

(i) MIP Design

- computer-based modelling,
- analytical spectroscopic techniques,

(ii) MIP Synthesis & Analysis.

- combinatorial synthesis techniques,
- HPLC analysis.

(i) MIP Design

Computational Studies.

Computational modelling was undertaken during the design phase of the project to sequester information about:

- The electronic structure of the two target species (TCA & TCP).

- The nature of potential interactions between the target species and a range of commercially available functionalised monomer units.

High-level software packages (including *Macspartan Plus, SPARTAN - running on a Silicon Graphics O2 R500 Work Station,* and the *Cerius 2* suite of programs) were utilised to obtain an electronic profile (electrostatic and hydrophobic potentials) and energy minimised conformations for TCA and TCP in combination with a range of commercially available monomers. The modelling packages were also used to design and test the interactions of custom monomer units with each of the targets. It is of some interest to note that the use of computational software has now become an integral aspect of MIP design, with modelling packages containing libraries of monomers units now available commercially[47,48].

Spectroscopic Studies.

Nuclear magnetic resonance (n.m.r.) spectroscopy is a powerful tool analytical tool that is routinely used in synthetic organic chemistry to characterise the structure of materials. Titration-based n.m.r. techniques[49,50] - a process used to examine the strength and

nature of complimentary interactions between molecules were employed during preliminary studies to attempt to confirm target-monomer interactions predicted by modelling studies. A typical procedure is described below.

Solutions of template and monomer (concentration = 0.05 M) were prepared in deuterated solvent. The template was progressively added to the monomer solution in aliquot's containing 0.5 equivalents. Additions were made up to and including 2.0 equivalents or until peak movement ceased. The mixtures were allowed to spin for 10 minutes prior to analysis via proton n.m.r. over 16 scans in CD_3CN.

While slight changes in the spectra were observed during the course of individual titration experiments, the magnitude of these changes was small, indicating that the interactions were in general extremely weak. The technique therefore appears to be of limited value as a diagnostic tool for determining the exact nature of non-defined target-monomer interactions in solution.

(ii) Preparation of Functionalised Monomers.

Two custom monomer units were prepared in the course of these studies. Their preparation is outlined below.

Synthesis of 2,6-Bisacryamido pyridine.

The compound was prepared according to the method of Yane *et. al.*[51]. Triethylamine (24.216g, 240 mmol) and 2,6-diaminopyridine (10.927g, 100 mmol) were dissolved in 300 ml of chloroform and chilled at 0°C. Acryloyl chloride (21.840g, 240 mmol) was added dropwise over a 12 hour period while stirring. Solvent was removed under reduced pressure and the precipitate dissolved in 400 ml of methanol. 200 ml of the methanol solution was gradually added to 1600 ml of distilled water while stirring. The resulting mixture was filtered and dried to yield the desired product (52%) as a white solid.

Synthesis of 2,4,6-Trichlorophenyl Acrylate (TCPA)[52 53]

Measured amount of acryloyl chloride was slowly added to equimolar amounts of 2,4,6-TCP and triethylamine in dichloromethane with constant stirring in an ice bath. The mixture was kept in the ice bath for about 3 hrs and allowed to react overnight at room temperature. The reaction mixture was washed with water, 5% sodium hydroxide (NaOH) solution, again with water then dried over Na_2SO_4. Evaporation of the solvent

gave the desired product as a yellow oil which was used without further purification. ^{1}H NMR δ(ppm): 6.04-6.07 (d, =CH), 6.26-6.35 (q, =CH), 6.61-6.67 (d, =CH-CO), 7.32 (s, 2 aromatic H's). Yield:80%.

(iii) MIP Synthesis & Analysis

Non-covalent MIP preparation.

The preparation of the imprinted polymers was undertaken in test tubes sealed with rubber septa. All polymerisations were conducted under an inert atmosphere of nitrogen gas. A combinatorial approach to synthesis, involving a range of commercially available and specifically designed functionalised monomer units, crosslinking agents and porogens in specified ratios (generally 1:4:20, target: functionalised monomer: crosslinker) was employed. Benzoyl peroxide or 2,2'-azobisisobutyronitrile (AIBN) were used as initiators for all reactions. For each system, a non-imprinted control polymer containing the same combination and ratios of functionalised monomer and crosslinker but without the target molecule was prepared. This is a key aspect of the molecular imprinting strategy as it allows specific binding within the cavities of the imprinted polymer to be separated from non-specific (i.e. background) sorption taking place at the polymer surface.

Polymerisation was generally allowed to occur over 24 hours. Thermally initiated reactions were conducted at 60°C or 80°C depending on the initiator utilised. When complete, the resulting bulk polymer was removed from the reaction vessel and crushed to a fine powder using a coffee grinder. A ball mill was then employed to reduce the polymer particles to their final size (<38 μm). The target molecule was removed from the bulk polymer by Soxhlet extraction using a polar solvent such as methanol. Samples of the solvent were routinely examined by HPLC to determine the percentage of the target recovered. Further grinding and extraction was undertaken at this stage if target recovery was deemed to be too low. Extraction was continued until no further leeching of the target from the polymer was observed.

Following extraction, the polymer was dried under vacuum and dry sieved through graded sieves using a mechanical shaker. Particles with a diameter of ≤38 μm were collected and used in batch rebinding studies to assess the polymer's performance.

Covalent MIP preparation.

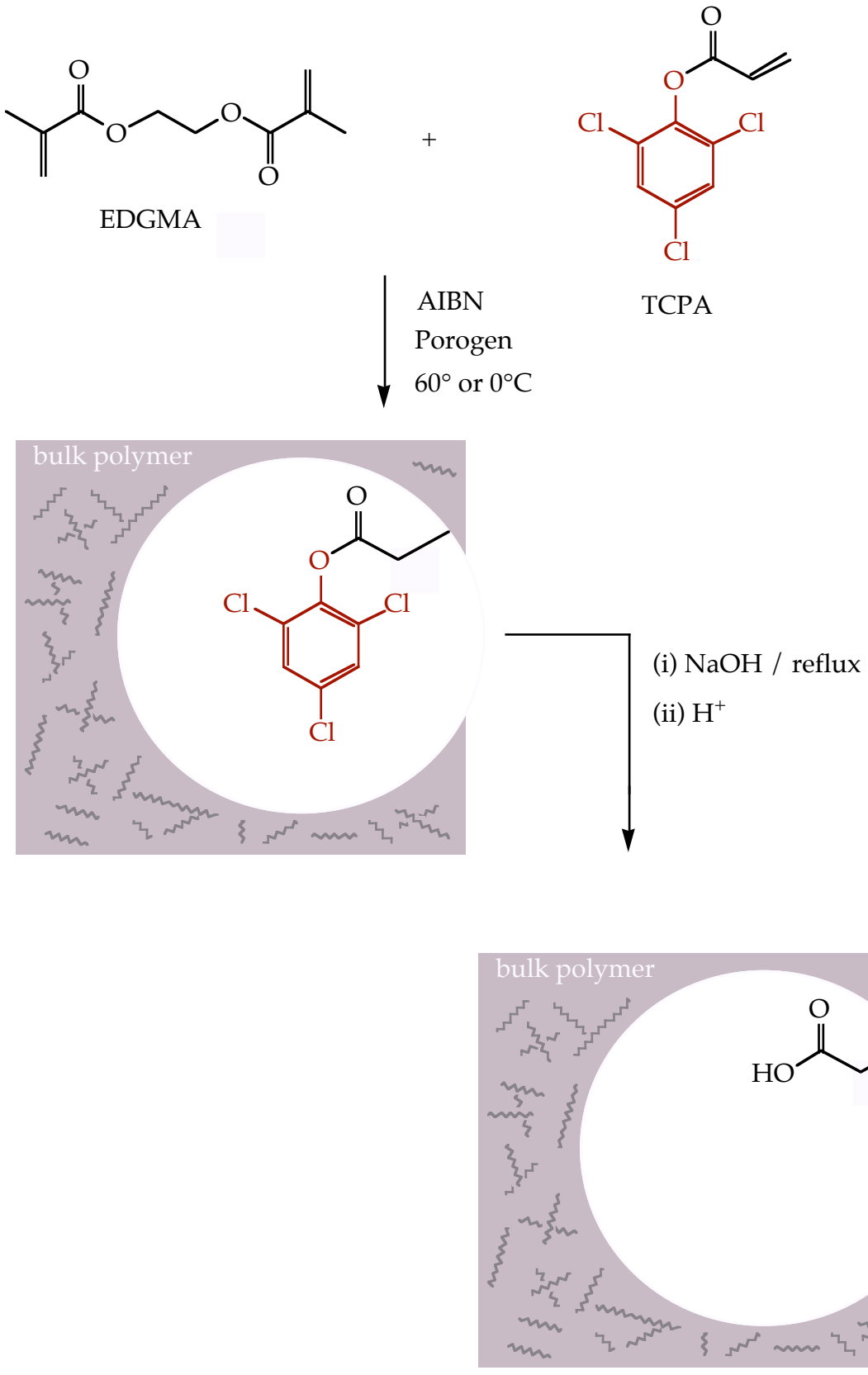
A typical polymer reaction mixture based on reported studies[54,55] consisted of the following: 36 mg (0.022 mmol, 1 mol% of crosslinker and monomer) of AIBN, 0.267 g (0.106 mmol, 5 mol% of crosslinker) of 2,4,6-trichlorophenyl acrylate (TCPA), 4.204 g (4.00 mL, 2.12 mmol) of ethyleneglycol dimethacrylate (EGDMA), and 10.0 mL (2 mL/g of starting materials) of porogen. Polymerisation was allowed to proceed at 60°C for 24 hrs, polymerisation at 0°C was effected by UV irradiation at 365 nm for 3 hrs. In all cases, blank polymerisation (non-imprinted, NIP) was performed. The resulting polymer was ground to fine powder and stirred in MeOH and dichloromethane to remove unreacted substances, for at least 2 hours. After air drying the polymer was ground to a uniform size (≤ 38 □m). Hydrolysis of TCP from within the polymer cavities was achieved by refluxing the polymer in 1M NaOH for 5 hours. The solution was then neutralized with dilute HCl and washed with methanol until UV analysis of the solvent showed no trace of the target (absorbance <0.01 at 300 nm). The polymers were dried *in vacuo* for at least 6 hrs. This general procedure is outlined in scheme 5.2.

Batch Rebinding Analysis by HPLC.

The affinity of the imprinted polymer for the target species was determined by comparing the quantity of target adsorbed (w/w polymer) from a standard solution with that adsorbed by the non-imprinted counterpart in a process known batch rebinding analysis. HPLC analysis is ideally suited to this purpose.

Maximum binding capacity of an imprinted polymer was determined by comparing target sorption levels with that its non-imprinted counterpart. A known mass of the control and imprinted polymer was added to separate vials. A solution containing the target species at a predetermined concentration was then added. The vials were then placed on a shaker table and allowed to equilibrate for several hours. An aliquot of the solution was then withdrawn from the vial and the concentration of the target remaining in solution determined by HPLC. The change in the concentration of the solution allowed the mass of target sorbed by the polymer (either imprinted or non-imprinted) to be determined. Comparison of the quantity of target material sorbed by the imprinted polymer relative to the non-imprinted control defines the efficiency of the imprinted polymer. Higher overall efficiencies reflect stronger interactions between the target and functionalised monomer units during the pre-association step, which leads to the formation of more defined cavities within the bulk polymer.

Scheme 5.2: Preparation of TCP polymers by Covalent Imprinting



Our original proposal planned to assess the relative binding efficiencies of imprinted and non-imprinted polymers by using them as solid phase components of a HPLC column. The approach is somewhat similar to that outlined previously, with the relative efficiency of the imprinted polymer being estimated as a function of the time taken for the target molecule to elute form the column the retention time). Longer retention times reflect greater interactions between the target and the polymer. Such a system required the use of highly specialised column packing equipment that was unavailable locally. Consequently the approach was not pursued.

Batch Rebinding Analysis by UV Spectroscopy.

Measured amounts of the polymer (10-100 mg) were mixed with 5.0 mL of a known concentration (~ 10^{-3} M) of TCP in the porogen solvent and shaken at 200 rpm for a predetermined amount of time (2 or 24 hrs). After removal from the shaker, aliquots (1.0 -2.5 mL) of the solution were filtered and diluted to 10.0 mL, in volumetric flasks. Solution absorbances were then determined at 300 nm and the resultant TCP concentrations calculated. The difference in solution concentration before and after shaking corresponds to the amount of bound TCP.

Other Diagnostic Methods

Diagnostic tools such as Fourier transform infra-red (FTIR) spectroscopy and n.m.r. spectroscopy proved to be of limited value in analysing the polymers. In the case of FTIR spectroscopy, no distinct bands pertaining to the target molecule could be clearly identified, while poor solubility resulting from the high degree crosslinking in the polymers prevented the use of n.m.r. techniques. It is possible that solid-state n.m.r. analysis may provide more useful insights into the nature of the binding interactions of the target within the polymer cavities, however, such instrumentation was unavailable to us.

Titration experiments involving n.m.r spectroscopy were used in the study of second generation MIP systems to identify potential target – monomer interactions. While the magnitude of spectral shifts were relatively small, the changes were consistent with the H-bonding and π-π stacking interactions predicted by computational modelling studies.

6. Results & Discussion

Non-covalent Imprinting Studies

Molecular imprinting is a technique that is becoming increasingly common as a means to generate polymers with recognition sites of predetermined selectivity[56]. The process relies on the formation of a complex between a chosen template and a monomer with complimentary functionality. Polymerisation in the presence of a cross-linker yields a rigid polymer from which the template may be extracted, producing recognition sites that contain complimentary size, shape and functionality to the template.

Interest in molecular imprinting lies not only in their potential as media for chromatographic separation, receptor, antibody and enzyme mimics or affinity and sensing materials[57,85,58,59] but also with their stability in a wide range of conditions (temperature, pressure, organic solvents, acidic or basic solutes etc).

To date, molecularly imprinted polymers (MIPs) that display good recognition properties generally contain well-defined functional groups (-COOH, -NH-, etc) that produce strong complimentary interactions with monomer units when polymerisation is conducted in relatively non-polar organic solvents (such as chloroform or toluene)[57]

As this research aimed to develop MIPs for poorly functionalised molecules such as 2,4,6-trichloroanisole (TCA) and 2,4,6-trichlorophenol (TCP) (both), we were interested in the approaches used by previous investigators. Recently, reports documenting the use of weak, non-covalent interactions such as π-π stacking and Van der Waals forces have been exploited to produce molecularly imprinted polymers for environmental pollutants such as herbicides, dioxins and polychlorinated biphenyls[60,61]. However, at the commencement of this research, few protocols for the imprinting of alcohols or other poorly functionalised molecules were known in the literature[58].

A preliminary computer-based molecular modelling study of TCA and TCP was undertaken to gain a better understanding of the electronic structure of the target molecules (Figure 6.1 and Table 6.1). The results revealed the presence of highly defined regions of electron potential because of the presence of electronegative chlorine atoms on the benzene rings, which withdraw electron density from the π system, polarising the attached ring carbon atoms, thereby inducing a positive charge.

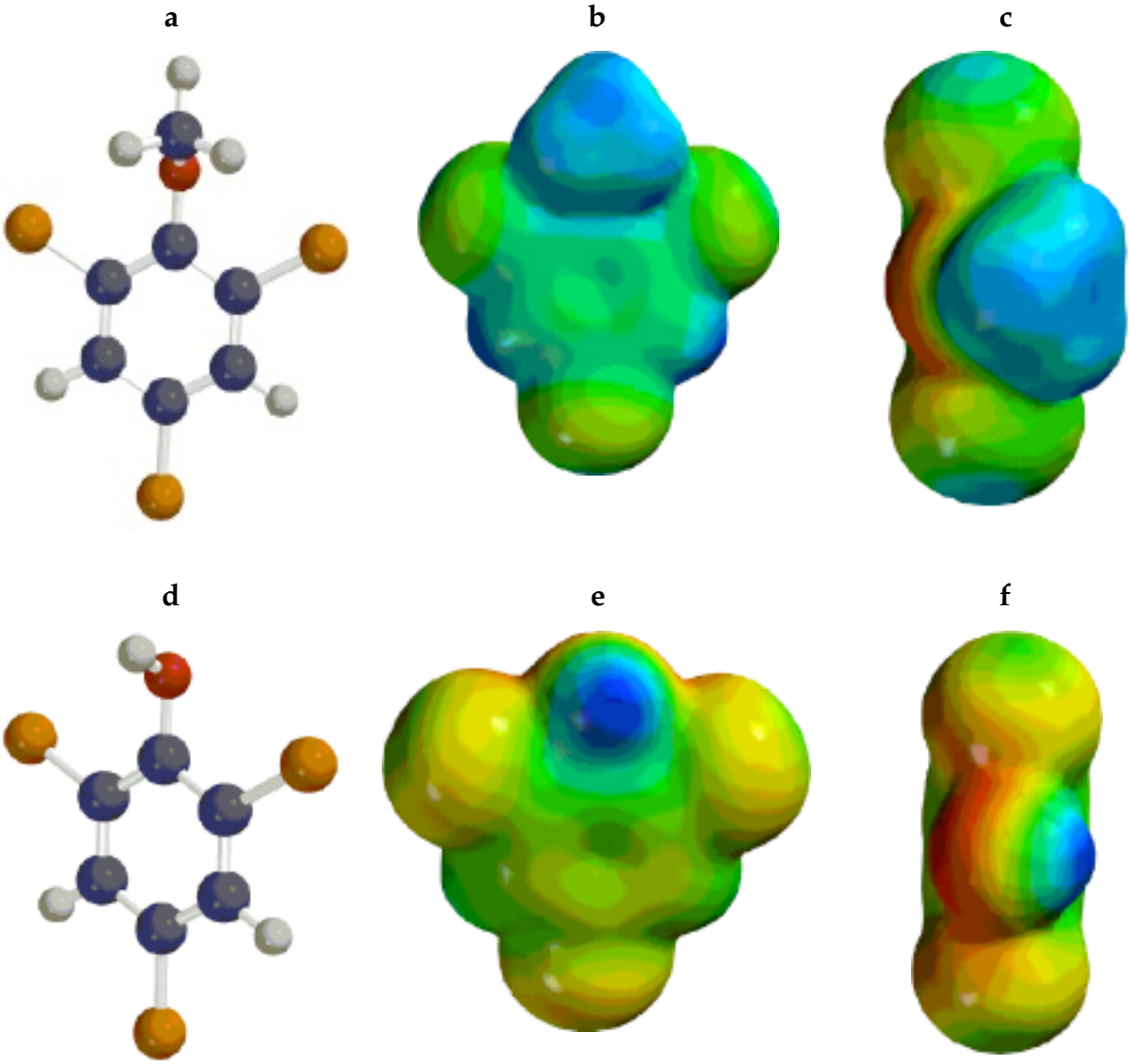


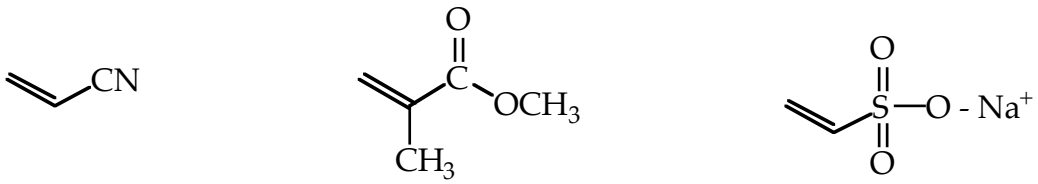
Figure 6.1: *Ab initio* calculations on the surface potential's of (a) trichloroanisole ((b) is the top view with blue indicating regions of low potential, and red indicating regions of high potential, (c) side view); (d) tricholorophenol (e) is the top view with blue indicating regions of low potential, and red indicating regions of high potential, (f) side view). Calculations conducted at the 6-31G* level of theory.

Table 6.1: Point charges calculated for TCA and TCP at the 6-31G* level of theory

TCA atom	Charge	TCP atom	Charge
O-CH_3	-0.64	O-H	-0.74
ortho-Cl	0.04	ortho-Cl	0.04
para-Cl	0.02	para-Cl	0.03

The distinct charge separation on both molecules suggested that weak associative interactions might develop with monomer units containing complimentary functional groups prior to polymerisation. To investigate this possibility, a number of TCA-based MIPs were prepared from commercial monomers possessing a range of electronic characteristics (Figure 6.2).

Figure 6.2

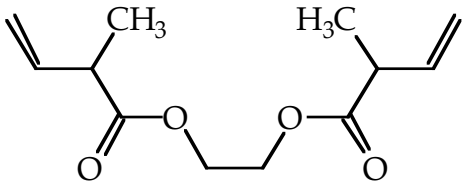


Acrylonitrile (ACN) | Methyl methacrylate (MMA) | Vinyl sulfonic acid (VSA)

The nitrile group (CN) of acrylonitrile possesses high electron density and a lone electron pair on the nitrogen atom, which, it was hoped, would interact favourably with electron deficient regions on TCA. Likewise, the oxygen atoms on methyl methacrylate and sodium vinylsulfonate possess similar high electron densities, which were also expected to present favourable interactions.

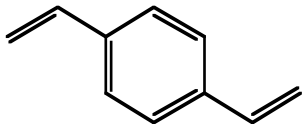
Polymers (Table 6.2) were prepared using a template: monomer : crosslinker ratio of 1:18:72, proportions which previous studies have shown to produce the highest levels of target rebinding[62]. Two crosslinking agents – ethylene glycol dimethacrylate (EGDMA) and divinyl benzene (DVB) were employed (Figure 6.3).

Figure 6.3



ethyleneglycol dimethacrylate

(EDGMA)



Divinylbenzene

(DVB)

The purpose of the cross-linker is to provide structural integrity in the resulting polymer to enable the shape of target cavities formed during polymerisation to remain defined following the removal of the target molecule[63]. It has been reported that EGDMA promotes a greater binding specificity than divinylbenzene[58] because the glycol oxygen atoms may contribute to target recognition within the binding site. Divinylbenzene may however prove useful in promoting hydrophobic □-□ stacking interactions within the target cavity.

Benzene was selected as the porogen (solvent) for all polymerisations because of its non-polar nature and low dielectric constant (e = 2.284 dB). This solvent characteristic is particularly important in molecular imprinting as it ensures that interactions between the target molecule and the functional groups of the monomer unit are maximised during pre-association[59].

With the exception of the sodium vinylsulfonate preparations, which produced a yellow resin, all polymers formed as hard blocks. Following removal from the reaction vessel (a sealed test tube), the polymers were ground to a fine powder using a combination of coffee grinder and ball mill, then dried at reduced pressure (~1 mm Hg) and extracted for 12 hours with hexane using a Soxhlet apparatus. The sodium vinylsulfonate polymers proved to be unsuitable for extraction and were not investigated further.

Table 6.2: Polymers (Imprinted and Non-Imprinted) Prepared by Thermal Initiation (80°C) Using Benzoyl peroxide (BP).

Polymer #	TCA (g)	Monomers	Crosslinker	Initiator	Solvent
14	0.1	VSA	EGDMA	BP	Benzene
14a	-	VSA	EGDMA	BP	Benzene
18	0.1	ACN	EGDMA	BP	Benzene
18a	-	ACN	EGDMA	BP	Benzene
20	0.1	MMA	EGDMA	BP	Benzene
20a	-	MMA	EGDMA	BP	Benzene
23	0.1	MMA	DVB	BP	Benzene
23a	-	MMA	DVB	BP	Benzene
24	0.1	ACN	DVB	BP	Benzene
24a		ACN	DVB	BP	Benzene
25	0.1	ACN	EDMA + DVB	BP	Benzene
25a	-	ACN	EDMA + DVB	BP	Benzene

All polymerisations were conducted at 80°C using a radical initiator (benzoyl peroxide). Low temperature initiators such as azobisisobutyronitrile (AIBN) are now carry restricted import status in Australia and were unavailable for this study. Several reports indicating that low temperature conditions (~ 0°C) for polymerisation enhance template – monomer binding affinities have appeared in the literature[63]. However, these appear largely to be restricted to MIPs involving biological species such as enzymes and proteins. While the temperature of the reaction undoubtedly exerts some influence over the strength of the interactions, it is unlikely to be the major factor dictating selectivity of the resulting polymer in such rigid molecules as TCA and TCP. It is instead likely that in the case of biological species, temperature control relates specifically to the maintenance of a preferred conformation by the target during polymerisation.

Control polymers, that is, polymers containing identical ratios of monomer and cross-linker but not the target molecule, were prepared for each MIP system (denoted with an

"a" in Table 6.2. The non-imprinted polymer (NIP) is a key aspect of molecular imprinting as it provides a benchmark against which target by rebinding by the MIP is normalised.

The amount of TCA removed from the imprinted polymers was determined by UV spectrophotometry (λ_{max} = 288 nm). Table 6.3 shows TCA recovery rates from the MIP systems. In general, template recovery was poor. This was attributed to a number of factors including variations in particle size and the high level of crosslinking within the polymer, both of which lead to trapping of target molecules deep within the polymer matrix.

Table 6.3: Percentage of TCA extracted from the polymer matrix.

Polymer #	TCA extracted (g)	% TCA recovered
18	0.47	47
20	0.432	43.2
23	0.442	44.2
24	0.51	51
25	0	0

The choice of hexane as the extracting solvent over a more polar system may also have contributed to the low levels of recovery, although the approach seemed justifiable given the non-polar nature of the target. Whitcombe *et a.l*[58] used a solution of 1 molar sodium hydroxide in methanol, heated to reflux over a 6 hour period for target extraction, while Matsui *et al.*[64] employed a strategy of continuous washing with a mixture of methanol and acetic acid for 24 hours to achieve recovery. Wet-sieving techniques using a sintered glass filter funnel in combination with polar solvents have also been successfully employed[65].

Rebinding Studies

Two methods for performing recognition or binding studies have been developed, the most common of which is to prepare the imprinted polymer as the stationary phase on a high performance liquid chromatography column (HPLC). A solution of known concentration of the template molecule is then passed along the column. The retention

time relative to that recorded on an identical non-imprinted polymer is then used to determine binding affinity.

The second method, developed by Whitcombe *et al.*[58,66] involves shaking a known mass of imprinted polymer in a solution containing a predetermined quantity of the target species. The solution is then analysed by GC or HPLC to determine the amount of target sorbed by the MIP.

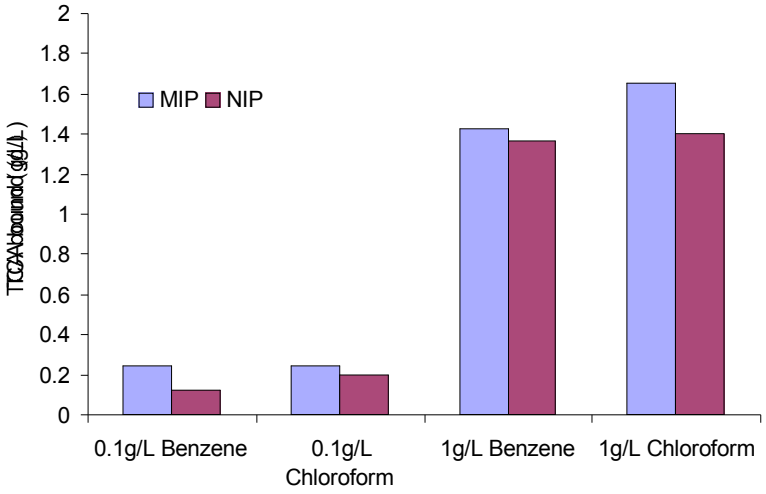
Method II was adopted to assess the binding affinities of the TCA MIPs. Samples of each MIP and its corresponding control polymer (100 mg) were shaken in TCA solutions (5.0 mL) at two concentrations (0.1 g L^{-1} and 1.0 g L^{-1}) overnight at room temperature (Table 6.4). All solutions were then analysed by gas chromatography. Samples were prepared in both benzene and chloroform to examine the effect of solvent properties on binding affinity.

Table 6.4: TCA rebinding conditions for Imprinted and Non-imprinted Polymers.

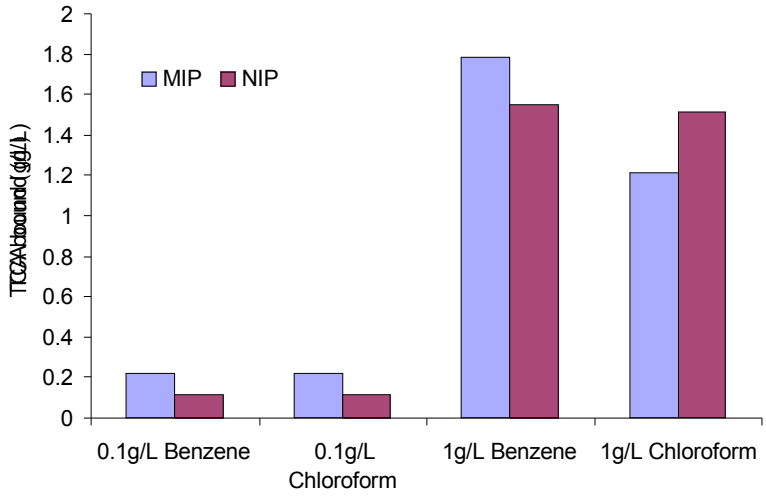
Polymer	Mass (mg)	Rebinding Solvent		[TCA] (gL^{-1})	
18	100	Benzene	$CHCl_3$	0.1	1.0
18a	100	Benzene	$CHCl_3$	0.1	1.0
20	100	Benzene	$CHCl_3$	0.1	1.0
20a	100	Benzene	$CHCl_3$	0.1	1.0
23	100	Benzene	$CHCl_3$	0.1	1.0
23a	100	Benzene	$CHCl_3$	0.1	1.0
24	100	Benzene	$CHCl_3$	0.1	1.0
24a	100	Benzene	$CHCl_3$	0.1	1.0
25	100	Benzene	$CHCl_3$	0.1	1.0
25a	100	Benzene	$CHCl_3$	0.1	1.0

TCA uptake for selected polymers is shown in figures 6.4 and 6.5. In general, target discrimination between the imprinted and control polymers was poor, with both systems showing high levels of non-selective surface binding.

Figure 6.4: Amount of TCA (gL^{-1}) bound by 100 mg of a) polymer **20** (MMA/EGDMA); and b) polymer **23** (MMA/DVB) in benzene and $CHCl_3$ rebinding solvents containing 0.1g/L and 1g/L TCA.

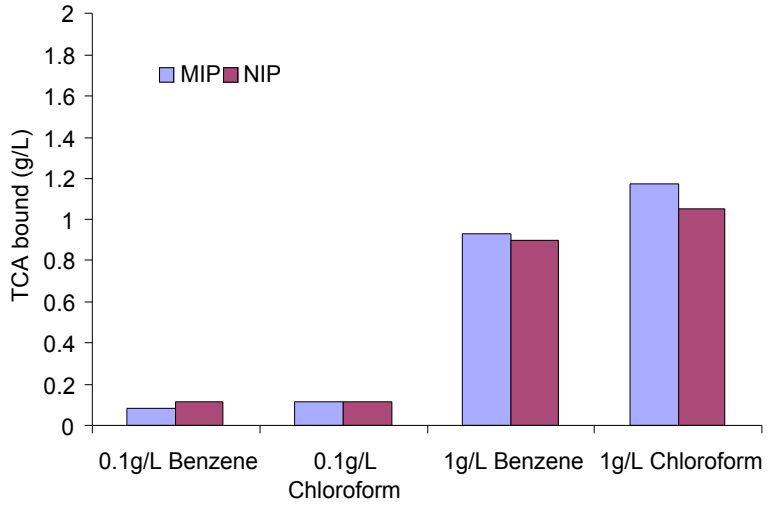


a)

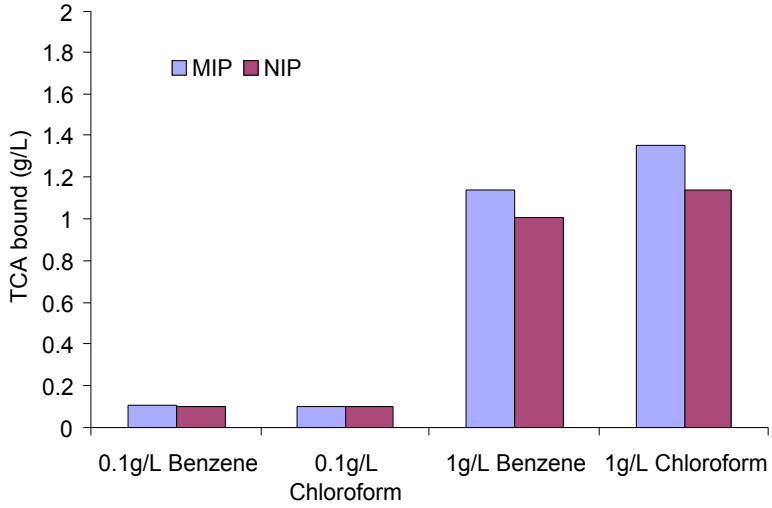


b)

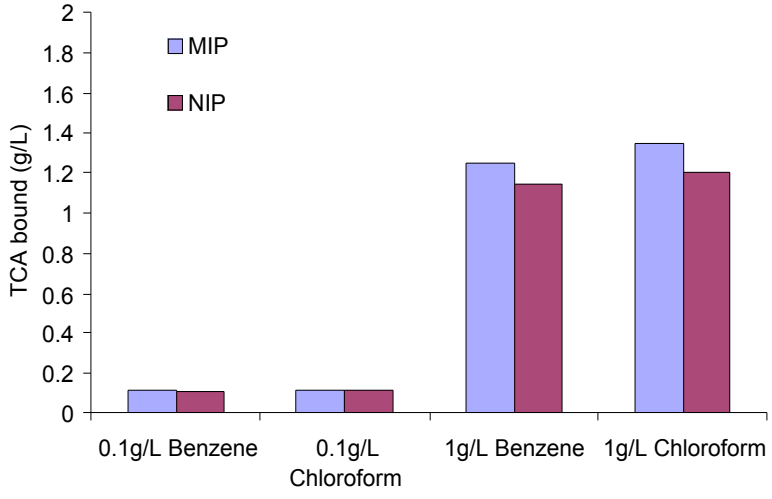
Figure 6.5: TCA bound to polymers (100 mg) a) **18** (acrylonitrile/EGDMA); b) **24** (acrylonitrile/DVB); and c) **25** (acrylonitrile/DVB/EGDMA).



a)



b)



Polymers crosslinked with divinyl benzene (**24** and **20**) appeared to exhibit slightly higher levels of both selective and non-selective binding for TCA than the more polar systems crosslinked with EGDMA. It is possible that the higher binding affinity results from complimentary target - polymer associations based on π-π stacking interactions. This phenomenon is an important aspect of conformational control in biological systems and is the result of cohesive forces between aromatic surfaces arising from favourable electrostatic interactions between the respective π orbitals of adjacent ring systems.

The rebinding results also exhibited slight variability between chloroform and benzene. Target recognition is generally found to be most efficient when rebinding is performed in the solvent used for polymer formation. Solvent is known to play an important role in developing the character of the final polymer by influencing important features such as porosity and cavity size[67] . Solvents possessing high dielectric constant values disrupt functional group interactions and result in poorer levels of specificity[63,68]. The results for polymer **18** seem to reflect this, with binding specificity lower in chloroform (e = 4.906 dB) than in benzene (e = 2.284 dB).

Perhaps the most puzzling observation arising from these results was the increase in TCA concentrations over the [0.1 gL^{-1}] and [1.0 gL^{-1}] TCA solutions that were introduced during rebinding. Two theories account for this observation.

The first is that TCA not previously removed during the extraction step has leeched from the polymer cavities during rebinding. That is to say, the initial extraction procedure may have been inadequate. It is possible that shaking the polymers, particularly in a different porogen (such as chloroform), result in new conformational changes within the polymer structure, thereby permitting previously inaccessible TCA to be released.

The second theory relates to cavity swelling as a result of a rapid influx of solvent molecules. They become trapped within the polymer matrix, effectively reducing their "free" concentration in solution[63]. In doing so, the mole fraction of free solvent molecules is reduced, and the target (TCA) mole fraction is therefore increased.

Both the imprinted and non-imprinted forms exhibited raised levels of TCA at both concentrations investigated. As the control (non-imprinted) polymers contained no TCA

initially, leeching cannot account for the increase in TCA concentration within the rebinding solution. Previous studies conducted on the swelling of insoluble imprinted polymers show that up to a four fold difference in percentage volume increase may occur when different solvents are employed as porogens.[68]

Preliminary studies into TCA imprinted polymers were unsuccessful at achieving molecular recognition. The findings indicated that more defined target – monomer interactions were required. Further computational analysis was therefore undertaken to analyse potential $\pi - \pi$ stacking and hydrogen bonding interactions with a range of aromatic monomer units.

2nd Generation MIPs

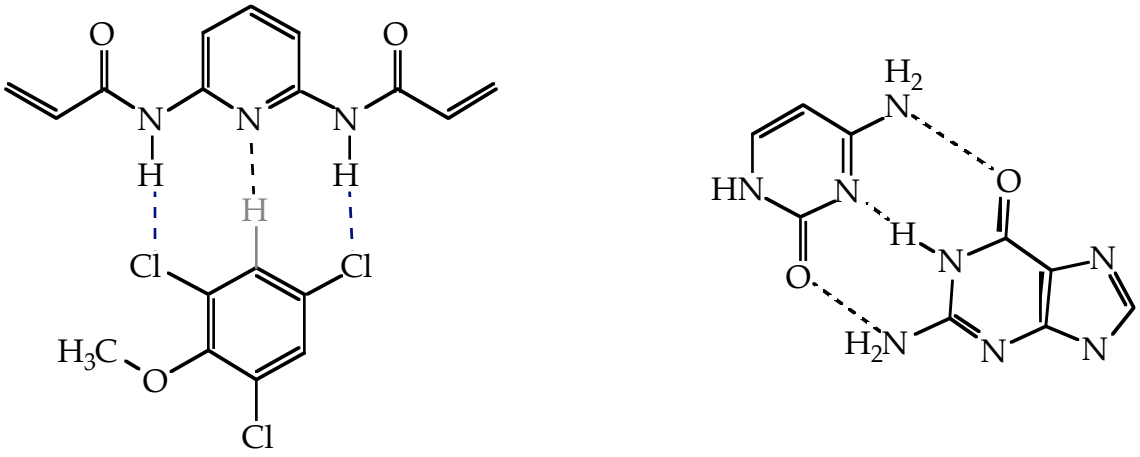
The non-covalent approach towards molecular imprinting was seen to be the most flexible strategy in terms of the choice of functional monomer and possible template molecules. However the weak non-covalent interactions formed during the pre-association stage of polymerisation were seen to be somewhat limiting[60].

At this stage of the research, our aim was to further explore a combination of electrostatic, hydrophobic interactions and hydrogen bonding interactions. With this in mind, a second target molecule was incorporated into our investigations to determine the effects of stronger interactions (particularly hydrogn bonding) on polymer affinity. 2,4,6-trichlorophenol (TCP) (Table 6.5), is structurally identically to TCA with a single but important exception. TCP contains an acidic phenolic hydrogen ($pK_a \approx 7.2$) that could be utilised to form a strong pre-association complex with a range of functional groups.

A range of commercially available and custom designed monomer units based on the pyridine ring systems were identified as being potentially capable of forming suitable interactions with both target species. The pyridine is heterocyclic aromatic ring system comprised of five carbon atoms and a nitrogen atom. The difference in electronegativity between nitrogen and carbon results in the molecule possessing a strong dipole moment. The ring nitrogen also possesses a lone electron pair (which does not participate in p bonding) that gives rise to its base like properties.

Monomers such as 4-vinyl pyridine (4-VP) (Table 6.5) have been previously used in MIP studies to compliment acidic and hydrophobic groups on targets including 2,4-dichlorophenoxy acetic acid[69], 2,4,5-trichlorpheoxyacetic acid[35] and 4-nitrophenol[70,71] through electrostatic and hydrogen bonding interactions. It was further postulated that 4VP might act as a complimentary electron rich π donor for the π deficient TCA and TCP rings, thereby resulting in complimentary π - π stacking interactions[51].

A custom monomer, 2,6-bis(acrylamido)pyridine (BAAP) (table 6.5) was also prepared. This system offers multiple binding through hydrogen bonding associations between the amide hydrogen atoms and the ring chlorine atoms of the targets (Figure 6.6a), although the strength of these associations is undoubtedly weak because of the more diffuse nature of the 3p orbitals on the chlorine atoms. The resulting template/monomer complex does bear some semblance (albeit somewhat tenuous), recognition elements observed in nucleotide systems found in nature (Figure 6.6b).



a) **b)**

Figure 6.6: a) Potential recognition elements between TCA and BAAP monomer and b) the nucleotide bases cytosine and guanine.

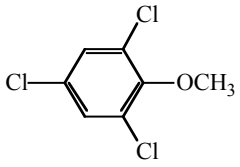
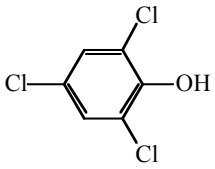
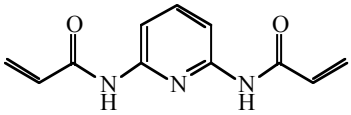
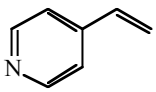
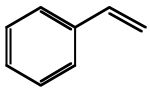
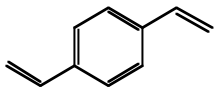
Modelling

For each monomer/template system a semi-empirical calculation with geometry optimisation was used with an AM1 force field, organic solvent, and with the number of maximum and optimum cycles restricted to 5000 and the energy convergence to 1 x 10^{-4} kJ/mol. The properties selected included MO's, dipole, muliken, natural, and bond order. Solvation selected was AM1-SM2. LogP selected was Ghose-Crippen, and

population selected was electrostatic charges. For the generation of surface, selections included – density, elpot property and medium resolution. No preset distance parameters were enforced for any target-monomer combination.

Modelling studies reveal that the ring nitrogen induces a strong dipole moment in 4-VP (μ = 2.247 D), when compared with styrene (μ = 0.010 D). This, coupled with the dipoles of TCA (1.574) and TCP (1.584), suggest that complimentary interactions between TCA/TCP and 4-VP should prevail in solution.

Table 6.5 Target and monomers used together with calculated dipole moments (μ).

	Compound	Structure	μ (Debye)[a]
1	Trichloroanisole		1.574
2	Trichlorophenol		1.584
3	2,6-Bisacrylamidopyridine		4.533
4	4-Vinylpyridine		2.247
5	Styrene		0.010
6	*p*-Divinylbenzene		0.034

[a]Dipole values do not indicate dipole vector which will influence the composition of the prearrangement complex prior to polymerisation.

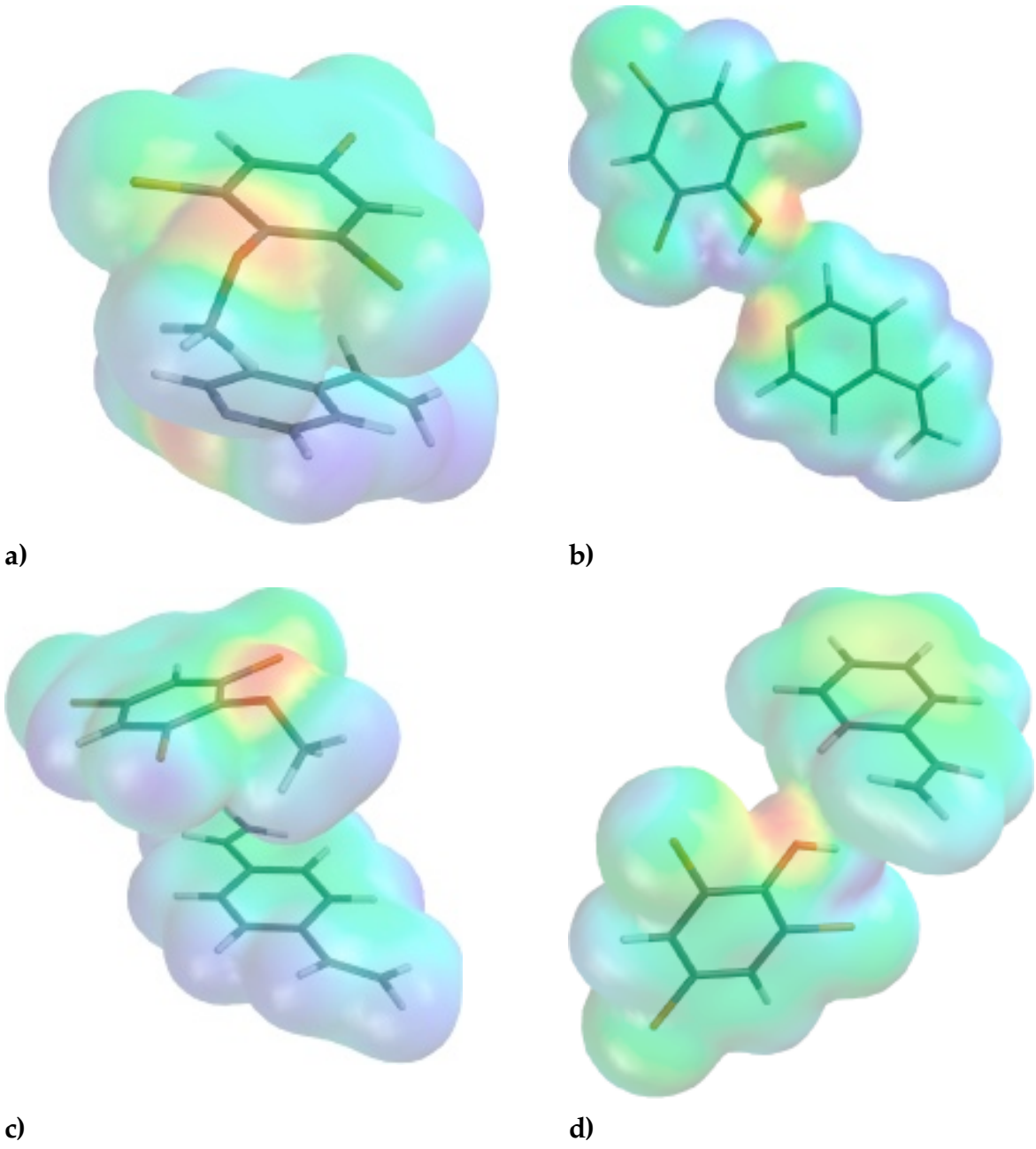
Figure 6.7 displays the predictive modelling of the templates with the 2 monomers; styrene and 4-VP with TCA. Only weak interacts are observed between TCA and 4-VP

(Figure 6.7a), while π-π stacking interactions predominate with styrene (Figure 6.7c). The strength of these interactions is summarised in Table 6.6 as a functions of complex dipole moment. For TCP (Figure 6.7 b), there clear hydrogen bonding interactions are observed between the acidic phenolic hydrogen and the ring nitrogen as well as the oxygen and nearby ring hydrogen. This may result in relatively strong interactions in solution that result in the development of recognition sites within the resultant polymer.

Somewhat surprisingly, the interactions between TCP and styrene do not appear to be the result of π-π stacking. Again there appears to be a preference for hydrogen bonding although the overall dipole of the complex is less than that for 4-VP (Table 6.6).

Table 6.6: Energy of formation and Dipole moments for the Template / monomer systems.

Target	Monomer	ΔH_f (KJ/mol)	μ (Debye)
8 TCA	BAAP	-203.455	1.961
9 TCA	4-VP	14.983	2.768
10 TCA	Styrene	35.235	1.607
11 TCA	DVB	33.285	1.024
12 TCP	BAAP	-259.146	2.116
13 TCP	4-VP	-25.888	2.569
14 TCP	Styrene	-5.114	1.067
15 TCP	DVB	4.005	1.055
16 Anisole	BAAP	-6.056	5.411
17 Phenol	BAAP	-26.452	4.269

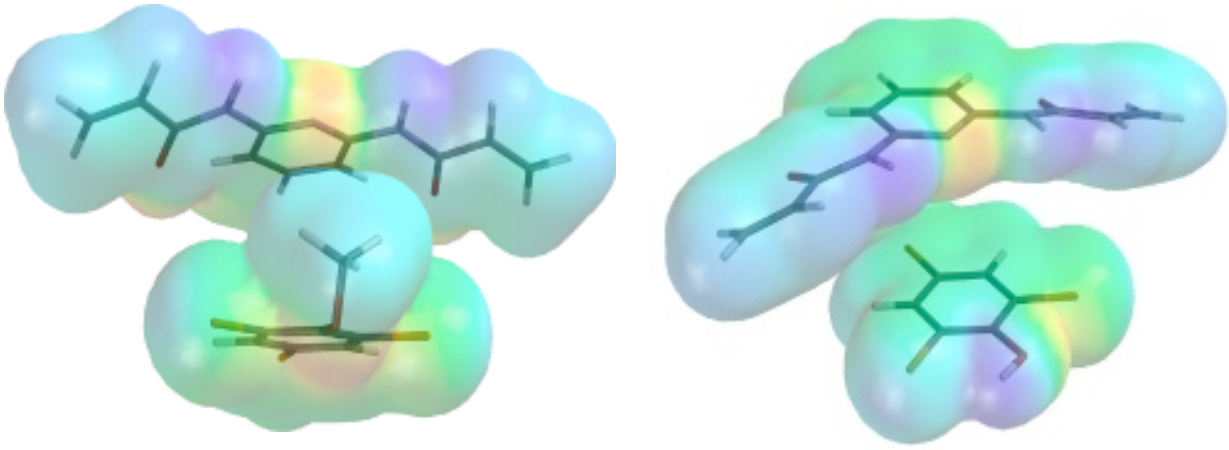


a) **b)**

c) **d)**

Figure 6.7 Geometry minimisation calculations between a) TCA and 4-VP; b) TCP and 4-VP; c) TCA and Styrene; and d) TCP and Styrene.

Modelling studies were also performed on TCA / TCP complexes with 2,6-bis(acrylamido)pyridine (BAAP) in order to examine if multiple points of interaction are observed, and thus feasible to then synthesise the monomer and subsequent polymer systems (Figure 6.8).



a) **b)**

Figure 6.8: Optimised geometries for a) TCA + BAAP; and b) TCP + BAAP.

Energy minimisations for both systems resulted in associations that appeared quite similar in nature. The electronegative O-CH_3 and OH groups on both targets were shown to align in a stacked formation directly below the nitrogen in the pyridine ring system, indicating possible combination H - bonding or π - π interactions. The high negative heats of formation for the BAAP complexes (Table 6.6) predict that they should form more stable associations than other modelled systems.

A series of ^{1}H n.m.r. titration experiment were then performed in an attempt to confirm the predicted interactions. This technique has been previously described in the literature as a method for evaluating template – monomer interactions in MIP systems[72].

Titration of BAAP with TCA and TCP (Figure 6.9 & 6.10) were typical of findings overall, showing only minor spectral shifts that could not be meaningfully interpreted. Similar results were observed for TCA and TCP titrated with 4-VP. No changes were observed for titrations involving styrene. Attempts to repeat the BAAP titrations in a less polar solvent (the polar nature of CH_3CN can mask potential hydrogen bonding interactions) were unsuccessful because of solubility problems.

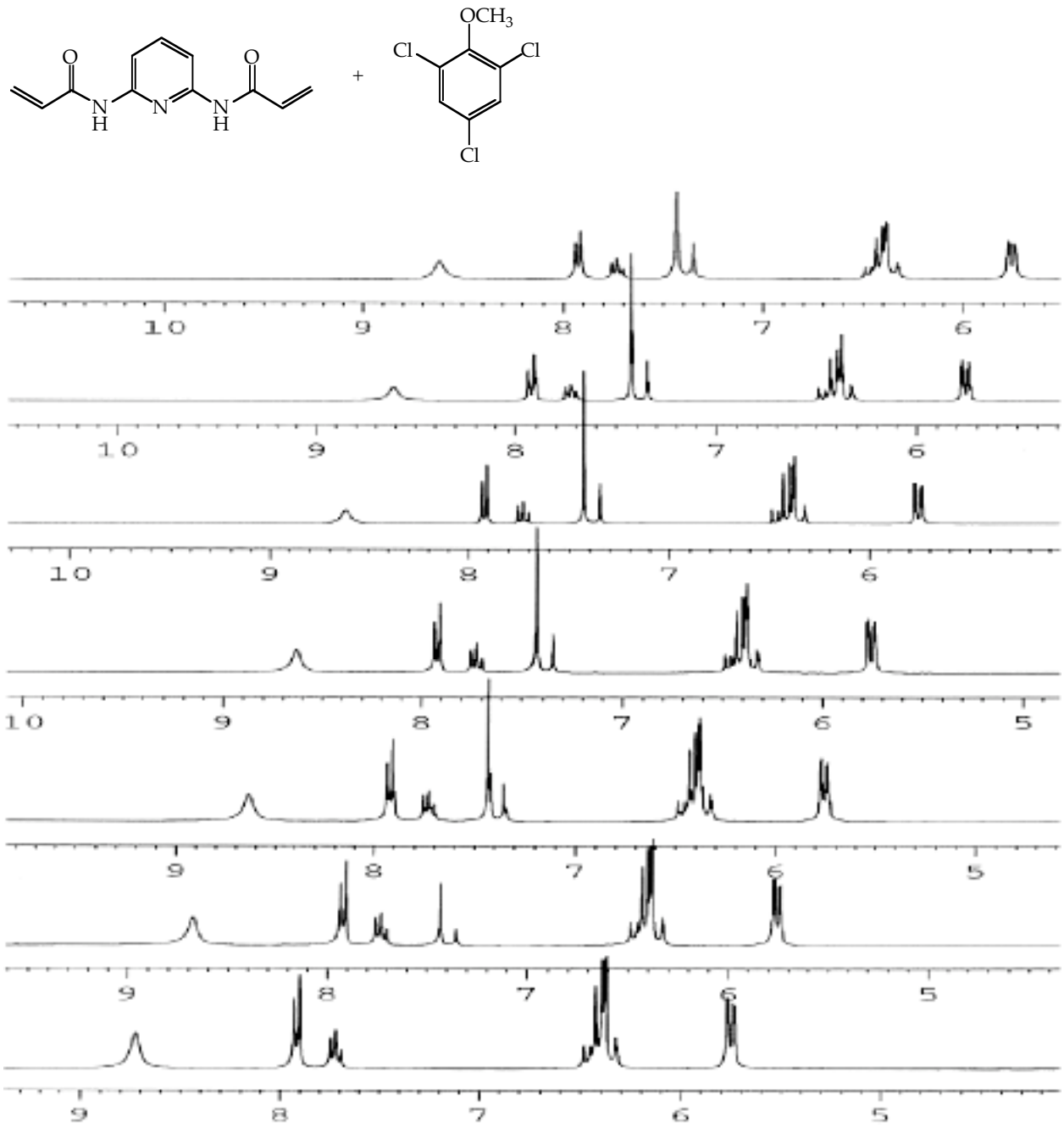


Figure 6.9: ^{1}H nmr Titration of 2,6-bisacryloamidopyridine with TCA in CD_3CN (entry **8**) monitored by ^{1}H nmr spectroscopy. Zero TCA added (bottom) to 3.0 equivalent (top). The gradual upfield movement of the NH resonance (8.6 ppm) is discernible.

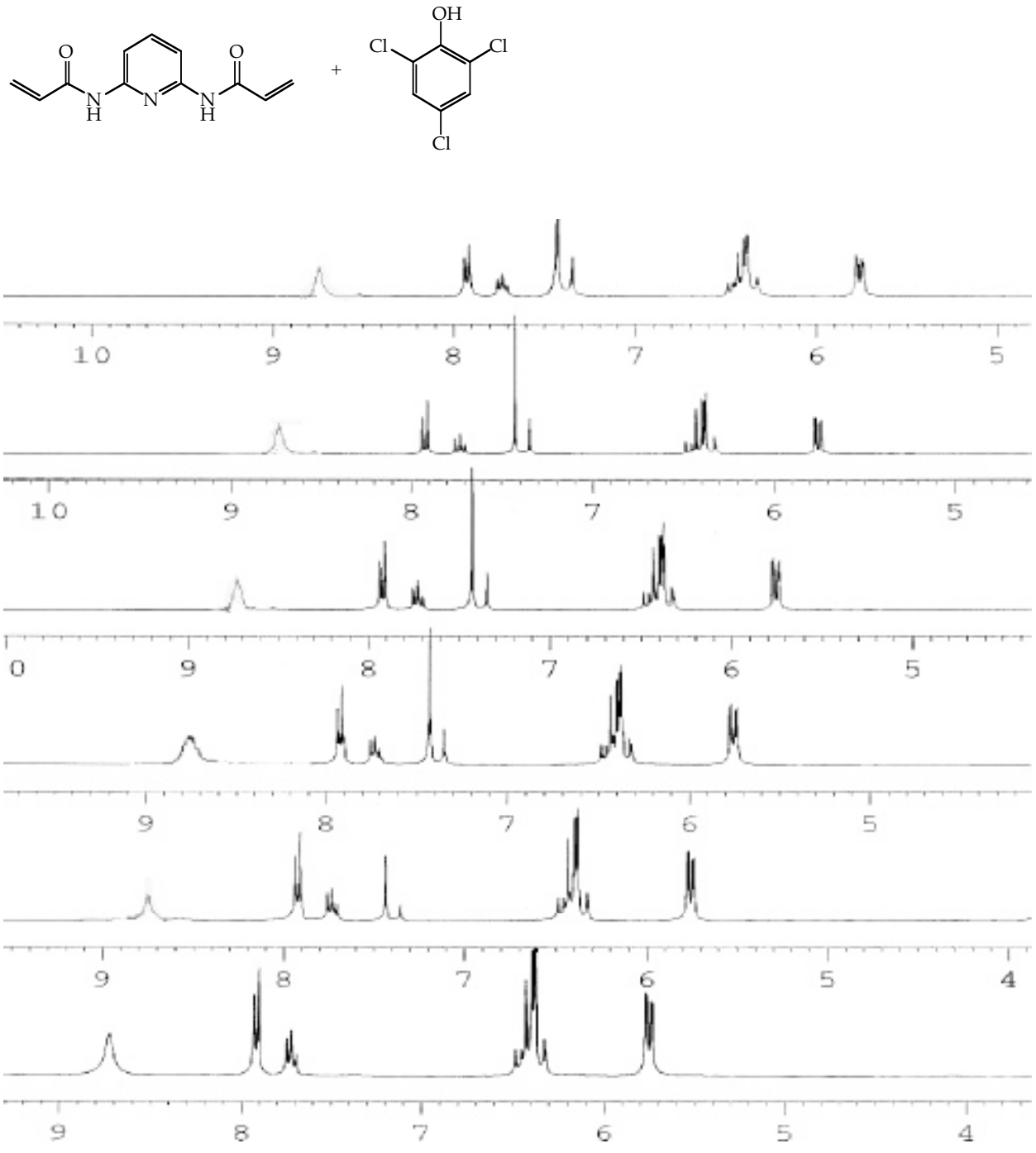


Figure 6.10 : ^{1}H nmr titration for TCP with BAAP in CD_3CN. No change in shifts.

2,6-Bis(acrylamido)pyridine Based MIPs.

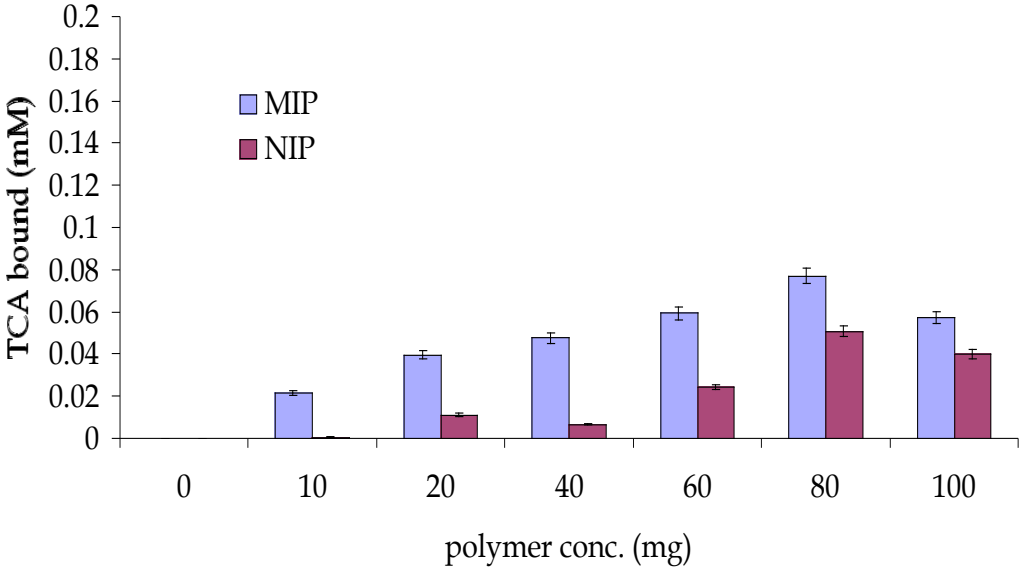
TCA and TCP imprinted polymers (and controls) were prepared using 2,6-bis(acrylamido)pyridine as the functional monomer in 80% aqueous methanol. The imprinted and control polymers were prepared in a ratio of 1:4:20 of template:functional monomer:crosslinker in $MeOH/H_2O$ (80:20) (5 ml) porogen[60], with EGDMA used as the crosslinking agent.

Batch rebinding studies were conducted on a range of polymer concentrations from 0-100 mg in [0.2] mM TCA solution in two solvent systems: $MeOH/H_2O$ (60:40) and CH_3CN. Samples were shaken for 2 hours before being filtered and the solution analysed by reversed phase HPLC to determine the change in TCA concentration.

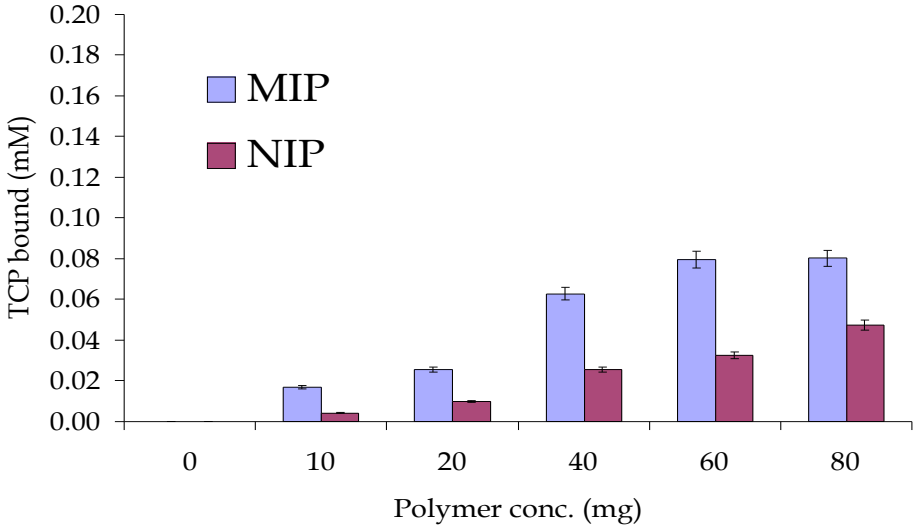
Figure 6.11 shows the results obtained from the initial analysis of the two polymers conducted in the formation porogen. They are the average of three replicate runs. Recognition in both cases is moderate, with TCP producing a slightly better overall result. Given the highly polar nature of the solvent system, it lis likely that that hydrophobic interactions (π-π stacking) dominate template – monomer recognition in the polymer cavities. It is well documented that π-π stacking is strongest in the aqueous environment.

Appreciable levels of non-specific surface binding (>50% at maximum sorption) was observed for both polymers. The slightly lower levels observed in the TCP system may reflect the presence of hydrogen bonding interactions which, in conjunction with π-π stacking may result in more defined cavities.

Binding efficiencies were also analysed in acetonitrile to examine the effect of solvent on recognition. Figure 6.12 shows a clearly show a loss of recognition in both systems, a result that is consistent with previous literature reports suggesting that solvent is an important aspect of recognition efficiency.

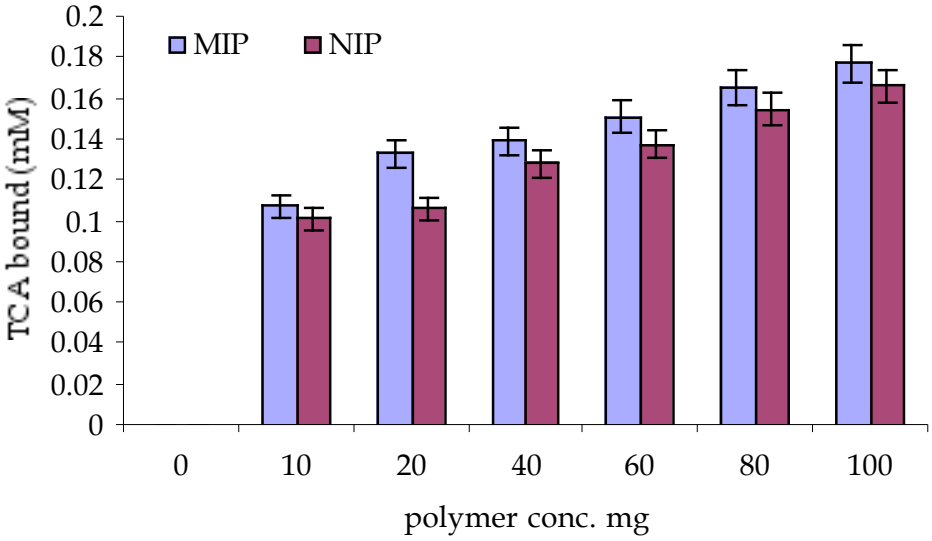


a)

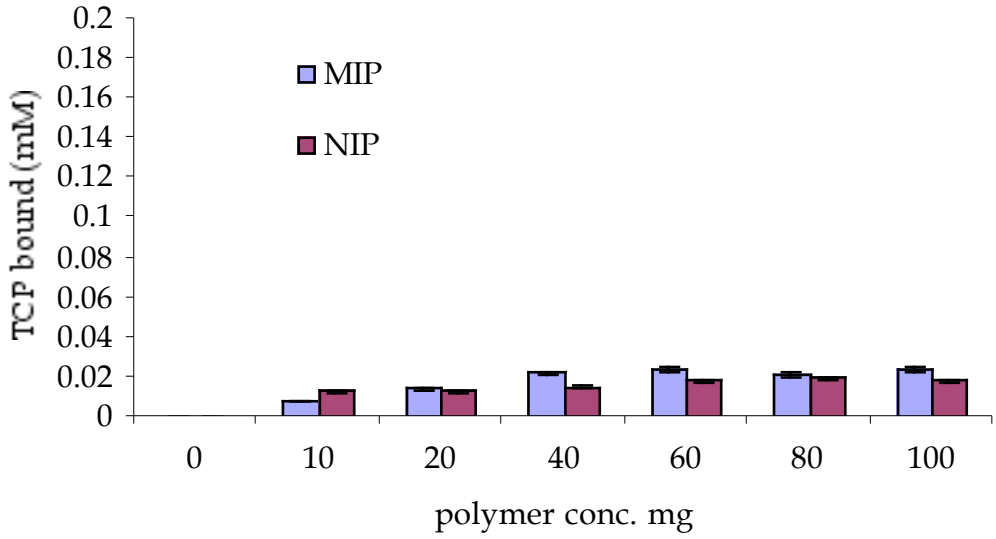


b)

Figure 6.11: Rebinding data for polymers imprinted with a) TCA; and b) TCP using 2,6-bis(acrylamido)pyridine monomer in MeOH/H_2O (80:20).



a)



b)

Figure 6.12: Rebinding data for MIPs imprinted with a) TCA; and b) TCP prepared in MeOH/H_2O porogen and analysed in CH_3CN.

4-Vinylpyridine Based MIPs

Molecularly Imprinted Polymers were prepared for the templates 2,4,6-trichloroanisole and 2,4,6-trichlorophenol in various porogens (Table 6.7) to examine the effect of different solvents on the imprinting effect. All polymers were prepared with 4-vinlypyridine as the functional monomer and ethyleneglycol dimethacrylate as the crosslinker (EGDMA) in a ratio of 1:4:20 mmol.

Table 6.7: Porogens used in 4-VP Based MIP synthesis

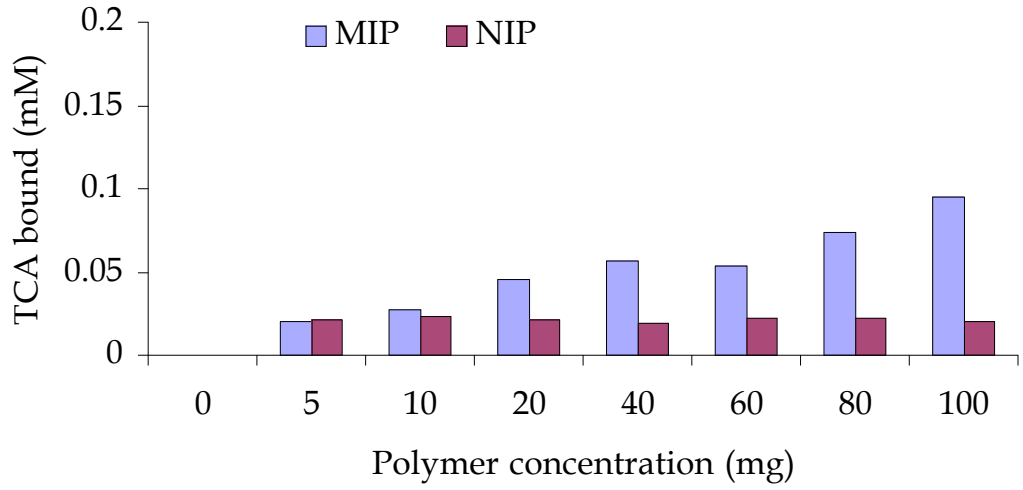
	Template	**Porogen**	**Dielectric constant**	**Template adsorbed mM per mg polymer**	
				MIP	NIP
1	TCP	CH_3CN	37.50	$1.197x10^{-3}$	$6.578x10^{-4}$
2	TCP	CH_2Cl_2	9.08	$9.810x10^{-4}$	$9.300x10^{-5}$
3	TCP	Hexane	1.89	$1.895x10^{-3}$	$1.348x10^{-3}$
4	TCA	CH_3CN	37.50	$3.182x10^{-4}$ 4	$1.388x10^{-}$
5	TCA	CH_2Cl_2	9.08	$9.542x10^{-4}$	$1.996x10^{-4}$
6	TCA	Hexane	1.89	$2.788x10^{-4}$	$1.384x10^{-4}$

Analysis of the molecularly imprinted polymers was performed using a Batch Rebinding Process. 1mL of [0.2mM] template solution was added to imprinted and non-imprinted polymer samples over a concentration range of 0-100 mg. Samples were shaken at 200 rpm on an orbital shaker for 2 hours followed by centrifugation (14000 rpm), filtration through a 0.2 □m membrane and reversed phase HPLC analysis.

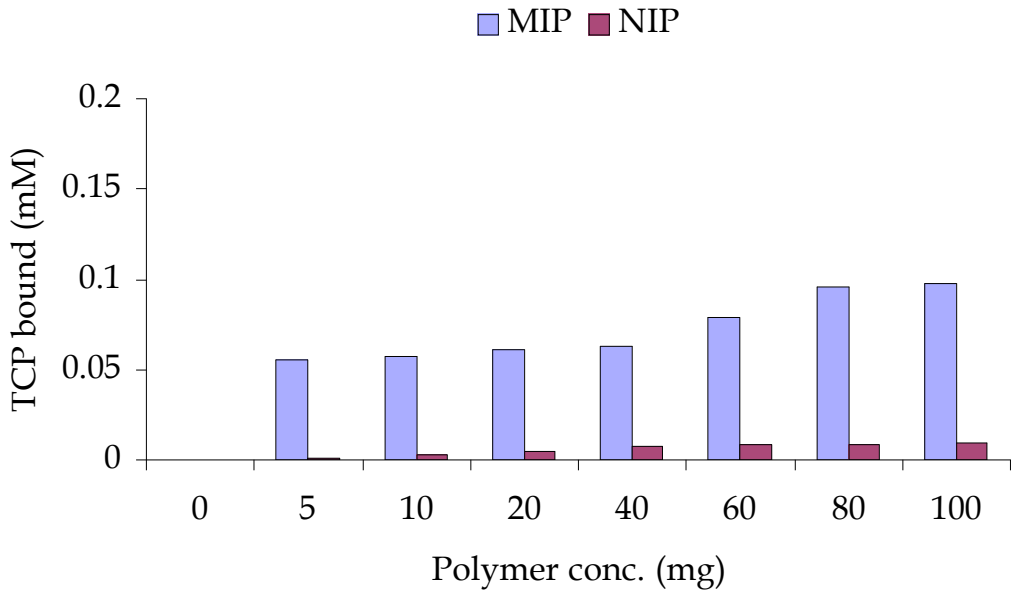
Selective recognition was achieved for TCA and TCP MIPs prepared and tested in dichloromethane (Figure 6.13). The high levels of selectivity can be attributed to the low dielectric constant of the solvent, which results in stronger associative interactions – particularly hydrogen bonding.

Selective recognition was also achieved for the MIPs prepared and tested in hexane (Figure 6.14b), a non-polar porogen that likewise enhances complimentary template - monomer interactions. However, significantly higher levels of surface binding were observed. The lower levels of specific binding observed for the hexane probably reflect the distinct differences between the two solvent systems. Size in particular would be expected to exert significant effects at the molecular level, particularly on factors such as cavity formation.

Binding affinities for the dichloromethane prepared MIPs were then assessed as a function of the rebinding solvent. Affinities in both cases (Figures 6.15) were found to be highest in dichloromethane reflecting the importance of solvent in ensuring that cavity swells correctly and regains the shape required to accommodate the target. shows the difference in binding capacity between the imprinted and non-imprinted polymers. The TCP-imprinted MIP showed higher levels of recognition in both the non-porogen solvents indicating again that presence of potential hydrogen bonding interactions involving the phenolic OH group. Loss of selectivity was encountered in both cases when acetonitrile was employed as the rebinding solvent.

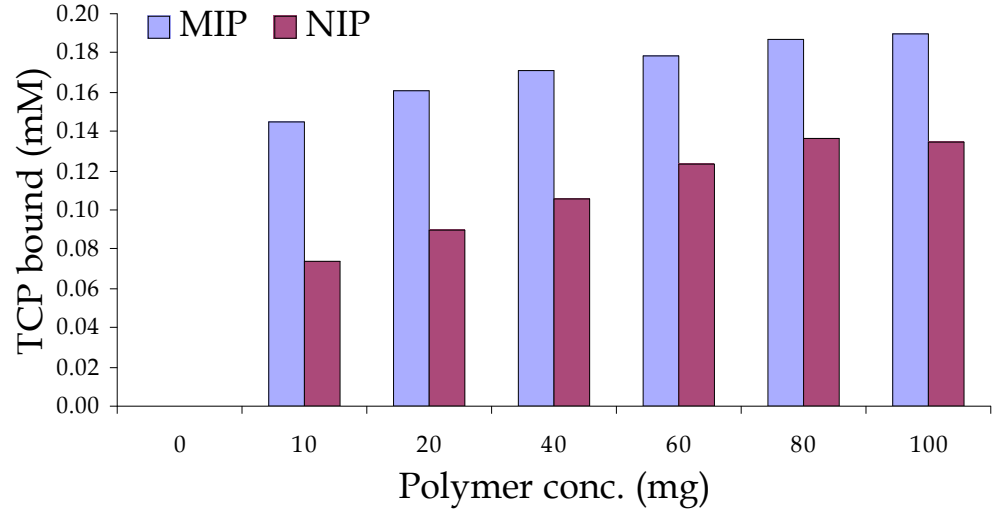


a)

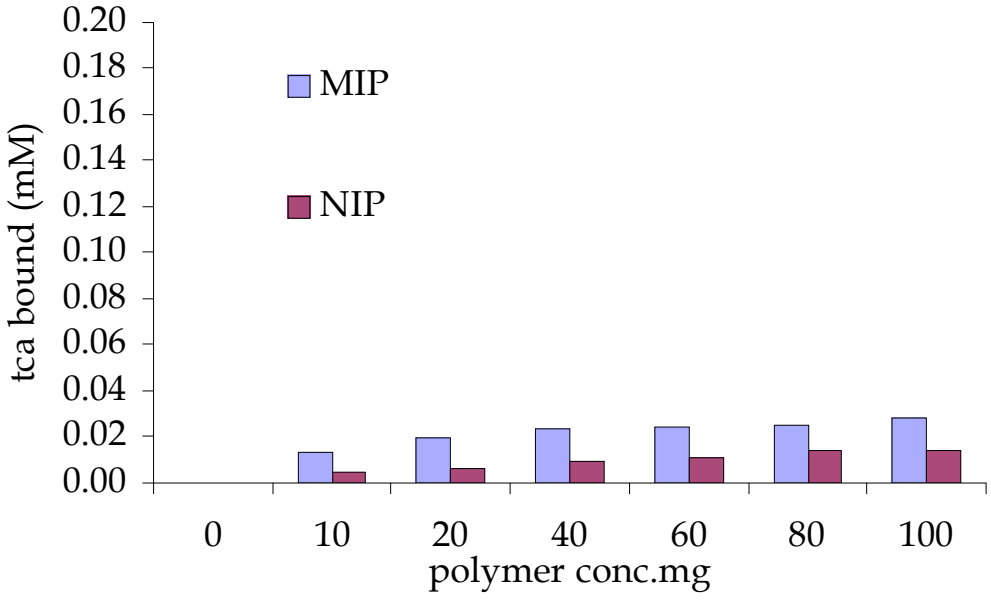


b)

Figure 6.13: Rebinding data for polymers imprinted with a) TCA; and b) TCP prepared and tested CH_2Cl_2 porogen.

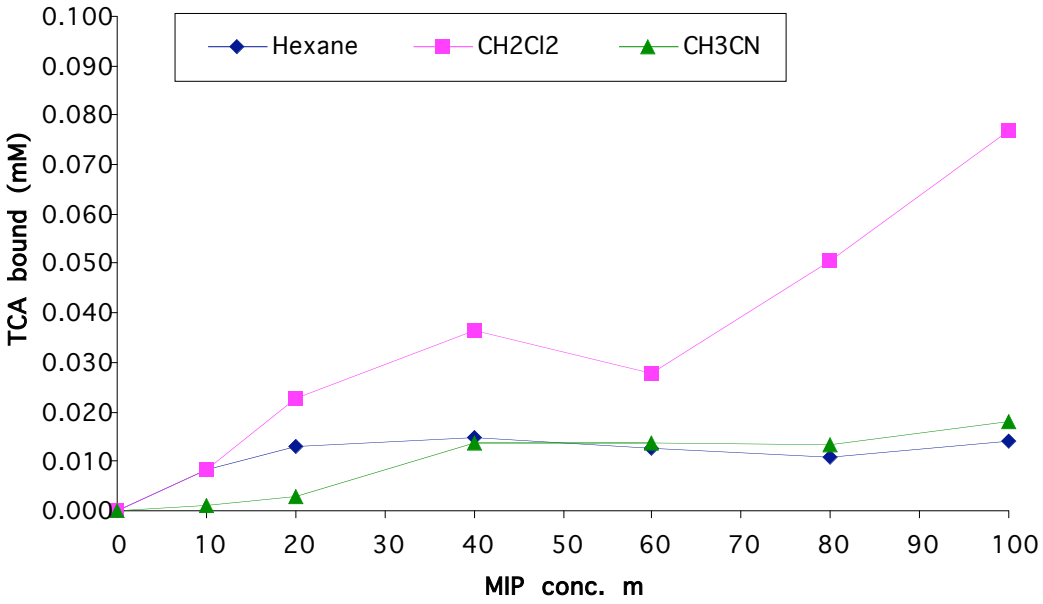


a)

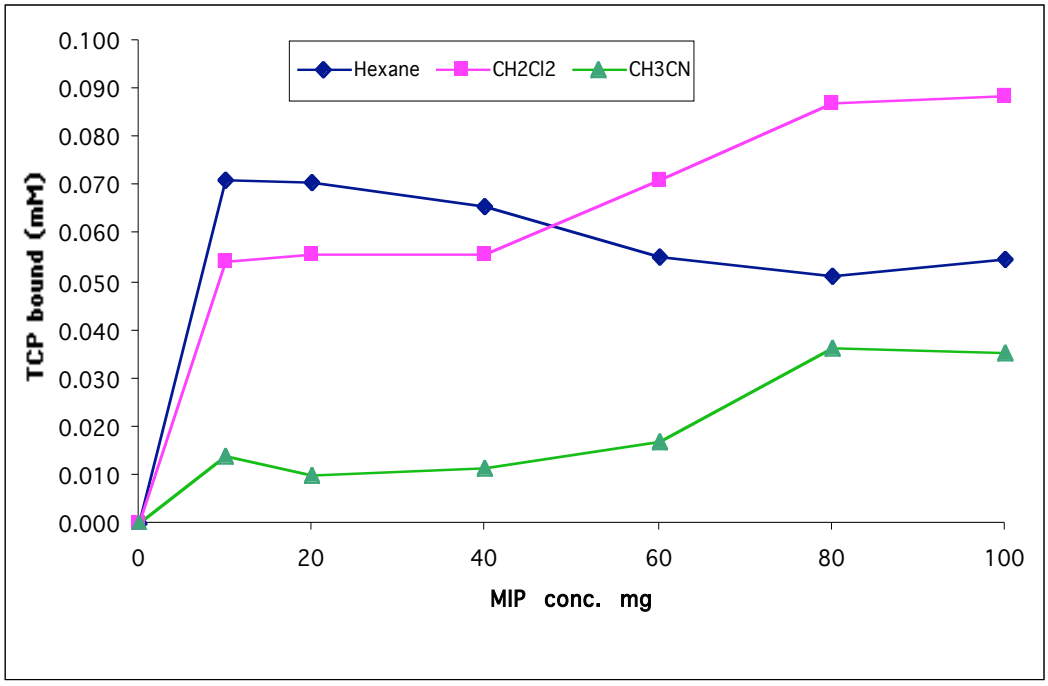


b)

Figure 6.14: Rebinding data for the polymers imprinted with a) TCA and b) TCP prepared and analysed in the porogen hexane.



a)



b)

Figure 6.15: Binding affinities (MIP – NIP) of TCA (a) and TCP (b) MIPs (CH_2Cl_2) in various porogens.

A polymer was imprinted with TCP in toluene was prepared in order to investigate binding recognition in a solvent of similar size and structure to the target. The results, not unexpectedly (Figure 6.16), showed poor levels of recognition. This suggests that the high level of π-character present in the solvent creates sufficient confusion in solution prior to polymerisation that it effectively prevents the suitable target – monomer associations from forming.

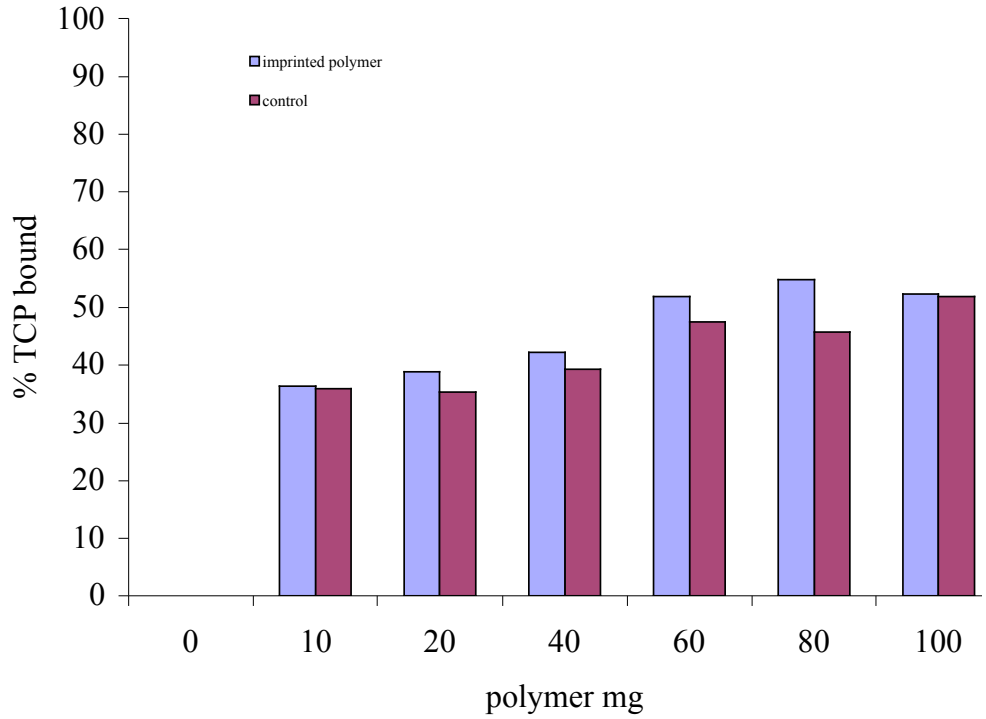
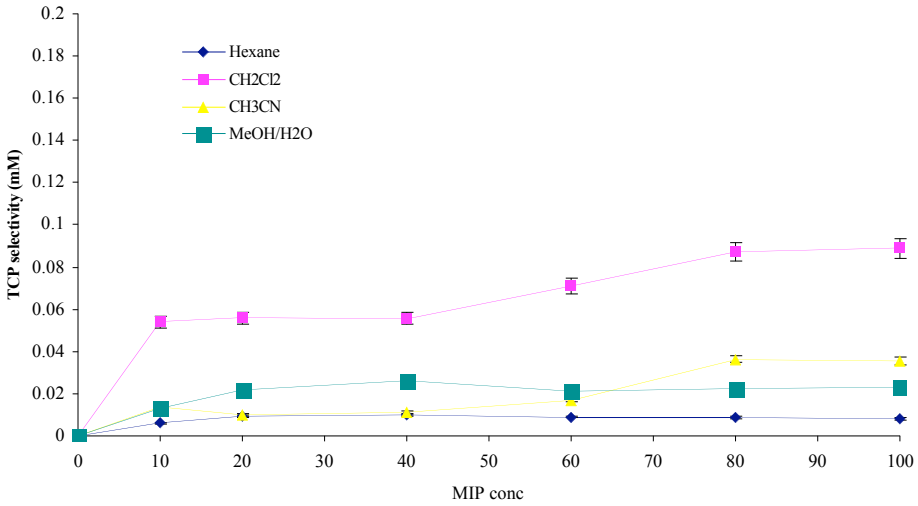


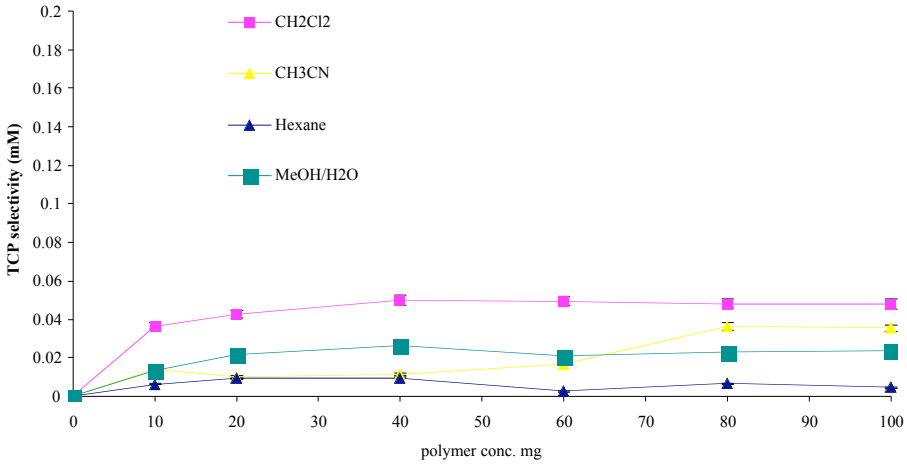
Figure 6.16 Polymer imprinted with TCP prepared and analysed in toluene.

The choice of rebinding solvent has been reported to exert significant effects on the efficiency of the imprinted polymers. It is commonly held that MIPs perform to a higher standard when the rebinding studies are carried out in the original porogen[73]. To examine this effect, TCA and TCP imprinted MIPs were prepared in porogens of differing polarities and their operational efficiencies assessed in various solvent systems.

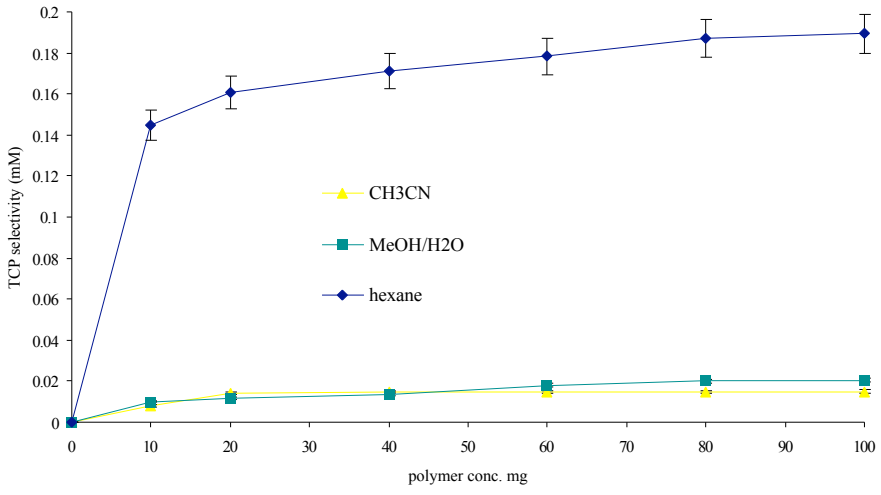
Figure 6.17 shows the batch rebinding results for three TCP MIPs prepared in dichloromethane, acetonitrile and hexane respectively. Both dichloromethane and hexane preparations (Figures 6.20a and c) displayed distinctly higher binding affinities in their respective porogens. By contrast, the acetonitrile MIP (Figure 6.20b) showed poor recognition qualities in all solvents.



a)



b)



c)

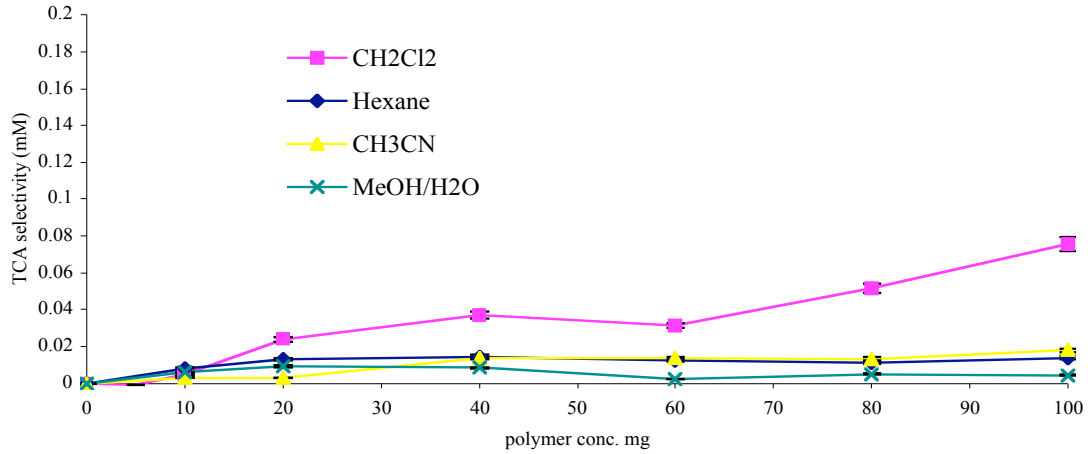
Figure 6.17c): Binding specificities for a TCP – 4 VP imprinted polymer formed in (a) CH_2CL_2, (b) CH_3CN and (c) hexane conducted in a range of solvents. Selectivity was measured as a function of MIP – NIP (mM TCA).

The higher affinities shown in the less-polar solvents is attributed to the ability of the solvent to stabilise the ion pair within the binding sites thus favouring weak hydrogen bonding[74] It further reinforces speculation that hydrogen bonding is the major recognition element present in the cavities. The small degree of recognition in the polar and aqueous solvents may indicate that weaker π-π interactions are being favoured.

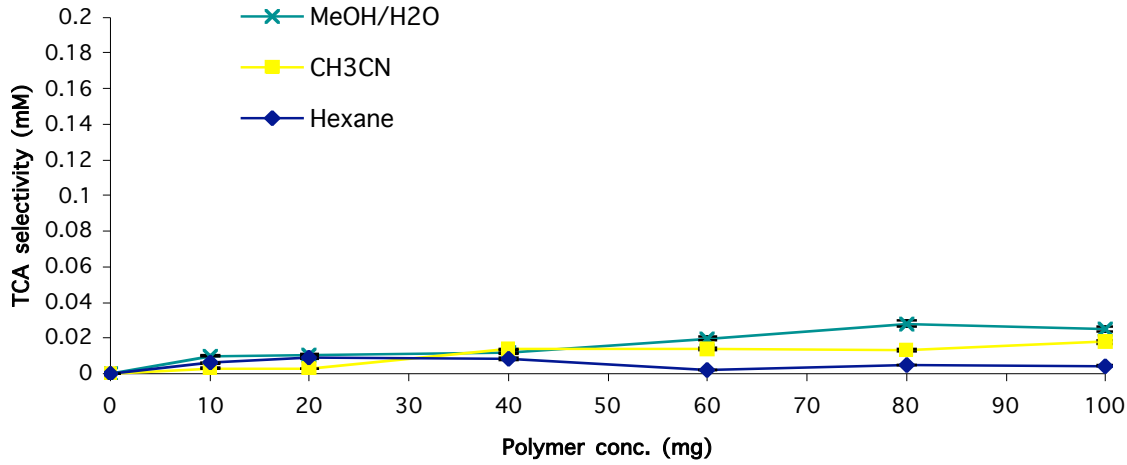
None of the polymers displayed significant recognition under highly polar aqueous conditions (MeOH/H_2O 60:40) suggesting a disruption of hydrogen bonding interactions between the acidic phenol and the basic pyridine group. Baggiani *et al*[35] has reported similar reductions in binding affinity in the more polar solvent conditions for similarly structured templates.

Rebinding results conducted on TCA imprinted polymers (Figure 6.18) were consistent with previous preparations, with moderate levels of TCA recognition displayed by the dichloromethane preparation when evaluated in dichloromethane. Hexane and acetonitrile preparations showed no selectivity irrespective of the porogen or evaluation solvent used.

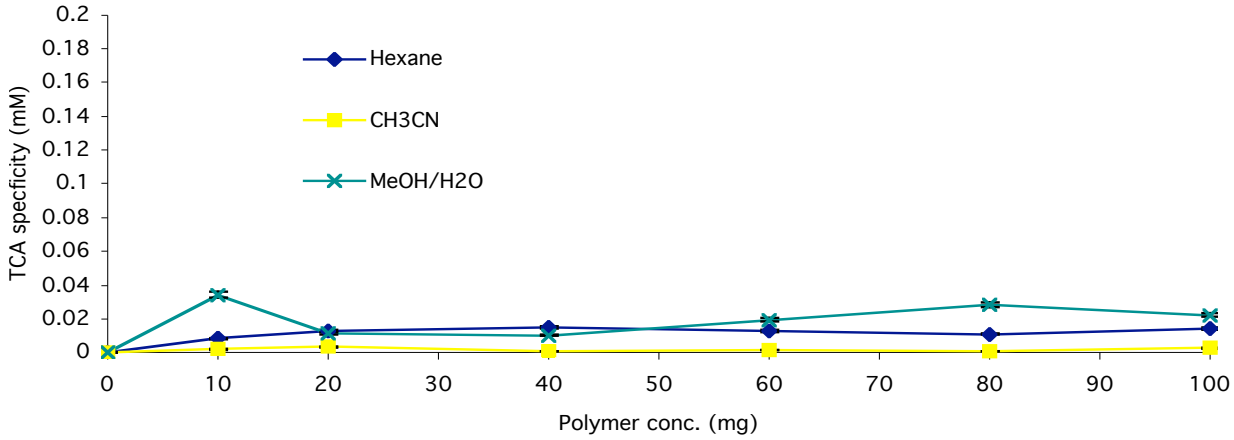
The absence of higher levels of non-selective surface binding in more polar solvent conditions was somewhat surprising given that it was expected given the non-polar character of TCA. However, the presence of large numbers of highly electronegative oxygen atoms present in the crosslinking agent may help to suppress Van der Waals interactions between the target and the surface of the polymer..[75,54]



a)



b)



c)

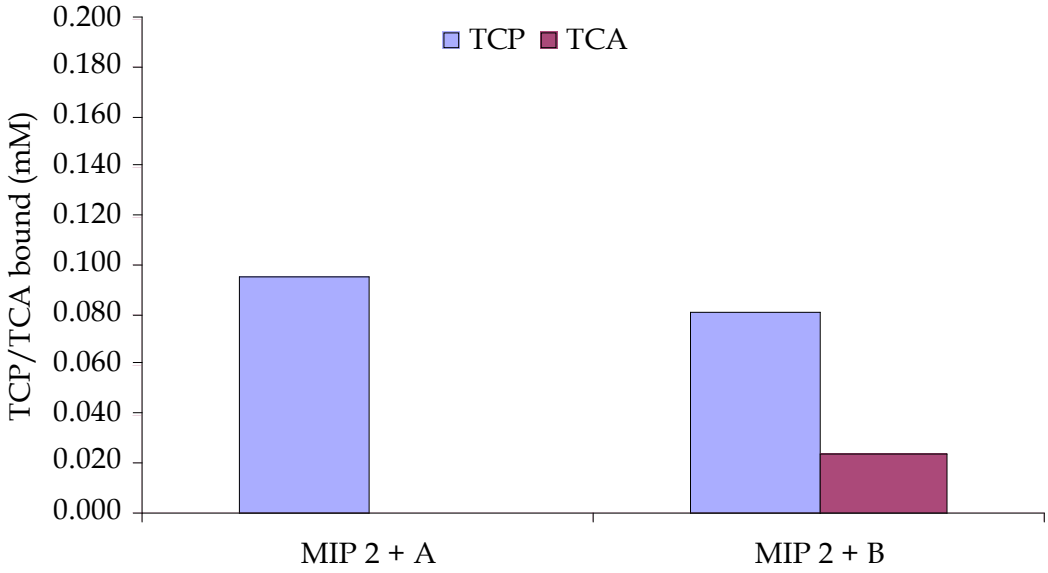
Figure 6.18: Binding specificities for a TCA – 4 VP imprinted polymer formed in (a) CH_2CL_2, (b) CH_3CN and (c) hexane conducted in a range of solvents. Selectivity was measured as a function of MIP – NIP (mM TCA).

Competitive Binding Studies

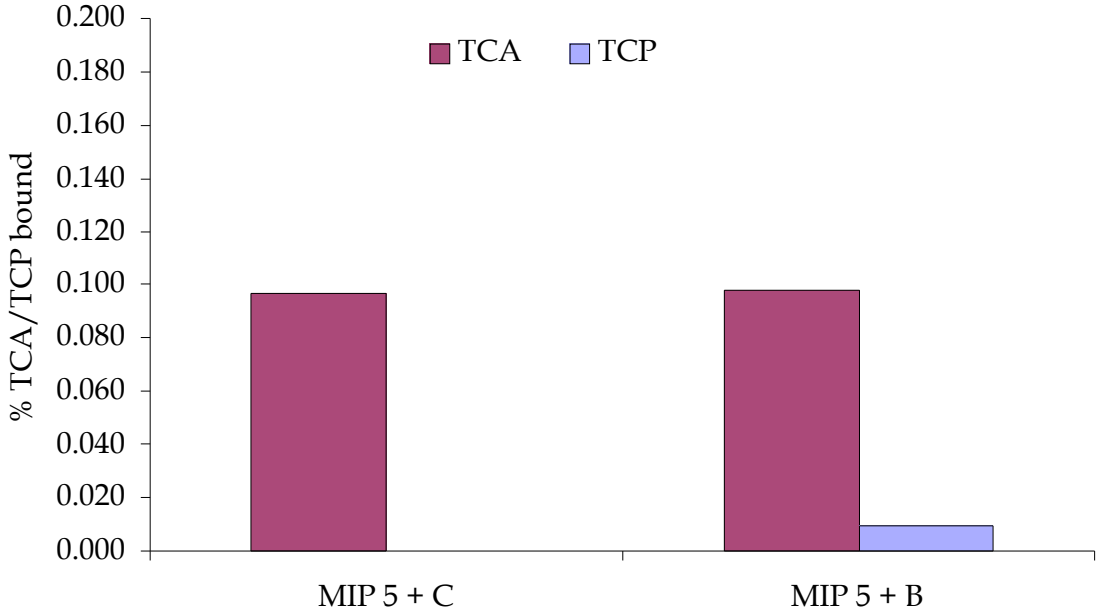
Competitive binding assays are designed to show the ability of an imprinted polymer system to discriminate the target molecule from structurally similar analogues. The procedure involves measuring the quantity of each species sorbed by the MIP from solution containing the target and analogue(s). Such studies have been previously decribed for aromatic target molecules by a number of authors[70,54,75].

The results from competitive binding assays between TCA and TCP (Figure 6.19) show that both imprinted polymers were capable of distinguishing between the two compounds despite their similar structures. Surprisingly, polymer **5** (TCA / CH_2Cl_2) showed little affinity for the TCP competitor despite its smaller footprint (Figure 6.19b). Given the similar electron potentials of the two targets, one would assume significant TCP uptake if $\pi - \pi$ stacking interactions are the dominant recognition element leading to TCA imprinting. Clearly this is not the case. It is likely that steric factors related to the presence of the methyl substituent are of some importance.

Minor amounts of TCA binding were observed for polymer **2** (figure 6.19a) suggesting that in this case hydrogen bonding is a prevalent recognition element. Proportionately higher TCA uptake of the larger TCA molecule suggests that there is some flexibility within the cavity, but this does not result in a significant loss of selectivity. In this case, the hydrogen bonding appears to be dominant recognition element.



a) 4-VP - TCP Imprinted MIP (**2**)

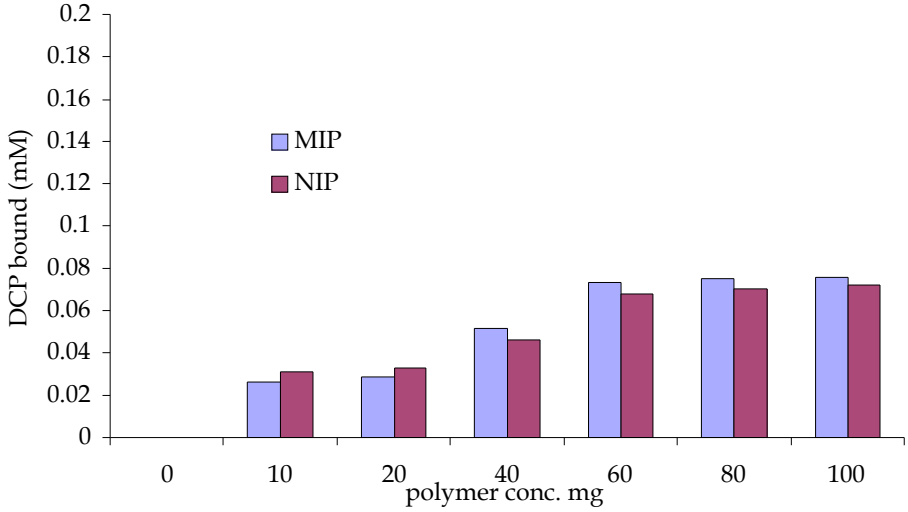


b) 4-VP - TCA Imprinted MIP (**5**)

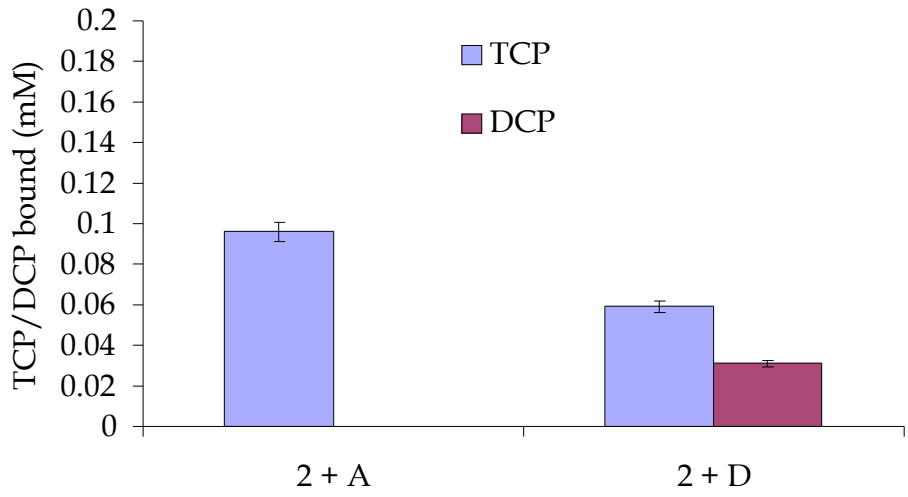
Figure 6.19 Competitive binding assays of 4-VP MIPs **2** (TCP / CH_2Cl_2), (a) and **5** (TCA / CH_2Cl_2) (b). Selectivity of each MIP was assessed in combinations of TCA and TCP solutions:

SolutionA: TCP [0.2mM] in CH_2Cl_2;
Solution B: TCA [0.2mM] + TCP [0.2mM] in CH_2Cl_2;
Solution C: TCA [0.2mM] in CH_2Cl_2.

To determine importance of the ring chlorine atoms as recognition elements, competitive binding assays were also performed on polymer **2** using 2,4-dichlorophenol (2,4-D)[60]. The results (Figure 6.20) show that 2,4-D competitively occupies binding sites within the imprinted polymer. Further competitive binding assays are in progress to more fully assess this issue.



a)



b)

Figure 6.20 a) Binding affinity of 2,4-DCP for **2** in [0.2mM] DCP in CH_2Cl_2; b) Competitive binding assay between TCP and DCP with TCP imprinted polymer synthesised in CH_2Cl_2 (**2**) in solutions of: A, [0.2mM] TCP; D, [0.2mM] TCP + [0.2mM] DCP in CH_2Cl_2 on 80 mg of polymer.

Covalent Imprinting Studies

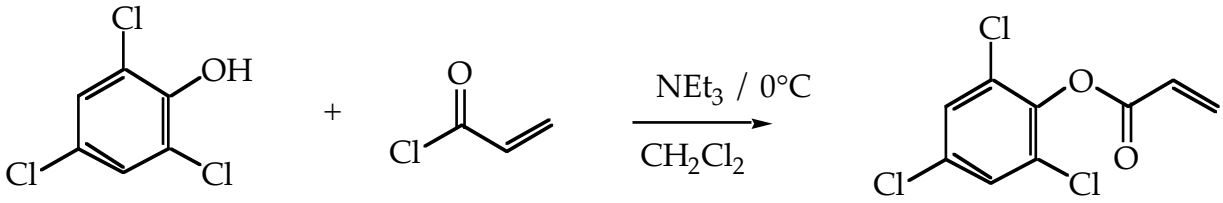
Introduction

Molecular imprinted polymers can be prepared either by covalent or non-covalent techniques. Although non-covalent imprinting has been shown to be more versatile and template extractions more straightforward, covalent imprinting has been successfully applied in a number of studies with high levels of specificity[76,77].

This section of the project was focused on the preparation of TCP imprinted polymers prepared by covalent techniques. The key aspect of the covalent imprinting strategy is the temporary derivatisation of the target molecule by direct reaction with the functionalised monomer prior to polymerisation. In this way, cavity formation relies not upon the presence of weak target – monomer interactions but on formal covalent interactions. Following polymerisation, the target is liberated from the bulk polymer by reversing the initial bond forming reaction, leaving a well-defined target cavity.

Acryloyl chloride was chosen as the functionalised monomer for these studies because of its commercial availability. The OH group of TCP was found to react smoothly with acryloyl chloride in the presence of triethylamine to give the corresponding acrylate ester in good yield (scheme 6.1). The product was found to be pure by n.m.r. analysis and was used without further purification.

Scheme 6.1



Preparation of Covalent MIP's

TCP-imprinted polymers were prepared in a range of porogens (hexane, toluene, dichloromethane and acetonitrile) to assess the effects of their differing characteristics (e.g. polarity) on polymer specificity. A polymer was also prepared in the absence of a porogen to evaluate the contribution that the solvent makes in providing access pathways through the bulk polymer to the resulting cavities.

The quantities of reactants used in individual preparations were based on optimum proportions reported in previous studies involving covalent imprinting[1,2]. Several MIP's were also prepared using other reagent ratios to address factors that could affect template binding such as the effect of the degree of crosslinking and the presence of a second monomer (copolymerisation).

Typically, molecular imprinted polymers containing higher proportions of crosslinking agent (up to 80% mol / mol) impart greater affinity, capacity and selectivity because of the greater rigidity that is imparted to the resulting bulk polymer. Recent studies however, show that specificity of MIP's can also be retained when substantially lower crosslinker ratios (as low as 20 mol %) are employed[78,79]. This is particularly advantageous for MIP's systems designed for use in aqueous media. Hydrogels for example, normally only contain less than 5 mol % per mol of crosslinker[80].

The use of mixed monomers can potentially improve the binding capabilities of the MIPs - especially if strong interactions exist between the second monomer and the template, as in the case of 4-vinylpyridine and TCP. This is particularly important for the development of MIPs capable of operating under aqueous conditions. A recently published example shows that MIP recognition of 2,6-pyridinedicarboxylic acid in polar solvent conditions was substantially improved when a 4-vinyl pyridine copolymer was incorporated into the acrylamide based system[81].

Polymerisations were conducted thermally at 60ºC, and photochemically at 0ºC to investigate the impact (if any) of thermodynamic factors on the selectivity of the resulting polymers. The formation of recognition sites is largely dependent on the order of molecular association between the crosslinker and functional monomers in the presence of a solvent. As molecular disorder increases with increasing temperature, a higher degree of order is expected at lower temperatures, resulting in more stable recognition sites and enhanced template binding, a feature previously identified in non-covalent imprinting[76, 81]

A summary of the covalently imprinted polymers prepared for this study is outlined in Table 6.8.

Table 6.8: List of TCP-templated Polymers Prepared.

Code NIP/MIP	**Initiation Temp (°C)**	**Porogen**	**% Yield**		**Remarks**
			NIP	**MIP**	
THB/THI	Thermal (60°C)	Hexane (C_6H_{14})	76	88	36 mg AIBN
TTB/TTI		Toluene ($PhCH_3$)	95	90	
TDB/TDI		Dichloromethane (CH_2Cl_2)	69	65	0.267 g TCPA, 5 mol% of crosslinker
TAB/TAI		Acetonitrile (CH_3CN)	100	100	95mol % EGDMA
TBB/TBI		None	60	67	
PHB/PHI	Photolysis (0°C)	Hexane	to be determined		10 mL porogen, 2 mL/g of reaction mixture
PTB/PTI		Toluene			
PDB/PDI		Dichloromethane			
PAB/PAI		Acetonitrile			
PBB/PBI		None			
TAI-180	Thermal (60°C)	Acetonitrile	to be determined		180 mg AIBN (not 36 mg)
TAI-25					25 mol % 2,4,6-TCPA (not 5 mol %)
TAI-VPy					50/50 mol % ratio of 2,4,6-TCPA and 4-vinylpyridine

Target Extraction

TCP removal from the polymer systems was achieved by hydrolysis of the ester linkage between the target and carboxyl group of the acryloyl monomer (scheme 5.2). Grinding of all polymers prior to hydrolysis was undertaken to ensure that the maximum number of cavities were exposed to base. Final removal of the target is then achieved by repeatedly washing the ground polymer with a polar solvent such as methanol. A final washing with weak acid was then undertaken to ensure that all acid moieties with the polymer cavities were protonated to maximise hydrogen bonding interactions during rebinding studies.

Batch Rebinding Studies

Choice of Method: Detection of TCP

HPLC has been an accepted and tested method for the measurement of very small concentrations of substances bound in imprinted polymers. The widespread use of HPLC as an analytical tool for batch rebinding analysis stems from historical interest in developing MIPs to act as solid phase agents for the separation of racemic mixtures. HPLC based analysis has been used reliably in our laboratory for the evaluation of non-covalent MIP's for TCP and TCA.

This technique is by no means the only method employed in batch rebinding studies. Radioligand binding is a particularly sensitive method involving the use of target species containing radioactive tracer atoms such as tritium (^{3}H) or carbon (^{14}C). Monitoring of changes in the radioactivity of the polymer permits the binding characteristics to be estimated using only minute quantities of material. The technique is however only suited to target species where isotopic labelling is both affordable and synthetically viable.

For these investigations, TCP sorption from solution was monitored using a fibre optic ultra-violet (UV) probe. UV spectroscopy is a highly sensitive technique that is particularly suited to the quantification of the levels of aromatics present in solution. Many aromatic compounds exhibit strong absorbance characteristics in the UV region of the electromagnetic spectrum. The instrument design, which incorporates the detector unit in the probe head permits rapid sample evaluation and requires no specialised containment apparatus such as quartz cells.

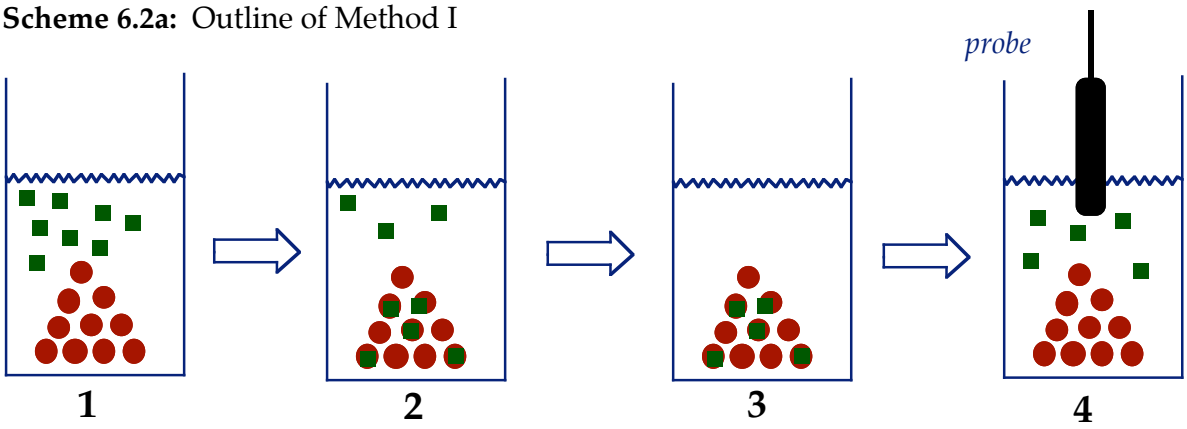
In all solvents tested, TCP exhibited maximum absorbance at 300 nm. Excellent linear correlation ($R^2 \geq 0.99$) for standard solutions was obtained for concentrations $\leq 1 \times 10^{-3}$ M. The method was found to be more economical and considerably faster than chromatographic analysis.

Two approaches to measure the quantity of TCP bound to the imprinted polymers were investigated (Schemes 6.2a and 6.2b). Method I involved the addition of a known concentration of TCP solution to vials containing pre-weighed amounts of polymer. The mixture was then shaken for a predetermined time, whereupon the polymer was filtered and transferred to a known amount of fresh solvent and shaken again overnight. The quantity of TCP present in the fresh solvent was then evaluated.

While TCP uptake was found to occur, re-extraction of the target from the polymer during the second shaking step proved extremely difficult and could only be achieved through multiple extractions, making the procedure impractical.

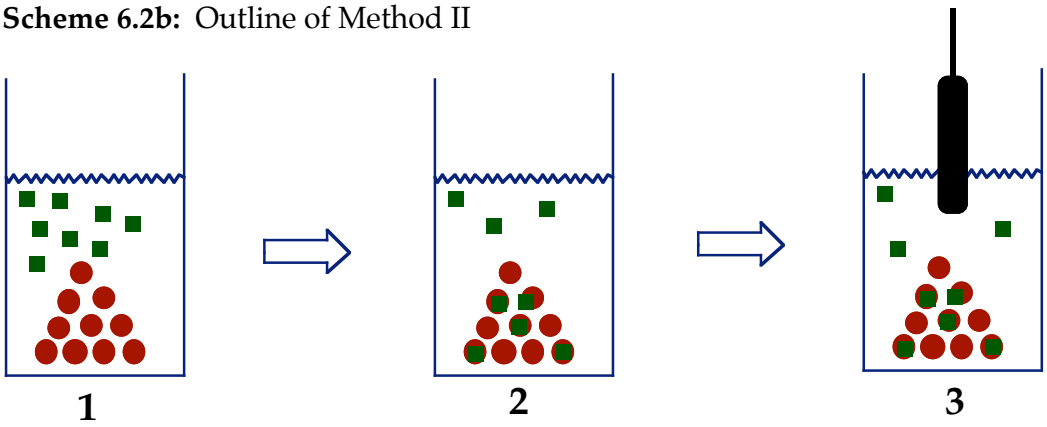
Method II proved more practical, as it directly measured the change in TCP concentration in the test solution *before and after* rebinding. This difference corresponded entirely to the amount of polymer bound. The difference in uptake between the imprinted polymer and its control could then be used to provide an overall estimate of binding specificity. Solution concentrations of $\leq 1 \times 10^{-3}$ M were required to observe a significant uptake of the target species. Method II was used determine all results shown in Table 6.9.

Scheme 6.2a: Outline of Method I



1. MIP placed in TCP solution of known concentration and shaken.
2. Equilibration results with a proportion of TCP sorbed by the polymer.
3. MIP with bound TCP is transsferred to fresh solvent.
4. A second round of shaking is undertaken to release the TCP from the MIP. The TCP concentration in solution is then determined by UV spectroscopy and uptake expressed as a proportion of the original TCP concentration.

Scheme 6.2b: Outline of Method II



1. MIP placed in TCP solution of known concentration and shaken.
2. Equilibration results with a proportion of TCP sorbed by the polymer.
3. TCP concentration in the solution is then determined by UV spectroscopy and uptake expressed pressed as a proportion of the original concentration.
a proportion of the original TCP concentration.

Evaluation of Rebinding Capacity

Table 6.9 shows the amounts of TCP sorbed by polymer samples (60 mg) after 2 and 24 hours respectively. A control polymer consisting of an equivalent amount of polymer immersed in pure solvent was used to detect potential TCP leeching. None was observed.

Polymers imprinted in the absence of a solvent showed no marked difference in TCP uptake relative to their non-imprinted controls. This reflects the key role played by the porogen molecules in forming passages within the polymer superstructure during the polymerisation.

After 24 hours, NIP binding was observed to be greater than or equal to that of MIP in the case of polymers prepared and rebound in hexane, toluene and dichloromethane. Petcu, *et al.*[54] observed this same trend with polymers covalently imprinted with the *propofol*, a phenolic anaesthetic widely used in veterinary science that is a persistent low level contaminant in milk. In this case, loss of selectivity by the imprinted polymer occurred (relative to the non-imprinted control) when exposure times were extended from 15 minutes to 24 hours. The changes was attributed to an increased incidence of non-specific target binding, presumably through strong hydrogen bonding associations between the phenol OH group and the oxygen atoms present in the glycol crosslinker used in polymer formation. Non-specific sorption was also found to be heavily dependent upon the size of the polymer particles, with smaller particle sizes exhibiting higher levels of specificity, presumably through increased access to recognition sites. This did not form part of our investigations, as it required the use of resources that were unavailable to our group.

Interestingly, TCP to MIP's prepared and rebound in hexane and toluene showed no marked increase in the binding capacity over the same time period, a result which appears to indicate that maximum binding capacity was achieved within the first 2 hours of contact with the TCP solution. The control polymers showed only low levels of TCP uptake during the same time period, indicating that sorption by the MIP occurs exclusively at specific binding sites within the polymer. This rapid uptake also indicates high levels of porosity within the polymer superstructures, which allow TCP to permeate rapidly to the recognition sites. By contrast, polymers prepared in dichloromethane (TBB/TBI), showed a higher rate of TCP uptake by the control

polymer during the first 2 hours of contact. The result appears to reflect a slow rate of permeation of TCP into the polymer superstructure, which leads to a dominance of non-specific surface binding.

Differential TCP binding rates may also be explained by a combination of concentration effects and the relative strength of the polymer-template and solvent-polymer interactions. Uptake of template is favourable when the polymer-template interactions are stronger than those between the solvent and polymer as in the case of polymer preparations THB/THI and TTB/TTI. However, in dichloromethane, TCP competes less effectively resulting in a slower uptake.

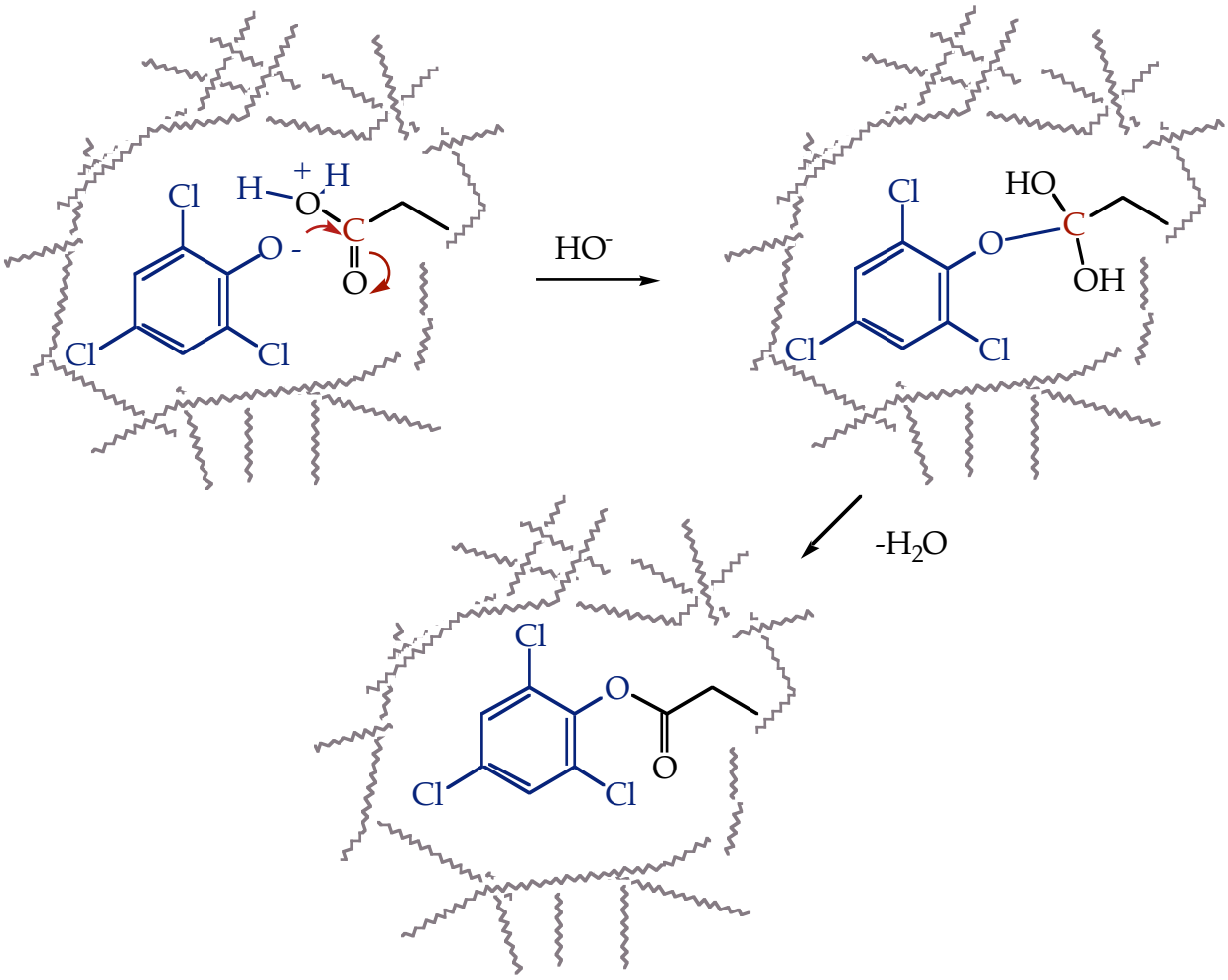
This solvent effect is not limited to imprinted polymers alone. Non-imprinted polymers also experience binding competition between the solvent and template even in non-specific binding on the polymer. This is expected to be lower for non-polar polymer systems immersed in polar solvents, because for the reduced importance of Van der Waals interactions. The results in Table 6.9 are consistent with this trend, with NIP binding in dichloromethane lower than that observed in toluene and hexane after 24 hours.

Table 6.9. Amount (in mmol) of TCP bound to 60 mg of imprinted (MIP) and non-imprinted (NIP) polymers in 2 and 24 hours.

Sample Code NIP/MIP	Solvent	Bound TCP (mmol)					
		2 hours			24 hours		
		NIP ($\times 10^{-4}$)	MIP ($\times 10^{-4}$)	Bind (MIP-NIP) ($\times 10^{-4}$)	NIP ($\times 10^{-4}$)	MIP ($\times 10^{-4}$)	Bind (MIP-NIP)
THB/THI	Hexane	1.4	5.4	4.0	5.8	5.8	0
TTB/TTI	Toluene	3.4	6.6	3.2	>10	6.8	> -3
TDB/TDI	CH_2Cl_2	1.5	0.7	-0.7	1.7	1.3	-0.4
TAB/TAI	CH_3CN	evaluation in progress					
TBB/TBI	Hexane	3.0	0.6	0.8	8.2	8.2	0
	Toluene	>10	>10	0	>10	>10	0
	CH_2Cl_2	0.0	3.3	3.3	3.1	0.0	-3.3
	CH_3CN	3.7	3.7				

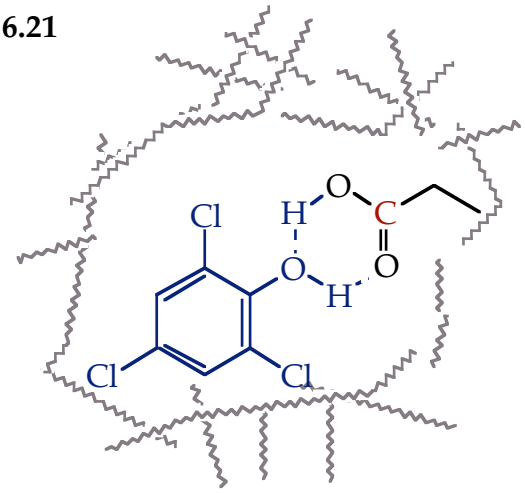
The lack of specificity observed in the covalent imprinting of TCP may also result from difficulties associated in the size of the resulting cavities. Recognition of the TCP is within the polymer cavity is unlikely to occur as a result of reforming of an ester linkage, as this would require a temporary change in hybridisation state of the carboxyl carbonyl group from sp^2 to sp^3 back to sp^2. (see Scheme 6.3). While cavity swelling resulting from solvent uptake might make the change in hybridisation state more likely, the thermodynamic of the phenolate ester formation by the Fischer type mechanism remain unfavourable.

Scheme 6.3

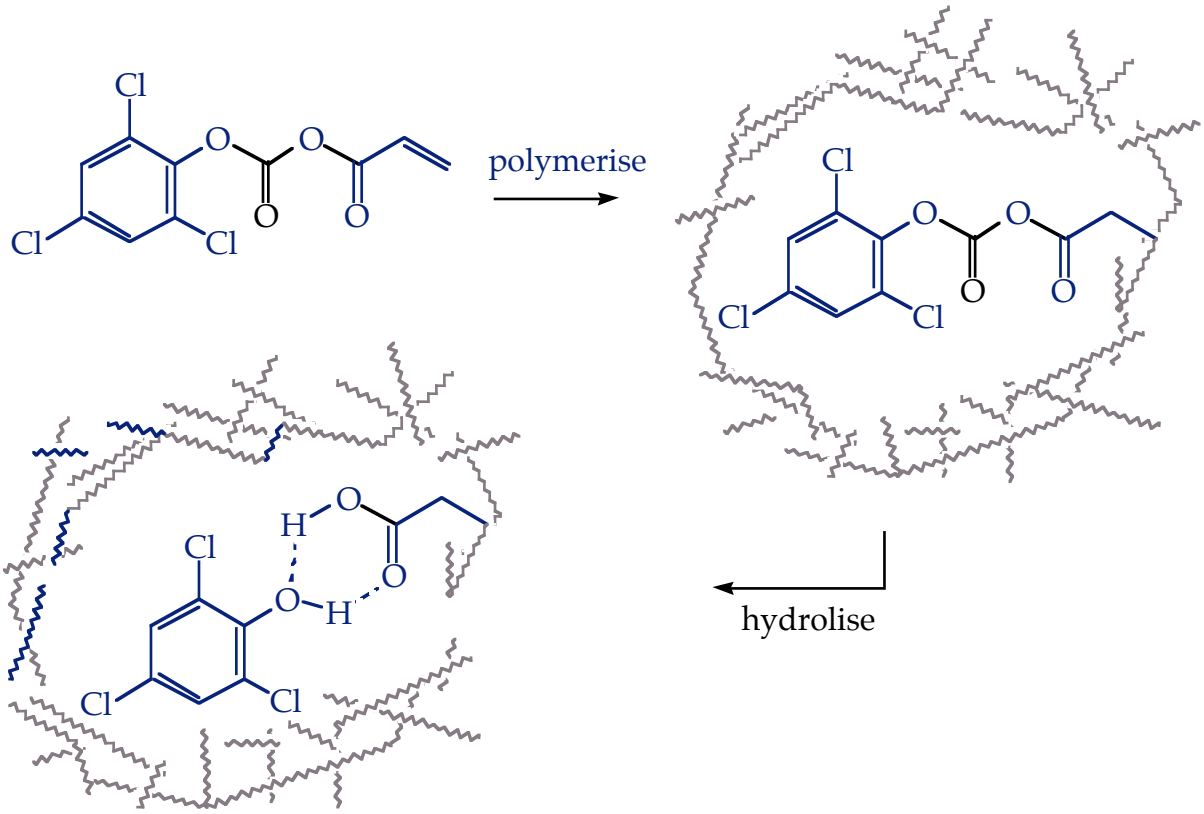


It is more likely that some form of hydrogen bonding interaction predominates within the cavity. Such an arrangement would require some flexibility within the polymer cavity that may be accommodated by swelling the geometry would be by no means fully optimised (Figure 6.21). Differential rates of swelling may however represent a plausible explanation to account for the different rates of target between the polymers shown in table 6.9.

Figure 6.21



More satisfactory hydrogen bonding interactions might be achieved by incorporating a temporary spacer unit between the target and functionalised monomer during MIP formation (Scheme 6.4). This would result in the formation of a larger cavity that would permit optimal hydrogen bonding interactions to be achieved. Investigations into the feasibility of such a system are currently underway.



Future Work

Most of the problems and limitations involved in the handling of covalent MIP systems and TCP measurement have already been dealt with and most solutions are already in place, hence, this work would hopefully be proceeding smoothly in the future. Several areas of research require further attention. These include:

- Further evaluation of the effect of shaking time on binding specificity, in order that full optimisation of MIP systems is achieved.
- Evaluation of TCP rebinding under aqueous conditions (EtOH/water, 10:90)
- Binding rebinding analysis on polymers prepared at 0°C.

Preparations for this work is currently underway.

Synthesis of TCPA network in aqueous medium.

Several studies have indicated that optimum template binding can be achieved when the rebinding and porogenic solvents are the same. Hence, in order to obtain a higher degree of TCP binding in aqueous medium, the MIP should preferentially be prepared in the same medium. Reported studies have shown successful synthesis of less hydrophilic vinylic polymers in MeOH/water[10] and pure water[11].

7. Outcomes & Conclusions

Planned outputs and performance targets for the project were achieved within the specified timeframe. Key outputs arising from the project are summarised below.

The successful development of imprinted polymer systems for TCA and TCP.

The key objective of this research project was to investigate the feasibility of developing molecularly imprinted polymers for the selective binding of TCA and TCP. At the commencement of the project, there was little literature evidence to suggest that molecular imprinting could be successfully extended to incorporate poorly functionalised species such as polychlorinated aromatics. This apparent lack of interest was somewhat surprising, given the toxic nature of these substances, and the potential health impacts associated with their presence in the environment. A number of publications on this topic have however recently appeared in the literature[82,83,84,85,86].

Our findings demonstrate that TCA and TCP can be successfully imprinted in a range of solvents using a 4-vinly pyridine monomer as the functional monomer unit. The selectivity and rebinding capacity of the polymers varies significantly with changes in the chemical characteristics of the porogen and to perform best when assessed in the solvent used to originally form the polymer.

The exact nature of the target – monomer interactions could not be unequivocally defined using conventional spectroscopic techniques, a fact that reinforces the extremely weak nature of the interactions determining recognition. Likewise, the collapse of selectivity that is observed with a change in solvent suggests that solvent morphology is of critical importance. Overall however, experimental findings appeared to reinforce the results of modelling studies that suggest that weak, non-covalent interactions based on complimentary differences in electron potential are the dominant factor controlling selectivity.

The high levels of selectivity for the target species that were exhibited by the polymers in competitive binding studies suggest that the polymers might be readily integrated existing analytical technologies such as solid phase extraction. Further exploration of this technology may present some immediate benefits to the wine industry in the form of;

- Improved efficiencies in current testing protocols used to evaluate cork taint in wine.

- The potential development of new on-line protocols to assess TCA and TCP concentration in corkwood by headspace analysis.

- The development of specific sensory devices for the detection and quantification of TCA and TCP in corkwood and wine samples.

A wider discussion of the possible implications of MIP technology for the wine industry is presented in section 8.

The goal of achieving TCA and TCP based imprinted polymer exhibiting good levels of selectivity under aqueous conditions could only be partially realised within the timeframe of the project. Initial attempts to adapt imprinted polymers synthesied in non-polar solvents to operate in aqueous conditions was somewhat variable, with some systems (such as those synthesised in dichloromethane) exhibiting an almost total loss of selectivity, while others (such as those synthesised in hexane) retaining modest levels of target recognition. The exact reasons for these inconsistencies are difficult to explain and are the subject of ongoing research.

Perhaps the most significant outcome of the project lies with our investigations relating to the production of MIPs capable of exhibiting selectivity under aqueous conditions. While such systems have been reported[8788,89,90,91], their ability to retain selectivity in water is generally attributed to the presence of well-defined functional groups on the target. Recent investigations indicate that these same strategies may apply to less well-defined species such as TCP and TCA. For example, MIP systems capable of operating under aqueous conditions have been synthesised for structurally similar molecules such as the herbicide 2,4,5-trichlorophenoxyacetic acid (2,4,5-T), using emulsion techniques (MeOH – H_2O, 3:1,v/v) [92]. Investigations aimed at adapting this approach to TCP and TCA based polymers has commenced and trials to assess cross-reactivity of the polymer are presently underway.

Development of scientific expertise in the field of molecular imprinting.

The generous financial support of the GWRDC has enabled Mr Lachlan Schwarz to undertake postgraduate studies in an exciting and rapidly evolving field of applied scientific research (molecular imprinting). Mr Schwarz's dissertation is due to be submitted for final review in December 2002. To the best of our knowledge, he will be the first graduate from an Australian university with expertise in this particular field of supramolecular chemistry.

Communication of Findings within the Scientific Community.

A manuscript describing selected results from the project was submitted to the international chemical journal *Analytica Chimica Acta* in late 2000. The manuscript was returned mid 2001 with a request for additional experimental information in a number of a number of areas. This work has now been completed and the manuscript is currently undergoing a final review prior to re-submission.

Presentations at two international conferences gatherings (Cardiff – 2000 and Christchurch 2002.) were used to communicate achievements during the course of our studies. These are detailed in Appendix 1 of this document.

8. Recommendations

Wine making is an industry that is steeped tradition and highly attuned to the perceptions of its conservative consumer market. Nowhere is the reluctance to alter the *status quo* of this relationship more evident than in the continued use of cork as a sealant for bottled wine in preference to more modern and hygienic sealing technologies. The trend continues to defy the mounting body of literature evidence highlighting the enormous economic costs associated with this practice.

The wine industry is in effect a victim of its own marketing success. The time-honoured custom of *popping the cork* is one that has been cultured over many centuries and is now so firmly engrailed in the psyche of wine drinkers - both the connoisseur and novice alike that it cannot be ignored by advocates of modern processing techniques. Perseverance with cork closures therefore places the wine industry in the unenviable position of being forced to wear the yoke of a traditional association that undermines improvements in the organoleptic qualities of wine through improved methods of production.

The phenomenal increase in popularity of wine drinking in Australia over the past two decades has resulted in an increase in public awareness of the cork taint issue and the causes that manifest the problem. Despite this, no rapid and cost effective method to evaluate the presence of TCA and / or TCP in corkwood has yet been devised. Consequently, there is a clear need for the wine industry to invest in fundamental research to replace oenologically-based evaluation methods. Two possible strategies may be pursued. These are:

- The development and integration of new technologies with existing diagnostic techniques to enhance current productivity levels.

- The development of alternative, robust and low cost technologies to facilitate the of rapid on-line assessment protocols.

The results from this preliminary study show that MIP technology holds promise for the above strategies. Resourcing of fundamental and applied MIP research may result in the emergence of new technologies across a number of areas that may be of long-term value to the wine industry. These are discussed below.

(i) New Solid-Phase Extraction Technologies

Solid phase extraction (SPE) and solid phase microextraction (SPME) are modern commercial analytical techniques used with increasing frequency for field and laboratory analysis. They are an attractive alternative to conventional liquid extraction techniques because they involve significantly reduced sample preparation times. Both systems employ a polymeric solid phase to concentrate volatile and semi-volatile components from either liquid or gaseous samples.

A number of experimental MIP-based SPE systems been investigated. Results to date are highly encouraging, with excellent recovery rates of the target species from solution occurring in the majority cases[93,94,95]. SPME / GC analysis represents an alternative approach to oenological evaluation of wine taint that offers excellent empirical results. Quantification of TCA levels in tainted wines (~10 ng L^{-1}) by SPME analysis of headspace has been investigated[96]. While excellent rates of recovery were reported, the method was hampered by relatively long sample equilibration times (100% ~60 min. @ 20°C). Shorter sampling times (down to 30 minutes) were achieved, but incurred poor signal to noise ratios during analysis phase. Attempts to directly evaluate TCA levels in moistened corkwood by headspace analysis proved unreliable because of slow diffusion of the non-polar target into the aqueous phase.

Replacing the non-imprinted polydimethylsiloxane solid phase used in these investigations with a TCA MIP could profoundly improve the efficiency of the technique. A collection system based on molecular recognition would enrich the concentration of the analyte, resulting in greater selectivity, increased sensitivity and reduced equilibration times, all of which could lead to the development of a viable on-line batch analysis protocol. The system could be fully automated using existing auto-sampling technology, which would further reduce operating costs.

(ii) Sensor Technology

Recent advances in chemical technology and microelectronics has facilitated the development of robust sensors exhibiting high selectivity and long-term stability. A range of transducer techniques including quartz-crystal microbalance (QCM)[97,98] surface-acoustic wave (SAW) [99] and field-effect transistor (FET)[100] have been used to modulate change in analyte concentration.

The realisation that imprinted polymer surfaces enriched with receptor sites produced a physico-chemical response when the bound to the target molecule has led to a rapid coalescence of molecular imprinting research with sensory science. While the sensitivity of biomimetic devices was initially reported to be substantially lower than biosensors, recently published findings suggest at least equivalent detection limits can now be achieved[101].

Electronic nose technology is a branch of sensory science that has aroused widespread interest in the food and beverage industries as a non-invasive quality control technique. The system mimics the biological olfaction system by producing a characteristic two-dimensional fingerprint (a so-called AromaMap), which results from sensors (either polymeric or metal oxide in nature) interacting with volatiles in the sample headspace. The output is then compared with an authentic map to confirm the authenticity or overall quality of the sample. Aromascan technology is currently used in the food industry industry for a variety of purposes including analysis of raw materials, quality of essential oils, detection of adulterants and food spoilage.

The technology has been successfully applied in the wine industry[102] to analyse a variety of factors including:

- Comparison of wines from different winemaking grapes.
- Comparisons between different wines from the same winemaking grape.
- Detection of blends of wines made from different grape varieties.
- Ethanol content.
- Cork taint.

The incorporation of MIP technology into such devices offers the possibility of developing stand-alone low-cost detection systems that can be tailored to specific tasks such as the analysis of as polychlorinated phenolics in corkwood. With the rapid evolution of microelectronic and microcomputing technologies the realisation of such devices would seem inevitable, given appropriate research support.

Appendix 1 : Communications

Conference Presentations

- Schwarz, L., McCluskey, A.; Bowyer, M.C.; *Selective Detection of 2,4,6-TCA and 2,4,6-TCP using Molecularly Imprinted Polymers*. - IUPAC-ICOS 14th International Conference on Organic Synthesis, Christchurch, New Zealand July 14-19, 2002.

- Schwarz, L., McCluskey, A.; Bowyer, M.C.; *Preliminary Study towards the Selective Detection of 2,4,6-TCA and 2,4,6-TCP using Molecularly Imprinted Polymers*. - MIP2000 - 1st International Workshop On Molecularly Imprinted Polymers. Cardiff, July 3-5, 2000.

Manuscripts

Schwarz, L.; McCluskey, A; Bowyer, M.; *Preliminary Study towards the Selective Detection of 2,4,6-TCA and 2,4,6-TCP using Molecularly Imprinted Polymers*.; In preparation.

Appendix 2: Intellectual Property

Appendix 4: List of Staff

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Appendix 5: Raw Data

The work outlined in this summary report forms part of a Masters thesis by Mr Schwarz that is currently in preparation. A Copy of the final thesis containing all data will be forwarded to the GWRDC upon final submission. Copies may also be requested directly from the University of Newcastle Library.

Appendix 6: Budget Reconciliation

Full details of expenditure relating to the project will be forwarded separately to the GWRDC by the Faculty Accountant.

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