3. RESULTS & DISCUSSION

Although the results of the individual experimental parts of the Thesis have been discussed above in their respective sections, the present section discusses the most important results as a whole. This section is divided into two main parts; the first deals with MIP synthesis and the second with the application of MIPs as sorbents in SPE.

As far as the synthesis is concerned, Table 3.1 summarizes the optimum conditions for preparing the imprinted polymers used in the present Thesis. As can be seen in this table, the protocol used to prepare the MIPs is the non-covalent approach because it is relatively straightforward. Nevertheless, the MIP imprinted with 4-NP was also prepared with a semi-covalent approach, which allowed the imprinting effect on both MIPs to be compared. The stability of the complex template-functional monomer used to obtain a MIP via a semi-covalent approach makes it possible to obtain well-defined cavities. Nevertheless, the imprinted effect of the semi-covalent MIP prepared in this case was diminished because an additional functional monomer (styrene) was added so that the template/functional monomer/crosslinker ratio used in the previous non-covalent MIP was constant. This decrease is because the styrene increases the hydrophobicity of the MIP and the binding sites of the MIP offer only one point of attachment. Moreover, it is also important to note that it is not always possible to find the appropriate template-functional monomer complex to prepare a MIP via a semi-covalent approach, and when it is possible, the strong interactions during the synthesis can hinder the template removal when the polymer has been synthesised. This means that it is more likely for the resulting MIP to bleed when it is used as a SPE sorbent, although in our case no bleeding was observed.

As can be seen in Table 3.1 several porogens, functional monomers, T:M:X ratios and polymerisation conditions were used. It should be noted that these parameters depended on the characteristics of the template molecule used in each case (e.g. the polarity, type and number of functional groups, and the stability of the template under the UV-light). Nevertheless, the crosslinking agent and the initiator used were the same in all the MIPs prepared. EGDMA was the

crosslinking agent used because it makes possible to obtain MIPs with good thermal and mechanical properties and is compatible with nearly all the organic solvents. In this Thesis it has also been demonstrated that EGDMA can be used when the porogen is a mixture of water and an organic solvent such as methanol, and the methanol/water ratio is lower or equal to 4:1 as has been previously explained for the 1-NS MIP. AIBN was used as the initiator in all the syntheses because it can react thermally and under UV light, and can therefore be used in both polymerisation methods.

Table 3.1 Optimum synthesis conditions for the different MIPs prepared in the present Thesis

Template	Porogen	Functional Monomer	T: M:X[a]	Polymerisation	
				UV + Thermic	Thermic
4-nitrophenol (4-NP)	ACN	MAA[+]	1:4:20	-	X
4-nitrophenil methacrylate	ACN	Styrene	1:4:20	-	X
4-chlorophenol (4-CP)	ACN	4-VP[*]	1:4:20	-	X
1-naphthalene sulfonic acid (1-NS)	MeOH/H_2O (4:1)	4-VP[*]	1:4:20	X	
Ibuprofen	Toluene	4-VP[*]	1:4:20	X	
Naproxen	Toluene	4-VP[*]	1:4:20	X	
Oxytetracycline (OTC)	DMSO	MAA[+]	1:8:40		X
Enrofloxacin (ENRO)	DCM	MAA[+]	1:8:40	-	X
Enrofloxacin (ENRO)	Chloroform	MAA[+]	1:8:40	-	X
Ciprofloxacin (CIPRO)	DCM	MAA[+]	1:8:40	-	X

[a]template:functional monomer:crosslinker ratio   [+]Methacrylic acid   [*]4-vinylpyridine

Whether a polymerisation was performed thermally or under UV light with a subsequent thermal polymerisation depended on the template stability under UV light. Lower temperatures produced better defined imprinting sites, so the polymers were obtained under UV polymerisation when the template was stable under such conditions.

This table also shows the porogens used to prepare the MIPs, which were chosen depending on the solubility properties of the template molecule. One example is the MIP imprinted with 1-NS, which requires a mixture of methanol/water to solubilise the template molecule completely. Nevertheless, other template molecules with less polar characteristics, such as naproxen or ibuprofen, can be solubilised without any problem in toluene, which is considered to be a good porogen because its dielectric constant is low and it is a non-polar and non-protic solvent. Although a wide variety of porogens were used, some of them good porogens (DCM, $CHCl_3$ or toluene) and others not so good (ACN, DMSO or $MeOH/H_2O$), it has been shown in the present Thesis that they all provide a good imprinting effect.

All the MIPs were prepared by conventional solution polymerisation; consequently the MIPs are obtained as a monolith. This polymerisation method allows MIPs to be prepared relatively easily and quickly even though the polymers have to be ground and sieved before they are used.

In order to check the selectivity of the MIP for the template molecule, the MIPs and their respective control polymers were packed in chromatographic columns and a chromatographic evaluation was performed. When an analogue to the template was injected in these columns, the chromatographic evaluation also made it possible to determine the selectivity of the MIP for other structurally related compounds of the template molecule. The normalised retention indices (RI) for the templates and their respective analogues are shown in Table 3.2. The RI value for the template is 1 by definition, and if the RI value for the analogue of the template is also close to 1, then the MIP shows high levels of cross-reactivity. This value determined whether the individual MIPs were used for one application or another. For example, the cross-reactivity of the ibuprofen MIP was used for extracting a mixture of four non-steroidal anti-inflammatories from river water while the naproxen MIP was used as selective sorbent to extract naproxen from a mixture of four NSAIDs (one of them ibuprofen) in urine samples.

Table 3.2 Selectivity shown by some MIPs for several template analogues

Template	Analogue	RI $_{template}$	RI $_{analogue}$
4-CP	4-NP	1	0.93
Ibuprofen	Naproxen	1	0.90
Naproxen	Ibuprofen	1	0.75
ENRO (DCM)	Ciprofloxacin	1	4.33
ENRO (CHCl$_3$)	Ciprofloxacin	1	1.33
CIPRO	Enrofloxacin	1	0.47

The MIP imprinted with 4-CP is another clear example of cross-reactivity, which was not shown by the 4-NP MIP. Thus, the 4-CP MIP was able to selectively extract 4-NP and a group of 4-chlorosubstituted compounds from a mixture of eleven nitro- and chlorophenols from river water samples. From the RI$_{analogue}$ value obtained for the ENRO MIP prepared in DCM it was also expected to selectively extract ENRO and CIPRO and this was confirmed when the MIP was applied in tissue samples. In some cases, it was not possible to perform the chromatographic evaluation of the MIP. In this situation, the cross-reactivity of the MIP was evident when it was used for the SPE. This was observed for the 1-NS MIP, which was able to selectively extract a mixture of eight NSs from a mixture of other polar compounds.

Throughout the present Thesis, it has been demonstrated that all the MIPs prepared can be applied in SPE and some of them have been used in an on-line system with liquid chromatography. Nevertheless, it was not always possible to develop this coupled system because the mobile phase of the chromatographic system sometimes could not disrupt the selective interactions between the template and the MIP and, when the appropriate elution solvent was used as the mobile phase sometimes the analytes could not be separated or the chromatographic column deteriorated. Table 3.3 summarises the extraction

system used, the sample analysed and the most important experimental conditions for each of the MISPE studies.

Table 3.3 Summary of the MISPE applications performed with each MIP synthesised in the present Thesis

Template	Sorbent (mg)	MISPE	Sample	Sample Volume (ml)	Clean-up	Eluting solvent
4-NP	40	On-line	River water	10	DCM	ACN/acetic acid (99:1)
4-nitrofenil metacrilat	40	On-line	River water	20	DCM	ACN/acetic acid (99:1)
4-CP	40	On-line	River water	10	DCM	ACN/acetic acid (99:1)
1-NS	200	Off-line	River water	500	MeOH/ pyridine	MeOH/H_2O(10% NaOH 1M) (4:1)
Ibuprofen	200	Off-line	River water	1000	DCM	ACN/acetic acid (99:1)
Naproxen	200	Off-line	Human urine	25	ACN	ACN/acetic acid (99:1)
OTC	500	Off-line	Pig kidney	25	ACN	MeOH (10% KOH 1M)
ENRO (DCM)	200	Off-line	Human urine and pig liver	25	ACN/HAc	ACN/formic acid (4%) (1:4)
ENRO ($CHCl_3$)	200	Off-line	Human urine	25	chloroform	ACN/formic acid (4%) (1:4)
CIPRO	200	Off-line	Human urine	25	DMF	ACN/formic acid (4%) (1:4)

This table shows that nearly all the MISPE studies were performed in off-line mode, which allows a wide variety of eluting solvents to be used. The MISPE studies were applied to the extraction of several analytes from river water and biological samples. Depending on the matrix sample, different sample pre-

treatments were used. For river water, the sample was just filtered through a membrane filter before being analysed. Then, the river water was directly applied through the MIP sorbents, which proved to have high affinity for the target analyte/s even in aqueous samples.

Only a few of the studies made of the extraction of compounds from river water samples were developed in the on-line mode because the mobile phase used was the same as the eluting solvent of the analytes from the MIP. Although in the ibuprofen MISPE study the mobile phase and the eluting solvent were also the same, it should be noted that the MISPE study was performed in an off-line mode because in this way it was possible to increase the amount of sorbent and subsequently to increase the capacity of the MIP. Therefore, a mixture of four NSAIDs was extracted from 1000 ml of river water with good recoveries for all the analytes. This volume of water was directly applied through the MIP cartridge, which makes this MIP interesting because MIPs usually show low capacity when working with these samples. In the experimental part, it has been demonstrated that the 1-NS MIP, was also able to extract a mixture of eight NSs with good recoveries from 500 ml of river water.

Throughout the experimental work described in the previous chapter, it has been demonstrated that in some cases, a clean-up step with an organic solvent is necessary before the elution step to enhance the selectivity of the MIP. As can be seen in Table 3.3, DCM was the most widely used washing solvent. This is because this solvent is able to remove the non-selectively retained compounds on the MIP while the target analyte/s is still retained because the selective interactions, which are mainly hydrogen bonding, are not disrupted. Nevertheless, in the study that used the 1-NS MIP, a mixture of MeOH/pyridine had to be used as washing solvent. In spite of the polar and protic solvent (MeOH), the selective interactions between the NSs and the MIP were not disrupted because they were electrostatic interactions. It should be mentioned that in some studies it was very important to dry the MIP before the clean-up step so that the clean-up was selective.

The MISPE of analytes from biological samples was mainly applied to urine and tissue samples, as can be seen in Table 3.3. Urine samples were filtered through a syringe filter and directly applied to the MIP cartridge. However, tissue samples first had to be homogenized and centrifuged to remove proteins and lipids. The liquid extract obtained after this pre-treatment was then applied through the extraction system.

The MISPE studies applied to biological samples were performed in an off-line mode so that a wider variety of eluting solvents could be used. The biological samples analysed in the present Thesis were also aqueous and, as in the analysis of river water, the urine and the liquid extract arising from the tissue were also directly applied to the MIP, except in the ENRO and CIPRO MISPE studies in which the sample was first passed through a commercial (OASIS) sorbent. This two-step SPE was necessary because in aqueous samples the analytes were not retained in their respective MIPs, so the loading solvent had to be changed. In this way, the analytes were first retained on the OASIS and eluted in MeOH. This methanolic solution was then passed through the MIP, which enhanced the selectivity for the target analyte.

The sample volume percolated in the studies on the extraction of compounds from urine samples was 25 ml and the liquid extract obtained after the tissue treatment passed through the MIP was also 25 ml. This sample volume can be considered to be high for the analysis of biological samples, in which analytes are usually extracted from a few ml of sample. In the MISPE study of ENRO and CIRO, the methanolic solution passed through each MIP was 10 ml.

For the MISPE of biological samples, the clean-up step was very useful, mainly when tissue samples were analysed. To remove the matrix sample from the MIP, solvents such as ACN were used. This is a polar but non-protic solvent which removes the matrix interferences without disrupting the selective interactions between the naproxen or the OTC, and their respective MIPs. Sometimes, modifiers such as acetic acid were added to the washing solvent to decrease the cross-reactivity of the MIP, as was observed in the ENRO MIP

when urine samples were analysed. However, the cross-reactivity of this MIP was very useful when fluorinated quinolones had to be extracted from tissue samples because in this way the MIP selectively extracted CIPRO and ENRO and both analytes could be quantified as specified by the European legislation. $CHCl_3$ and DMF were also used as washing solvents (see Table 3.3). After an optimization step, it was seen that they were the best washing solvents for removing the matrix sample and enhancing the selectivity of the MIP. One example is the CIPRO MIP in which clean extracts were obtained and directly injected into the mass spectrometer without signal suppression. In this way, the separation system was avoided and the time of analysis decreased.

The present Thesis has shown that MIPs can be prepared using several template molecules which can vary from environmental pollutants to drugs and the molecules can be big or small and have different degrees of polarity and different numbers of functional groups. It has also shown that analytes can be extracted from river-water samples and quantified without matrix interferences because humic and fulvic acids are removed during the clean-up step.

When MIPs are used to extract compounds from biological samples, biofluids are the most common samples analysed and only few studies have been made of tissue samples. However, because tissue is a very complex matrix, MISPE can be very useful for removing the sample matrix.