

Predicting the performance of molecularly imprinted polymers: Selective extraction of caffeine by molecularly imprinted solid phase extraction

Keith Farrington^a, Edmond Magner^b, Fiona Regan^{a,*}

^a School of Chemical Sciences, Dublin City University, Glasnevin, Dublin 9, Ireland

^b Materials and Surface Science Institute, Chemical and Environmental Sciences Department, University of Limerick, Limerick, Ireland

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Abstract

A rational design approach was taken to the planning and synthesis of a molecularly imprinted polymer capable of extracting caffeine (the template molecule) from a standard solution of caffeine and further from a food sample containing caffeine. Data from NMR titration experiments in conjunction with a molecular modelling approach was used in predicting the relative ratios of template to functional monomer and furthermore determined both the choice of solvent (porogen) and the amount used for the study. In addition the molecular modelling program yielded information regarding the thermodynamic stability of the pre-polymerisation complex. Post-polymerisation analysis of the polymer itself by analysis of the pore size distribution by BET yielded significant information regarding the nature of the size and distribution of the pores within the polymer matrix. Here is proposed a stepwise procedure for the development and testing of a molecularly imprinted polymer using a well-studied compound—caffeine as a model system. It is shown that both the physical characteristics of a molecularly imprinted polymer (MIP) and the analysis of the pre-polymerisation complex can yield vital information, which can predict how well a given MIP will perform.

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1. Introduction

One of the foremost applications of molecular imprinting has been in solid phase extraction [1–6]. In theory, molecular imprinting provides greatly increased selectivity and sensitivity over conventional solid phase extraction (SPE) packing materials. Furthermore due to the specific nature of the interactions between rebinding template and the chemical functionalities within the pores or cavities of the cross linked polymer matrix, it is expected that molecularly imprinted solid phase extraction has the ability to discriminate between closely related compounds.

The molecular imprinting technique is a specific chemical procedure for the generation of explicit nano-cavities that can mimic the behaviour (in terms of binding) of naturally occurring receptor sites [7]. The molecularly imprinted polymers (MIPs) are generated in the presence of a template molecule

(the analyte of interest). In non-covalent molecular imprinting (the most common approach) the template interacts with a functional monomer via hydrogen bonding, electrostatic or hydrophobic interactions. This pre-polymerisation complex is then incorporated into a polymer network by a cross linker. The polymerisation process itself is based on the free radical approach and is started by an initiator. The end result is the production of a rigidly cross linked polymer matrix monolith. After the process is complete the monolith is crushed and ground to a suitable size. The template can then be removed by a mechanism of washing involving as solvent (usually methanol) and an acid or base. Following the removal of the template, the resultant imprinted polymer now possesses the ability to recognise the template (or closely related structural analogues) with high selectivity and specificity.

The main advantage that MIPs possess over conventional solid phase extraction packing materials is their specificity [8]. Retention mechanisms of many of the commonly employed SPE materials are based on hydrophobic interactions and a major drawback of SPE can be the co-elution of interfering species. The nature of the MIP–template rebinding is such that non-specific

* Corresponding author. Tel.: +353 1 7005765; fax: +353 1 7005503.
E-mail address: fiona.regan@dcu.ie (F. Regan).

interactions such as hydrophobic should be minimised due to the fact that other compounds will not form the same interactions with the functional monomer. This is also in part owing to the nature of the shape arrangement of the complex. This is related to the character of the actual cavities (pores) themselves or “binding pockets” [9].

In this regard, the relative size distribution of the pores is critical to the arrangement of the binding site locations, which are located within the pores [10,11]. A crucial aspect of engineering MIPs with pores of defined size distribution is the choice of porogen (solvent) [12]. Typical pore forming solvents include acetonitrile, benzene, ethylacetate whereas non-polar solvents such as chloroform and toluene tend to not form a porous matrix with high surface areas. As a general rule by increasing the volume of porogen used in the polymerisation process or decreasing the amount of cross linker will lead to a greater pore size distribution (meso–macropores). Decreasing the volume of porogen or increasing the amount of cross linker will lead to polymers of smaller average pore sizes.

In this respect it would be advantageous to engineer the polymerisation conditions and process to optimise the efficiency of the resultant MIP in terms of rebinding. Traditionally, the production of imprinted polymers has been based on the trial and error approach. This method involved the generation of numerous MIPs for a particular template. Typically, relative ratios of template to functional monomer were varied. In addition, the volume of cross linker is varied together with the porogen itself. All of the polymers constructed are washed, sieved to appropriate size and tested for effectiveness in rebinding studies. This process is labour intensive, laborious and wasteful of laboratory resources. Hence, to improve the efficiency of selection of suitable MIPs attention is now turning towards a more calculated approach to the design of imprinted polymers. This method has been termed the rational design approach [13,14]. In order for this to be effective, a greater level of understanding of the molecular level events surrounding the formation of the pre-polymerisation complex is required. Furthermore, a knowledge of the stability of the complex during the early stages of the polymerisation is desirable [15].

Within the field of molecular imprinting NMR spectroscopy is now being widely used to predict the ratios of template to functional monomer [16]. Furthermore it has also been used to elucidate information on selective recognition [17], and also to determine association constants [15,18]. Molecular modelling software is a relatively newer tool in this regard. Its potential has been recognised in various recent reports [19–21]. Here the Hyperchem 7.5 molecular modelling software has been employed.

In this study, we show the application of NMR spectroscopy and molecular modelling software in elucidation of the nature of non-covalent interactions present during the formation of the pre-polymerisation complex. Firstly, a number of MIPs for caffeine were synthesised based on the data acquired from the NMR and modelling studies and assessed (in terms of rebinding) by solid phase extraction. Secondly, and based on the SPE results, two MIPs were selected for analysis by BET, a MIP which had performed well and one which had been unsuccessful. Both the

surface areas and the pore size distributions were analysed by BET.

This paper shows that the data obtained from the modelling and NMR studies allied to the post-polymerisation pore size data allows the engineering of functional MIPs. The basis of this engineering is an enhanced understanding of the nature of complex formation and behaviour of the complex during polymerisation.

2. Experimental

Acetonitrile, methanol, water (HPLC grade), caffeine, theophylline, theobromine, methacrylic acid (MAA), ethylene glycol dimethacrylate, 2-vinylpyridine, sodium phosphate were purchased from Sigma–Aldrich (Dublin, Ireland). Azoisobutyronitrile was purchased from Fisher Scientific (UK). Deuterated acetonitrile, methanol and chloroform were purchased from Apollo Scientific (Bredbury, UK) and used as supplied. 2-Vinylpyridine, methacrylic acid and ethylene glycol dimethacrylate (EGDMA) were purified by vacuum distillation prior to use in order to remove inhibitors. The 25 μm mesh size sieve used was purchased from Retsch.

2.1. MIP preparation

One mmol of template (caffeine 194.19 mg) was dissolved in 5 ml acetonitrile or chloroform in a borosilicate test tube. To this was added 4 mmol of either MAA (0.344 mg) or 2Vpy (0.421 mg). The mixture was stirred at room temperature for 5 min. EGDMA, 20 mmol (4167 μl) was then added followed by AIBN, 50 mg per polymerisation. The ratio of EGDMA to functional monomer was kept below 10:1. The mixture was stirred for a further 10 min to ensure complete dissolution of all the components. The mixture was then sonicated for 10 min. In order to completely degas the solution it was sparged with N_2 for 5 min. The tubes were then plugged under vacuum and gradually heated to 60 $^\circ\text{C}$ in a water bath. A 16 h polymerisation time was used. Following polymerisation, the tubes were smashed and the polymer was removed. The polymer was broken up before being ground with a mortar and pestle and finally ground by hand.

The particles were sieved to <25 μm in acetone. Repeated cycles of grinding and sieving were necessary in order to acquire enough of the polymer at size <25 μm . Smaller particle (<5 μm) were removed. Typically, 100 ml of acetone was added to the polymer in a graduated cylinder. The polymer was allowed to settle for ~ 1 h and then the acetone was removed. This was repeated four to six times per polymer. Control polymers were prepared in the same way with the omission of template. The MIPs were washed in a solution of hot methanol with 10% acetic acid. The solution was stirred vigorously. This procedure was repeated numerous times until no trace of caffeine was detected in the washings. HPLC was used to test the washing solutions. During the solid phase extraction procedure (Section 2.6) the packed cartridge was washed before conditioning with both methanol and acetonitrile.

2.2. NMR analysis

All NMR spectra were recorded on a Bruker 500 MHz instrument at 25 °C. The volume for analysis was 750 µl and the concentrations of both caffeine (template) and functional monomer were 0.04 M. The deuterated solvents used were acetonitrile and chloroform. Processing of spectra was performed on a silicon graphics workstation operating off a Unix platform. Further processing was performed using the Mestrec software available from <http://www.mestrec.com>.

2.3. Molecular modelling

The molecular modelling software program used for this study was Hyperchem 7.5 (Hypercube Inc., Gainsville, FL). The structure of caffeine, theophylline and the functional monomers (methacrylic acid and 2-vinylpyridine) were drawn in the Hyperchem program and minimised to the lowest energy conformation allowed by the molecular mechanics (MM+) method and then refined using the semi-empirical mechanic (PM3) method. The conformation of lowest energy was refined with an ab initio (3–21 G) quantum mechanic basis set. To analyse possible interactions between template and functional monomer and for calculation of binding energies, the Amber MM method was used. The force field was set up with constant dielectric and van der Waals and electrostatic scale factors as 0.5. The calculation of the binding energies (between template and monomer) was performed using Eq. (1) shown below:

$$\Delta E = [E_{\text{complex}} - E_{\text{caffeine}} - E_{\text{monomer}}] \quad (1)$$

2.4. UV–vis mole ratio analysis

A molar ratio plot was constructed by the systematic variation of the relative molar ratio of caffeine and methacrylic acid. The initial concentration of both compounds was 0.4 M. The concentration of the caffeine was kept constant and the ratio of methacrylic acid was increased from 1:0 to 1:5. The total volume for each analysis was 3 ml and all UV–vis spectra were recorded at 272 nm.

2.5. SPE studies

Empty plastic solid phase extraction cartridges were used. A 200 mg aliquot of the dry polymer was placed into the cartridges with frits at either end. The system was washed through thoroughly with acetonitrile to remove any air bubbles that may affect solvent flow. The packing was further washed with methanol to ensure the absence of caffeine and the effectiveness of the washing procedure. The extraction experiments involved of loading the MIP-SPE column with 50 µg ml⁻¹ of caffeine or a mixture of caffeine analogues (theophylline and theobromine). All SPE experiments were performed on a VacMaster 20 SPE processing system. Samples were loaded in 50 mM sodium phosphate. A first wash in the same buffer (1 ml) was followed by a second in 1% TEA in acetonitrile also 1 ml. Elution was

performed in 1 ml of 1% acetic acid in acetonitrile. All solutions were evaporated to dryness under N₂ and reconstituted in 800 µl methanol.

2.6. Sample preparation

Samples of Red Bull™ a high caffeine soft drink containing 32 mg per 100 ml amongst other ingredients was diluted 1/100 with water and applied directly (1 ml) to the MIP SPE column. Also, 1 ml of the undiluted Red Bull was applied to the column.

2.7. HPLC determinations

HPLC analyses were conducted on a Hewlett Packard series 1050 system with a Rheodyne injection valve using Alltech BRAVA BDS column (25 cm × 4.6 mm, particle size 5 µm). The mobile phase was a 80:20 (v/v) mixture of 0.05 M sodium phosphate:methanol and the flow was maintained at 1.0 ml/min. A 10 µl injection volume was used for all analyses. Separations were performed at 25 °C (isocratic elution). The wavelength used for detection was 272 nm.

To facilitate quantitative determinations in extracts a calibration curve was constructed. Reference standard solutions of caffeine and theophylline at concentrations 0.1, 0.5, 1, 5, 20, 50, 100 and 200 µg ml⁻¹ were determined in triplicate and the peak areas were plotted against the concentrations.

2.8. Pore size distribution and surface area analysis

Pore size distribution and surface areas of the washed polymers were analysed by Brunauer–Emmett–Teller (BET) analysis performed on an ASAP 2010 from RMIT Applied Chemistry (Micromeritics). A 300 mg quantity of the dry polymer was used for analysis. Relevant information was obtained as follows. A plot of pore size versus incremental pore volume gave pore size distribution. A plot of pore size versus pore volume gave total pore volume. The surface areas and total pore volumes of the polymers were also obtained.

3. Results and discussion

3.1. Molecular modelling of template–monomer interaction

In order to study the interactions between monomers and caffeine, a series of molecular modelling interaction studies was performed using the Hyperchem 7.5 software package (Gainsville, FL). Molecular modelling is useful in this regard as it allows the calculation of the partial charges on each of the atoms of the molecule [22]. Fig. 1 presents the partial charges calculated for the caffeine molecule. From this it can be seen that there are several sites on the molecule capable of undergoing electrostatic or hydrogen bonding interactions. In particular the two nitrogens of the five-membered ring and both of the oxygens are likely to become hydrogen bond acceptors with a proton present in a functional monomer.

Dong et al. [20] have used the calculation of partial charges on the theophylline molecule as an indication of the hydrogen bond-

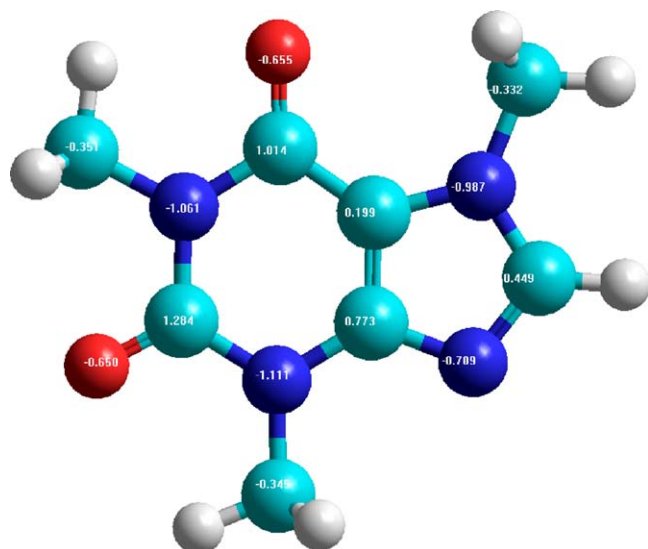


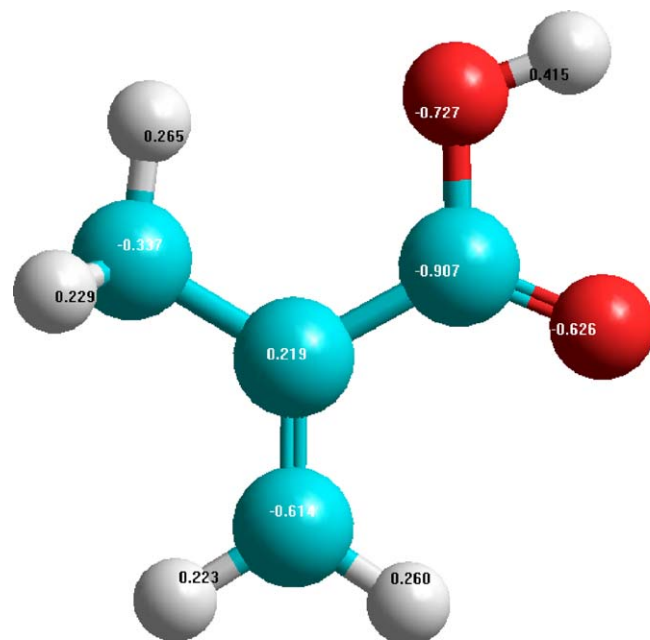
Fig. 1. The partial charges obtained by molecular modelling of atoms on the caffeine molecule. Nitrogens are represented in blue, carbons in green and oxygens in red. Hydrogens are shown in white and always have partial charges between 0.2 and 0.29 (for interpretation of the references to colour in this figure legend, the reader is referred to the web version of the article).

ing capacity of each of the oxygen, nitrogen and hydrogen atoms (in terms of being a donor or acceptor.) The molecular modelling method employed here shows that there is some degree of delocalisation of pairs of electrons of the nitrogen atoms on the five-membered ring. Looking at the model of methacrylic acid shown in Fig. 2a, it can be seen that the nitrogens (of caffeine) may participate in hydrogen bonding with the proton on the hydroxyl group. Fig. 2b shows the model of 2-vinylpyridine and due to the absence of functional protons on the molecule it is unlikely to undergo interactions with caffeine. In order to evaluate the possibility of template monomer interactions, the binding energies (ΔE) of caffeine with methacrylic acid, 2-vinylpyridine and 4-vinylpyridine were calculated and are shown in Table 1.

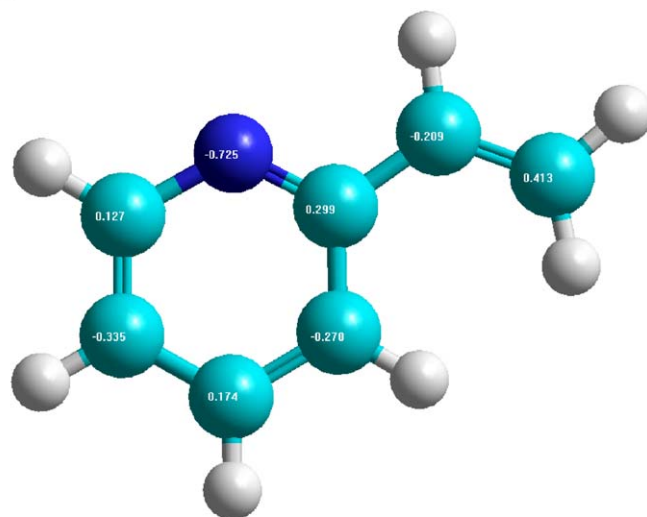
It is documented [13] that when a library of functional monomers is screened against a template using molecular modelling software that the monomers giving the highest binding energy are more likely to form strong complexes. Table 1 shows that $\Delta E(\text{MAA}) \gg \Delta E(2\text{Vpy}) > \Delta E(4\text{Vpy})$. This is indicative of complex formation between caffeine and methacrylic acid being stronger than with either 2- or 4-vinylpyridine. Hence, the Hyperchem study predicts that a MIP for caffeine generated

Table 1
Calculated binding energies (ΔE) of caffeine with methacrylic acid, 2-vinylpyridine and 4-vinylpyridine in a vacuum

Molecules	Energy (kJ mol^{-1})	ΔE (binding energy) (kJ mol^{-1})
Caffeine	-24.74	-
Methacrylic acid	-36.63	-
2-Vinylpyridine	-12.52	-
4-Vinylpyridine	-4.86	-
Complex (methacrylic acid)	-77.27	-38.41
Complex (2-vinylpyridine)	-56.04	-17.95
Complex (4-vinylpyridine)	-38.41	-9.87



(a)



(b)

Fig. 2. (a) The Hyperchem calculated partial charges for methacrylic acid (MAA) and (b) the partial charges for 2-vinylpyridine (2Vpy). Nitrogen = blue, carbon = green, oxygen = red, hydrogen = white (for interpretation of the references to colour in this figure legend, the reader is referred to the web version of the article).

with methacrylic acid is more likely to have greater rebinding and selectivity than a MIP manufactured with either of the vinylpyridines.

In order to establish the conformation of the complex post-minimisation, the PM3 semi-empirical method was used [21,22]. According to Stuart [23], the PM3 method is very efficient. Furthermore PM3 has been shown to be superior to AMI in modelling of biochemical interactions [24]. This is due to an improvement in the treatment of non-covalent interactions and in particular hydrogen bonding, van der Waals forces and interestingly, steric effects. The latter is good rationale for the selection of the PM3 method for the modelling of imprinted polymer pre-

Table 2

Calculated binding energies on addition of further molecules of methacrylic acid to the system (in vacuum)

Amount of methacrylic acid molecules	ΔE (binding energy) (kJ mol ⁻¹)
1	-38.41
2	-60.28
3	-65.84
4	-52.12

polymerisation complexes. The flexibility of the PM3 method is demonstrated by Deng et al. [25] in the capillary electrophoretic (CE) separation and theoretical study of inclusion complexes of cyclodextrins with estrogen hormones.

Furthermore, molecular modelling, in this case, can also be used to analyse the likely molar ratio of the interactions. Table 2 presents the binding energies (ΔE) for the further addition of molecules of methacrylic acid to the system. Interestingly, the binding energy increases significantly upon the addition of a second molecule of methacrylic acid, however the addition of a 3rd or a 4th molecule to the system produces little change in ΔE . This would indicate that the energy of the system is optimal and further additions of functional monomer will not lead to a greater level of complexation. This is in contrast to conventional thinking in non-covalent imprinting in which an excess of functional monomer is used in order to ensure that the template molecule is fully complexed [26]. In short, the Hyperchem molecular modelling studies point towards methacrylic acid forming a strong complex with caffeine with a 1:2 ratio of template: functional monomer. Fig. 3 shows the Hyperchem predicted complex structure between caffeine and the two molecules of methacrylic acid.

3.2. Spectral analysis of template–monomer interaction

3.2.1. NMR

The molecular modelling studies using Hyperchem have suggested the possibility of hydrogen bond formation between the –OH of methacrylic acid and the nitrogen atoms of the five-membered ring on the caffeine molecule. The interaction of the template and methacrylic acid and 2-vinylpyridine was further characterised by proton NMR. NMR techniques have been widely employed for the study of pre-polymerisation complexes in MIP rational design approaches [15–18]. To a solution of caffeine in deuterated acetonitrile (CD₃CN) or chloroform (CDCl₃) was added an equimolar concentration of methacrylic acid (or substituted with deuterated acetic acid—for clarity of the spectrum). Fig. 4a shows the ¹H NMR spectrum of caffeine and Fig. 4b shows the peak shift observed on addition of deuterated acetic acid.

NMR then has confirmed the existence of a hydrogen bond or electrostatic interaction between caffeine and deuterated acetic acid and by proxy methacrylic acid. Chemical shifts in NMR can be affected by the local electric fields arising from charged or polar groups, e.g. –OH. Positive charges usually deshield nearby protons while negative charges generally perturbate shielding [15]. Given that the proton, which is shown to shift upfield is

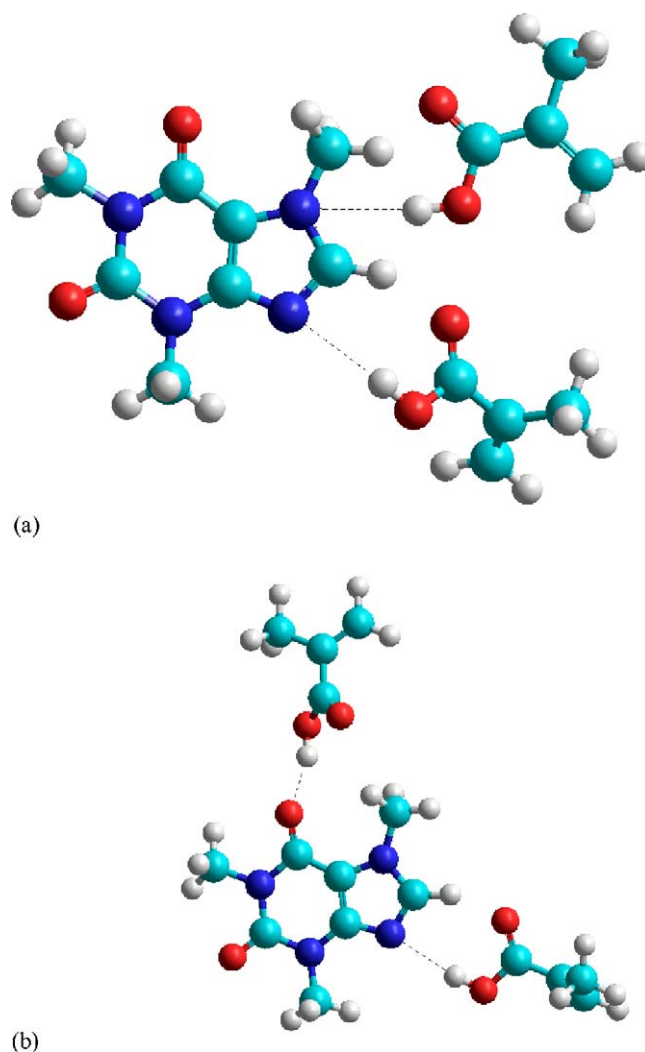


Fig. 3. (a) The Hyperchem derived structure of the complex formed between the caffeine molecule and two molecules of methacrylic acid. The presence of hydrogen bond is indicated by the dashed white lines. (b) An alternative possible configuration.

not directly involved in the template–monomer interaction. It is likely that the interaction causes an electron delocalisation effect forcing the proton upfield. Fig. 4b also shows the proposed mechanism of interaction between the template (caffeine) and the functional monomer (methacrylic acid). Importantly though, the prediction of interaction between caffeine and methacrylic acid postulated by Hyperchem has now been confirmed by NMR. Given the delocalisation effects suggested and the fact that the upfield shift was seen in CD₃CN, the nature of the interaction is unlikely to be hydrogen bonding. Acetonitrile is a solvent with medium to strong ability to form hydrogen bonds.

3.2.2. UV–vis

UV–vis spectroscopic studies on pre-polymerisation complex formation have been conducted previously with notable success [27]. In terms of further investigating the stoichiometry of the complex formation, a UV–vis mole ratio plot was used. The procedure was conducted in both chloroform and acetonitrile. The concentration of caffeine was kept constant while the

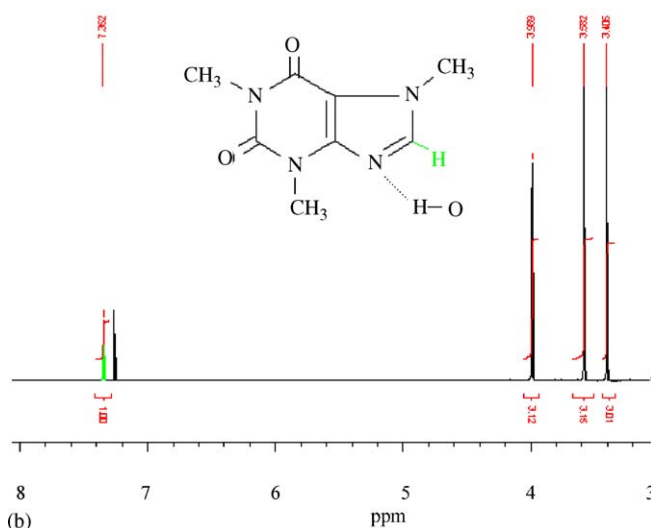
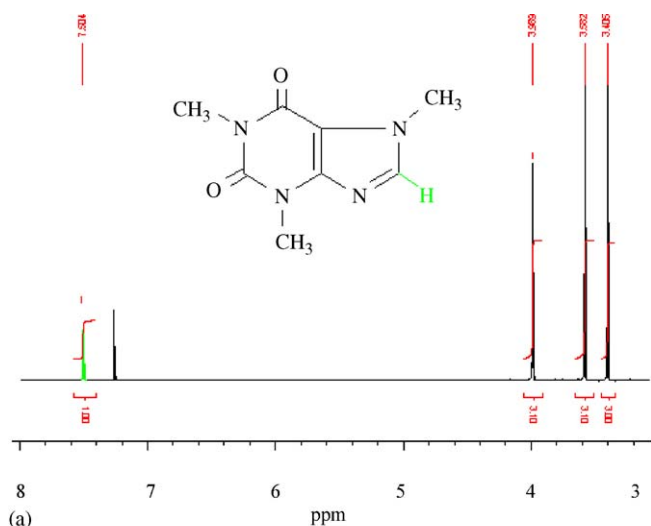


Fig. 4. (a) The ^1H NMR spectrum of caffeine and (b) that of caffeine as part of the pre-polymerisation complex. The highlighted peak at 7.504 ppm is seen to shift upfield to 7.362 ppm on complexation with the deuterated acetic acid. Note the reference CDCl_3 signal at 7.24 ppm.

ratio of caffeine: methacrylic acid was increased from 1:0 to 1:5. Fig. 5 shows the plot of absorbance at 272 nm against the mole ratio. Using a mole ratio plot, the point on the plot at which two lines intersect corresponds to the mole ratio of the complex. As can be seen from Fig. 5 when the mole ratio plot is performed in chloroform (above) an optimal ratio of caffeine:methacrylic acid of 1:1 is observed whereas in acetonitrile (below) the optimum ratio is 1:2.

Hence the UV–vis study has shown the presence of a second point of interaction, which was not identified by NMR. However, Table 2 shows that Hyperchem had predicted an optimal ratio of 1:2. This experiment emphasises the importance of the porogen itself in the polymerisation process [28]. Given that the second point of interaction is observed in acetonitrile and not in chloroform, it is indicative of that too being electrostatic in nature. However the 1:2 complex has more curvature at the stoichiometric ratio than is seen that the stoichiometric ratio of the

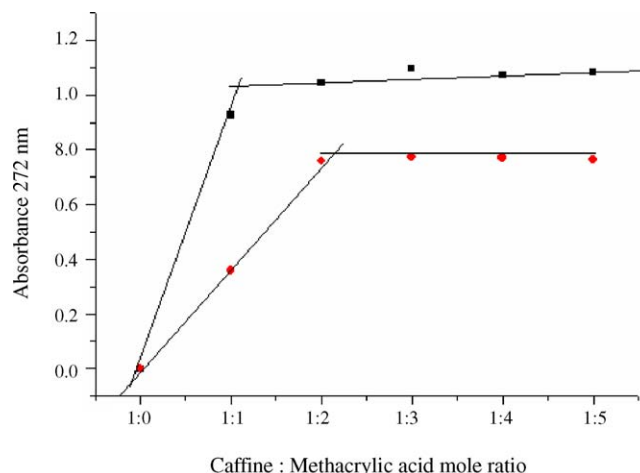


Fig. 5. UV–vis mole ratio plot of caffeine against methacrylic acid at 272 nm in (A) chloroform and (B) acetonitrile.

1:1 complex. This is indicative of a less stable complex. It is therefore probable that a second labile non-covalent interaction exists when acetonitrile is used as porogen. This interaction is not evident when chloroform is employed.

As concluded by Andersson and Nicholls [27] the self assembly of template with the functional monomer in the pre-polymerisation complex is in a constant state of molecular flux, where the extent of template complexation at equilibrium is subject to changes in ΔG . Complexation then, requiring the conformational configuration of two or more species will incur energetic penalties caused by barriers to rotation and translation. This is responsible for the instability of the second interaction observed in the mole ratio plot. Despite this, however, Table 2 shows a significant increase in binding energy at a 1:2 ratio. It is important to note however, that all Hyperchem studies are performed in a vacuum. It is probable that the inclusion of solvent effects would reduce the binding energy values.

3.3. Synthesis of the molecularly imprinted polymers

Given the data from the modelling and spectral studies, five imprinted polymers (plus corresponding controls) were synthesised and are listed in Table 3. Although, the modelling studies predicted a 1:2 ratio typically in molecular imprinting an excess of functional monomer is required [28]. MIPs produced at the predicted ratio were found to have high non-specific binding. Therefore, only ratios of 1:3 or 1:4 were used. Of the five MIPs shown in Table 3, it was expected that CAF 3 would perform

Table 3

Ratios of template:monomer:cross linker:porogen used in the generation of the imprinted polymers

MIP	Monomer	Monomer molar ratio	EGDMA molar ratio	Porogen	Porogen volume (ml)
CAF 1	MAA	4	20	CDCl_3	8
CAF 2	2Vpy	4	20	CH_3CN	4
CAF 3	MAA	4	20	CH_3CN	8
CAF 4	MAA	4	20	CH_3CN	4
CAF 5	MAA	3	20	CH_3CN	4

best in rebinding studies given that it was generated in a higher volume of acetonitrile. CAF 4 was expected to perform less well. Given the fact that there are specific interactions between monomer and template in chloroform and due to the amount of chloroform used, CAF 1 was expected to perform at least as well as CAF 4. CAF 2 was not expected to show significant rebinding whereas CAF 5 depending on the level of excess complexation of template required was an uncertainty.

3.4. Molecularly imprinted solid phase extraction (MISPE)

Given that MIPs perform well in terms of rebinding when used in the solvent they were manufactured in it was decided to use acetonitrile as the loading solvent for SPE. This is based on the likelihood of a reduced swelling of the polymer when exposed to the polymerisation conditions and hence the provision of an optimal environment for the template–functional site interaction [19]. However it was noted that the caffeine was not retained, most likely due to the characteristic of acetonitrile in being a good eluting solvent. Methanol showed similar problems to acetonitrile as it is also a highly polar solvent.

When chloroform was used a high degree of non-specific binding was observed (result not shown). This is likely due to the electrostatic nature of the template–functional monomer interactions. In non-polar solvent hydrophobic interactions will be more pronounced leading to the non-specific retention observed. Theodoridis and Manesiotis [8] noted similar problems. They decided upon the use of aqueous based loading conditions using 0.05 M $\text{CH}_3\text{COONH}_4\text{-NH}_3$ (aq.) at pH 9 and obtained high recoveries. The role of their buffer was to suppress non-specific interactions by masking the reactive acidic moieties on the surface of the polymer. For this study a sodium phosphate buffer at pH 10.5 has been used.

Fig. 6 shows a graph of percentage recoveries of caffeine using the five caffeine MIPs from a standard solution of 50 $\mu\text{g}/\text{ml}$ caffeine. In all cases the reproducibility was found to be good. Furthermore it was noted that the same cartridge could be loaded, washed and eluted several times (at least six) before any deterioration in performance was seen. At that amount of re-uses a drop

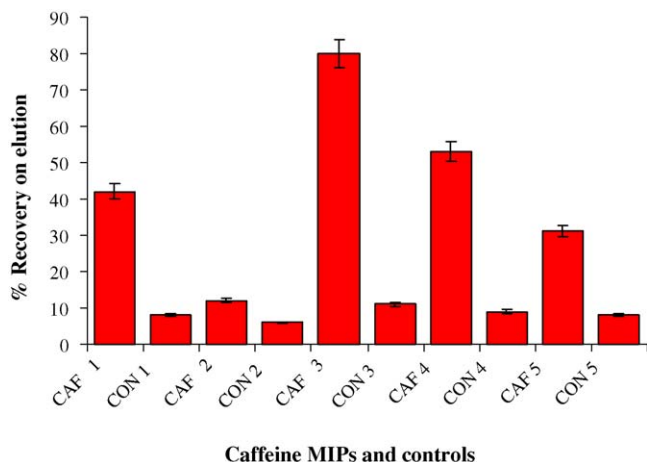


Fig. 6. The % recovery on elution for all five of the caffeine MIPs (CAF 1–CAF 5) in addition to the five control polymers (CON 1–CON 5).

in specific recovery of 5–10% was recorded. The recovery was found to be $80.6 \pm 2\%$. This compares well with conventional solid phase packing materials. Other authors have observed similar values for the recovery of caffeine by MISPE. A specific recovery of 87.6% was observed by Theodoridis and Manesiotis [8] for recovery of caffeine from a MIP. Furthermore, Puoci et al. [1] observed recoveries of up to 80% for MISPE determination of Sudan I in food matrices. The highest recovery was determined using an elution solution of 1% acetic acid in methanol.

3.5. Sample analysis

The potential of the MISPE procedure was evaluated using a real sample. The specific aim of the study was to demonstrate an efficient “first phase” clean up and extraction of caffeine from Red Bull™, a “high caffeine” soft drink. It was not key to the study, to develop an online SPE-HPLC assay for caffeine (many are available already) but rather to show the potential of a MIP in off line selective clean up of a beverage sample. This soft drink contains 32 mg of caffeine per 100 ml among other ingredients. The sample was adjusted to pH 10.5 to facilitate specific binding (as standard caffeine had been dissolved in the loading buffer at pH 10.5). The procedure was performed as described in Section 2. A 1 ml volume of Red Bull was loaded onto the SPE column (either diluted 1/100 or undiluted). Fig. 7 shows the high degree of clean up that was obtained for Red Bull using the CAF 3 MIP. The presence of a front on the caffeine peak indicates possible interferences. The ingredients of the soft drink are: carbonated water, sucrose, glucose, sodium citrate, taurine, glucuronolactone, caffeine, inositol, niacin, D-pantothenol, pyridoxine, HCl, vitamin B12, artificial flavours, colours. The other ingredients are not present in as high a concentration as caffeine. It is possible that trace levels of one of these compounds co-elutes with caffeine. The nature of the co-eluting species was not investigated at this point.

Based on the modelling data obtained, CAF 3 was expected to out-perform the other MIPs that were synthesised, and that was achieved. Furthermore, given the relative complexity of the sample, it is likely that many solid phase extraction materials

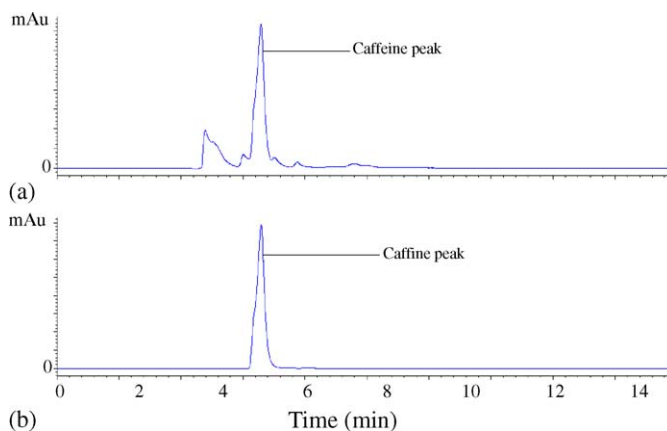


Fig. 7. Solid phase extraction clean up of caffeine from a Red Bull™ sample on an SPE cartridge packed with MIP CAF 3. (a) Raw sample, (b) extracted sample.

would struggle to extract caffeine alone from such a matrix. A % recovery of $65.8 \pm 2.4\%$ was obtained upon elution. For the control polymer a recovery of $3.2 \pm 0.7\%$ was determined.

3.6. The pore size distribution studies by BET

The study of certain traits of the polymers can yield information important to the physical characteristics of rebinding. Given that the importance of porogen has been demonstrated firstly by the NMR and UV studies and secondly by the MISPE, it can be concluded that the type and amount of porogen used can have an impact on the rebinding ability of the resultant MIP. To investigate this, two of the caffeine MIPs were selected (CAF 3 and CAF 5). These represented firstly a MIP which had performed well in the solid phase extraction studies (CAF 3) and secondly a MIP which had shown poor rebinding data (CAF 5). CAF 3 was prepared using 8 ml of acetonitrile as porogen and CAF 5 was prepared using 4 ml acetonitrile. Studies performed by Brüggemann [29], showed that a MIP may have over five times the surface area of the control MIP. Further to this, at least as an important forecast of how well a given MIP will perform is the pore volume and pore diameter. Typically pore sizes have been separated into three size categories [30], micropores (<2 nm), mesopores (2–50 nm) and macropores (over 50 nm). Table 4 shows the surface areas, total pore volumes, average pore sizes (diameter) for the MIPs CAF 3 and CAF 5 along with their corresponding controls.

It was found that by increasing the volume of porogen a major impact on the pore size distribution and pore volume was observed in otherwise identical polymers. As the average pore size diameter increases, the caffeine molecule (template) has more steric manoeuvrability within the pore and is more likely that there will be a higher rate of rebinding as attested by the solid phase extraction data. Furthermore the higher pore volume and surface area are indicative of a higher capacity and better-formed pores. One potential misinterpretation of this result is that it is possible that there is an apparent increase in rebinding which may not be caused by specific complexation. Template self-association can be a problem here in terms of aggregation of numerous caffeine molecules together within the same pore. An increase in the amount of diluent (porogen) causing an increase in the average pore size in the MIP should have the same effect in the control polymer though. If template molecules aggregate inside the pores of the MIP then they should do likewise inside the pores of the control and this should be reflected in an increase in rebinding. This is not seen however so it is likely that an

increase in average pore size means that there is a greater level of access to the functional groups for the template.

In terms of the surface area of the MIPs, CAF 3 produced a surface area of $345.59 \text{ m}^2/\text{g}$ whereas CAF 5 produced a surface area of $301.42 \text{ m}^2/\text{g}$. From this it can be seen that doubling the amount of porogen increased the surface area by over 12%. Typically, a higher surface area and pore volume is indicative of more well formed and uniform pore distribution. It would be expected that increasing the size of the pores would have led to a larger decrease in surface area. However there is still a relatively large population of pores in the micro- and lower mesoporous range. This may also help to explain the recoveries in the SPE studies. Given that the highest % recovery on elution as with CAF 3 and that amounted to approximately 80% (conventional SPE cartridges would be in the region of 80–85%) it is likely that a further increase in average pore size would lead to better recoveries in the SPE procedure.

4. Conclusion

The objective of this study was to provide an insight into the engineering molecularly imprinted polymers for specific analysis. The rationale was based on gaining an intrinsic understanding of the nature of complex formation between template and functional monomer with a view to exploiting this knowledge in terms of rationally designing a procedure for MIP production. Furthermore, the physical characterisation of the polymer in terms of the pore sizes could predict the performance of the MIPs regarding rebinding.

The caffeine molecule is a well characterised compound and was selected for study so the method could be compared and contrasted with other available analytical techniques. Given the success of the rational design approach for caffeine it is expected that the same approach could be easily adapted employed for the development of a MIP against a more complicated molecule/biomolecule and herein lays the usefulness of the rational design approach. To develop a fully operational chemical sensor or analytical method will inevitably involve an element of trial and error however, this work shows that it is possible to significantly reduce the time and materials involved in such a process.

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Table 4

The surface area, total pore volume and average pore diameter data obtained from the BET studies

Polymer	Surface area (m^2/g)	Total pore volume (cm^3/g)	Average pore diameter (nm)
CAF 3	345.59	0.8487	12.02
CAF 3 control	301.42	0.4750	5.01
CAF 5	289.62	0.5155	8.08
CAF 5 control	290.99	0.3472	3.42

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