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Molecularly imprinted polymers for the determination of a pharmaceutical development compound in plasma using 96-well MISPE technology

C. Chassaing [a,*], J. Stokes [a], R.F. Venn [a], F. Lanza [b], B. Sellergren [b], A. Holmberg [c], C. Berggren [c]

[a] *Christophe Chassaing, Bioanalytical Group, Drug Metabolism, IPC 664, Pfizer Global Research and Development, Sandwich Laboratories, Ramsgate Road, Sandwich, Kent CT13 9NJ, UK*
[b] *University of Dortmund, INFU, Otto Hahn Strasse 6, 44221 Dortmund, Germany*
[c] *MIP Technologies AB, Research Park Ideon, 22370 Lund, Sweden*

Abstract

The use of molecularly imprinted polymers (MIPs) as sorbents for the solid phase extraction (SPE) of a pharmaceutical compound in development, prior to quantitative analysis was investigated. Three MIPs were synthesised using a structural analogue as the template molecule. Each polymer was prepared with different monomers and porogens. The MIPs were then tested for their performance both in organic and aqueous environments, the final aim being to load plasma directly onto the polymers. At an early development stage, there is a limited amount of compound available. Due to this limitation, reducing the amount of template required for imprinting was investigated. A MIP capable of extracting the analyte directly from plasma was produced. The specificity of the polymer allowed the method to be validated at a lower sensitivity than a more conventional SPE assay. For the first time, MIPs were packed into 96-well blocks enabling high throughput analysis. The analytical method was fully validated for imprecision and inaccuracy down to 4 ng/ml in plasma.

Keywords: Molecularly imprinted polymers

1. Introduction

Molecularly imprinted polymers (MIPs) are highly cross-linked polymer matrices bearing specific recognition sites for individual analytes. Their preparation is based on the co-polymerisation of functional and cross-linking monomers around a target molecule, which acts as a molecular template. After removal of the template, the three-dimensional complementary binding sites, which remain in the host, can rebind the molecule with an affinity similar if not better than those of natural antibody–antigen systems. Due to an exponential growth in the interest of molecular imprinting in the past 10 years, a broad range of potential applications involving MIPs have emerged. This includes antibody mimics [1,2], enzyme-like catalysis [3], chiral stationary phases for HPLC [4] or capillary electrochromatography (CEC) [5,6], biosensors [7,8] and solid phase extraction (SPE) materials [9,10].

Solid phase extraction is routinely used to pre-concentrate drugs present at low levels of concentration and to remove interfering components from complex matrices prior to quantitative analysis. Much of method development work is spent on optimising SPE-based assays that yield cleaner extracts in the analysis of biological fluids and new strategies are called for to eliminate interferences in the subsequent matrix. One approach is to implement MIPs as chromatographic material in SPE, potentially allowing a higher degree of specificity to be achieved.

The introduction of MIPs into SPE [11], a technique commonly referred to as MISPE, is fast emerging as a very popular tool capable of overcoming these restrictions. The fact that MIPs can bind a particular analyte from a mixture of similar structures makes MISPE a very desirable technique for the development of selective and sensitive methods for trace analysis. During recent years there have been several publications describing the success of MISPE for the extraction of a whole range of compounds from different biological matrices. These include, amongst

* Corresponding author. Tel.: +44-1304-644136; fax: +44-1304-651987.
E-mail address: christophe_chassaing@sandwich.pfizer.com (C. Chassaing).

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many, the extraction of triazine herbicides [12], steroids [13], nicotine [14], clenbuterol [15,16], propranolol [17], darifenacin [18], local anaesthetics [9,19], sameridine [20], caffeine [21], coumarins [22] and even nerve agents degradation products [23]. Some applications were also reported for on-line SPE [24,25].

The overall aim of this study was to investigate the potential use of MIPs as SPE sorbents for the direct extraction of a Pfizer development compound from plasma. This work investigates the behaviour of three MIPs produced using different monomers and porogens. The first aim was to overcome limitations associated with template leaching by using a structural analogue capable of being chromatographically separated from the analyte of interest. The second aim was to use the MIPs to extract a Pfizer development compound directly from plasma. The use of a minimal amount of template to imprint with, either by using lower template-to-monomer ratios, or less actual polymer for each extraction was also investigated. The optimised MIP was used quantitatively in a 96-well SPE plate format for the first time. A high throughput assay was fully validated using MISPE as an extraction method. The sensitivity of the assay was compared with a more conventional C18 solid phase extraction.

2. Experimental

2.1. Materials

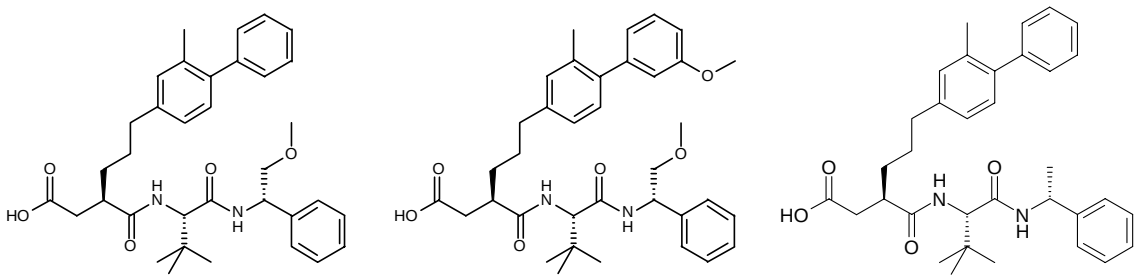
The target compound A and its structural analogues, the template compound B and the internal standard (IS) (see Fig. 1) were synthesised at Pfizer Global R&D, Sandwich, UK. Methacrylic acid (MAA), methacrylamide (MAM), ethylene glycol dimethacrylate (EDMA), dichloromethane (DCM) and acetonitrile (ACN) for the MIP synthesis were purchased from Sigma–Aldrich, Germany. 2-Vinyl-pyridine (VPy) was purchased from Merck (Darmstadt, Germany) and the initiator azobisisobutyronitrile (AIBN) was obtained from Janssen, Germany. Trifluoracetic acid HPLC grade (TFA) was obtained from BDH Lab Supplies, UK. Formic acid, methanol (MeOH) and tetrahydrofuran (THF) HPLC grade as well as propan-2-ol (iPrOH), triethylamine (TEA) and ammonia were purchased from Sigma–Aldrich, UK. Acetonitrile Super purity HPLC grade was purchased from Riedel-de-Haen, UK. Chloroacetic acid (MCA) was obtained from Acros, UK. Acetic acid (AcOH) HPLC grade, ammonium formate, ammonium acetate and ammonium phosphate were obtained from Fisher Scientific, UK. Control dog plasma was obtained from Charter House, UK. The 96-well SPE blocks (C18, 25 mg) were obtained from Varian, UK. The 96-well collection blocks were purchased from Porvair, UK. The MCA solution was prepared by dissolving 1 mole of MCA in 1 l of 90/10 (v/v) water/methanol.

2.2. Methods

2.2.1. Synthesis of the MIPs

Compound B was chosen as template (Fig. 1). Its structure was close enough to the target, such that any polymer imprinted with compound B would also recognise selectively compound A. The difference in lipophilicity between target and template was sufficient to obtain a good separation between compounds A and B by reversed-phase HPLC. Three MIPs were synthesised using different monomers and porogens (Fig. 2), partly inspired by recent optimisation studies [29]. The choice of the monomers was determined according to a miniMIP screening approach described elsewhere [30]. Several polymers with different functional monomers were synthesised in the presence of the template. The selection criteria were then based on the amount of released template in the same solvent used as porogen during polymerisation. Thus, for a particular MIP, a quantitative release of the template indicates that it will not recognise the template to a significant degree and the MIP may thus be discarded. In the second step, the rebinding of the template to the miniMIPs and to blank non-imprinted polymers was compared. The miniMIPs that rebound the template selectively were then synthesised in a large scale version.

A previously described procedure [4,26] for the synthesis of the MIPs on a large scale was adopted, but the template-to-monomer ratio used was reduced. MAA (4 mmole) was used as monomer for the preparation of MIP1, MAM (2 mmole) and VPy (2 mmole) were used for MIP2, Vpy (2 mmol) and MAA (2 mmol) for MIP3. The template-to-monomer ratio was 1/80 (50 μmole) for MIP1, 1/20 (200 μmole) or 1/80 (50 μmole) or 1/200



Target (compound A) **Template (compound B)** **Internal standard (IS)**

Fig. 1. Structures of the target, the template and the internal standard.

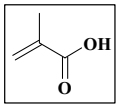
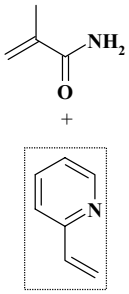
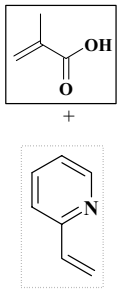
	MIP1	MIP2	MIP3
Monomers	 Methacrylic acid (MAA)	 Methacrylamide (MAM) 2-Vinylpyridine (VPy)	 Methacrylic acid (MAA) 2-Vinylpyridine (VPy)
Porogen	Dichloromethane	Acetonitrile	Acetonitrile

Fig. 2. Monomers and porogens used to prepare the MIPs.

(20 μmole) for MIP2 and 1/20 (200 μmole) for MIP3. EGDMA (20 mmole), the monomer(s) (4 mmole) and the initiator (AIBN, 0.25 mmole) were mixed with approximately 6 ml of the porogen. The solvent used as porogen was DCM for MIP1 and acetonitrile for MIP2 and MIP3. The solutions were transferred to a glass tube (14 mm i.d.). The polymerisation mixture was degassed with nitrogen for 5 min. The tubes were sealed, transferred to a water bath and the mixture was polymerised thermally at 45 °C. After 24 h the tubes were crushed, the polymers were extracted in a Soxhlet for 17 h with 99% acetic acid, washed with water to remove the acid and dried at room temperature. The polymers were then ground (with a ball mill) and sieved by hand under water to 25–36 μm grain size fractions (Resch stainless steel sieves). Crushing and sieving were repeated in cycles until all polymer had passed the 36 μm sieve.

MIP1 was produced at Dortmund University, Germany (Fig. 2) and was received in a loose powder form, which was manually packed into 1 ml SPE cartridges (25 mg). MIP2 and MIP3 were produced by MIP Technologies in Lund, Sweden and were packed by IST, UK, into 96-well SPE blocks (25 mg). This is the first reported use of MIPs in a 96-well SPE format, making them available for high throughput analysis. Some loose powder was also sent to carry out method development in a cartridge format. Blank reference polymers (RP) were synthesised by using the same procedure as the corresponding MIP, but without template.

2.2.2. SPE extraction from plasma

Dog plasma (500 μl) was spiked at different concentrations of compound A into 12 ml polypropylene tubes. The internal standard solution (IS, Fig. 1) (1 μg/ml, 20 μl) and the template solution (1 μg/ml, 20 μl) were added to all samples, except to the double blanks. A different extraction was used for each of the three sorbents: C18 SPE, MIP2 and MIP3.

2.2.3. C18 SPE

The calibration standards and replicates in plasma (500 μl) were diluted with 750 μl of MCA solution. The 96-well SPE block (25 mg, Varian C18) was conditioned with iPrOH (0.5 ml) followed by MCA solution (1 ml). The samples were transferred onto the SPE block with Pasteur pipettes and were loaded using a weak vacuum. The SPE block was washed with 1ml of water followed by 1 ml of 40/60 (v/v) methanol/water. Full vacuum was applied for approximately 5 min to ensure the SPE block was fully dry. Two aliquots of iPrOH (2 × 0.5 ml) were sequentially added to each well, a light vacuum was applied and the eluates were collected into a 96-well (2 ml) block.

2.2.4. MIP2

The calibration standards and replicates in plasma (500 μl) were diluted with 500 μl of 75/25 (v/v) 25 mM ammonium acetate/acetonitrile. The MISPE 96-well block (25 mg per well) was conditioned with methanol (1 ml), water (1 ml) and 75/25 (v/v) 25 mM ammonium acetate/acetonitrile. The samples were transferred onto the block with Pasteur pipettes and were loaded using a weak vacuum. The block was washed with water (1 ml) followed by four aliquots (4 × 0.5 ml) of 60/40 (v/v) 50 mM ammonium phosphate/acetonitrile. Full vacuum was applied for approximately 5 min to ensure the block was fully dry. The compounds were eluted, by adding to each well 1 ml of methanol containing 2% TFA. The eluates were collected into a 96-well (2 ml) block.

2.2.5. MIP3

Cold acetonitrile (0.5 ml, 4 °C) was added to calibration standards and replicates in 0.5 ml of plasma, causing protein to precipitate. The mixture was centrifuged at 2000 × *g* for 10 min at 4 °C. The supernatant was then diluted with

1.5 ml of MCA solution and loaded onto the MIP3 96-well SPE block (25 mg per well). The MISPE block was preconditioned with methanol (1 ml), water (1 ml) and 1 ml of MCA solution. The samples were transferred onto the block with Pasteur pipettes and loaded using a weak vacuum. The block was washed with water (1 ml) followed by five aliquots (5 × 1 ml) of 65/35 (v/v) 5 mM ammonium formate (pH =?)/acetonitrile. Full vacuum was applied for approximately 5 min to ensure the block was fully dry. The compounds were then eluted with 1ml of methanol containing 2% TFA. The eluates were collected into a 96-well (2 ml) block.

The eluates from the C18 SPE, the MIP2 and MIP3 blocks were evaporated to dryness under heated nitrogen at approximately 55 °C. The residues were reconstituted in 100 μl of 44/56 (v/v) water/acetonitrile containing 0.2% TFA. The block was vortex-mixed thoroughly and centrifuged at approximately 2000 × *g* for 15 min at 4 °C. The reconstituted samples were then injected onto the HPLC system (50 μl injection volume).

2.2.6. HPLC

A Varian ProStar 230 tertiary pump (Varian, Walton-on-Thames, Surrey, UK) delivered the mobile phase at a 0.5 ml/min flow rate onto a C18 Phenomenex Luna 150 mm × 3 mm i.d. HPLC column, with 5 μm packing. The composition of the mobile phase was 44/56 (v/v) water/acetonitrile containing 0.2% TFA. A Jasco AS-950-10 autosampler (JASCO, Great Dunmow, Essex, UK) injected 50 μl aliquots of reconstituted plasma extracts. The template (compound B), the target (compound A) and the internal standard (IS) were eluted at approximately 10.7, 12 and 14.5 min, respectively (Fig. 13). The overall run time was 22.5 min including a 2 min wash with 10/90 (v/v) water/acetonitrile containing 0.2% TFA and a 4 min re-equilibration period. A LaChrom L-7480 fluorescence detector (Merck-Hitachi-VWR, Poole, Dorset, UK) was used for the detection of the analytes. The excitation and the emission wavelengths were set at 255 and 312 nm, respectively.

3. Results and discussion

3.1. Synthesis of the MIPs

A limitation associated with the use of MIPs is the continual leaching of template from the polymer even after extensive washing. The necessary extent of removal depends on the application in which it is to be used. Template leaching has been identified in the past as one of the main limitations for the use of MIPs in quantitative trace analysis and has hindered the development of applications such as MISPE for many years. Many groups have tried various approaches to remove it all including thermal annealing, controlled in situ decomposition of template, and microwave assisted extraction all failing to get the leaching below that required for trace analysis. These attempts have been published recently [27]. An alternative approach can be used in order to avoid limitations associated with template leaching. Rather than imprinting with the actual analyte of interest, a structural analogue is used, and providing the two can be separated chromatographically then template leaching does no longer get in the way of quantification [28]. The so-called 'target-analogue' approach is very effective in preventing that any template contamination coming from the MIP during the extraction step could not interfere with the analysis of the target during the chromatographic step [20]. The three MIPs prepared during this study were imprinted with a structural analogue to the target analyte (see Section 2).

3.2. Evaluation of the MIPs

It was expected that a greater imprinting effect would be obtained if the analyte was dissolved in the porogen before being loaded onto the SPE cartridges. MIP1 was demonstrating a good imprinting effect towards the analyte dissolved in DCM, compared to RP (Fig. 3). Most of compound A was eluted from RP during the washing steps, whilst being selectively retained on MIP1. Strong acidic conditions were required to elute compound A from MIP1. The final aim of this work was to obtain a MIP capable of retaining

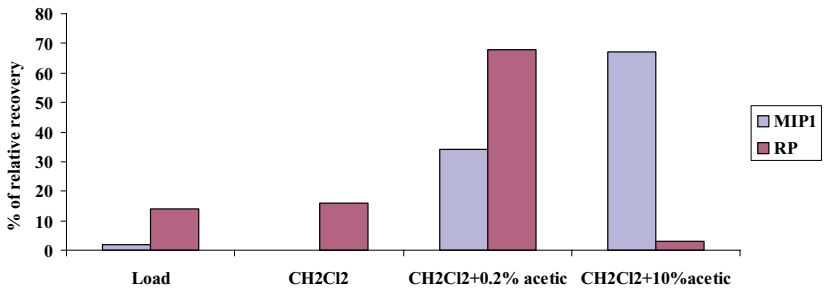


Fig. 3. Recovery profile for MIP1 in dichloromethane. The cartridges were preconditioned with methanol (1 ml), dichloromethane with 0.2% acetic acid (1 ml) and dichloromethane (1 ml). Compound A (0.5 ml, 300 ng/ml solution in dichloromethane) was loaded onto the cartridges. The cartridges were then washed with two aliquots of dichloromethane (2 × 1 ml) followed by 1 ml of dichloromethane with 0.2% acetic acid. Compound A was then eluted with two aliquots of dichloromethane with 10% acetic acid (2 × 1 ml).

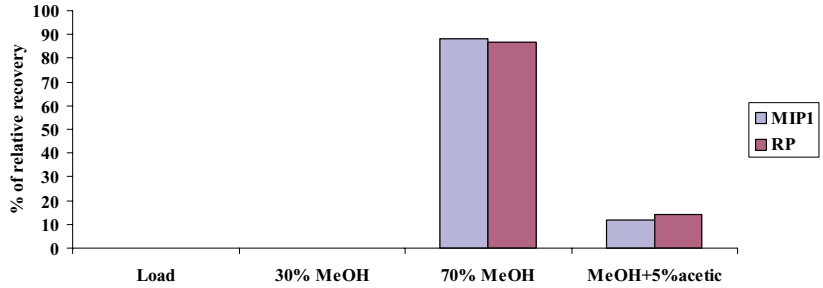


Fig. 4. Recovery profile for MIP1 in aqueous conditions. The cartridges were preconditioned with two aliquots of methanol (2 × 1 ml) and two aliquots of water with 10% of methanol (2 × 1 ml). Compound A (0.5 ml, 300 ng/ml solution in water with 10% methanol) was loaded onto the cartridges. The cartridges were then washed with water containing 30% methanol (1 ml) and 70% methanol (1 ml). Two aliquots of methanol containing 5% acetic acid (2 × 1 ml) were used for the elution step.

selectively compound A directly from an aqueous biological sample, such as plasma. Compound A was dissolved in a mainly aqueous solution and loaded onto MIP1 (Fig. 4). In aqueous conditions, MIP1 appeared to behave very similarly to RP and did not show any imprinting effect towards the target. The compound did not break through on the load, but was eluted with a weak wash, indicating that compound A was most probably bound to the polymer by non-specific interactions. MIPs generally express better recognition abilities towards the target when the SPE is carried out in the same environment as the one used for the polymer synthesis [31]. This was the case for MIP1, which was performing well in dichloromethane, but not in water. Extracting drugs directly from mostly aqueous biological fluids using MIPs is a very challenging process. The selective interactions between template and monomers vary according to their environment, and are based mostly on normal phase interactions. In most cases the selective binding occurs in organic solvents, where hydrogen bonding and dipole–dipole interactions predominate. When loading the analyte onto a MIP, it interacts via both specific and non-specific interactions and the extraction process is designed to try to maximise the specific binding. When loading aqueous samples (i.e. plasma) hydrophobic interactions come into play and this generally increases the amount of non-specific binding, reducing the imprinting effect.

MIP2 was synthesised by using acetonitrile as a porogen (Fig. 2). Acetonitrile, which is miscible with water, can be used in either purely organic or aqueous solvent mixtures. The presence of the porogen in the aqueous load was expected to enable the polymer to interact selectively with the target. MIP2 was first tested in pure acetonitrile (Fig. 5). MIP2 showed some imprinting effect in that a lot more of the analyte was breaking through in the loading step for RP. However, the compound was eluted from MIP2 as well as RP during the first few weak wash steps. MIP2 did not perform as well as MIP1 in presence of the porogen.

MIP2 was then evaluated in aqueous mode. Compound A was loaded onto the cartridges using two buffers, one containing a percentage of porogen, the other being 100% aqueous. By using 25% of acetonitrile in the loading buffer, the polymer can generate interactions similar to its imprinting environment, favouring analyte binding. Any more than 25% of acetonitrile would result in protein precipitation when plasma is diluted with the buffer before loading onto the block. As seen in Fig. 6, the effect of acetonitrile in the load greatly improves the imprinting effect. Without acetonitrile, MIP2 shows poor imprinting effect and in fact behaves almost identically to RP (Fig. 6A). In the presence of acetonitrile during the loading step (Fig. 6B), MIP2 behaves selectively with most of the compound being eluted from RP during the wash stages whilst most of it is retained on MIP2 until the elution stage. The relative recovery values given in Fig. 6 represent the percentage of the total amount of compound eluted from the polymer during the overall process. Experiments were also performed in order to op-

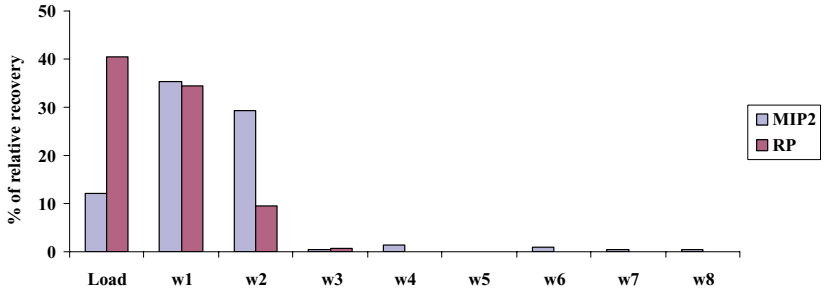


Fig. 5. Recovery profile for MIP2 in acetonitrile. The cartridges were preconditioned with two aliquots of methanol (2 × 1 ml) followed by acetonitrile (1 ml). Compound A (0.5 ml, 300 ng/ml solution in acetonitrile) was loaded onto the cartridges. The cartridges were then washed with acetonitrile (1 ml, W1) followed by acetonitrile with 0.1, 0.3, 0.5, 1, 2, 5 and 10% acetic acid (1 ml each, W2–W8).

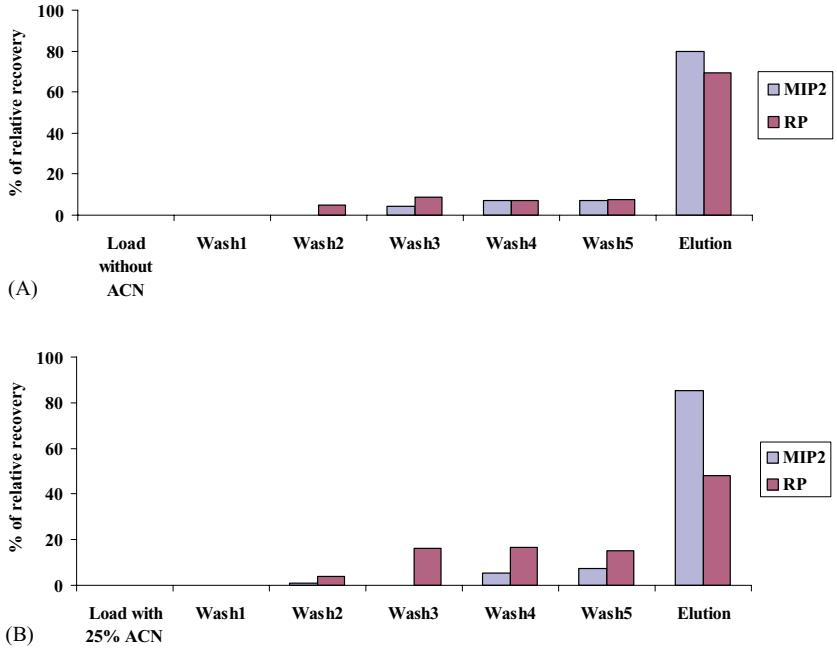


Fig. 6. Recovery profiles for MIP2 in aqueous mode with (B) or without (A) acetonitrile in the loading solution. The cartridges were preconditioned with methanol (1 ml) followed by 25 mM ammonium acetate buffer with (B) or without (A) 25% acetonitrile. Compound A (0.5 ml, 400 ng/ml in 25 mM NH4Ac with (A) or without (B) acetonitrile) was loaded onto the cartridges. The cartridges were then washed with water (1 ml) followed by four aliquots (4×0.5 ml, W2–W5) of a 60/40 (v/v) 50 mM ammonium phosphate/acetonitrile. The elution was carried out with two aliquots (2×1 ml) of methanol containing 5% formic acid.

timise the composition of the elution solvent. A range of strong bases and strong organic and inorganic acids were used in combination with methanol in order to increase the analyte recovery (Fig. 7). TFA (2%) gave the greater recovery with 54% of compound A coming off in the elution step. Another set of experiments used TFA as an organic modifier but this time in combination with other solvents (Fig. 7). It was expected that 2% TFA in acetonitrile might work better since acetonitrile was the original porogen. Still, methanol with 2% TFA seemed to give the best recovery results, may be due to the fact that methanol can disrupt

Fig. 7. Recovery of compound A using MIP2 with various elution solvents. Solvents a–e contained methanol with (a) 2% TFA, (b) 5% ammonia, (c) 5% triethylamine, (d) 5% formic acid and (e) 0.1 M HCl. Solvents f–j contained 2% TFA in (f) acetonitrile, (g) acetonitrile/methanol/water 60/35/5 (v/v/v), (h) THF/water 70/30 (v/v), (i) acetonitrile/water 70/30 (v/v) and (j) methanol/water (v/v).

hydrogen bonds. The extraction was also carried out with various mixtures of organic solvents as well as with a small percentage of water. The presence of water did not cause the polymer to swell and therefore did not help to release more compound from the polymer. Even with the optimised 98/2 (v/v) methanol/TFA mixture, only 54% of the analyte was recovered during the elution step, 26% of the analyte breaking through during the various washes and 20% being irreversibly retained on the polymer.

The main limitation with the preparation of MIPs is the amount of template required for the imprinting process, leading to a high cost for the production of the polymer. One way to avoid this is to use lower ratios of template-to-monomer. All the experiments shown so far have been done using a 1/80 ratio (MIP1) or a 1/20 ratio (MIP2). MIP2 was also produced with the two lower template-to-monomer ratios 1/80 and 1/200. The chromatograms in Fig. 8 show, as expected, an increase in template leaching with higher ratios. The analyte was loaded in buffer containing 25% acetonitrile, onto the three different MIP2 cartridges: 1/20, 1/80 and 1/200 ratio. The results are shown in Fig. 9. For the 1/20 ratio, a good imprinting effect was obtained in aqueous conditions. Most of the analyte was eluted from RP in the wash stages but retained on MIP until the strong acidic elution. The elution recovery in buffer was approximately 54% from MIP2 1/20 ratio and only 24% from the RP. The results obtained for the two lower ratios were not as clear. MIP2 1/80 ratio and MIP2 1/200 ratio seemed to behave the same way as MIP2 1/20 ratio during the load and washing steps but not

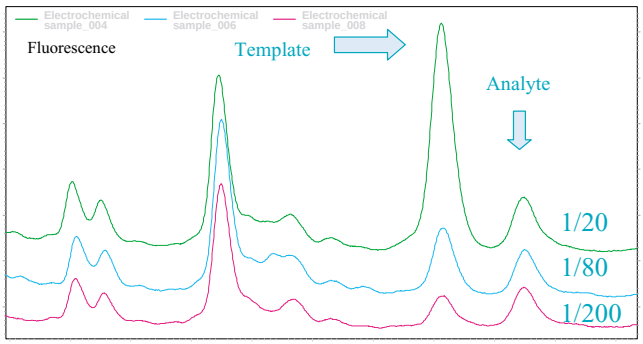


Fig. 8. Differences in template leaching from the three different template-to-monomer ratios: 1/20, 1/80 and 1/200.

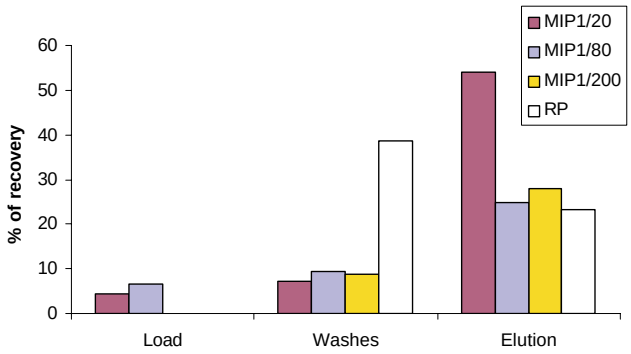


Fig. 9. MIP2 performance using three different template-to-monomer ratios: 1/20, 1/80 and 1/200 (same conditions as Fig. 6B).

during the elution step. The overall recoveries obtained with MIP2 1/80 and 1/200 ratio were quite low, with approximately 60% of the loaded compound seemingly still retained on the MIP. A low template-to-monomer ratio generates less recognition cavities and may result in a higher polymer density not in favour of an efficient mass transfer. Alternatively, the template sites may be better defined per se due to the reduced influence of template self-association during their formation. Stronger elution solvents failed to release any more compound from these lower ratios. Using stronger conditions, such as 10% acetic acid, also led to the degradation of the polymer itself and resulted in the blockage of the HPLC column upon analysis of the extracted samples. Due to the low recovery as well as the apparent poor imprinting effect obtained with lower template-to-monomer ratios, MIP2 with a 1/20 ratio was used for the rest of this study.

Another way of reducing the amount of template is to decrease the actual amount of MIP used per cartridge or per extraction well. All the experiments described so far were done using 25 mg of loose MIP powder packed into 1 ml SPE cartridges. Various other amounts (10, 15 and 20 mg) were tried to see if the same results could be obtained with less polymer (Fig. 10). The results indicate that the less MIP is used the more breakthrough of compound there is during the load and subsequent washes. In light of this, various increasing amounts of MIP2 (35, 45 and 55 mg) were used to see if the recovery could be further improved. The results suggest that at 55 mg almost 80% of the analyte is released during the elution step (Fig. 10). With lower amounts of MIP2, for example 10 mg, the analyte was breaking through in the loading and wash stages, whereas in the 55 mg experiment, the analyte was retained until the elution step. The possibility of increasing the recovery from 52 up to 80% would make it worthwhile using more polymer. However, to get a fair assessment of these experiments, the results obtained with MIP2 had to be compared with the ones generated with the same amount of reference polymer in order to check if the imprinting effect was affected (Fig. 11). Although there was a much higher recovery when using more MIP2, the imprinting effect seemed to be drastically reduced. This may be due to the fact that the height of the MIP packing in the 45 mg cartridge is almost double that of the 25 mg. Therefore the compound travels slower through the polymer and binds through non-specific weak interactions. A compromise between recovery and more importantly, imprinting effect was found by using 25 mg of polymer for all the following experiments.

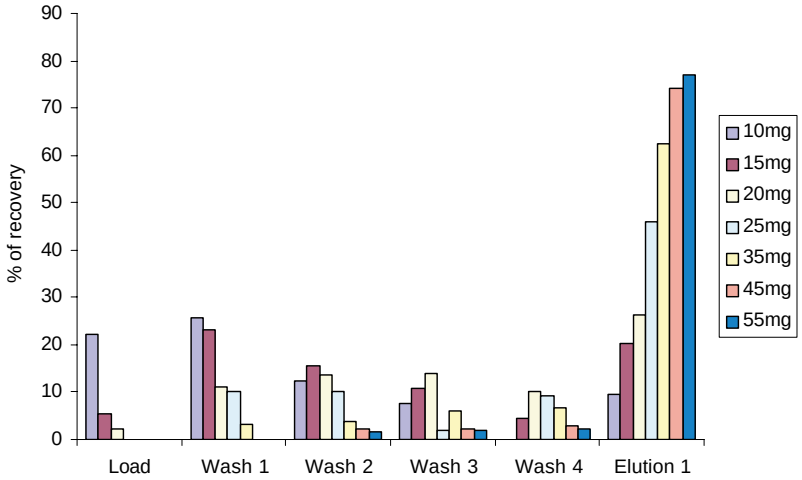


Fig. 10. Extraction profile using various amounts of MIP2 per cartridge (same conditions as Fig. 6B).

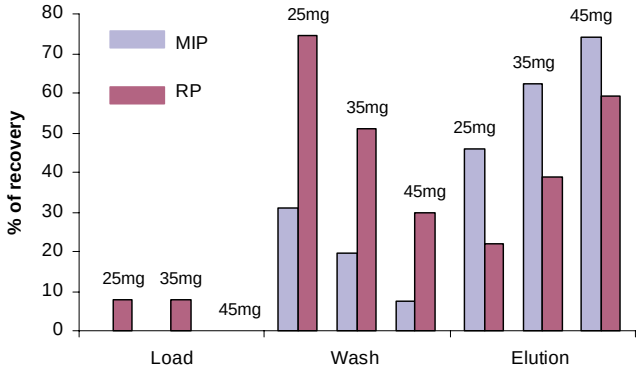


Fig. 11. Recovery profiles for three different amounts of MIP2 and reference polymer.

3.3. Plasma extraction with MIP2

Since MIP2 demonstrated a good imprinting effect in aqueous conditions, experiments involving the direct extraction of plasma were carried out. The same method as for the buffer samples was used (see Section 2). It was not possible to analyse all the extraction steps on the HPLC as the other stages were all containing too many protein residues to be directly injected in the analytical system. The recovery was only monitored in the last elution step but it was assumed that the load and wash steps behaved the same way as the buffer profiles. The recovery from plasma was approximately 43%, to be compared with the 54% obtained by loading the analyte in buffer only. The reduced recovery from plasma may be due to the high protein binding of the analyte.

3.4. MISPE validation

An assay involving HPLC with fluorescence detection was developed and validated to analyse plasma samples extracted with MIP2. The internal standard (IS) was a structural analogue to compound A (Fig. 1). Due to the fact that a structural analogue to the target was used as the template, it was essential that the two compounds, as well as the IS, could be separated chromatographically, such that any template bleeding would not interfere with the analytical and internal standard peaks.

A conventional C18 SPE was validated in order to compare the extraction results with the data generated with MISPE. The dynamic range of the assay was 5–500 ng/ml. Imprecision and inaccuracy, were determined on one occasion at four concentration levels spanning the calibration range. These were calculated using the formulae shown below:

$$\text{imprecision (\%R.S.D.)} = 100 \times \frac{\text{S.D.}}{\text{mean concentration}}$$

$$\text{inaccuracy (\%error)} = 100 \times \frac{\text{found concentration–spiked concentration}}{\text{spiked concentration}}$$

Table 1
Imprecision and inaccuracy data for C18 SPE extraction

Compound A Spiked concentration (ng/ml)	Found concentration		Imprecision (%R.S.D.)	Inaccuracy (%error)	n
	Mean (ng/ml)	(±S.D.)			
5	5.22	0.14	2.8	4.3	7
15	15.0	0.6	3.6	0.0	7
250	256	4	1.7	2.4	7
500	524	7	2.4	4.9	7

As shown in Table 1, the imprecision and inaccuracy did not exceed 5% at the top, middle, lower and bottom concentration levels. The lower limit of quantification (LLOQ) was 5 ng/ml. The coefficient of correlation for the calibration line was 0.998 (Fig. 12). The extraction recovery was close to 100%.

In order to compare the MISPE method with the more conventional C18 SPE, a validation was carried out using MIP2. The same LLOQ as the C18 SPE method was achieved, even though the recovery for the MIP assay was less than half the recovery of the C18 SPE. A cleaner baseline was obtained by using the MIP compared with the SPE, with less endogenous materials being retained on the MIP (Fig. 13). Also, an interference, which was eluting just after the analyte in the SPE extract, was selectively removed by MIP2. The LLOQ of an assay is determined by the sensitivity of the detection method and the recovery of the extraction, but also by the level of the background noise. Although the intensity of the peak was lower with MIP2 due to low recovery, the baseline noise was much lower than with the C18 SPE. The chromatogram of blank dog plasma extracts shown in Fig. 14 highlights the baseline difference between the MIP and the C18 SPE. Due to the specificity of the MIP extraction, high sensitivities were obtained with MIP2 despite the low recovery. The imprecision and inaccuracy results obtained with the MIP2 using dog plasma are given in Table 2. The values were well within the ±15% limits required for the assay to validate. The assay was linear over a 5–500 ng/ml range with a correlation coefficient of 0.997 (Fig. 15).

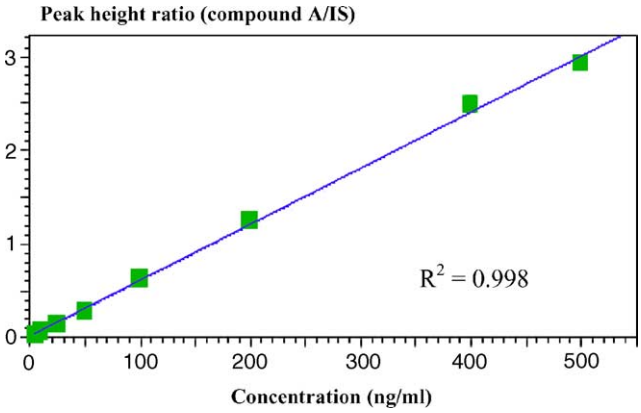


Fig. 12. Calibration line (5–500 ng/ml) of compound A in dog plasma extracted by C18 SPE.

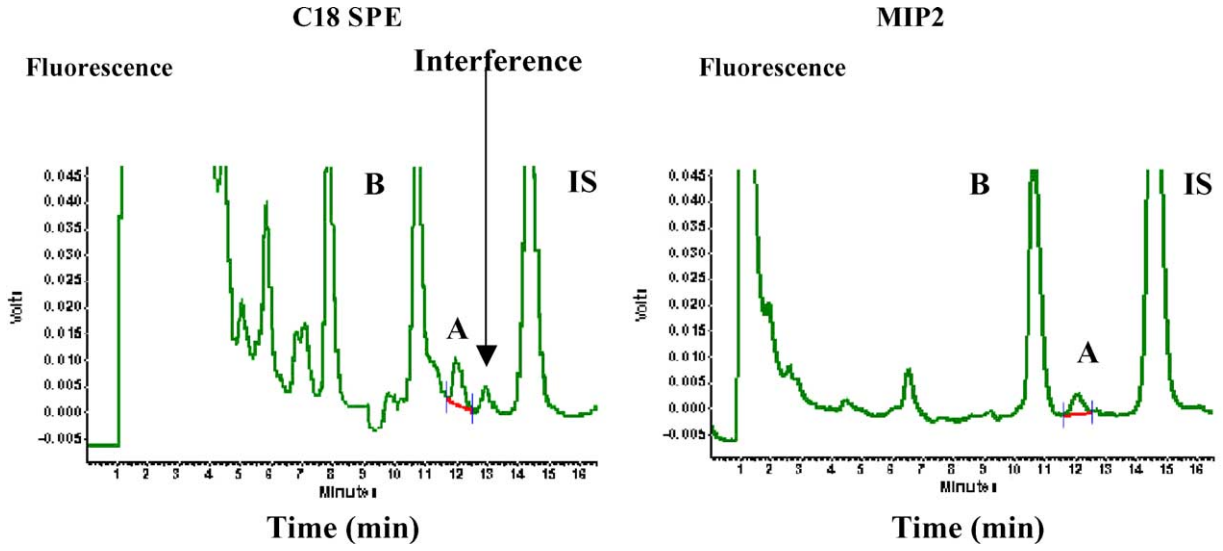


Fig. 13. Chromatograms of control dog plasma spiked at 5 ng/ml and extracted by C18 SPE and MIP2.

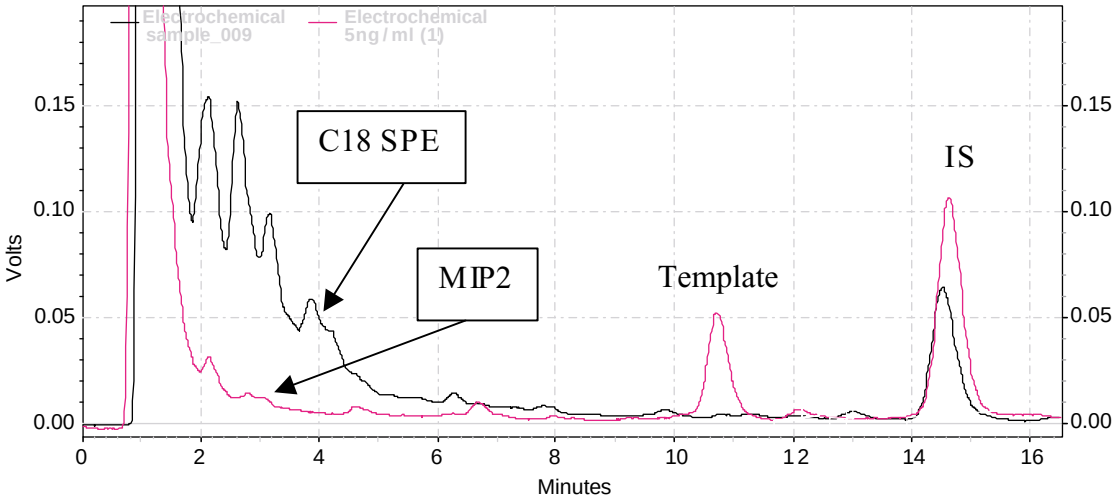


Fig. 14. Chromatograms of blank dog plasma spiked with IS and extracted by C18 SPE and MIP2.

3.5. Evaluation of MIP3

The aim of this new polymer was to build on the experience acquired with MIP2, which was working well in aqueous mode and to get a polymer that would also release more of the analyte. The recovery obtained with MIP2 was 43% only. Improved recovery would lead to higher assay sensitivity. MIP3 was tested in the aqueous mode and showed a good imprinting effect (Fig. 16). The elution recovery obtained with MIP3 when the analyte was loaded in an aqueous buffer containing 10% methanol, was over 90%, signifi-

Table 2
Imprecision and inaccuracy data for MIP2 extraction

Compound A Spiked concentration (ng/ml)	Found concentration		Imprecision (%R.S.D.)	Inaccuracy (%error)	*n*
	Mean (ng/ml)	(±S.D.)			
5	5.38	0.61	11.2	7.6	7
15	16.9	1.0	5.8	12.9	7
250	263	16	5.9	5.2	7
500	448	42	9.4	−10.4	7

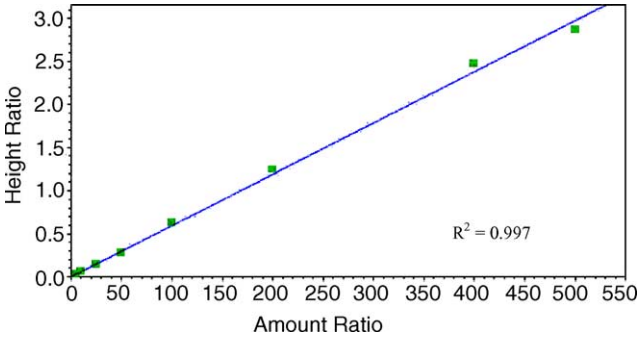


Fig. 15. Calibration line from validation of MIP2 extraction of compound A from dog plasma.

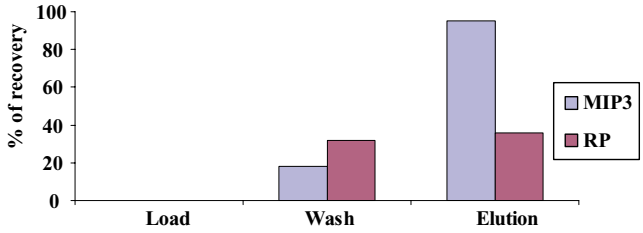


Fig. 16. Recovery profile for MIP3 in aqueous mode. The cartridges were preconditioned with methanol (1 ml), water (1 ml) and two aliquots of water with 10% of methanol (2 × 1 ml). Compound A (0.5 ml, 300 ng/ml solution in water with 10% methanol) was loaded onto the cartridges. The cartridges were then washed with water containing 30% methanol (1 ml) and methanol containing: 30% water (2 × 1 ml). Two aliquots of methanol containing 5% acetic acid (2 × 1 ml) were used for the elution step.

Table 3
Imprecision and inaccuracy data for MIP3 extraction

Compound A Spiked concentration (ng/ml)	Found concentration		Imprecision (%R.S.D.)	Inaccuracy (%error)	*n*
	Mean (ng/ml)	(±S.D.)			
4	4.27	0.53	12.3	6.8	7
8	8.68	0.53	6.0	8.4	7
250	187	5	2.6	−6.3	7
400	372	14	3.8	−6.8	7

cantly higher than MIP2. However, there was only approximately 10% of the analyte recovered in the elution step with spiked plasma, no matter how much the plasma was diluted with the buffer. Due to the promising performance observed with buffer, a protein precipitation step was carried out before loading the plasma. The supernatant was then diluted with buffer and loaded onto MIP3 (see Section 2). Using protein precipitation followed by SPE with MIP3 increased to 55% the extraction recovery, a 12% increase compared with MIP2. The MIP3 assay validated in dog plasma at 4 ng/ml, which is slightly more sensitive than, both MIP2 and the original C18 SPE method (Table 3). The assay was linear over a 4–400 ng/ml range with a correlation coefficient of 0.996 (Fig. 17). Although MISPE technology applied to compound A only provided half the recovery of conventional C18 SPE, it was possible to validate the assay down to more sensitive levels.

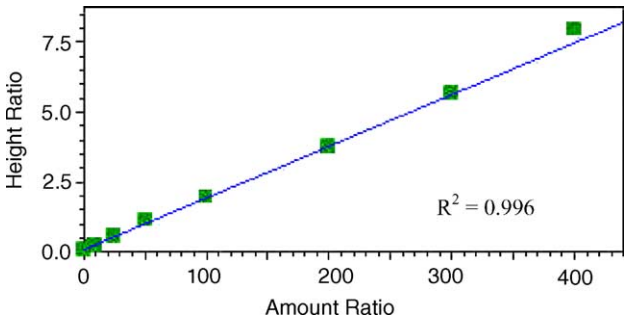


Fig. 17. Calibration line from validation of MIP3 extraction of compound A from dog plasma.

4. Conclusion

The results generated during this study highlight the strong potential of molecularly imprinted polymers as SPE sorbents. Three different polymers were investigated. MIP1 performed very well in dichloromethane but showed no imprinting effect in aqueous conditions. MIP2 was synthesised using a water miscible porogen. This allowed the polymer to be very selective in aqueous mode. It was a major breakthrough to be able to load plasma directly onto the polymer. An assay involving the extraction of the analyte from plasma with MIP2 was validated down to a limit of quantification of 5 ng/ml. It was directly comparable to the results obtained with a more conventional C18 SPE method, even though the analyte recovery with MIP2 was less than half of that seen with the C18 SPE. The fact that there was always 20% of analyte retained on this polymer, no matter how much it was washed, limited the performance of MIP2. A third MIP was prepared in order to overcome the poor recovery issue observed with the previous polymer. MIP3 was designed using a combination of monomers from MIP1 and MIP2. MIP3 was working perfectly in aqueous buffer with recoveries close to 100%. However, when plasma was loaded onto MIP3, the recovery fell to less than 10% no matter how much the plasma was diluted with buffer. A protein precipitation step was included before the MISPE extraction in order to prevent this recovery loss in plasma. The protein precipitation step did increase the recovery from 43% with MIP2 to approximately 55% with MIP3, and subsequently the assay was able to validate at a slightly more sensitive level of 4 ng/ml, performing better than both MIP2 and conventional C18 SPE.

Much cleaner baselines were obtained by using a MISPE approach, leading to low background noise. Hence a higher sensitivity even with lower analyte recovery was obtained. The attempt to reduce further the amount of template needed for imprinting in order to reduce costs was not successful. The performance of the lower ratios of template-to-monomer was poor compared to that of the 1/20 ratio. Reducing the amount of template by using less polymer was not suitable since it resulted in recovery loss. Imprinting with a high template-to-monomer ratio, becomes a real issue at an early development stage when large quantities of compounds are not available yet.

MISPE technology was used in a 96-well block format for the first time allowing for the high throughput extraction of samples. This 96-well format contributed to the good results obtained for inaccuracy and imprecision. It improved the reproducibility of the technique and for the first time allowed a MISPE method to be used quantitatively in a fully validated assay. This study is one of the few cases reported whereby a MIP made in an organic porogen is successfully used in aqueous conditions, allowing for the extraction of a compound directly from plasma, which was the main aim of this work. This demonstrates the potential of MIPs as a valuable alternative tool for sample extraction prior to quantita-

tive analysis. Work in progress involves the use of MISPE with mass spectrometry as an end point instead of fluorescence detection. The fact that the MIP baselines are so clean means that there would be very low ion suppression on the mass spectrometer, such that a more sensitive assay can be obtained. Future work will also involve trying new methods to completely remove the template irreversibly bound to the MIP. This would allow to imprint with the actual analyte of interest and also to recycle the polymer.

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